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THE ROLE OF AUTOPHAGY IN CANCER: FOCUS ON THE ONCOSUPPRESSOR BECLIN 1

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Introduction

CHAPTER 1

OVARIAN AND BREAST CANCER

1.1.A Ovarian Cancer

Ovarian cancer is the fifth leading cause of cancer death among women in the U.S., and the prime cause of deaths from gynecological malignancies among women in the Western world. The causes and natural history of these cancers are the least understood among major human malignancies. Furthermore, in spite of intensive research, the 5-year survival from ovarian cancers has remained at only about 40% for the last 50 years *(Cancer Genome Atlas Research Network, 2011)*.

The poor survival rate is related to the diagnosis made in the advanced stages of the disease *(Jemal et al., 2008)*. Over 85% of ovarian malignancies are categorized as epithelial ovarian cancers, and among these, the most common and most lethal are the high grade serous ovarian carcinomas (HGSOCs) *(Auersperg, 2013)*.

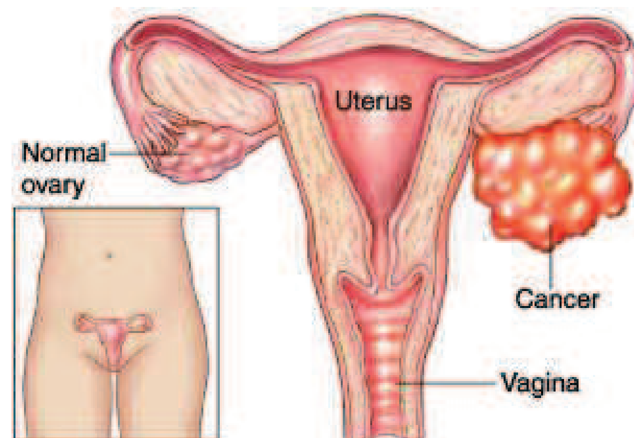

Fig.1 *Ovarian Cancer*

1.2A Diagnosis of Ovarian Cancer

Generally, ovarian cancer is detected by increased abdominal volume. The symptoms are indistinct and frequently overlooked by the patient and even by the doctor, with

gastrointestinal conditions and conditions originated in the urinary tract. Thus, 79% of cases are diagnosed at advanced stages, when the malignant ovarian tumor become apparent. At this point the woman experiences intense gastrointestinal symptoms, with lower abdominal or pelvic pain, and periodic constipation, diarrhea and vaginal bleeding.

The diagnosis of malignant ovarian tumor can be made during a gynaecological investigation and sometimes because of twisting of the ovarian pedicle, which requires an investigative laparotomy for acute abdomen, mostly with no indication for oncological surgery.

Once the ovarian tumor has been diagnosed as malignant, the prognosis for survival will be related to the following factors:

- Age and menopause status.
- Tumor size.
- Tumor stage.
- Characteristics of the tumor by imaging (ultrasound, CT, MRI).
- Presence or absence of symptoms.
- Tumor marker values.
- Unilateral, compared with bilateral.

In the childbearing age of the women, the ovary normal size is 3.5 cm. The menopausal ovary undergoes atrophy, it measures 2 cm and in late menopause less than 2 cm. Therefore in menopausal and post-menopausal age the presence of a palpable ovary is presumptively a neoplasm. Similarly, an ovary of more than 3.6 cm diameter and of solid consistence in the fertile age is suspicious.

Patients with suspected ovarian cancer, following the initial history-taking, physical examination, laboratory results, and tests for the tumor marker CA-125, should be investigated in depth for the contents of the abdominal cavity. In general, the pelvic examination, the level of CA-125 determination and the ultravaginal ultrasound make possible an early ovarian cancer diagnosis.

Therefore, imaging studies must be conducted, such as ultrasound CT, MRI, and special techniques of radioactive isotope scanning; none of these give details of the correct staging of the tumor, and at the end of the study an abdominal CT, chest x-ray, and bone scan must be carried out.

The recommended first-line ultrasound procedure for a suspected single adnexal tumor is by the vaginal route, using color Doppler. This has a sensitivity of over 93.5% and specificity of 91.5%. MRI is appropriate for adnexal investigation, particularly if there is suspected tumor

activity outside the pelvis. It has a sensitivity of 91.1% and specificity of 84%. CT has shown 87.2% sensitivity and 84% specificity *(Dodge et al., 2012)*. Serum levels of CA-125 tumor-associated antigen can be high in other malignant tumors such as: breast cancer, pancreatic cancer, colon cancer, bronchogenic cancer, and endometrial cancer. Therefore, it is not recommended as a single or standard method for diagnosing ovarian cancer; when the result is elevated to three times its normal value, it is considered to have 78.7% sensitivity and 77.9% specificity.

Elevated CA-125 can be found in various benign ovarian disorders, particularly in premenopausal women with disorders related to infertility, such as endometriosis, endometrial cyst, pelvic inflammatory disease, hepatitis, pregnancy, menstruation, peritonitis, and after recent abdominal surgery. Until this investigation finds evidence of disease outside the abdominal cavity, a protocol-based exploratory laparotomy is an essential part of the initial investigation for a patient *(Vargas, 2014)* .

Findings of epidemiological studies have shown that the risk of ovarian cancer is reduced by states of anovulation, such as pregnancy or the use of oral contraception *(Tsilidis et al., 2011)*; or through tubal ligation-reduced reflux of menstrual products onto the ovary *(Sieh et al., 2013)*. Supraphysiological ovarian stimulation for treating infertility has been implicated, but not proven, to increase the risk of borderline ovarian tumors *(Stewart et al., 2013)*.

Endometriosis is sometimes found adjacent to endometrioid or clear cell ovarian cancers, and findings of some prospective case-control studies have shown an increased incidence of ovarian cancer in women with documented endometriosis *(Pearce et al., 2012)*. Human papilloma virus *(Anttila et al., 1999)*, perineal talc *(Gertig et al. 2000)*, and smoking *(Beral et al., 2012)* have been discounted as causes of the disease, whereas findings of epidemiological and genetic analyses have shown that epithelial ovarian cancer is a major element in several germline genetic mutation syndromes.

1.3A Histopathological and Molecular Classification of Ovarian Cancer

Ovarian cancer is a heterogeneous disease, in fact it is associated with family history of cancer, genetic risk, and histopathology of this disease. The most common histology is epithelial ovarian cancer comprising 95% of invasive cancers. Among the invasive epithelial ovarian cancer there are several subtypes. The most common subtype is the serous followed by endometrioid, clear cell, and mucinous cancer.

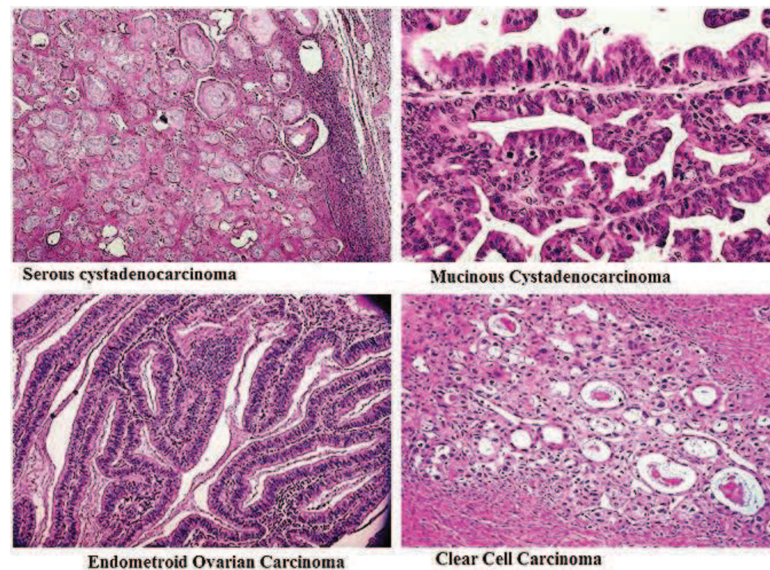

Fig.2 *Ovarian Cancer Histology*

Serous ovarian carcinomas, by definition, exhibit serous (oviductal differentiation), in contrast to other epithelial ovarian carcinomas. Thus, epithelial ovarian cancers mimic the derivatives of the Mullerian ducts, that are the epithelia of the oviduct, uterus and cervical canal. Clear cell carcinomas are an exception since they look like renal clear cell carcinomas. In recent years, evidence has accumulated indicating that endometrioid and clear cell carcinomas may arise in sites of endometriosis and the composition of mucins in ovarian mucinous carcinomas resembles mucins of the gastro-intestinal tract, suggesting that some of these neoplasms may be derived from extraovarian sources. Forms of ovarian cancer other than epithelial are sex cordstromal tumors which include granulosa cell tumors, and rare types such as germ cell tumors *(Köbel et al. 2008)*.

The prognostic classification of ovarian cancer based on the classical clinical pathological parameters (FIGO stadium, histological type, differentiation, residual tumor after surgery), is considered insufficient.

The epithelial ovarian cancers comprise a variety of subtypes, defined by their morphology and differentiation. They are also characterized clinically by different progression, responses to therapy and prognosis. The histologic subtypes of epithelial ovarian cancers can further be classified on the basis of molecular profiles.

Two groups have been defined on the basis of specific combinations of mutations and gene inactivation *(Shih and Kurman, 2004):*

- Type I tumors include low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas. These tumors are slow growing, frequently discovered at low stages, and share lineages with preinvasive and benign precursors.
- Type II tumors include the high-grade serous, high-grade endometrioid, and undifferentiated carcinomas, with the great majority being classified as serous. These carcinomas are usually detected late, don't have well defined precursors and account for most deaths due to ovarian cancer.

As a group, type I tumors are genetically more stable than type II tumors and display specific mutations in the different histologic cell types. KRAS, BRAF, and ERBB2 mutations occur in most low-grade serous carcinomas, whereas TP53 mutations are rare in these tumors. Low-grade endometrioid carcinomas have mutations of CTNNB1, PTEN and PIK3CA, while most mucinous carcinomas have KRAS mutations and clear cell carcinomas exhibit PIK3CA activating mutations.

In contrast, the high-grade serous carcinomas, the predominant type II tumors, are highly unstable genetically, are characterized by p53 mutations in over 90% of cases, but rarely contain the mutations found in type I tumors *(Kurman and Shih, 2010)*.

Integrated genomic analysis of ovarian cancer in several hundred tumors, further delineated four transcriptional subtypes, and identified somatic mutations in NF1, BRCA1, BRCA2, and CDK12 *(Shih and Kurman, 2004)*. Importantly, homologous recombination repair of DNA damage is defective in roughly 50% of high grade serous cancer and NOTCH and FOXM1 signaling are implicated in the pathophysiology of serous tumors *(Cancer Genome Atlas Research Network, 2011)*.

Combination of molecular and histopathological classification permits to distinguish the following subtypes:

- High-grade serous and endometrioid ovarian cancers

- Low-grade serous and endometrioid ovarian cancer

- Clear-cell ovarian cancer

- Mucinous ovarian cancers

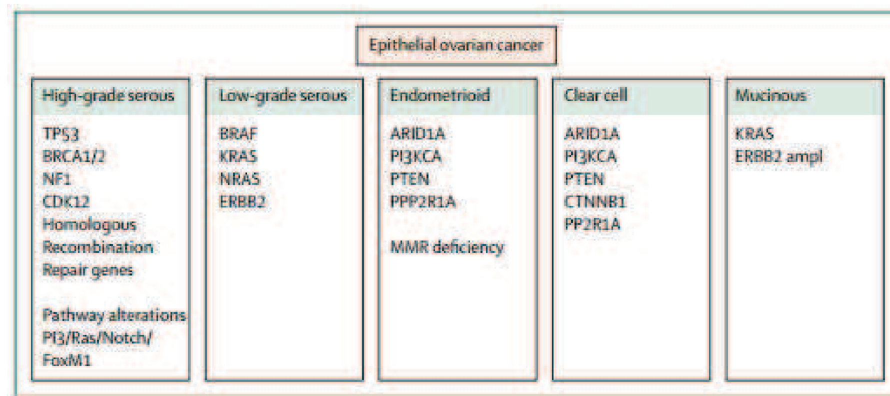

Fig.3 *Epithelial subtypes and associated mutations (Jayson et al., 2014)*

Most patients with epithelial ovarian cancer have high grade serous cancer, a disease that is characterized by nearly universal p53 gene abnormalities *(Kobel et al., 2010; Ahmed et al., 2010)* also seen in endometrioid and other high-grade undifferentiated histologies.

High-grade serous cancer is characterised by genomic instability, DNA copy number abnormalities, but few distinct and recurrent mutations. Nearly all ovarian cancers related to deleterious mutations in BRCA1 and BRCA2 are high-grade serous cancer. Genomic studies have subdivided high-grade tumors into four subgroups termed proliferative, immunologic, mesenchymal, and differentiated *(Tothill et al. 2008)*. High-grade cancers are characterized by initial chemo sensitivity with subsequent acquisition of increasing resistance at each recurrence.

Low-grade serous ovarian cancer shows more indolent behavior and retrospective studies describe low response rates to cytotoxic and hormonal agents. Mutations in PI3KCA, BRAF, and KRAS, are prevalent and although MEK inhibitors seem promising *(Farley et al., 2013)*.

Clear-cell and low-grade endometrioid epithelial ovarian cancer bear frequent mutations in the ARID1A gene *(Wiegand et al.2010)* and might be associated with endometriosis. Clear cell cancers respond poorly to chemotherapy and clinical trials are being developed for this type of ovarian cancer to take advantage of its kinase-inducing pseudo hypoxic drive *(Anglesio et al., 2011)*, and the PIK3CA gene mutations found in a third of cases.

Mucinous ovarian cancers are most commonly diagnosed at an early stage. The incidence of true advanced ovarian mucinous tumors has reduced through the application of immunohistochemistry for the cytokeratins CK7 and CK20, which help histopathologists to

distinguish ovarian from the more common gastrointestinal source of these cancers. This rare type of cancer has nearly 100% KRAS mutation and a high frequency of HER2 amplification. Overall, the availability of high quality immunohistochemistry with specialist histopathology coupled with tissue or germline mutational analysis defines distinct types of ovarian cancer that now affect clinical management.

1.4A Chemoresistance in Ovarian Cancer: Molecular Mechanisms

In addition to the difficult screening, the lack of early presenting symptoms and the late diagnosis, over 70% of high recurrence rate, especially for platinum-resistant tumor, keeps the 5-year survival rate for patients with advanced epithelial ovarian cancer below 30%, despite enormous efforts to develop anti-cancer drugs *(Suh et al 2013)*. In early stage disease, chemotherapy adjuvants about 20% of epithelial ovarian cancer *(Trimbos et al., 2003)*. Regimens containing platinum have been the standard of care for almost 40 years worldwide, although recent findings have started to affect the options for first-line treatment. In one study *(McGuire et al., 1996)*, survival was improved when paclitaxel was added to cisplatin, as a result, six-three-weekly cycles of the less toxic carboplatin is now given in combination with a paclitaxel or docetaxel *(Ozols et al., 2003; Vasey et al., 2004)*. Most patients with recurrent disease receive second-line chemotherapy, but a subset can be considered for second surgery. The choice of chemotherapy regimen for recurrent ovarian cancer is dictated to a large extent by the interval from the last cycle of platinum-containing treatment to the point of disease progression *(Markman et al., 1991; Eisenhauer et al., 1997)*. Platinum-resistant disease has been defined by retrospective studies as progression within 6 months of the last platinum-containing regimen, with a less than 15% potential for response to re-treatment with platinum. Patients with a platinum-free interval of 6 to 12 months have partly platinum-sensitive disease, with increasing sensitivity for those whose recurrence occurs after 12 months *(Eisenhauer et al., 1997; Markman et al., 1991)*. Findings of randomized trials have shown that platinum-sensitive recurrent disease is best treated with a combination of platinum-containing drugs such as carboplatin with paclitaxel, gemcitabine *(Parmar et al., 2003; Pfisterer et al., 2006)*, or pegylated liposomal doxorubicin *(Pujade-Lauraine et al., 2010)*. Chemosensitivity assays or genetic screening arrays that establish drug sensitivity have been studied, but remain unproven *(Cree et al., 2007)*.

Several chemotherapeutic drugs, including cisplatin, the most commonly used in ovarian cancer, have an effect on apoptotic pathways. p53 is an important regulator of the apoptotic cell death. Loss or mutation of p53 function is the one of the most frequent genetic abnormalities in ovarian cancer, and is observed in 60-80% of both sporadic and familial cases *(Bast et al., 2009)*. p53 mutation has been reported be associated with chemoresistance *(Kigawa et al., 2001; Suh et al., 2011)*. Furthermore, TP53 mutation and consequent overexpression are observed more frequently in advanced stage than in early-stage and are associated with the poor survival *(Havrilesky et al. 2003; Lavarino et al., 2000)*. It is possibly not only due to more aggressive phenotype but also due to the resistance to chemotherapy-induced apoptosis *(Reles et al., 2001; van der Zee et al., 1995)*.

1.1B Breast Cancer

Breast cancer is the most commonly diagnosed cancer in women. The latest world cancer statistics calculated by the International Agency for Research on Cancer (IARC) revealed that 1,677,000 women were diagnosed with breast cancer in 2012 and 577,000 died *(Miller et al., 2014)*. Improvements in chemotherapy, surgery, lymph node evaluation and hormone receptor blocking therapy have successfully doubled the survival of breast cancer patients. The evolution of genomic research enabled the genetic and molecular profiling of cancers, which also revealed the profound complexity and heterogeneity of breast cancer *(Lehmann et al., 2011, van de Vijver et al., 2002)*. Different molecular subtypes of breast cancer have various prognoses and responses to therapy *(Van't Veer et al., 2002)*. Such complexity makes it challenging for clinicians to keep abreast of new knowledge and novel.

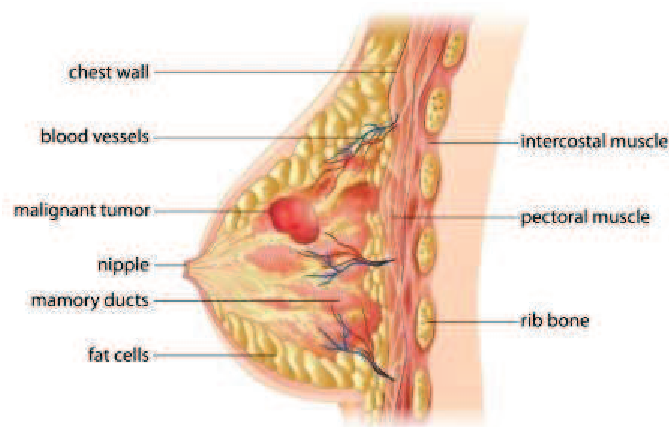

Fig.4 *Breast Cancer*

1.2B Diagnosis of Breast Cancer

Emerging genomic and proteomic technologies have changed the approach to the diagnosis of breast cancer. Molecular imaging is currently one of the most powerful non-invasive techniques used in clinical diagnosis. Mammography and ultrasound are the most common methods used for diagnosis and guided intervention in breast disease. Multiple diagnostic techniques, including tomosynthesis, mammography and ultrasound contrast elastography, 3D ultrasound, diffusion and perfusion and breast spectroscopy, have also been developed.

There are three breast cancer biomarker used in routine clinical practice worldwide: ER, PR and HER2 *(Rakha et al., 2010)*.

The ER was identified by *Ellwood Jensen* in the late 1950s *(Jensen, 2012)*, and ER status has been used since the mid-1970s, both to predict responsiveness of the tumor to endocrine therapy and as a prognostic factor for early recurrence and long-term outcome. Around 80 % of breast cancers are ER-positive *(Anderson et al., 2002)*. The actions of oestrogen and the ER are complex with binding followed by dimerization and translocation to oestrogen responsive elements in the promoter region of genes and subsequent modulation of transcription. Anti-oestrogen therapy used in the management of breast cancer modulates growth through antagonism of the ER (tamoxifen) or through suppression of oestrogen production (aromatase inhibitors).

The action of the ER is also influenced by growth factor receptors such as HER2 (via src or MAPK) as well as by cyclin/CDK complexes which modulate phosphorylation and hence activity. The progesterone receptor gene is regulated by oestrogen, and thus, its expression is taken to indicate an intact and functioning ER pathway *(Horwitz et al., 1978; Lanari et al., 2009)*. Approximately 40% of ER-positive tumors are PR-negative. The regulation of the PR is also complex with modulation by growth factor receptors. Tumors that are PR-negative may reflect aberrant growth factor signalling. It is known that ER-positive/PR-negative tumors are generally less responsive to tamoxifen than those that are positive for both receptors *(Rakha et al., 2010; Arpino et al., 2005; Ferno et al., 2000)*.

The demonstration of HER2 gene amplification/protein over-expression is used in clinical practice as an indicator of poor prognosis and as a predictor of response to systemic treatment with the humanized anti-HER2-specific monoclonal antibody (trastuzumab; Herceptin).

HER2 positivity is seen in 13-20 % of invasive breast cancers, and more than half of these tumors are hormone receptor-negative *(Danadachi et al., 2002; Slamon et al., 1987)*.

Approximately 10-15 % of breast cancers are negative for ER, PR and HER2, so-called triple-negative breast cancers. They represent a distinct group of tumors with characteristic morphology, presentation, behavior and outcome.

1.3B Histopathological and Molecular Classification of Breast Cancer

The classic histological classification divided breast cancer in two principal type: in situ (ductal and lobular) and invasive disease. There are more than 21 subtypes of invasive breast carcinoma defined in the fourth edition of the WHO Classification of Tumors of the Breast *(Lakhani et al., 2012)*. The most frequent is Invasive Carcinoma of No Special Type (NST), also known as invasive ductal carcinoma NST, and this comprises 40-75 % of cases. The remaining tumor types are morphologically distinct in several subtypes including invasive lobular, tubular, mucinous and metaplastic carcinoma and carcinoma with medullary, neuroendocrine or apocrine features. Some subtypes of breast cancer are associated with specific genetic signatures and clinical behavior in addition to the characteristic morphology, thus correct histological categorization provides important prognostic and predictive information *(Weigelt et al., 2008)*.

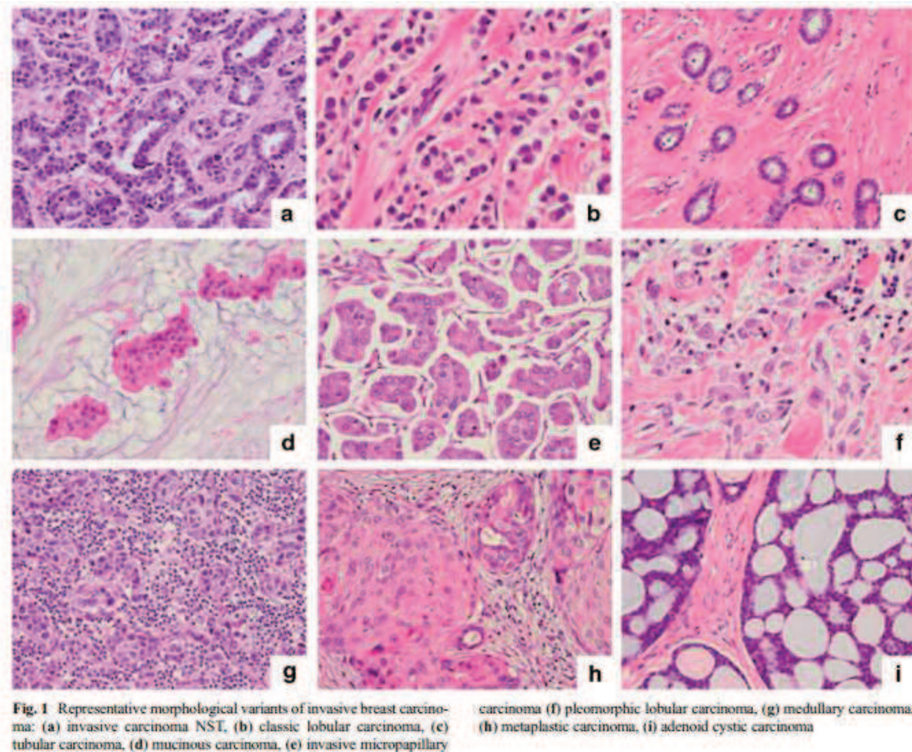

Fig.5 *Histological Classification of Breast Cancer (Vuog et al.,2014)*

Breast carcinomas are histologically graded using the Bloom and Richardson systems *(Bloom et al., 1957)*, modified by Elston and Ellis *(Elston and Ellis, 1991)*. Scores are assigned for the proportion of tubule formation (score of 1-3 with 3 being poor tubule formation), the degree of nuclear pleomorphism (1–3 with 3 showing a high degree of pleomorphism) and the mitotic count (1-3, 3 being a high mitotic count), the exact number dependent on the size of the microscopic field). The scores are combined to give a grade of 1 (total score 3 to 5), 2 (scores 6 or 7) or 3 (score 8 or 9), where grade 1 tumors are the most differentiated and grade 3 are the least. Breast cancer is staged using the TNM system published by the American Joint Committee on Cancer/Union for International Cancer Control *(Lakhani et al., 2012)*. It uses both clinical and pathology information including tumor size (T), the status of regional lymph nodes (N) and spread to distant metastatic sites (M) *(Edge et al., 2010)*.

Several efforts have been made to classify breast carcinomas based on molecular features. ER and PR status has been used since the 1970s to classify breast tumors *(Osborne et al., 1980)*. Immunohistochemical staining for these two receptors is the foundation for clinicopathological stratification of breast cancer and guides the use of endocrine therapy *(Hammond et al., 2010)*. Measurement of human epidermal growth factor receptor (HER) 2 status has for more than a decade been performed routinely in breast cancer clinical samples *(Ross et al., 2010)*. Amplification and/or overexpression of the HER2 gene (also known as

ERBB2) in breast cancer is associated with adverse prognosis *(Chia et al., 2008; Slamon et al., 1987)*, which encouraged development of the monoclonal antibody trastuzumab for adjuvant treatment of HER2-positive breast cancer. With the introduction of gene expression microarrays, additional studies on molecular classification of cancer were carried out. A set of pivotal studies on gene expression patterns of human breast tumors led to the identification of five molecular subtypes associated with differences in patient survival *(Perou et al., 2000; Sorlie et al., 2001)*. This system of molecular subtyping was based on the transcriptional profiles of the protein-coding genome of each tumor. Most of these genes do not much with in their expression patterns and are assumed not to contribute to the biological phenotype of the disease. Five main clusters emerged, termed luminal A, luminal B, basal-like, ERBB2+ and normal breast-like subtypes *(Perou et al., 2000)*. In invariable survival analysis, it was shown that the subtypes were associated with significant differences in overall survival *(Sorlie et al., 2001)*, and they were identified in independent cohorts of patients with breast cancer from whom gene expression data were available *(Sorlie et al., 2003)*. These subtypes were constructed based on sets of genes with similar behavior in gene expression variation across a set of tumors.

Tumors of the luminal A subtype typically highly express the ER, along with the oestrogen-regulated gene SLC39A6 (solute carrier family 39 (zinc transporter), member 6), transcription factors GATA3, FOXA1 and XBP1, and luminal cytokeratins KRT8 and KRT18 *(Perou et al.,2000; Sorlie, 2004)*. Luminal A tumors show a relatively low mutation rate but a diverse spectrum, the most frequently mutated genes being PIK3CA (phosphatidyl inositol-4,5-bisphosphate 3-kinase, catalytic subunit α), GATA3 and MAP3K1 (mitogen-activated protein kinase (MAPK) kinase kinase 1) *(Cancer Genome Atlas Network, 2012)*. Cyclin D1 is frequently overexpressed in luminal A tumors *(Agarwal et al., 2009; Natrajan et al., 2010)*. Luminal tumors show a gradient of expression of the ER and the luminal B subtype is on the lower end of this scale, exhibiting low expression of ER and other oestrogen-regulated genes *(Perou et al., 2000; Sorlie, 2004)*. Luminal B tumors are more proliferative, of higher grade, more often show aneuploidy and frequently display mutations in the TP53 and PIK3CA genes, and alterations affecting the retinoblastoma and MAPK pathways*(Cancer Genome Atlas Network, 2012; Russnes et al., 2010; Creighton, 2012; Sorlie, 2007)*. A proportion of luminal B tumors show overexpression of the ERBB2/HER2 oncogene *(Perou et al., 2000; Wirapati et al., 2008)*. The ERBB2+ or HER2-enriched subtype is characterized by high expression of ERBB2 and additional genes located in the same chromosome region, including GRB7, MED24 and MED1 (peroxisome proliferator activated receptor binding protein,

PPARBP) *(Perou et al., 2000)*. These tumors are typically negative for expression of luminal epithelial genes and show high expression of genes associated with cell cycle progression *(Sorlie, 2004)*.

HER2-enriched tumors have relatively few whole arm aberrations, but high levels of more complex rearrangements on 17q16. Of note, tumors of this subtype don't necessarily show amplification of the ERBB2 oncogene itself, but have similar overall expression profiles to those of ERRB2-amplified cases *(Perou et al., 2000; Sorlie et al., 2001; Prat and Perou, 2011)*, suggesting a similar effect on downstream pathways.

Basal-like tumors are characterized by high expression of the basal cytokeratins 5 (KRT5) and 17 (KRT17), and other genes typically expressed in basal/myoepithelial cells such as LAMC1 (laminin γ1), ANXA8 (annexin A8) and CDH3 (cadherin 3, type 1) *(Perou et al., 2000; Sorlie et al., 2001)*. These tumors do not express ER, or other luminal epithelial genes, are negative for ERBB2, and often overexpress EGFR (epidermal growth factor receptor). Basal-like breast cancers frequently display complex genomic rearrangements and TP53 mutations, and are most often aneuploid tumors of high grade *(Russnes et al., 2010; Sorlie, 2007; Curtis et al., 2012; Kwei et al., 2010)*. BRCA1 mutation carriers develop basal-like tumors *(Sorlie et al., 2003; Foulkes et al., 2003)*, and it may be speculated that impaired double-stranded DNA repair mechanisms are central in the development of basal-like tumors *(De Summa et al., 2013)*.

Finally, the normal breast tissue-like subgroup was identified and named based on similarities in gene expression patterns to those of normal epithelial cells, adipose tissue and other non-epithelial cell types *(Sorlie et al., 2001)*. Although tumors of this subtype are clearly carcinomas that have gene expression features common to both basal-like and luminal subtypes, they show no expression of proliferation-associated genes *(Sorlie, 2004)* and it is speculated that these tumors may have low tumor cell percentage.

Intrinsic subtype	Characteristics	Immunohistochemical definition	Recommended treatment
Luminal A	ER+, highly express luminal epithelial genes, *PIK3CA* mutations, diploid, low grade, cyclin D1 overexpression, whole chromosome arm aberrations	ER+ and/or PR+ HER2− Ki-67 low	Hormone therapy
Luminal B	ER+ (low), proliferative, high grade, whole chromosome arm aberrations and complex rearrangements, *TP53* and *PIK3CA* mutations, alterations in retinoblastoma and MAPK pathways, some are HER2+	ER+ and/or PR+ HER2− (or HER2+) Ki-67 high	Hormone therapy, chemotherapy, anti-HER2 if HER2+
HER2-enriched	ER−, most tumours show *HER2* amplification, overexpression of genes on 17q22, highly proliferative, *TP53* mutations, focal high-level amplifications	HER2+ (amplified or overexpressed) ER−	Anti-HER2, chemotherapy
Basal-like	ER−, HER2−, highly express basal keratins, express EGFR, highly proliferative, aneuploid, high grade, *TP53* mutations, complex genomic rearrangements, WNT pathway activation increased	ER−, PR−, HER2− (triple negative)	Chemotherapy
Normal-like	Express basal and myoepithelial genes, adipose tissue-specific genes	Not relevant	Not relevant

ER, oestrogen receptor; PIK3CA, phosphatidyl inositol-4,5-bisphosphate 3-kinase catalytic subunit α; PR, progesterone receptor; HER, human epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor.

Fig.6 *Molecular subtypes of breast cancer (Norum et al., 2014)*

1.4B Chemoresistance in Breast Cancer: Molecular Mechanisms

The identification of HER2 in breast cancer pathogenesis has led to the development of therapies targeting this receptor. Trastuzumab is a monoclonal antibody that has demonstrated improved survival in the first-line setting in combination with chemotherapy in patients with HER2+ advanced disease *(Slamon et al., 2001; Marty et al., 2005)* and improved disease-free survival and overall survival in patients with HER2+ early breast cancer when used in combination with or sequentially after adjuvant chemotherapy *(Piccart-Gebhart et al., 2005)*. Cross-talks between HER-2 and Hormone Receptor (HR) pathways may intervene in trastuzumab and lapatinib resistance *(Thery et al., 2014)*. An increase in HR signaling was observed in patients with HER-2-positive/HR-positive tumor treated with lapatinib monotherapy *(Munzone et al., 2006; Liu et al., 2009)*. These data suggest that HR-positive status might be also a marker of lower sensitivity to anti-HER-2 therapies. Recently, ER pathways have been postulated as means of escape to HER-2 directed therapy. *Wang et al.* showed that, following lapatinib and trastuzumab treatment, ER and its downstream effectors increased in all but one ER positive/HER-2 positive cell lines, and the acquisition of HER-2 directed agents resistance is mediated by activation of ER pathway, via Bcl2 family members *(Wang et al., 2011)*. Another recent study investigated the cross-talks between HER-2 and ER pathways and the effect of HER-2 blocking agents on the tyrosine kinase effector transcription factor Myc, showing that elevated Myc protein was inversely associated with pCR. In HER-2 positive cells trastuzumab can repress Myc transcriptional activity, inhibiting

its target gene survivin, and this correlates with favorable response. Conversely, the co-expression of ER leads to upregulation of survivin expression and increased ER transcriptional activity, with subsequent lower response *(Collins et al., 2014)*.

General mechanisms of resistance to HER2-targeted therapies occur at three levels *(Rexer and Arteaga, 2012)*. The first includes mechanisms intrinsic to the target, such as molecular changes in the target receptor *(Mayer et al., 2013)*; the expression of p95HER2, which is a truncated HER2 receptor *(Scaltriti et al., 2007; Scaltriti et al. 2010)*; and HER2 gene amplification *(Mittendorf et al., 2009)*. Resistance involving parallel signaling pathways bypassing HER2 inhibition, such as increased activation of HER3 *(Juntilla et al., 2009; Sergina et al., 2007)*, aberrant activation of pathways downstream of the receptor *(Hurvitz et al., 2013)*, and compensatory crosstalk with other pathways, might also occur *(Mayer et al., 2013; Keck et al., 2012; Bender and Nahta, 2008)*. Resistance from defects in the apoptosis pathway in tumor cells or in extrinsic host factors participating in the action of the drugs is another potential mechanism of resistance to HER2-targeted therapy *(Rexer and Arteaga, 2012)*. Lapatinib and trastuzumab emtansine (T-DM1) are licensed treatments for use in the setting of trastuzumab resistance *(TYKERB, 2013; KADCYLA, 2013)*. Lapatinib is a dual HER2 and epidermal growth factor receptor (EGFR)/HER1-specific tyrosine kinase inhibitor that binds to the intracellular domain of HER2, allowing it to inhibit both full-length HER2 and truncated p95HER2 *(Scaltriti et al., 2007; Wood et al., 2004)*. Lapatinib monotherapy and lapatinib in combination with capecitabine were shown to provide the same clinical benefit, including progression-free survival (PFS), clinical benefit rate, and overall response rate (ORR), regardless of p95HER2 expression in breast tumors from the first- and second-line lapatinib clinical development program *(Scaltriti et al. 2010)*. Lapatinib in combination with capecitabine is approved for patients with HER2+ metastatic breast cancer that has progressed with trastuzumab, based on a phase-3, randomized study of 324 patients with HER2+, advanced or metastatic breast cancer who received previous treatment with an anthracycline, a taxane and trastuzumab (ClinicalTrials.gov identifier NCT00078572) *(TYKERB, 2013; Geyer et al. 2006)*. Patients were randomly assigned to receive either lapatinib plus capecitabine or capecitabine alone *(Geyeret al. 2006)*. Several investigational treatments to manage resistance to HER2-targeted therapies are under development.

CHAPTER 2

TUMOR RECURRENCE: DORMANCY AND CANCER STEM CELL HYPOTHESES

Tumor dormancy is a common biological property of malignancies and a leading factor in treatment failure, metastasis and tumor recurrence. Metastases arise from residual disseminated tumor cells (DTCs). This can happen years after primary tumor treatment because residual tumor cells can enter dormancy and evade therapies. Several studies support the notion that disseminated tumor cells undergo proliferative arrest upon infiltrating a target organ because they find themselves deprived of appropriate adhesive and signaling interactions *(Liu et al., 2002; Shibue et al., 2012; Shibue and Weinberg, 2009)*. This suggests that dormancy is induced by maladaptation and must be resolved by genetic or epigenetic alterations that increase the fitness of dormant cells within a specific tissue microenvironment. In agreement with this notion, enhanced survival signaling appears to be a prerequisite for dormancy. Tumor dormancy was originally defined by *Willis* in the late 1940s and then redefined by *Hadfield* in the early 1950s as a temporary mitotic arrest *(Hadfield, 1954)* and a growth arrest. Dormancy was later divided into three categories *(Klein, 2010)*:

- cellular dormancy, where intrinsic and/or extrinsic mechanisms drive solitary or small groups of DTCs to enter quiescence;
- angiogenic dormancy, where the tumor mass is kept constant by a balance between dividing cells and cells that die due to poor vascularization;
- immune-mediated dormancy, where the immune system keeps a proliferating tumor mass constant via a persistent cytotoxic activity.

These categories are not static, as processes that affect single cells may share underlying mechanisms with processes that affect the tumor mass. Clinical evidence supports the idea that DTCs are non-proliferative, as determined by the lack of proliferation markers when DTCs are profiled at the single-cell level *(Pantel et al., 2009; Klein, 2009)*, arguing that they enter cellular dormancy. Current therapies target proliferating tumor cells with varying success. For example the DTCs could be kept dormant or could be eradicated while dormant, this would constitute a novel strategy to prevent metastasis *(Sosa et al. 2011)*. Reduced PI3K-

AKT signaling has been linked to dormancy-like phenotypes *(Jo et al., 2008; Schewe and Aguirre-Ghiso, 2008)*. In the presence of nutritional stress, cancer cells secrete factors that inhibit the PI3K pathway, resulting in quiescence and autophagy induction *(Jo et al., 2008)*. In tumor cell spheroids, loss of adhesion and nutrient deprivation promoted short-term growth arrest. This arrest was linked to epidermal growth factor receptor (EGFR)-Y1086 autophosphorylation, which inhibited AKT activation and cyclin D1 induction *(Humtsoe and Kramer 2010)*. Tumor cells detached from the extracellular matrix (ECM) can survive by inhibiting AKT signaling and by inducing autophagy and an antioxidant response *(Avivar-Valderas et al., 2013)*. Autophagy might allow dormant DTCs to maintain metabolic fitness while inhibiting PI3K-AKT signaling, although reducing this signal may not always inhibit mTOR activation *(Schewe and Aguirre-Ghiso, 2008).* The ability of normal adult stem cells to balance self-renewal with the production of differentiated progeny is governed by complex adhesive and signaling interactions that occur within specialized niches *(Alvarez-Buylla and Lim, 2004; Hsu and Fuchs, 2012; Morrison and Spradling, 2008)*. Recent studies suggest that metastasis-initiating cells enter into dormancy and undergo reactivation in response to niche signals, which are similar to those that affect normal adult stem cells. Some studies have suggested that carcinoma cells can establish a permissive niche in the target organ even prior to seeding. In this model, primary tumors release systemic factors that upregulate the production of fibronectin by fibroblasts residing in the target organ, leading to the recruitment of VEGFR1+ hematopoietic progenitor cells expressing the a4b1 fibronectin-binding integrin. The hematopoietic cells in turn mold the local microenvironment within the premetastatic niche by secreting MMP-9 and other factors and promoting angiogenesis *(Psaila and Lyden, 2009)*. The relevance of these observations in dormancy and reactivation has not been examined, but one envisions that failure to establish a premetastatic niche may delay adaptation, thereby favoring dormancy. In agreement with this hypothesis, whereas adhesion to the abluminal surface of mature blood vessels induces metastatic breast cancer cells to become dormant, angiogenic sprouts create a local microenvironment that facilitates reactivation *(Ghajar et al., 2013)*.

Like normal tissues, tumors require a functional vasculature. When a DTC grows into a micrometastasis it recruits a new vasculature. If angiogenesis fails, cell death proceeds and equilibrium between proliferation and apoptosis can keep a small tumor mass constant and clinically dormant *(Holmgren et al., 1995)*. This could persist until genetic, epigenetic or microenvironmental signals trigger the angiogenic switch. However, there is no evidence to support the persistence of angiogenic dormancy for years or decades in patients. A study of

angiogenic-poor dormant tumor masses *(Almog et al., 2009) in vivo* models of breast cancer, gliobastoma, osteosarcoma and liposarcoma that remain dormant in immunodeficient mice for >90 days *(Almog et al., 2006)* showed that thrombospondin (TSP) and angiomotin were upregulated in these tumors, although their functional relevance was unclear.

In spite of significant improvements in early diagnosis, many cancer patients who have been treated with surgery eventually develop distant metastases and succumb to the disease. The efficacy of adjuvant therapy is predicated upon its ability to eradicate tumor cells that have undergone dissemination prior to surgery. The realization that the tumor cells that are fated to eventually outgrow into metastases are at least functionally equivalent to cancer stem cells and experience a prolonged period of dormancy suggest two distinct approaches to the prevention of metastasis.

First, because dormant tumor cells are critically dependent on signaling pathways that enhance their survival, interfering with the operation of these pathways may improve the efficacy of adjuvant therapy. Based on existing evidence, it would be informative to conduct preclinical studies in mouse models of dormancy with inhibitors of Src, Akt, or TOR alone or in combination with chemotherapy or oncogene-targeted therapy. Second, if the metastasis-initiating cells are or resemble cancer stem cells, they may be similarly refractory to chemotherapy as well as to targeted therapies. Finally, increased understanding of the mechanisms underlying dormancy and reactivation may lead not only to the identification of additional therapeutic targets, but also to changes in the schedule of administration of adjuvant therapy.

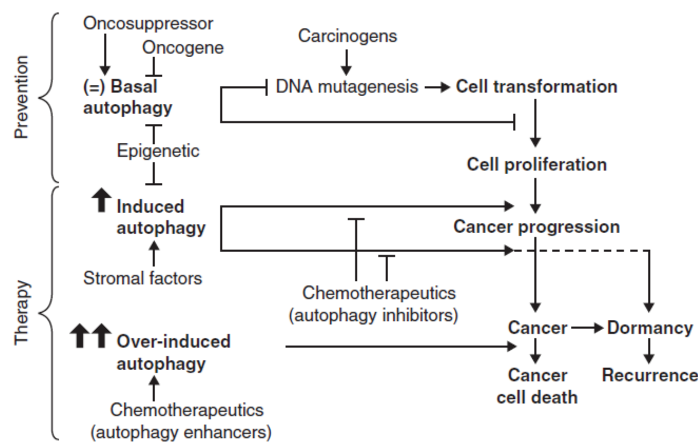

Fig.7 *Role of genetics and epigenetics in autophagy regulation in its effects in cancer progression (Morani et al, 2013)*

CHAPTER 3

AUTOPHAGY

3.1 Autophagy

The term autophagy (in Greek "self-eating") has been coined at the CIBA Foundation Symposium on Lysosomes by *Christian de Duve* in 1963 to describe a process in which cytoplasmic components are degraded by the lysosome *(De Duve et al. 1966).*

It involves the readjustment of the subcellular membranes to sequester macromolecules, such as proteins, glycogens, lipids, and nucleotides and organelles such as mitochondria, peroxisomes, and the endoplasmic reticulum for delivery to the lysosome where the sequestered material is degraded and recycled *(Mizushima 2011, Levine and Klionsky, 2004).*

Three different pathways of autophagy have been identified:

- macroautophagy, characterized by the formation of the autophagosome, a unique double-membrane organelle;
- microautophagy where lysosomes engulf cytoplasmic materials by internal invagination of the lysosomal membrane
- chaperone-mediated autophagy. Mediated by the chaperone hsc70, co-chaperones, and the lysosomal-associated membrane protein type 2A *(Mizushima and Komatsu, 2011; Kaushik and Cuervo, 2012).*

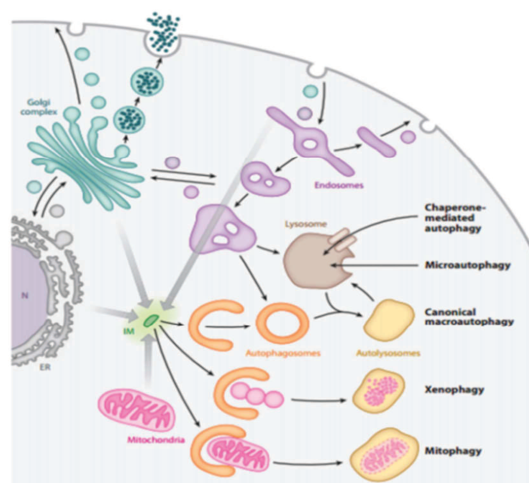

Fig.8 *Different models of autophagy (Mizushima et al. 2011)*

The most analyzed type of autophagy is the macroautophagy. The process of autophagy has been divided into five stages, including induction, vesicle nucleation, vesicle elongation and completion, docking and fusion, then followed by degradation and recycling (*Wen et al., 2013*).

It starts with the phagophore, the initial sequestering compartment *(Seglen et al., 1990)*, which results in the formation of a double-membrane structure, called autophagosome *(Dunn, 1990)*.

After the maturation the autophagosome fuses with the endosomes and then finally fuses with lysosomes, and the materials inside the autophagosome are degraded by lysosomal enzymes, resulting in further degradation and recycling.

Recently different types of macroautophagy have been discovered, including mitophagy (clearing of the dysfunctional mitochondria), pexophagy (peroxisome degradation), and xenophagy (intracellular pathogens elimination) (*Klionsky et al,. 2007*).

Genetic studies in yeast have identified more than 30 autophagy-related (ATG) genes, which are required for various types of autophagy (*Suzuki and Ohsumi, 2007; Xie and Klionsky, 2007*). Many of which have known orthologs in higher eukaryotes ATG genes are conserved in higher eukaryotes (*Longatti and Tooze, 2009*).

Autophagy plays an important role in the response of the cell to starvation conditions. In nutrient rich conditions, autophagy is suppressed. Under conditions of stress, autophagy is upregulated maintaining cellular integrity by regenerating metabolic precursors and clearing subcellular debris *(Levine and Kroemer, 2008; Ravikumar et al., 2010; Tsukada and Ohsumi, 1993; Kuma et al., 2004)*.

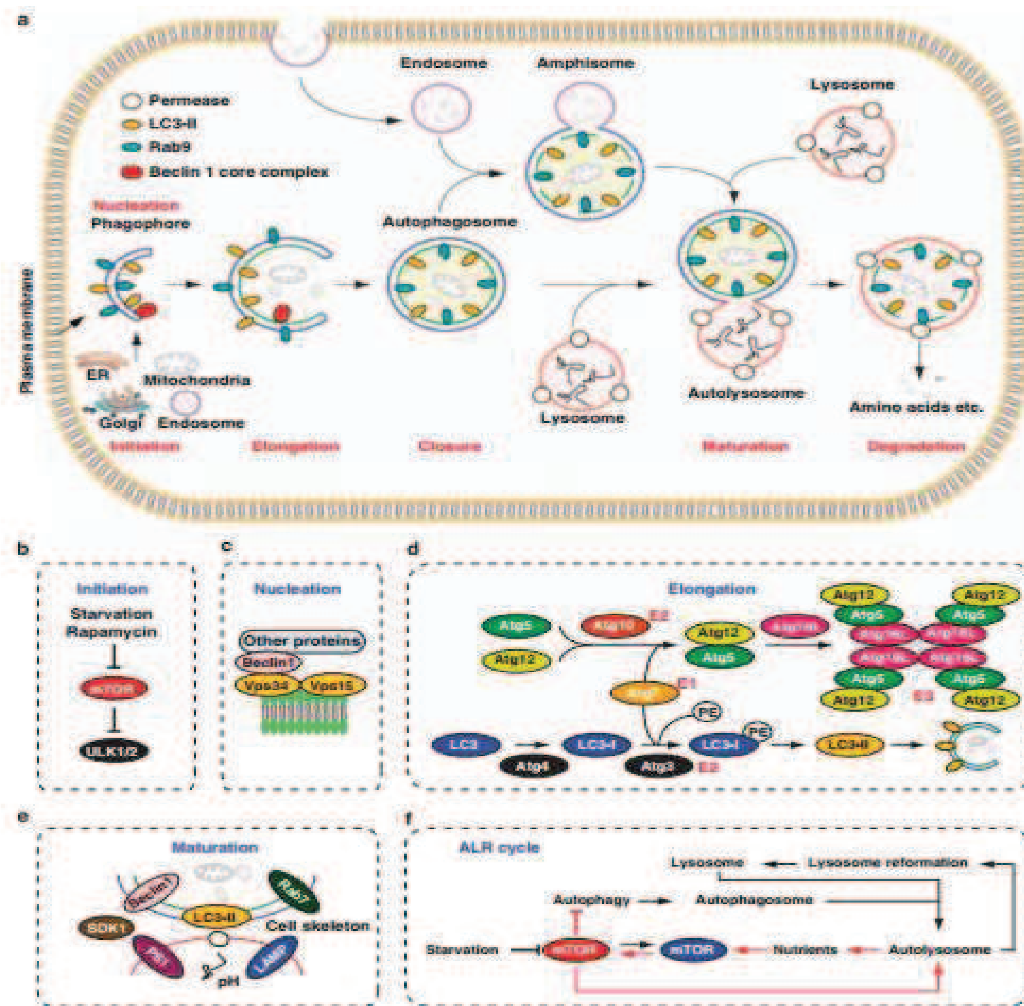

Fig. 9 *Autophagy Stages (Kang et al. 2011)*

Autophagy initiation is coordinated by two kinases, ULK1/ATG1 a Ser/Thr protein kinase and VPS34/PIK3C3 a class III phosphoinositol 3-kinase. Induction of autophagy is initiated by the proteins of the ULK complex, composed of the mammalian Atg1 homologues ULK1 or ULK2, Atg13, focal adhesion kinase family interacting protein of 200 kDa (FIP200) and Atg101 (*Jung et al., 2009*). As part of larger protein complex, ULK1 is activated following nutrient depletion upstream of VPS34 *(Itakura and Mizushima, 2010; Russell, 2013).* Activation of both ULK1 and VPS34 drives the recruitment of additional ATG proteins to phagophore membranes and promotes autophagosomal maturation. As well as regulating autophagy, ULK1 and VPS34 are known to influence signal transduction through mTORC1.

Nutrient deprivation stimulates ULK1 in complex with ATG13-FIP200 and initiates autophagy via ULK1 autophosphorylation and ATG13 and FIP200 phosphorylation *(Jung et al., 2009; Ganley et al., 2009).* ULK1 is inhibited by mTORC1. In fact treatment with

rapamycin, the mTOR inhibitor, upregulates the kinase activity of ULK1. Rheb actives mTORC1 and represses ULK1 *(Jung et al., 2009).*

By the activation of the class III PI3K/BECLIN-1 complex occurs the vesicle nucleation, through the recruitment of proteins and lipids to construct the autophagosomal membrane (*Yang and Klionsky, 2010*). Ubiquitin-like protein conjugation is required for vesicle elongation and autophagosome completion, mediated by proteins Atg3, Atg5, Atg7, LC3, Atg10, Atg12 and Atg16L, to fully encapsulate the cytosolic cargo (*Ravikumar et al., 2010*) .

Autophagy receptors such as p62 have emerged promoting degradation of misfolded proteins by autophagy, through the regulation of ubiquitin–proteasome system and autophagy. p62 has a duple binding sites for ubiquitin chains and LC3 (*Kirkin et al., 2009*). In mammalian cells, p62 plays a crucial role of adaptor between LC3-decorated autophagosomes and ubiquitin-conjugate protein aggregates (*Itakura and Mizushima, 2011; Ichimura et al., 2008*).

Finally, docking and fusion are required for the disassembly of Atg protein complexes from mature autophagosomes, regulated by Atg2, Atg9 and Atg18 (*Liu et al., 2011*).

3.2 Autophagy Regulation by Nutrient Signaling

The maintenance of metabolic homeostasis of the cells in responses to nutrient withdrawal is dependent by the up-regulation of autophagy. mTORC1 and AMPK are the two kinases essential for the nutrient sensing of the autophagy pathway *(Russell et al., 2014).*
Autophagy is responsive to amino acids changes. In 1977 *Schoworer et al*., showed that perfusion of rat livers in the absence of amino acids, in particular leucine, were responsible for the repression or protein turnover and autophagy *(Maortimore and Schoworer, 1977; Li et al., 1978; Seglen et al. 1980).* mTOR forms two functionally distinct complexes in mammals called mTORC1 and mTORC2. One of the most important effectors of amino acid-mediated autophagy repression is mTORC1. It is able of integrating signals from many stimuli including amino acids, energy levels and oxygen *(Laplante and Sabatini, 2012)*.
The mTORC1 activation by amino acids is due to the activation of the Rag GTPase complex that binds mTORC1 to the lysosome *(Kim et al., 2008; Sancak et al., 2008)*. The recruitment of mTORC1 to the lysosome brings it into proximity with another small GTPase Rheb that is absolutely required for mTOR activation *(Garami et al., 2003; Tee et al., 2003)*. Rheb is

negatively regulated by the tuberous sclerosis complex (TSC1/2). In the presence of growth factor, the TSC complex is inactivated by the PI3K pathway through multiple mechanisms including direct repression of TSC by AKT mediated phosphorylation *(Inok et al., 2002; Potter et al., 2002)*. Therefore, full activation of mTORC1 can only be in the presence of both amino acids and growth factors.

To maintain metabolic homeostasis, the cell must generate and consume ATP, the level of ATP:ADP:AMP is an important indicator of cellular energy levels. These nucleotides are directly sensed by AMPK. AMPK is a trimeric serine/threonine kinase, that contains three subunity α, β and γ. AMP and ADP activate AMPK by preventing the subunity α dephosphorylation *(Oakhill et al., 2011; Xiao et al., 2011)*. Reduction in cellular ATP levels, caused by glucose withdrawal or other stressors such as mitochondrial dysfunction initiates a cellular metabolic response through AMPK targets that seek to generate energy by increasing glucose uptake and glycolysis and stimulating lipid catabolism *(Hardie, 2007)*.

Oxygen is an essential nutrient that is required for key metabolic processes within the cell. Oxygen along with the electron transport chain in the mitochondria is necessary for generating ATP through oxidative phosphorylation *(Kaelin and Ratcliffe, 2008)*. Hypoxia results in a reduction in ATP levels, at least transiently, which activates AMPK and inactivates mTOR *(Liu et al., 2006, Marsin et al. 2002)*. One of the best-characterized events of the hypoxic response is stabilization of the HIF1α transcription factor *(Kaelin and Ratcliffe, 2008; Maxwell, 2005)* and accumulates in the nucleus where it activates the transcription of a wide array of genes that are necessary for metabolic adaptation to reduced oxygen levels *(Maynard and Ohh, 2005).*

3.3 Epigenetic Regulation of Autophagy

In the last years, progress has been made in understanding the molecular and cellular process of autophagy. During stress situations, the post-transcriptional and translational controls play important roles, in response to growth conditions changes *(Zhai et al., 2013)*.

Recently has been associated the contribution and involvement of non-coding miRNAs in this process. miRNAs are highly conserved class of small (18-25 nucleotides), non-coding RNAs, which regulate post-transcriptional gene expression by translational arrest or mRNA cleavage, most likely, through interaction mainly at the 3'-UTRs of the target mRNAs *(Lee et al., 1993; He and Hannon, 2004)*. Because of the autophagy involvement in cancer progression,

emerging data point to the role of miRNAs as regulators of autophagy gene expression. In fact, the immediate and acute modulation of protein expression mediated by miRNAs plays a fundamental role in the adaptive response of the cell metabolism to environmental stresses such as nutrient shortage, hypoxia, and genotoxic stress. Deregulation of miRNAs has been associated with cancer development and progression, and miRNAs have emerged as a new research frontier for understanding cancer development at the post-transcriptional and translational level *(Ryan et al., 2010)*. Most of the past efforts of studying autophagy focused on key proteins with critical roles in the direct autophagy processing and the signaling pathways involved in transcriptional activation. The contributions of post-transcriptional and translational controls of autophagy mediated by miRNAs emerged just recently *(Zhai et al., 2013)*.

Autophagy could be regulated by miRNAs targeting the mRNA of key molecules that indirectly induce or suppress autophagy, as, for instances, miR-504 that negatively regulates p53 *(Hu et al., 2010)* or miR-20b that negatively regulates the expression of HIF-1α *(Lei et al., 2009)* or any miRNA implicated in the regulation of the PI3k-(PTEN)-AKT-mTOR pathway as is, for instance, the case of miRNAs targeting PTEN *(He, 2010)*. More recently, miRNAs specifically targeting the mRNA of autophagy proteins are being identified *(Zhai et al., 2013)*.

The first to report the involvement of miRNA in autophagy and cancer was *Zhu et al.* by providing experimental evidence that miR‑30a targets BECLIN-1, a critical scaffold protein for autophagosome formation *(Zhu et al. 2009)*. They demonstrated that miR‑30a downregulates BECLIN-1 expression, which mimics blunted activation of autophagy induced by rapamycin. Most recent reports further support the functional significance of miR‑30a-mediated autophagy by enhancing Imatinib activity against human chronic myeloid leukemia cells *(Yu et al., 2012a; Yu et al., 2012b)* miR‑30a also sensitizes tumor cells to cisplatin by suppressing BECLIN-1-mediated autophagy *(Zou et al., 2012)*. These results support a new treatment development strategy of overcoming chemoresistance by modulating miRNA-mediated autophagy. Recently, *Jegga et al*. used a system biology-based approach to define the complex regulatory and functional networks of genes controlling the autophagy-lysosomal pathway and found miR-130, miR-98, miR-124, miR-204, and miR-142 as putative post-transcriptional regulators of this pathway at various levels *(Jegga et al., 2011)*.

Certain miRNAs can target both the autophagy and the apoptosis pathways and therefore can influence the cross-talk between these two processes and determine whether the cancer cell

will resist or succumb to the toxic drug. For instance, miR-199a-5p was shown to increase chemoresistance by simultaneously promoting autophagy and suppressing apoptosis. By down-regulating BECLIN-1 expression, miR-30a and miR-376b down-regulate not only autophagy but also apoptosis since the level of free antiapoptotic BCL-2 protein in the cell will increase. Thus, miRNAs can act as molecular switches to turn on or off either or both of the autophagy and apoptosis processes. These findings provide the rationale for designing novel therapeutic approaches combining the conventional anticancer drugs with miRNAs targeting the autophagy process *(Titone et al., 2014)*.

3.4 Autophagy and Cancer

Modulation of autophagy has a great impact on the carcinogenesis process. Recent research has shown that autophagy can act both as a tumor suppressor and as a tumor promoter. In fact, depending on whether it is considered at the pre-cancerous or at the advanced stage, up or downregulation of autophagy may elicit either tumor promoting or tumor-suppressive effects *(Degenhardt et al., 2006; Rosenfeldt and Ryan, 2009).* Deletions of several autophagy-specific genes are commonly found in human malignancies. Autophagy has a great impact on the progression of tumors and on the response to therapeutic treatments, and therefore influences the prognosis. Consistently, certain autophagy-related proteins have been shown to be of prognostic value. For instance, the hyperexpression of BECLIN 1 and of LC3 in general associates with a better prognosis in patients with glioblastoma *(Pirtoli et al., 2009)*, colorectal cancer *(Li et al. 2009, Koukourakis et al. 2010)*, lymphomas *(Nicotra et al., 2010; Huang et al., 2011),*or duodenal adenocarcinoma *(Wu et al. 2013)*. Conversely, low expression of BECLIN 1 or of LC3 associates with poor prognosis in patients with hepatocarcinoma *(Ding et al., 2008)*, glioblastoma *(Huang et al., 2010)*, colorectal cancer *(Koukourakis et al. 2010)*, lymphoma *(Nicotra et al., 2010)*, or lung carcinoma *(Won et al., 2012)*.

Thus, autophagy may be a tumor-suppressor pathway, and its decreased activity may contribute to the development of human cancer. Consistent with this theory, tumor-suppressor genes that are frequently mutated in human cancer (p53, PTEN) turn autophagy on, and genes that are frequently activated in cancer, such as those encoding class I PI3K and AKT, turn it off. The conflicting pro-survival and pro-death functions of autophagy make the connection to cancer treatment more complex. The pro-survival function may help cancer cells to survive in nutrient-limited environments, and to resist ionizing radiation and chemotherapies. But the

pro-death function may help to kill cancer cells, either spontaneously or when they are exposed to radiation or chemotherapy *(Levine, 2007)*.

How can autophagy prevent cancer?

There are several possible ways:

- Tumor suppressor functions through autophagy death-promoting effects.

- DNA damage prevention, through its cellular housekeeping role in removing sources of oxidative stress such as defective cellular organelles (mitochondria or endoplasmic reticulum).

- Negative regulation of the cell growth. The effects of autophagy on cell growth are potential pro-death and can play a pivotal role in tumor suppression.

How can autophagy promote cancer?

- In all eukaryotic organisms, autophagy-specific genes promote the survival of normal cells during nutrient starvation. Similarly, autophagy might enhance the survival of rapidly growing cancer cells that have outgrown their vascular supply and face oxygen shortage or metabolic stress.
- By promoting the survival of cancer cells by targeting damaged mitochondria and other organelles for lysosomal degradation, thereby buffering oxidative stress that can be triggered by activated cancer-causing genes or by cancer treatments *(Levine, 2007)*.

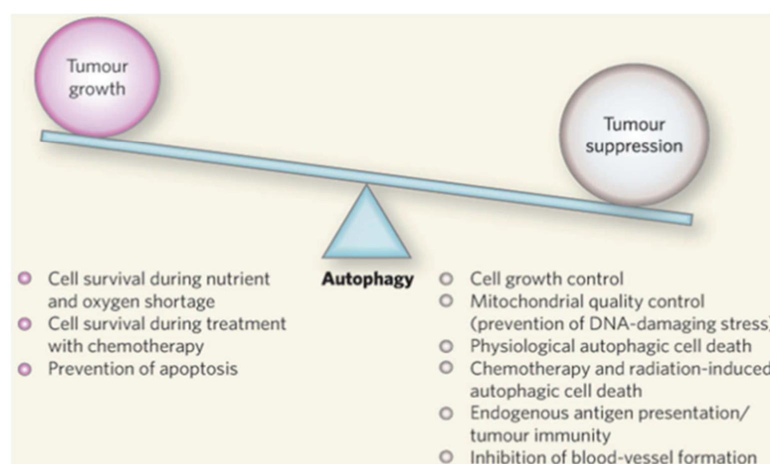

Fig.10 *Conflicting effects of autophagy on cancer (Levine, 2007)*

Several mechanisms have been proposed for the antitumorigenic effects of autophagy. *Eileen White and colleagues* showed that the management of oxidative stress is the key mechanism how defects in autophagy promote cancer development. The group demonstrated that autophagy protects cells from genotoxic stress, thus helping to maintain genomic integrity. In response to metabolic stress, autophagy-defective cells show an increase in DNA double-strand breaks and gene amplification *(Degenhardt et al., 2006; Karantza-Wadsworth et al, 2007)*. Thus, defective autophagy was suggested to cause increased mutations in cells that survive metabolic stress, which subsequently promotes tumorigenesis.

Defective autophagy causes the accumulation of the scaffold protein p62/SQSTM1 that is specifically turned over via autophagy *(Bjorkoy et al., 2005).* p62 is a multidomain protein implicated in the autophagic clearance of aggregate-prone proteins and in the activation of the transcription factor NF-kB. p62 is required for tumor formation, and the clearance of p62 by autophagy suppresses tumorigenesis *(Mathew et al., 2009)*. Elevated levels of p62 were shown to cause the production of reactive oxygen species (ROS), accumulation of endoplasmic reticulum (ER) chaperones (indicating ER stress), and DNA damage. Preventing the accumulation of p62 or ROS diminished the damage in autophagy-defective cells *(Mathew et al., 2009).*

Autophagy may function as a tumor suppressor by preventing tumor-cell necrosis. In apoptosis-incompetent cells, defective autophagy tends to cause necrosis in response to metabolic stress *(Degenhardt et al., 2006)*.

Finally, autophagy is also required for oncogene-induced senescence, an irreversible cell cycle arrest that limits the proliferation of transformed cells *(Marino et al., 2007; Bingle et al., 2002)*. Autophagy was shown to be activated during senescence induced by oncogenes and DNA damage, and depletion of Atg genes favored the ability to escape the senescence. Thus, during oncogenic stress, autophagy may play a role in restricting cell proliferation via senescence.

Since autophagy is a stress response and survival mechanism, it is conceivable that it also contributes to the survival of established tumor cells under various stress conditions. Upregulation of autophagy can give tumor cells an advantage over normal cells, which promotes aggressiveness and resistance to cancer therapy. Especially in poorly vascularized tumors, cells often experience metabolic stress and hypoxia. A recent study demonstrated that pancreatic cancers show increased levels of autophagy *(Yang et al. 2011)*. Moreover,

inhibition of autophagy by silencing Atg proteins or inhibiting autolysosomal degradation suppressed pancreatic tumor growth by causing accumulation of ROS, DNA damage and defective mitochondrial functions.

Autophagy may also promote tumor cell metastasis. It has been observed that autophagy protects epithelial cells from anoikis, which may promote metastasis. *Jayanta Debnath's* group showed that separation from the extracellular matrix induced autophagy in epithelial cells. The results also indicated that RNA interference-mediated depletion of Atg proteins inhibited detachment-induced autophagy, enhanced apoptosis, and reduced clonogenic recovery after anoikis *(Fung et al., 2008).*

Autophagy can promote tumor cell survival, metastasis and resistance to cancer therapy. Numerous studies have shown that tumor cells can survive anti-cancer treatments by activating autophagy. The majority of these studies indicates that downregulation or inhibition of autophagy by silencing Atg proteins or utilizing chemicals to inhibit autolysosomal degradation, respectively, sensitizes tumor cells to cancer therapies *(Morselli et al., 2009)*.

Several classes of anticancer drugs including DNA damaging agents, microtubule-targeted drugs, antimetabolites, death receptor antagonist, hormonal and antiangiogenic agents, proteasome histone deacetylase and kinase inhibitors have all been shown to effect autophagy.

Microtubule inhibitors often block autophagy *(Shen et al., 2011)*. Although autophagosomes are formed throughout the cytoplasm, lysosomes tend to be localized around the nucleus and autophagosomes use the microtubule network to migrate to lysosomes, so disruption of the microtubule dynamics by drugs like vinblastine prevent autophagosome fusion with the lysosome *(Köchl et al., 2006)*. Drugs like chloroquine (CQ) and hydroxychloroquine (HCQ), the agents currently used deliberately to inhibit autophagy in active clinical trials *(Amaravadi et al., 2011)*, are weak bases that accumulate in the lysosomes causing a rise in lysosomal pH and preventing fusion with autophagosomes. CQ accumulation in lysosomes can also lead to inhibition of mTORC1, leading to induction of genes by the transcription factor TFEB that stimulates lysosomal biogenesis and autophagy *(Settembre et al., 2012)*. Thus, CQ can potentially both inhibit and stimulate autophagy simultaneously. For the more common case whereby anticancer drugs induce autophagy, different mechanisms apply: some that directly affect the core autophagy machinery and others that regulate autophagy indirectly. Perhaps the best example of anticancer agents that directly affect the core machinery comes from mTOR inhibitors, such as rapamycin, temsirolimus, or similar inhibitors. These drugs induce

autophagy by directly inhibiting mTORC1, which is a core negative regulator of the autophagy machinery.

Other drugs directly target the core autophagy machinery itself. A good example comes from agents, such as ABT737 and similar drugs, that were designed to inhibit Bcl-2 family proteins by acting as BH3 mimetics *(Oltersdorf et al., 2005)*.

Direct regulation of the BECN1 complex is also responsible for autophagy induction by tyrosine kinase inhibitors, such as erlotinib, that target the epidermal growth factor receptor (EGFR) *(Wei et al., 2013)*. The mechanism in this case is the inhibition of direct phosphorylation of BECN1 by EGFR that changes the BECN1 interactome to switch it from an autophagy-inhibiting to an autophagy-inducing state.

Different kinds of DNA damaging agents activate autophagy, but it is quite unclear how they do so. This is important because a better understanding of how different kinds of anticancer agents can induce autophagy may provide a way to selectively interfere with drug-induced autophagy; this might allow selective inhibition of the autophagy that limits anticancer drug action rather than general autophagy (*Eskelinen*, *2011)*.

CHAPTER 4

THE AUTOPHAGY PROTEIN BECLIN 1

4.1 BECLIN 1: an Oncosupressor that Regulates Autophagy

Studies of the functions of BECLIN 1 revealed the connection of impaired autophagy and cancer development. BECLIN 1 is monoallelically deleted in 75% of ovarian, 50% of breast and 40% of prostate cancers and in several cancer cell lines *(Aita et al. 1999)*. Studies showed that monoallelic disruption of BECLIN 1 in mice increased the incidence of epithelial and hematopoietic tumors, indicating that BECLIN 1 protein level is crucial for the tumor suppressor function *(Qu et al., 2003)*. Moreover, the other autophagy promoting components of the BECLIN 1/Vps34 complex also show anti-proliferative or tumor suppressive properties. Here are the examples, ATG14L maps to a locus frequently lost in multiple cancers *(Sun et al. 2009)*. Depletion of Ambra 1 decreases autophagy and increases cell proliferation by reducing the BECLIN 1-Vps34 interaction, while overexpression of Ambra 1 decreases cell proliferation *(Fimia et al. 2007)*. UVRAG-mediated activation of the BECLIN-Vps34 complex promotes autophagosome maturation and suppresses the proliferation and tumorigenicity of cancer cells *(Liang et al., 2006)*. Loss of Bif-1 suppresses autophagy and enhances the development of spontaneous tumors in mice *(Takahashi et al., 2007)*. Moreover, the antiapoptotic proteins BCL-2 and BCL-XL inhibit autophagy by blocking the binding of Vps34 to BECLIN 1 *(Pattingre et al., 2005; Hoyer-Hansen et al., 2007)*. Thus BCL-2 and BCL-XL may promote tumorigenesis not only by blocking apoptosis, but also by inhibiting autophagy.

BECLIN 1 is the first downstream autophagy-execution gene linked to human cancer, it is important to note that certain oncogenes (e.g., PI3K and AKT) are already known to function in the negative regulation of autophagy *(Petiot et al., 2000, Arico et al., 2001)*, and that the PTEN tumor-suppressor gene is known to function in the positive regulation of autophagy (*Arico et al., 2001)*. Furthermore, rapamycin analogs that inhibit mTOR, a downstream target of class I PI3K and Akt, induce autophagy in yeast and mammalian cells *(Cutler et al., 1999)*; reduce neoplasia in tumors that arise in PTEN-deficient mice *(Podsypanina et al., 2001)*; and have yielded encouraging results in early clinical cancer trials *(Huang and Houghton, 2002;*

Elit, 2002). It has been commonly assumed that the mechanisms by which the class I PI3K signaling pathway promotes oncogenesis involve the induction of cell growth in response to mitogenic signals, the inhibition of apoptosis, or the promotion of angiogenesis. The precise mechanisms by which the autophagy function of BECLIN 1 contributes to tumor suppression are not known. In the late 1970s, several studies demonstrated that the rate of cell growth represents a balance between the amount of protein synthesized and the amount of autophagic protein degradation and established autophagy as a lysosomal pathway involved in cell-growth control *(Gunn et al., 1977; Tanaka and Ichihara, 1978, Amenta et al., 1980)*. Autophagy may also contribute to tumor suppression by degrading specific cellular organelles and long-lived proteins that are essential for regulating cell growth, thereby functioning as a brake on cell growth in response to mitogenic signals.

4.2 BECLIN 1 as a Tumor Prognostic Marker

Recent studies document that BECLIN 1 expression is inversely correlated with the tumor size and the primary tumor stage in squamous cell carcinoma and adenocarcinoma of the lung *(Won et al. 2012)*. The decreased expression of BECLIN 1 was found associate with pelvic lymph node metastasis and histological grade of cervical *(Wang et al., 2011)* and ovarian *(Duan et al., 2007)* cancers. There were significant associations between increased BECLIN 1 expression and the absence of lymphatic invasion and low rate of distant metastasis of pancreatic ductal adenocarcinoma *(Kim et al., 2011)*. *Chen et al.* found that BECLIN 1 expression was significantly correlated with depth of invasion, lymph node metastasis, and clinical stage of esophageal squamous cell carcinoma *(Chen et al., 2009)*. *Pirtoli et al.* found that high cytoplasmic expression of BECLIN 1 protein score was positively correlated with apoptosis and negatively with cell proliferation in high-grade glioma *(Pirtoli et al., 2009)*.

We have studied the biological and clinical significance of BECLIN 1 and LC3 in ovary tumors of different histological subtypes. The positive expression of BECLIN 1 was correlated with the presence of LC3-positive autophagic vacuoles. We found that type I tumors, which are less aggressive than type II, were more frequently expressing high level of BECLIN 1. Of note, tumors of histologic grade III expressed low level of BECLIN 1. Consistently, high level of expression of BECLIN 1 and LC3 in tumours is well correlated with the overall survival of the patients. The present data are compatible with the hypotheses that a low level of autophagy favors cancer progression and that ovary cancer with

upregulated autophagy has a less aggressive behavior and is more responsive to chemotherapy *(Valente et al., 2014)*.

In a recent work we interrogated the effects of BECN1 in breast cancer patients. We studied the mRNA expression of BECN1 from two different datasets: The Cancer Genome Atlas (TCGA) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC). In both datasets, low expression of BECN1 was more common in HER-enriched and basal-like breast cancers compared to Luminal A/B intrinsic tumor subtypes, and was also strongly associated with TP53 mutations and advance tumor grade. Then low expression of BECN1 was associated with poor prognosis. These findings suggest that decreased mRNA expression of the autophagy gene BECN1 may contribute to the pathogenesis and progression of HER2-enriched, basal-like, and TP53 mutant breast cancers *(Tang et al., 2014)*.

4.3 BECLIN 1 Structure and Function

The mammalian autophagy protein BECLIN 1, an orthologue of the Atg6/vacuolar protein sorting (Vps)-30 protein in yeast, was cloned through a yeast two-hybrid screen in 1998 by *Beth Levine*'s group *(Liang et al., 1998)*. It is highly conserved among different species, with orthologues being identified in diverse organisms indicating an important biological function. *(He and Levine, 2010; Huang et al., 2012)*.

The BECN1 gene is composed of 11 introns and 12 exons and is localized on chromosome 17q21*, (He and Levine, 2010; Tangir et al., 1996)* which is deleted in a several number of human cancers *(Aita et al. 1999).* The gene translates in a protein of 450 amino acid long and a molecular weight of 60-kDa *(Levine and Klionsky, 2004).*

In mammalian cells BECLIN 1 protein has been shown to be ubiquitously expressed *(Wang et al., 2009),* and is mainly localized within the cytoplasm and organelles such as mitochondria, endoplasmic reticulum and trans-golgi network. *(Kihara et al. 2001; Levine and Klionsky, 2004).*

BECLIN 1 contains three principal functional domains:

- an N-terminal Bcl-2 homology 3 (BH3) domain (amino acids 108-127), by this domain BECLIN 1 interacts with Bcl-2 family proteins *(Oberstein et al., 2007)*;

- a coiled–coiled domain (CCD; amino acids 174-266) *(Li et al., 2012),* an interaction platform to cofactors (Atg14L, UVRAG, Bif-1 and Rubicon) which leads to activation of autophagy *(Liang et al., 2006);*

- the C-terminal evolutionary conserved domain (ECD; amino acids 244-337) *(Huang et al., 2012)*, essential for interaction with Vps34 complex *(Furuya et al., 2005).*

BECLIN-1 also contains a nuclear export signal sequence, poorly conserved, leucine rich, necessary for the nuclear export and its cytoplasmic localization *(Liang et al. 2001).*

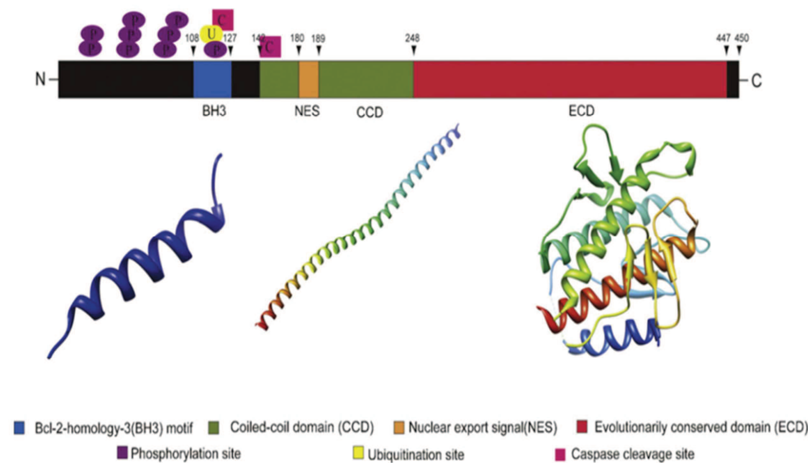

Fig.11 *BECLIN 1 Structure and Domains*

BECLIN 1 plays a crucial role during the autophagic initiation process that involves the phagophore formation. First BECLIN-1interacts with Vps34, AMBRA1, Vps15 and ATG14L. When this complex is active starts the generation of phosphatidylinositol 3-phosphate, an early step in autophagosome formation downstream of the ULK1 complex *(Hamacher-Brady, 2012).*

In response to nutrient starvation, ULK1 phosphorylates AMBRA1 to release it from its interaction with LC8/DLC1, and it is then translocated to the ER together with the BECLIN 1-class III PI3K complex. Downstream of ULK1/MTORC1 occurs the starvation-dependent activation of BECLIN 1 complex.

In a different complex, UVRAG substitutes ATG14L and BECLIN 1 controls the formation and maturation of autophagosomes. Rubicon, which contains a conserved RUN domain, down-regulates the maturation of autophagosomes when is present in a complex with

UVRAG. Rubicon has been also identified in BECLIN 1 complexes with ATG14L, thus the possibility cannot be excluded that Rubicon may also control the initiation of autophagy. Many cellular proteins have been shown to interact with BECLIN 1, and to modulate autophagy. Initial studies by the *Beth Levine's* group reported that Bcl-2 blocks starvation-induced autophagy by interacting with the BH3 domain of BECLIN 1 *(Pattingre et al., 2005)*.

The dissociation of this complex is dependent on the phosphorylation of a triad of amino acids present in the non-structural loop of the cellular form of Bcl-2 by c-jun N-terminal kinase 1 (JNK1) *(Wei et al,. 2008)*. JNK1-dependent dissociation of the BECLIN 1/Bcl-2 complex is also triggered by other mediators of autophagy such as the sphingolipid ceramide *(Pattingre et al., 2009).* Following on from this study, it has been shown that BH3 proteins and BH3 mimetics disrupt the interaction between BECLIN 1 and Bcl-2/Bcl-xL *(Maiuri et al,. 2007)*. The interaction can also be disrupted by phosphorylation of the BH3 domain of BECLIN 1 by death-associated protein kinase (DAPK) *(Zalckvar et al., 2009).*

The BECLIN 1-class III PI3K core complex is recruited to microtubules via the dynein light chain 1(LC8/DLC1) by two non-mutually exclusive mechanisms *(Di Bartolomeo et al., 2010; Luo et al., 2012)*. The protein BECLIN 1 interacts with the BH3-only protein Bim that bridges the BECLIN 1–LC8/DLC1 interaction. Under starvation conditions, the phosphorylation of Bim by JNK abolishes its interaction with LC8/DLC1, and dissociates the Bim-BECLIN 1 interaction *(Luo et al., 2012)*. LC8/DLC1 also recruits BECLIN 1-class III PI3K via its interaction with the BECLIN 1 interacting protein, AMBRA1 *(Di Bartolomeo et al., 2010)*.

Starvation-induced autophagy is also regulated by BECLIN 1 phosphorylation at serine 90, by two different kinases, members of the p38 mitogen-activated protein kinase (MAPK) signaling pathway MAPKAPK2 (MK2) and MAPKAPK3 (MK3). The phosphorylation site serine 90 is the one that is blocked in vitro and in vivo by BCL2, negative regulator of BECLIN 1. Consequently, the MAPK signaling pathway actives the autophagic function of BECLIN 1 *(Wei et al. 2015)*.

Akt is a serine/threonine protein kinase frequently mutated and activated in cancers *(Manning and Cantley, 2007)*. Akt down-regulate the PI3K complex I *(Wang et al., 2012)*. It phosphorylates BECLIN 1 favoring its interactions with 14-3-3 proteins and vimentin. Akt can inhibit autophagy via several pathways, indirectly by activation of the MTOR pathway *(Codogno and Meijer, 2005)* upstream of the autophagic machinery or by direct inhibition of

the PI3K complex I *(Wang et al., 2012)*. Class III PI3K in the PI3K complex I is a substrate for cyclin-dependent kinases (Cdk1 and Cdk5) *(Furuya et al., 2010)*. Cdk-dependent phosphorylation of class III PI3K down-regulates its interaction with BECLIN 1.

The activity of BECLIN 1 in autophagy can also be inhibited as result of proteolytic cleavage by caspases *(Djavaheri-Mergny et al., 2010)*. Experimental evidence show that the C-terminal fragment of BECLIN 1 localizes to the mitochondria and sensitizes cells to apoptosis *(Wirawan et al., 2010)*.

BECLIN 1 is involved in different processes, in addition to its role in autophagy, development, tumor suppression and the clearance of protein aggregates, it is also an important regulator in Programmed Cell Death (PCD) *(Yu et al., 2004)*.

Bcl-2 inhibits BECLIN 1-dependent autophagy, and thus autophagic cell death upon starvation *(Mauri et al., 2007; Pattingre et al., 2005)*. However, overexpression of Bcl-2 or Bcl-XL in apoptosis-defective bax−/− bak−/−mouse embryonic fibroblast cells treated with etoposide, an apoptosis-inducing agent, stimulates BECLIN 1-dependent autophagic cell death *(Shimizu et al., 2004)*. These findings suggest that Bcl-2 has a different effect on autophagy and autophagic cell death depending on the cell type and stimulus, and are in agreement with the general observation that autophagy can act in both cell survivor and cell death functions. Inhibition of autophagy with small interfering RNA to reduce levels of BECLIN 1, or other autophagy proteins, leads to apoptotic cell death, and the reduction in autophagy sensitizes cells to apoptotic stimuli, suggesting some type of crosstalk between these pathways *(Boya et al., 2005; Daniel et al., 2006)*. Probably, autophagy acts initially as a cytoprotective mechanism, and when autophagy is suppressed, cells are more prone to apoptosis. On the other hand, if cell death occurs when apoptosis is blocked, it is likely to involve an autophagic mechanism.

Aim of the work

The purpose of my studies was to understand the effects of the autophagic oncosuppressor protein BECLIN 1 in human cancer. In particular we focused on two kind of cancer: ovarian and breast cancer.

Ovarian and breast cancers are respectively the fifth and the second most deadly tumors in women. The TCGA Research Network uncovered genomic similarities between the basal-like subtype in breast cancer and the serous ovarian cancer. Computational analyses show that Basal-like breast cancer and serous ovarian cancer might both be susceptible to agents that inhibit blood vessel growth, cutting off the blood supply to the tumor, as well as to compounds that target DNA repair, which include chemotherapy drugs such as cisplatin. These molecular similarity give us the opportunity to compare treatments and outcomes across these poor prognosis subtypes.

Several studies show the role of autophagy in cancer development, progression, and treatment. Autophagy is a mechanism for the degradation of cytoplasmic material, damaged organelles and aggregate proteins in lysosomes. Recent evidence indicates that autophagy is a tumor suppressor mechanism, which is connected to its role in the clearance of the scaffold protein p62/SQSTM1 and prevention of oxidative stress and genomic instability. Cancer cells can also use autophagy to survive nutrient limitation and hypoxia that often occur in solid tumors. Tumor cells can also upregulate autophagy as a response to cancer treatment.

The BECLIN 1 protein plays a pivotal role in the autophagic initiation process that involves nucleation of the autophagic vesicle (formation of phagophore). Has been shown that the BECN1 gene is a tumor suppressor, and an important prognostic marker for a variety of cancers in clinical settings. We demonstrate that high BECLIN-1 expression levels are associated with good overall survival rate in patients with ovarian and breast cancer.

The aim of the doctoral work was to analyze the role and regulation of autophagy in human cancer focusing:

1. The molecular and epigenetic mechanisms that regulate the expression of autophagic genes in cancer
2. mRNA and protein expression of autophagic proteins, such as BECLIN 1 and LC3 as possible marker for human cancer
3. New anticancer therapies targeting the autophagy pathway

Results

INVOLVEMENT OF AUTOPHAGY IN OVARIAN CANCER

1. - Epigenetic control of Autophagy in Cancer: the role of microRNAs

Chiara Vidoni, Rossella Titone, Federica Morani, Carlo Follo and Ciro Isidoro

- Epigenetic Control of Autophagy by MicroRNAs in Ovarian Cancer

Rossella Titone, Federica Morani, Carlo Follo, Chiara Vidoni, Delia Mezzanzanica and Ciro Isidoro

MicroRNA are small non-coding RNAs involved on the gene expression post-transcriptional regulation. Considering the implications of both miRNAs and autophagy in cancer related processes and given the lack of current evidence linking these two rapidly growing fields of research, we prompted to review miRNAs regulating autophagy. In principle, autophagy could be regulated by miRNAs targeting the mRNA of key molecules that indirectly induce or suppress autophagy.

In these reviews, we focus on those miRNAs that are either up or down expressed in cancer and that potentially regulate autophagy. Advance in detection and functional evaluation of miRNAs is expected to strongly contribute to unravelling the network of apoptosis and autophagy regulation in this complex disease. In the near future, miRNA signatures associated with autophagy in cancer, thus posing the basis for the possible harnessing of these miRNAs as therapeutic targets, as well as possible diagnostic-prognostic tools.

Personal Contribution

In the paper *Vidoni et al. 2014,* I contributed to the literature search.

In the paper *Titone et al. 2014,* I contributed to the literature search, in silico analysis of microRNAs targeting autophagy-related genes and writing of the report.

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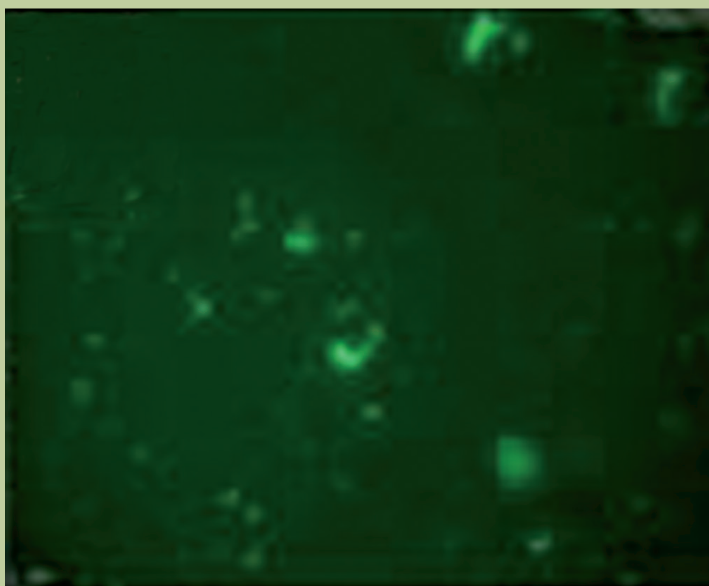

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Epigenetic control of autophagy in cancer: the role of microRNAs

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Autophagy is a cellular process for the lysosomal degradation of redundant, aged or damaged self-constituents. Autophagy is controlled by thirty known autophagy-related proteins, and is regulated by a complex signalling network of protein- and lipid-kinases, GTPases, and protein- and lipid-phosphatases. Owing to its housekeeping role in cell homeostasis, autophagy plays a crucial role in the cell death and cell survival decision, and therefore it is implicated in cancer development and progression. Accordingly, numerous oncogenes and tumor suppressors regulate autophagy. MicroRNAs (miRNAs) are 20-22 oligonucleotides non coding RNAs that post-transcriptionally silence gene expression by affecting mRNA translation and stabilization. MiRNAs are aberrantly expressed in cancer cells, adding to the epigenetic control of cancer cell phenotypes. Here, we briefly review the studies implying a role for miRNAs in the regulation of autophagy in cancer cells.

Key words: Autophagy - MicroRNAs - Neoplasms - Cell death - Apoptosis.

Autophagy is a process of degradation of self-constituents that occurs within the lysosomal compartment. Through the elimination of redundant, aged or damaged cell constituents, autophagy largely contributes to the maintenance of cell homeostasis and also helps the cell to face intra- and extracellular injuries.[1]

MicroRNAs (miRNAs) are small non-coding RNAs that hybridize to target mRNAs, thus hampering the expression of genes.[2] Because of their regulatory function on protein expression, miRNAs are involved in pathways that control all aspects of the cell life, including cell metabolism, cell differentiation, cell proliferation and cell death.[3, 4] MiRNAs are aberrantly expressed in cancer cells, adding to the epigenetic control of cancer cell phenotypes and response to therapy.[5-7]

In this review, we summarize the recent findings implying a role for miRNAs in the regulation of autophagy in cancer cells. In particular, we will focus on those miRNAs that target autophagy-related proteins with an obvious impact on the autophagy process and, consequently, on the autophagy-associated effects in cancer cells. We will not discuss the miRNAs that affect the expression of the several signalling molecules that regulate the induction and progression of autophagy. Examples of these epigenetic regulations of autophagy in cancer have been reported in other reviews.[8-10]

The autophagy process and its role in cancer

In order to keep the cell healthy, cellular self-constituents, including proteins, small portion of cytoplasm and of membranes, and entire organelles, are constitutively degraded and renewed in a process named "macromolecular turnover". The alteration in

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the equilibrium between the rates of degradation and synthesis of macromolecules and of cellular structures is a driving force in cancer development.[11, 12] By definition, "autophagy" refers to the degradation of cellular self-constituents that occurs within lysosomes.[13] Depending on the pathway to deliver the substrate to lysosome for degradation, autophagy is classified in micro-autophagy (in which small portion of cytoplasmic material is directly endocytosed by lysosomes), chaperon-mediated autophagy (in which a single-protein is internalized in the lysosome with the assistance of the chaperon HSC73) and macro-autophagy.[14-16] In this review, we will focus on macro-autophagy (now on simply referred to as "autophagy"), since this degradative process plays a major role in cell homeostasis and, as predictable, appears to be altered in cancer cells. In fact, numerous oncogenes and tumor suppressor genes are involved in the regulation of autophagy.[17]

The autophagy process is schematically depicted in Figure 1. Through this pathway it is possible to deliver to lysosomes for degradation big-sized protein aggregates, large portions of membranes and entire organelles.[13] Extracellular stressors or genetic mutations that lead to the production of altered and potentially harmful lipid/protein structures, including membranes and organelles, induce autophagy as a means for the clearance of such material. The degradative substrates are entrapped within a vesicle named the "autophagosome", which characteristically is made up of a double-membrane in which the lipidated form of Light Chain 3 (LC3-II) is post-translationally inserted. The presence of LC3 in a vacuolar structure is considered an hallmark for the definition of an autophagosome.[18] LC3-II arises from its precursor microtubule-associated protein (MAP), which is processed by an array of autophagy-related proteins (ATG proteins) to be covalently linked to phosphatydil-ethanolamine (PE) and subsequently inserted onto the autophagosomal membranes (Figure 1). The autophagosome eventually fuses with endosomes and lysosomes to form the autophagolyso-

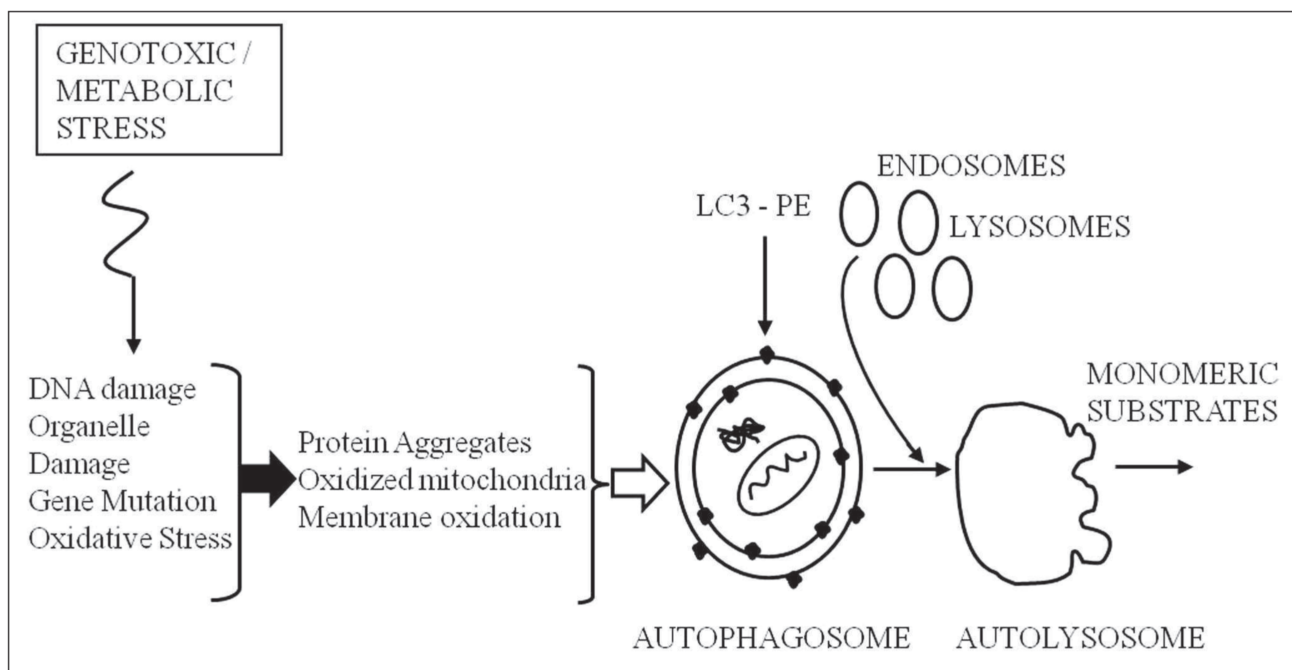

Figure 1.—Schematic representation of the autophagy process.
Stressful hits causing damages to DNA, proteins, or membranes and leading to the formation of protein aggregates or organelle mis-function induce autophagy as a pathway for their removal. Autophagy substrates are specifically targeted to the autophagosome by proteins (*e.g.* the sequestosome SQSTM1/p62) that have an interacting domain for LC3. The autophagosome then fuses with endosomes and lysosomes to form an autophagolysosome (or autolysosome) in which the autophagy substrates are degraded by the acid hydrolases. Finally, the monomeric substrates are exported in the cytoplasm for their utilization in the biosynthetic pathway.
Abbreviations: LC3, light chain 3; PE, phosphatidylethanolamine.

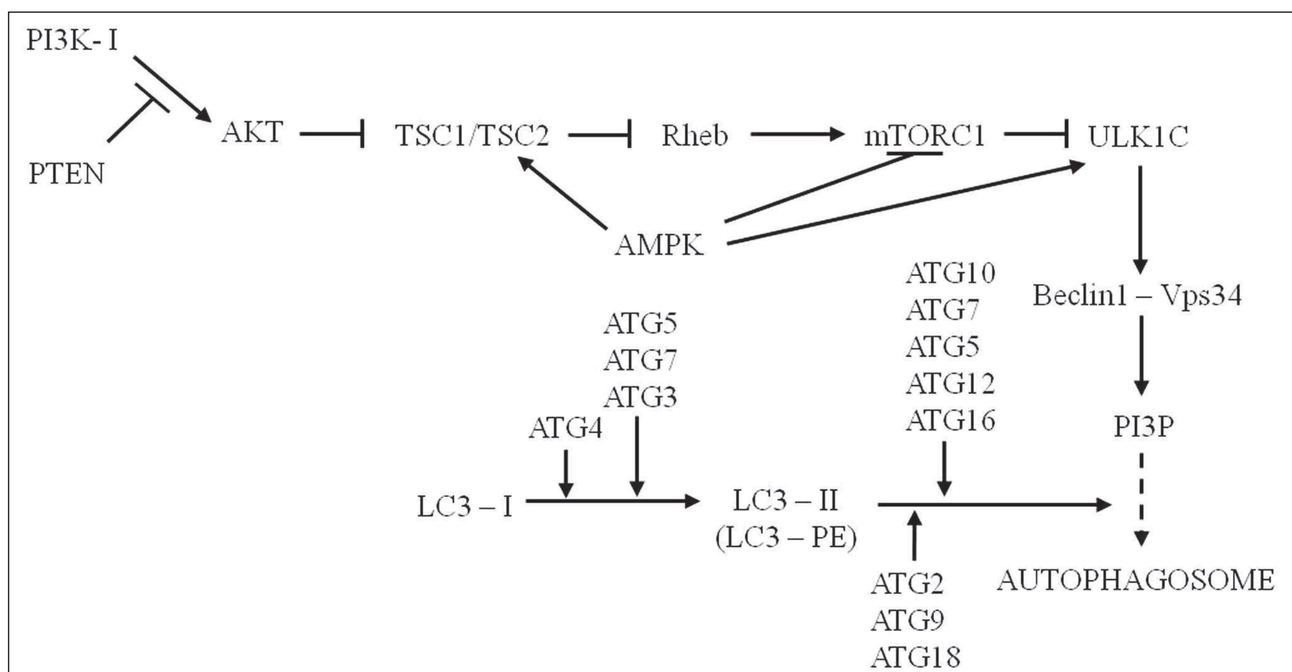

Figure 2.—Biochemical regulation of the autophagy pathway.
The principal (and canonical) signalling pathways that regulate the autophagy process are schematically represented in the Figure (see the text for explanatory details). The ATG (autophagy-related) proteins involved in the steps for the processing of LC3 and for the formation of the autophagosome are indicated. Abbreviations (see also legend to Figure 1): AMPk, adenosine monophosphate-activated protein kinase; MTORC1, mammalian target of rapamycin–raptor complex1; PI3k, Phosphatydil-inositol-3-kinase; PTEN, phosphatase and tensin homolog; RHEB, ras homolog enriched in the brain; TSC, tuberous sclerosis complex; ULK, Unc51-like kinase. PI3P, phosphatidyl-3-phosphate;

some (or autolysosome) in which full degradation of the autophagy material takes place thanks to the acid hydrolases.

The autophagy process is finely tuned and controlled by a network of signaling pathways made up of protein- and lipid-kinases,[19-24] protein- and lipid-phosphatases.[25-29] For a detailed description of the signalling pathways governing autophagy, the readers may refer to the recent excellent reviews.[30-33] A very simplified scheme of the main regulatory pathways is illustrated in Figure 2. Briefly, through activation of class I PI3k (phosphatydil-inositol- 3-kinase), growth factors and nutrients (essentially, glucose and aminoacids) maintain active the mTOR complex 1, that in turn keeps basal autophagy at low level by repressing the activation of the ULK1 complex. Downstream the ULK1 complex is the BECLIN 1- class III PI3k (also known as Vps34) complex that leads to the production of PI3P (phosphatydil-inositol-3-phosphate), which is crucial in initiating the autophagosome formation.[34] In the absence of nutrients and growth factors (starvation condition), the tonic inhibition by mTORC1 is relieved, and autophagy is induced to provide the substrates needed for the synthesis of pro-survival molecules and structures. Autophagy is also induced when it is necessary to eliminate un-folded protein aggregates and damaged membranes/organelles (*e.g.*, leaky mitochondria) formed in the cell because of extracellular hits (*e.g.*, cytotoxic drugs, oxidative stress) or intracellular genetic defects (*e.g.*, gene mutations). As such, autophagy has a pro-survival function and prevents the accumulation of toxic substances that could lead to cell death or cell transformation.[35-37] However, in established cancer autophagy may turn of advantage for cancer cells to survive under prohibitive conditions (*e.g.*, in hypoxic and starvation conditions) and to face the genotoxic stress of chemotherapeutics.[38] In an opposite fashion, autophagy could also be a means for inducing cell death if the self-eating process is up-regulated to a point-of-non-return, and this could be exploited for therapeutic purposes. The double-face role of autophagy in cancer development and progression

explains why either drugs over-inducing or repressing autophagy have been shown to exert anti-cancer effects.[39-41]

Regulation of autophagy by microRNAs in cancer

In this paragraph, we will discuss how miRNAs may impact on cancer development and progression and on the cancer therapeutic response through the regulation of autophagy. Recent reviews have addressed the links between miRNAs and autophagy in the development of specific cancers [8, 10] and in chemotherapy resistance.[42] Given the relative abundance of miRNAs in the cell, and considering that each single miRNA has many different potential targets, it is not surprising the fact that autophagy in a cancer cell is dynamically regulated by these small molecules.[9, 43, 44] In Table I we report a short list of the miRNAs that have been experimentally validated as regulators of autophagy in cancer cells.[45-49] In relation to the oncogenic consequences of their dys-regulation, miRNAs have been classified as oncomiRs, if they target the mRNA of oncosupressor proteins, or as tumor suppressive miRs, if they target the mRNA of oncogene proteins.[5, 50] Members of both these classes of miRNAs have been shown to target the mRNA of ATG proteins. For instance, the mRNA of BECLIN 1 (which is itself an oncosuppressor) is targeted by both the tumor suppressive miR-30a [45] and the oncogenic miR3-76b.[46] This latter miRNA was shown to also target the mRNA of ATG4, the cystein protease involved in the processing of LC3.[46] Of note, miR-30a could improve imatinib-induced apoptosis in chronic myelogenous leukemia cells by halting autophagy through the down-regulation of BECLIN 1 and ATG5 expression.[51] Very recently, miR-30d, another member of this family, was shown to down-regulate autophagy in vary human epithelial cancer cells by targeting the mRNA of BECLIN-1, ATG2, ATG5 and ATG12.[52] Autophagy induced under stressful conditions may confer resistance to the chemotherapeutic treatments in cancer cells, and miRNAs could contribute to the modulation of autophagy in these situations. For instance, Hsa-miR-181a-1 was shown to prevent the induction of autophagy in cancer cells subjected to starvation or to mTOR inhibition by down-regulating the expression of ATG5.[53] Down-regulation of ATG7 and ULK1 mediated by miR-290-295 prevented autophagic cell death and conferred resistance to glucose starvation in melanoma cells.[54] Recently, it has been shown that down-regulation of ATG7 by miRNA-17 improves the cytotoxic effects of Temozolomide and of radiation treatments in human glioblastoma cells.[55] In this same line, the treatment with cis-Platin was found to induce a chemoresistance-promoting autophagy through the down-regulation of certain miRNAs targeting ATG proteins, such as miR-119a-5p (which targets ATG7) in hepatocarcinoma cells,[56] and miR-181a and miR-374a (targeting ATG5), miR-630 (targeting ATG12) and miR-519 (targeting BECLIN-1, ATG10 and ATG16L1) in squamous carcinoma cells.[57] Finally, the oncogenic miR-183 was found up-regulated in metastatic medullary thyroid cancers and its down-regulation decreased the viability of cultured thyroid cancer cells while promoting the expression of the autophagy marker LC3.[58]

Conclusion and perspective

MicroRNAs are now recognized as master epigenetic regulators of gene expression that have a great impact on many malignant features of cancer cells, such as the propensity to metastasize and to resist to antineoplastic treatments.[50] Autophagy, as a homeostatic metabolic process, plays a crucial role in the carcinogenesis process. Both miRNAs and autophagy are now-a-day being considered as potential targets for the molecular therapy of cancer. Indeed, a cross-talk between autophagy and cell death exists, and

Table I.—*miRNAs experimentally validated as regulators of autophagy in cancer cells.*

microRNA	ATG Target	Effect	Reference
miR-30a	Beclin 1	no activation of Vps34	Zhu *et al*. Autophagy 2009 [45]
miR-376b	Beclin 1	no activation of Vps34	Korkmaz *et al*. Autophagy 2012 [46]
miR-376b	ATG4	no LC3 processing	Korkmaz *et al*. Autophagy 2012 [46]
miR-130a	ATG2B	stop elongation pre-autophagosome	Kovaleva *et al*. Cancer Res 2012 [47]
miR-204	LC3	stop autophagosome formation	Mikhaylova *et al*. Cancer Cell 2012 [48]
miR-375	ATG7	no LC3 insertion in autophagosome	Chang *et al*. Gastroent 2012 [49]

miRNAs, as controllers of both processes, may play a determinant role in the switch from a pro-survival to a pro-death function of autophagy in response to chemotherapeutic treatments.[59] Therefore, a more in depth characterization of how miRNAs control autophagy will hopefully open new avenues for the molecular therapy of cancer.

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Review Article

Epigenetic Control of Autophagy by MicroRNAs in Ovarian Cancer

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Autophagy is a lysosomal-driven catabolic process that contributes to the preservation of cell homeostasis through the regular elimination of cellular damaged, aged, and redundant molecules and organelles. Autophagy plays dual opposite roles in cancer: on one hand it prevents carcinogenesis; on the other hand it confers an advantage to cancer cells to survive under prohibitive conditions. Autophagy has been implicated in ovarian cancer aggressiveness and in ovarian cancer cell chemoresistance and dormancy. Small noncoding microRNAs (miRNAs) regulate gene expression at posttranscriptional level, thus playing an important role in many aspects of cell pathophysiology, including cancerogenesis and cancer progression. Certain miRNAs have recently emerged as important epigenetic modulators of autophagy in cancer cells. The mRNA of several autophagy-related genes contains, in fact, the target sequence for miRNAs belonging to different families, with either oncosuppressive or oncogenic activities. MiRNA profiling studies have identified some miRNAs aberrantly expressed in ovarian cancer tissues that can impact autophagy. In addition, plasma and stroma cell-derived miRNAs in tumour-bearing patients can regulate the expression of relevant autophagy genes in cancer cells. The present review focuses on the potential implications of miRNAs regulating autophagy in ovarian cancer pathogenesis and progression.

1. Introduction

The research fields of autophagy and microRNAs (miRNAs) are relatively new (less than 20 years from their definition and discovery) and our knowledge of these fields is in tremendous expansion; on the other hand, the ovary cancer remains a deadly disease since no significant improvement in overall survival was achieved in the last three decades [1]. Here we focus on the involvement of macroautophagy in the pathogenesis of cancer and on the molecular significance of miRNAs that potentially regulate this process. Targeting of the autophagy pathway is being under evaluation as a new anticancer therapeutic option [2, 3]. Therefore, unravelling the clinical implications of autophagy-miRNA interaction in ovary cancer might hopefully open the way to new diagnostic and molecular therapeutic approaches for this highly malignant disease.

2. MicroRNAs and Cancer

Over the last decade, several classes of molecules that form a complex transcriptional regulatory network are being identified and still their complete characterization is ongoing [4]. The most well-known small noncoding RNAs, discovered nearly 20 years ago, are the miRNAs, which posttranscriptionally regulate gene expression through base pairing with the 3′-untranslated region of target mRNAs [5]. MiRNA-mediated repression of gene expression occurs

through complex mechanisms not fully understood, including translational inhibition and mRNA degradation [6]. MiRNAs, as master regulators of gene expression, are among the major players in development, cell biology, and disease onset; in fact, it has been estimated that miRNAs can regulate the expression of more than half of protein-coding sequences in mammalian genomes. Accumulating evidence shows that miRNA expression is dysregulated in many types of cancer and that they can act either as oncogenes or tumour suppressors, depending on the cellular context and the expression of the miRNA targets in the particular tissue (reviewed in [7]). The effects of miRNA deregulation in cancer progression, diagnosis, and therapy have been extensively reviewed [8, 9].

3. Autophagy and Cancer

3.1. Morphological Aspects and Biochemical Regulation of Autophagy in Brief. Autophagy refers to a cellular process committed to the lysosomal degradation of self-constituents [10, 11]. Depending on the mechanism through which the substrate is delivered into the lysosome, autophagy is classified as macroautophagy, microautophagy, and chaperon-mediated autophagy [12–15]. However, macroautophagy (now simply referred to as autophagy) is the process mainly subjected to fluctuations to comply the needs for keeping cell homeostasis in response to stressful injuries. Autophagy, in fact, is the only pathway allowing the degradation of macromolecular aggregates, portion of cytoplasm, membranes, and entire organelles [16]. In this process, the autophagy substrate is sequestered within a newly formed vesicle (named autophagosome) that subsequently fuses with several endosomes and lysosomes to form autophagolysosomes (or autolysosomes) in which the autophagy substrate is fully degraded by the lysosomal acid hydrolases [17]. The substrates are selectively incorporated within the nascent autophagosomes through the intervention of proteins, such as p62/SQSTM1 (sequestosome), NBR1 (neighbour of BRCA1 gene 1), and Nix/BNIP3, that bridge the substrate and the membrane-bound LC3 [18–20]. LC3 (light chain 3; the mammalian orthologue of yeast atg8) derives from the posttranslational modification of MAP-LC3 (microtubule-associated protein-LC3) and is specifically associated with autophagosomal membranes [21]. The autophagosome originates from the nucleation and expansion of a preautophagosomal structure, a double-layered omega-shaped semicircle originating from the smooth endoplasmic reticulum [22]. Eventually, this structure closes up to form the autophagosome, which entraps the cargo. While being on formation, the lipidated isoform LC3-II is inserted onto the internal and external membranes of the autophagosome. The autophagosomes then move toward the microtubular organizing center, where they meet and fuse with the lysosomes [23]. The cargo is then completely degraded, along with the internal membrane of the autophagosome, within the acidic lumen of the autophagolysosome [24]. LC3-II present on the internal membrane of the autophagosome is also degraded, so that its consumption serves as readout of the autophagy flux [21]. Finally, the monomeric substrates are then pumped out in the cytosol for recycling purposes [25].

The autophagy pathway is controlled by a variety of signalling molecules [26, 27]. The ULK1 (Unc51-like kinase 1, the homolog of the yeast Atg1) kinase is believed to master the induction of autophagy [28]. Its function is under the control of two upstream kinases, AMPk and mTOR. Schematically, the class I PI3-k-AKT signalling pathway negatively impinges on autophagy through the activation of mTOR complex 1 (mTORC1), which inhibits the ULK1 complex, while the LKB-AMPk signalling pathway positively regulates autophagy through the inactivation of mTORC1 and the direct activation of ULK1 [29]. The activation of these pathways is influenced by intracellular and extracellular factors. The availability of nutrients (essentially, glucose and amino acids) and of growth factors activates the class I PI3k-AKT-mTORC1 pathway, thus repressing autophagy, whereas starvation strongly induces autophagy [30, 31]. On the other hand, energy depletion (i.e., shortage of ATP), oxidative stress, and DNA damage activate the LKB-AMPk pathway and therefore trigger autophagy [32–35]. The ULK1 complex signals to (also known as Vps34), which forms an active complex with Beclin-1 (also known as ATG6 or Vps30) [31]. This complex is recruited at the level of the preautophagosomal structure and locally produces PI3P (phosphatidyl -3-phosphate), the starting platform for the recruitments of membranes necessary for the biogenesis of the autophagosome [12].

3.2. The Pathophysiological Role of Autophagy in Cancer. The role of autophagy in cancer biology is not unequivocal. While basal (constitutive) autophagy prevents carcinogenesis through the constant elimination of damaged molecules and organelles that may increase the probability of oxidative stress mediated DNA mutation [36], induced autophagy can help cancer cells to face adverse situations such as the metabolic stress due to hypoxia and hyponutrition or the damaged provoked by anticancer treatments [37, 38]. In addition, the upregulation of autophagy may switch cancer cells into a dormant state, thus posing the basis for tumour relapse [39–41].

Many oncogenes and oncosuppressors regulate autophagy [42]. In general, oncogenes (e.g., *AKT, BCL2*) tend to repress autophagy, though for some of them (e.g., *RAS*) the final effect is cell context dependent [43–46]. It has been proposed that the abnormal expression of oncogenes favours the induction of prosurvival autophagy in cancer cells experiencing a metabolic stress. By contrast, oncosuppressors (e.g., PTEN, TSC1/TSC2, and DAPk) positively regulate autophagy and thus their lack reduces or abrogates the level of basal and inducible autophagy. Consistently, loss of function of the oncosuppressors Beclin-1 [47, 48] or PTEN [49, 50] predisposes to spontaneous cancers. The role of the oncosuppressor p53 in the regulation of autophagy in cancer cells appears ambiguous: while nuclear DNA-binding proficient p53 promotes the transcription of certain autophagy genes [51], p53 mutants that reside in the cytoplasm hamper autophagy [52, 53].

Besides, microenvironmental factors (hypoxia, pH, oxidative stress, nutrient availability, cytokines, hormones, and growth factors) and the physical-metabolic interaction of tumour cells with surrounding cells (inflammatory cells, fibroblasts) in the matrix greatly influence the actual level of autophagy in the cancer cells [54–56].

4. Ovarian Cancer Genesis and Progression: The Potential Role of MicroRNAs and of Autophagy

4.1. Involvement of Autophagy in the Pathogenesis of Ovarian Cancer. Based on the traditional view, ovarian tumours arise from subsequent metaplastic changes in the ovarian surface epithelium that lead to the development of four main histologic types: serous, endometrioid, mucinous, and clear cell (for a review see, [57]). More recently, the correlation of clinicopathological features with genetic studies has suggested a new paradigm for the pathogenesis and origin of epithelial ovarian cancer based on a dualistic model of carcinogenesis that classifies ovarian cancer in two types [58]. Type I tumours comprise low grade serous and endometrioid carcinomas, clear cells, and mucinous carcinomas which develop in a stepwise fashion from well-defined precursor lesions. They are indolent and relatively genetically stable, being characterized by a variety of somatic mutations or amplification/deletion of oncogenes or oncosuppressors including *K-RAS*, *B-RAF*, and *PTEN* [59, 60]. In contrast, type II tumours comprise high-grade serous and endometrioid carcinomas, malignant mixed mesodermal carcinomas, and undifferentiated carcinomas; they are rapidly growing and highly aggressive. Type II tumours are chromosomally unstable and express mutated *TP53* in more than 95% of the cases and *BRCA* inactivation in up to 50% of high-grade serous tumours (for a review see [61]). Besides these genetic abnormalities, also epigenetic alterations in the expression of critical genes may occur during cancer progression, and these changes are reflected in the signalling pathways that govern cell proliferation, cell migration, dormancy, and chemoresistance. At least 15 oncogenes and 16 oncosuppressor genes have been found deregulated in ovarian cancers because of genetic or epigenetic alterations [62–64]. Many of these oncogenes and oncosuppressors have also been involved in the regulation of autophagy [65]. Indeed, there is experimental evidence linking autophagy to ovarian cancer genesis. For instance, poorly differentiated and highly malignant ovarian cancer cells were shown to express very low level of the autophagosomal marker LC3, compared to benign hyperplastic tissues and borderline ovarian tumours [66]. The expression of the oncosuppressor *BECN 1*, which activates PI3k III-dependent autophagy (see above), was found downregulated in ovarian cancers, compared to benign lesions [66]. Also *DRAM* (damage-regulated autophagy regulator) 2, a p53-transcribed gene that positively regulates autophagy [67], was found to be expressed at very low level in aggressive ovarian tumours [68]. As many as 60 to 80% of both sporadic and familial ovarian cancers have been shown to bear mutations and deletions of the oncosuppressor *TP53* gene [64, 69, 70]. Deletion of *TP53* could favour high level of basal autophagy [71], whereas DNA-binding deficient p53 mutants, which are found in human ovarian carcinomas [72], are unable to sequester BCL-2 or BCL-XL and indirectly could inhibit autophagy [53]. On the other hand, the hyperactivation of mTOR, which results in suppression of basal autophagy, was associated with a poor prognosis in ovarian carcinoma patients [73]. Taken together, it seems that ovarian carcinogenesis associates with insufficient autophagy. Another interesting gene linking autophagy and ovarian cancer is the aplasia ras-homolog member 1 (*ARH1*; also known as *DIRAS3*), which codes for a ras-homolog 26 kDa GTPase. The expression of *ARHI* correlates with prolonged progression-free survival and has been found downregulated in more than 60% of ovarian cancers [74, 75]. *ARH1* is an imprinted oncosuppressor gene (one allele is inherited in a hypermethylated form), and therefore one single event (deletion, mutation, or epigenetic silencing) affecting the functioning allele is sufficient to cause the loss of function [76, 77]. ARH1 protein has recently been shown to modulate autophagy and dormancy in ovarian cancer cells [40]. It was shown that reactivation of *ARHI* by stromal factors could rescue dormant ovarian cancer cells through modulation of autophagy [40].

4.2. Modulation of Autophagy by MicroRNAs. Considering the implications of both miRNAs and autophagy in cancer-related processes and given the lack of current evidence linking these two rapidly growing fields of research, we prompted to review miRNAs regulating autophagy.

Recently, Jegga et al. used a system biology-based approach to define the complex regulatory and functional networks of genes controlling the autophagy-lysosomal pathway and found miR-130, miR-98, miR-124, miR-204, and miR-142 as putative posttranscriptional regulators of this pathway at various levels [78].

In principle, autophagy could be regulated by miRNAs targeting the mRNA of key molecules that indirectly induce or suppress autophagy, as, for instances, miR-504 that negatively regulates p53 [79] or miR-20b that negatively regulates the expression of HIF-1α [80] or any miRNA implicated in the regulation of the PI3k-(PTEN)-AKT-mTOR pathway as is, for instance, the case of miRNAs targeting PTEN [81]. More recently, miRNAs specifically targeting the mRNA of autophagy proteins are being identified [82]. For instance, members of the miR30 family can target Beclin-1, ATG2, ATG5, and ATG12 [83, 84]; miR-130a targets ATG2B [85]; mi-R181a-1 targets ATG5 [86, 87]; miR-290-295 targets ATG7 and ULK1 [88]; miRNA-17 and miR-119a-5p target ATG7 [89, 90]; miR376b targets ATG4 and BECLIN-1 [91]; miR-630 targets ATG12 [86]; and miR-519 targets Beclin-1, ATG10, and ATG16L1 [86].

Here, we will focus on those miRNAs that are either up- or downexpressed in ovarian cancers and that potentially regulate autophagy.

4.3. MicroRNAs Aberrantly Expressed in Ovarian Cancer. Comparative miRNAs expression profiling of ovarian cancer

Table 1: (a) Autophagy-related genes and their function identified as targets of the microRNA involved in ovarian cancer development and progression (miRanda release, August 2010; TargetScan release 6.2.). (b) Genes coding for autophagy-regulating molecules identified as targets of the microRNA involved in ovarian cancer development and progression (miRanda release, August 2010; TargetScan release 6.2.).

(a)

miRNAs (involved in ovarian cancer progression)	Predicted autophagy Target Genes	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-141 hsa-miR 200a	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
hsa-miR-199a -5p	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
	ATG4D	Processing of MAP1-LC3	Yes	Yes
	BECN1	BCL-2 interacting myosin/moesin-like coiled-coil protein 1, part of the class III PtdIns 3-kinase complex (activating macroautophagy)	Yes	Yes
hsa-miR-214	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
hsa-miR-182	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
	ATG16L1	A component of the ATG12-ATG5-ATG16 complex for the formation of autophagosome	Yes	Yes
	MAP1LC3B	Microtubule-associated protein 1 light chain 3, precursor of LC3-II inserted in autophagosomal membranes	Yes	Yes
hsa-miR-140-5p	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
hsa-miR-125b	*UVRAG*	Interacting with Beclin-1 and Bif-1 (activation and stimulation of macroautophagy)	Yes	Yes
hsa-miR-34a	*ATG4B*	Processing of MAP1-LC3	Yes	Yes
	ATG9A	A transmembrane protein involved in lipid transport for phagophore expansion	Yes	Yes
hsa-let-7a	*ATG4B*	Processing of MAP1-LC3	Yes	Yes
	ATG9A	A transmembrane protein involved in lipid transport for phagophore expansion	Yes	Yes
	ATG16L1	A component of the ATG12-ATG5-ATG16 complex for the formation of autophagosome	Yes	Yes
hsa-miR-15a hsa-miR-15b	*ATG13*	A component of the ULK1 complex needed for ULK1 kinase activity	No	Yes
	ATG9A	A transmembrane protein involved in lipid transport for phagophore expansion	Yes	Yes
	ATG14L	A component of the class III PtdIns 3-kinase complex	No	Yes
hsa-miR-210	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
hsa-miR-449b	*ATG4B*	Processing of MAP1-LC3	Yes	Yes

(b)

miRNAs (involved in ovarian cancer progression)	Predicted autophagy Target genes	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-141 hsa-miR 200a	*PTEN*	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR 200b hsa-miR 200c	*PTEN*	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
hsa-miR 21	*TSC1*	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
	BCL2	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes

(b) Continued.

miRNAs (involved in ovarian cancer progression)	Predicted autophagy Target genes	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-125b	*UVRAG*	Interacting with Beclin-1 and Bif-1 (activation and stimulation macroautophagy)	Yes	Yes
	BCL2	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes
hsa-miR-101	*MTOR*	Mammalian target of rapamycin (kinase) component of MTORC1 (that inhibits autophagy) and of MTORC2 (that phosphorylates Akt)	No	Yes
	RAB5A	Endocytic vesicle associated ras-homolog GTPase (involved in autophagosome formation)	Yes	Yes
hsa-miR-31	*RAB1B*	Endocytic vesicle associated ras-homolog GTPase (involved in autophagosome formation)	Yes	Yes
hsa-miR-34a	*BCL2*	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes
has-let-7a	*TSC1*	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR-15a hsa-miR-15b	*BCL2*	Interactor of BECLIN 1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
	FKBP1A	An immunophilin that forms a complex with rapamycin and inhibits mTOR activity	Yes	Yes
hsa-miR-155	*PDK1*	Kinase that phosphorylates AKT in Thr308	Yes	Yes
	RPTOR	Regulatory associated protein of mTOR (component of MTORC1)	No	Yes
hsa-miR-99a hsa-miR-100	*MTOR*	Mammalian target of rapamycin (kinase) component of MTORC1 (that inhibits autophagy) and of MTORC2 (that phosphorylates Akt)	No	Yes
hsa-miR-449b	*BCL2*	Interactor of Beclin-1 (represses autophagy) and of BAX (represses apoptosis)	Yes	Yes

and normal ovary epithelium specimens has been performed in several laboratories and the readers can refer to some excellent comprehensive reviews [92, 93]. The laboratory of Carlo Croce first reported on the differential expression of some miRNAs between normal and cancer ovary epithelial tissues, showing an upregulation of miR-200a/b/c, miR141, miR-21, miR-203, and miR-205 and a downregulation of miR-199a, miR-140, miR-145, miR-222, and miR-125b1 [94]. In another study, miR-21 was found as the most upregulated and miR-125b as the most downregulated miRNA in ovary cancer versus normal ovary epithelium tissues [95]. However, a clear consensus on the diagnostic and prognostic value of a miRNA signature has not been reached yet. One study reported the complete downregulation of 44 miRNAs (including the oncosuppressive miR-15a, miR-34a, and miR-34b) and the upregulation of miR-182 in late-stage ovary cancers [96]. Another group found miR-199a, miR-214, and miR-200a as the ones most upregulated and miR-100 as the most downregulated miRNA in high-grade and late-stage ovary cancers [97]. Also miR-200a, miR-34a, and miR-449b were found downregulated in late-stage ovary cancers [98]. Late-stage ovary cancers are associated with the acquisition of chemotherapy resistance and metastasis formation, with the latter resulting from the phenotypic transformation known as epithelial-to-mesenchymal transition (EMT). A miRNA signature of the mesenchymal-like phenotype of epithelial ovary cancer was shown to include miR-141, miR-200, miR-29c, miR-101, miR-506, and miR-128 [99]. Further, the response to chemotherapeutics (e.g., Platinum) was found to be associated with a particular miRNA signature that includes let-7i [100], hsa-miR-27a, hsa-miR-23a, and miR-378 [98, 101].

In searching for the molecular pathways responsible for the metabolic and phenotypic alterations associated with a certain miRNA signature, it must be taken into account that one single miRNA can target the mRNA of multiple genes and that one single mRNA can have multiple target sequences for different miRNAs. Recently, another level of complexity in the global regulation of gene expression by miRNAs has emerged. It was in fact shown that the overexpression of certain miRNAs could indirectly regulate the level of other miRNAs in ovarian cancer cells [102].

4.4. Regulation of Autophagy by MicroRNA Aberrantly Expressed in Ovarian Cancer. As stated above, the modulation of autophagy by environmental stressful conditions (nutrient depletion, hypoxia, oxidative stress, and

Table 2: Genes coding for proteins involved in the autophagy pathway identified as targets of microRNA involved in the cytotoxic response to cis-Platinum in ovarian cancer (miRanda release, August 2010; TargetScan release 6.2.).

miRNAs (involved in cis-Pt response)	Predicted gene(s) involved in autophagy	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-27a	*PDK1*	Kinase for the phosphorylation of AKT in Thr308	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR-23a	*UVRAG*	Interacts with Beclin-1 and Bif-1 (activation and stimulation macroautophagy)	Yes	Yes
	ATG12	A ubiquitin-like protein that modifies (autophagosome expansion)	Yes	Yes
	BCL2	Interactor of Beclin-1 (represses autophagy) and of Bax (represses apoptosis)	Yes	Yes
	PTEN	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
	RAPTOR	Regulatory associated protein of mTOR (component of MTORC1)	No	Yes
hsa-miR-378	—			
hsa-let-7i	*ATG4B*	Processing of MAP1-LC3	Yes	Yes
	ATG16L1	A component of the ATG12-ATG5-ATG16 complex for the formation of autophagosome	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes

chemotherapeutic drugs) and/or by genetic and epigenetic hints may confer resistance to the chemotherapeutic treatments in cancer cells and may also favour the EMT and metastasization of cancer cells [103]. MiRNAs could contribute to the modulation of autophagy in these situations. For instance, the treatment with cisplatin could induce chemoresistance-promoting autophagy through the downregulation of certain miRNAs targeting ATG proteins or the pathways that control autophagy. As an example, miR-214 was shown to confer cisplatin resistance in ovarian cancer cells by targeting PTEN [97], and PTEN is known to positively regulate autophagy [104]. PTEN expression is posttranscriptionally regulated by a set of miRNAs [81, 97, 105]. In ovarian cancers, overexpression of miR-21 correlated with late stage and metastasis and significantly decreased the expression of PTEN [106].

We have made an "in silico" search of the *ATG* genes that are potential target candidates of the most relevant miRNAs found aberrantly expressed in ovary cancers. In Table 1 we report the results obtained using two algorithms for the prediction of microRNA gene targets, namely, the "TargetScanHuman" [107] and the miRanda [108] software. We have considered three different sets of miRNAs: in Tables 1(a) and 1(b) are reported the miRNAs that were found aberrantly expressed (either up- or downregulated with respect to the normal ovary epithelium) in ovarian cancers and that are possibly involved in ovarian tumorigenesis and progression; in Table 2 are reported the miRNAs that apparently play a role in chemoresistance; in Table 3 are reported the miRNAs that were found involved in the epithelial-to-mesenchymal transition of the phenotype. For clarity, in Table 1 we have separately described the *ATG* genes coding for ATG proteins (a) and the genes coding for signalling molecules that directly or indirectly control the induction and progression of autophagy. (b) In the tables, we also describe the function of the proteins coded by the genes predictably targeted by the miRNAs. In general, the two algorithms agreed in the identification of *ATG* target genes for most of the miRNAs of interest. The main discordances between miRanda and TargetScan were relative to the recognition of ATG14L as target of miR-21, miR-214, miR-140, miR-15a, and miR15b, and of ATG13 as a target of miR-15a and miR15b.

For some of these miRNAs the *ATG* gene target has been validated in tumours other than ovarian cancer. Although these data should be considered with caution due to the possible context and tissue specificity of miRNA regulation, we can assume that some available information can be applied also to ovarian cancer. For instance miR-101, reported to act as inhibitor of autophagy in breast cancer by targeting STMN1, RAB5A, and ATG4D mRNAs [109], has been found downregulated also in ovarian cancer compared to normal tissue, and its reexpression exerted tumour-suppressive effects in ovarian carcinogenesis [110]. Of note, stathmin overexpression showed a significant association with poor prognosis in ovarian cancer patients [111]. In keeping with the potential of miR-101 to regulate autophagy and ovarian cancer progression, it is to be mentioned that its target RAB5A was shown to be upregulated and to promote cell

Table 3: Genes coding for proteins involved in the autophagy pathway identified as targets of the microRNA involved in the epithelial-to-mesenchymal transition process in ovarian cancer (miRanda release, August 2010; TargetScan release 6.2.).

miRNAs (involved in EMT)	Predicted gene(s) involved in autophagy	Function	Target prediction	
			miRanda	TargetScan
hsa-miR-141 hsa-miR 200a	*ATG7*	A ubiquitin-activating (E1) enzyme homolog that activates both ATG8/LC3 and ATG12	Yes	Yes
	PTEN	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes
hsa-miR 29c	*ATG14L*	A component of the class III PtdIns 3-kinase complex	No	Yes
	PTEN	Protein/lipid phosphatase that reduces the level of PIP3, thus limiting the activation of AKT	Yes	Yes
hsa-miR-101	*MTOR*	Mammalian target of rapamycin (kinase) component of MTORC1 (that inhibits autophagy) and of MTORC2 (that phosphorylates Akt)	No	Yes
	RAB5A	Endocytic vesicle associated ras-homolog GTPase (involved in autophagosome formation)	Yes	Yes
hsa-miR-506	—			
hsa-miR-128	*PDK1*	Kinase that phosphorylates AKT in Thr308	Yes	Yes
	TSC1	Tuberose Sclerosis Complex component that negatively regulates mTOR	Yes	Yes

proliferation in ovarian cancer [112]. Also, miR-30a, which negatively regulates the expression of Beclin-1 in ovarian cancer cells [113], was found deregulated in stage I ovarian cancer patients together with other miRNAs; in particular, it was downregulated in samples from relapsing patients [114, 115]. This result is in line with possible involvement of miR-30a in autophagy-dependent chemoresistance in ovarian cancers.

5. Conclusion: Clinical Implications and Future Perspectives

Modulation of autophagy has a great impact on the carcinogenesis process. In fact, depending on whether it is considered at the precancerous or at the advanced stage, up- or downregulation of autophagy may elicit either tumour-promoting or tumour-suppressive effects [116, 117]. The actual level of ongoing autophagy in the tumour cells is dictated by genetic mutations but also influenced by the epigenetic regulation of gene expression [65, 103]. In the context of the intricate involvement of autophagy in cancer progression, emerging data point to the role of miRNAs as regulators of autophagy gene expression. The immediate and acute modulation of protein expression mediated by miRNAs plays a fundamental role in the adaptive response of the cell metabolism to environmental stresses such as nutrient shortage, hypoxia, and genotoxic stress. Autophagy is one of the main stress response pathways. Therefore, the modulation of ATG proteins and/or of signalling molecules that regulate autophagy by miRNAs finally impacts the capability of the cell to overcome the stress. This aspect is of particular relevance when considering the cytotoxic response of cancer cells to a chemotherapeutic drug. Chemosensitivity could be rescued by manipulating the level of miRNAs targeting autophagy. In fact, certain miRNAs can target both the autophagy and the apoptosis pathways and therefore can influence the cross-talk between these two processes and determine whether the cancer cell will resist or succumb to the toxic drug. For instance, miR-199a-5p was shown to increase chemoresistance by simultaneously promoting autophagy and suppressing apoptosis. By downregulating Beclin-1 expression, miR-30a and miR-376b downregulate not only autophagy but also apoptosis since the level of free antiapoptotic BCL-2 protein in the cell will increase. Thus, miRNAs can act as molecular switches to turn on or off either or both of the autophagy and apoptosis processes. These findings provide the rationale for designing novel therapeutic approaches combining the conventional anticancer drugs with miRNAs targeting the autophagy process.

Autophagy is clearly deregulated in ovarian cancer (reviewed in [65]), and here we have highlighted the possibility that the miRNAs aberrantly expressed in ovarian cancer could be involved in such deregulation.

The miRNA landscape of ovarian cancer is in rapid progress [118] and advance in detection and functional evaluation of miRNAs is expected to strongly contribute to unravelling the network of apoptosis and autophagy regulation in this complex disease. In the near future, studies ongoing in our and other laboratories will likely identify the miRNA signatures associated with autophagy in ovarian cancer, thus posing the basis for the possible harnessing of these miRNAs as therapeutic targets, as well as possible diagnostic-prognostic tools.

Conflict of Interests

The authors declare that the present study was performed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interests.

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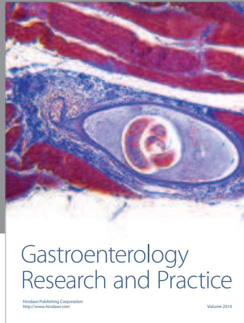

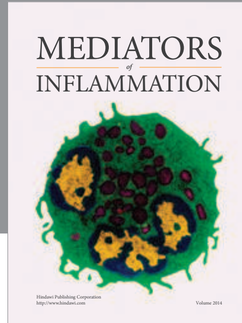

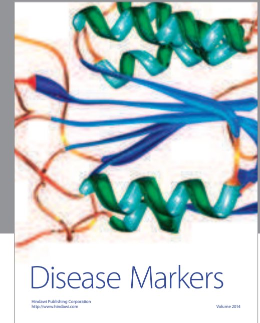

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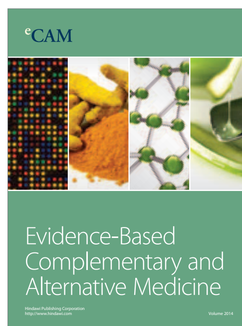

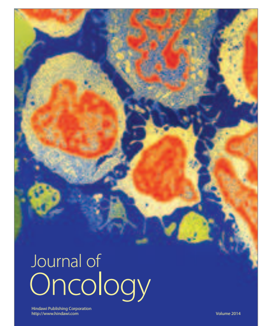

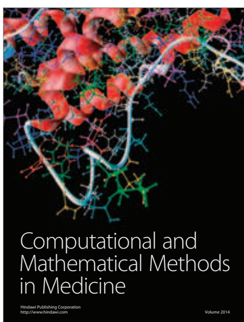

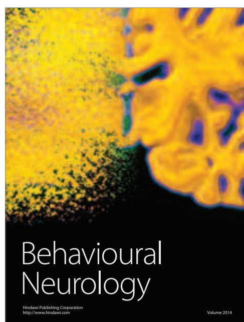

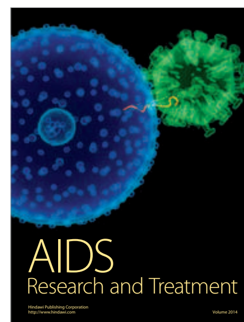

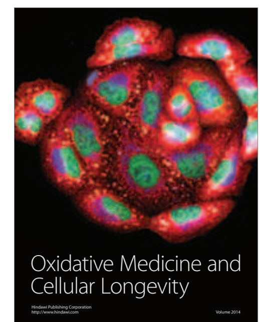

2. Single Amino Acid Arginine Deprivation Triggers Prosurvival Authophagic Response in Ovarian Carcinoma SKOV3

Galyna Shuvayeva, Yaroslav Bobak, Natalia Igumentseva, Rossella Titone, Federica Morani, Oleh Stasyk and Ciro Isidoro

Arginine plays an important role in the production of proteins, nitric oxide, polyamines, nucleotides, proline, and glutamate. The endogenous production of arginine is not sufficient for rapidly proliferating cells such as tumor cells, which require exogenous arginine to support their growth and survival. In this report we demonstrate that single amino acid arginine deprivation triggers profound prosurvival autophagic response in cultured human ovarian cancer SKOV3 cells. In fact, a significant drop in viability of arginine-starved SKOV3 cells was observed when autophagy was inhibited by either coadministration of chloroquine or transcriptional silencing of the essential autophagy protein BECLIN 1. Enzymatic arginine deprivation is a novel anticancer therapy undergoing clinical trials.

Personal Contribution

In this paper, I contributed to the assessment of autophagy by immunofluorescence (replicates of Figures 3 and 4; additional experiments not shown in the paper).

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Research Article

Single Amino Acid Arginine Deprivation Triggers Prosurvival Autophagic Response in Ovarian Carcinoma SKOV3

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Autophagy is a process of cytosol-to-lysosome vesicle trafficking of cellular constituents for degradation and recycling of their building blocks. Autophagy becomes very important for cell viability under different stress conditions, in particular under amino acid limitation. In this report we demonstrate that single amino acid arginine deprivation triggers profound prosurvival autophagic response in cultured human ovarian cancer SKOV3 cells. In fact, a significant drop in viability of arginine-starved SKOV3 cells was observed when autophagy was inhibited by either coadministration of chloroquine or transcriptional silencing of the essential autophagy protein BECLIN 1. Enzymatic arginine deprivation is a novel anticancer therapy undergoing clinical trials. This therapy is considered nontoxic and selective, as it allows controlling the growth of malignant tumours deficient in arginine biosynthesis. We propose that arginine deprivation-based combinational treatments that include autophagy inhibitors (e.g., chloroquine) may produce a stronger anticancer effect as a second line therapy for a subset of chemoresistant ovarian cancers.

1. Introduction

It is established that some types of tumours are deficient in the biosynthesis of certain amino acids and often exhibit elevated sensitivity to deprivation of a corresponding single amino acid (such as arginine, methionine, and asparagine), both *in vitro* and, importantly, *in vivo* (for recent reviews: [1–5]). This provided a rational basis for the development of metabolic anticancer therapies based on the application of recombinant amino acid degrading enzymes, such as asparaginase for the treatment of leukemias and other tumours [2, 5, 6]. First clinical trials with recombinant enzymes hydrolyzing amino acid arginine, human arginase I, and *Mycoplasma hominis* arginine deiminase have demonstrated therapeutic efficacy in controlling the growth of hepatocarcinomas and melanomas [7–9]. Recent *in vitro* studies also suggested that other types of cancers may be potentially sensitive to this therapy (pancreatic, prostate, renal carcinomas, and mesotheliomas) due to the transcriptional silencing of arginine anabolic enzyme of urea cycle, argininosuccinate synthetase (ASS) [10–13] (see Figure 2). It was also observed that the development of chemoresistance to platinum compounds in ovarian carcinomas leads to collateral appearance of arginine auxotrophy due to the downregulation of ASS [14], adding these tumours to the list of potential targets of arginine deprivation-based enzymotherapy.

Although metabolic enzymotherapy based on arginine deprivation is considered as nontoxic and selective, it is not free of certain limitations. One such limitation arises from the upregulation of ASS expression in many tumours in response to arginine starvation, leading to the appearance of the ASS-positive tumour relapse insensitive to the therapy [2]. Also, we recently observed that tumour cells become profoundly more resistant to arginine withdrawal in *in vitro* 3D spheroid models relative to respective monolayer cultures [15, 16]. This phenomenon is consistent with the results of animal studies and ongoing clinical trials which showed that arginine deprivation is effective in inhibiting tumour growth but not

in inducing tumour regression. The latter observation stimulates further search for more efficient rational combinational therapeutic approaches based on arginine deprivation.

Arginine, besides being required for protein biosynthesis, has other versatile functions in the cell as a precursor of nitric oxide, agmatine, and polyamines [17]. It was also demonstrated that arginine is an essential amino acid for cultured tumour cells due to their deficiency in arginine biosynthesis *de novo* [18]. Thus, arginine withdrawal profoundly affects tumour cell physiology. In this work we show that arginine deprivation strongly induces the autophagic process in ovarian carcinoma cells in monolayer culture. Autophagy, the selective process of lysosomal recycling of cell constituents, is known to have a prosurvival role under different stresses in tumour cells [19]. Therefore, we addressed the question whether inhibition of autophagy affects tumour cell survival upon arginine starvation. Such a strategy could be applied to enhance the therapeutic effect of enzymotherapy based on arginine deprivation.

2. Materials and Methods

2.1. Reagents. The following antibodies were used: polyclonal antibodies against MAP-LC3 (Novus Biologicals, CO, USA) and BECLIN 1 (BD Biosciences, CA, USA), mouse monoclonal anti-LAMP1 (BD) and anti-Golgin97 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), ph-4E-BP1 and ph-p70-S6k (Cell Signaling Technology, Beverly, MA, USA), FITC-conjugated polyclonal goat anti-rabbit (Santa Cruz) and Cy3-conjugated polyclonal goat anti-mouse (Santa Cruz), and horseradish peroxidise- (HRP-) conjugated polyclonal goat anti-mouse and anti-rabbit (both from Millipore Corporation, Bedford, MA, USA).

Monodansylcadaverine (MDC), 3-methyladenine (3MA), chloroquine (CQ), asparagine (Asn), and other bench chemicals were purchased from Sigma-Aldrich.

2.2. Cell Line and Culture Conditions. SKOV3 cells originating from human ovarian carcinoma tissues were obtained from ATCC (USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone Laboratories, Logan, Utah, USA) with 10% foetal bovine serum (FBS; PAA Laboratories GmbH, Pasching, Austria), 2 mmol/L glutamine, and 50 mg/L gentamycin (Sigma-Aldrich, Steinheim, Germany) and maintained in the incubator at 37°C with 5% CO_2. Where indicated, arginine-containing (0.4 mM; HyClone Laboratories, Logan, UT, USA) and arginine-free media were supplemented with 5% dialysed FBS (HyClone). To study the growth dynamics of ovarian carcinoma cells under standard and arginine-deprived conditions, the cells were seeded at a density of 20000 cells per well in regular medium in 96-well plates and allowed to adhere for 24 h; then the medium was aspirated and the cell monolayer was washed two times with PBS, and finally the cells were incubated with fresh complete medium or arginine-free medium (AFM). The cells were cultured for up to 96 h, and cell growth was assessed by counting the cells every 24 h in triple. Cell viability was assessed using the trypan blue (final concentration 0.05%) dye exclusion. Viable (unstained) and nonviable (blue-stained) cells were counted on a haemocytometer by light microscopy.

2.3. RT-PCR. Total RNA was isolated from cells by the method of Chomczynski and Sacchi [20]. First-strand cDNA synthesis was performed using First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and an oligo-dT primer according to the manufacturer's instructions. PCR was performed using a High Fidelity PCR Enzyme Mix (Fermentas) with the following primer pairs:

ASS-S, 5′-GGGGTCCCTGTGAAGGTGACC-3′;

ASS-AS, 5′-CGTTCATGCTCACCAGCTC-3′;

ASL-S, 5′-GAAGCGGATCAATGTCCTGC-3′;

ASL-AS, 5′-CTCTTGGTGAATCTGCAGCG-3′;

OTC-S, 5′-AATCTGAGGATCCTGTTAAACAATG-3′;

OTC-AS, 5′-CTTTTCCCCATAAACCAACTCA-3′;

GAPDH-S, 5′-CAAGGTCATCCATGACAACTT-TG-3′;

GAPDH-AS, 5′-GTCCACCACCCTGTTGCTGTA-G-3′.

PCR fragments were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The relative mRNA expression levels were estimated after normalization with GAPDH. The number of cycles was chosen at which PCR product amount was optimal and within the linear portion of the curve, well before saturation point.

ASS: number of cycles—26, size of the amplicon—448 bp,

ASL: number of cycles—27, size of the amplicon—502 bp,

OTC: number of cycles—40, size of the amplicon—1125 bp,

GAPGH: number of cycles—25, size of the amplicon—496 bp.

2.4. Visualization of Monodansylcadaverine-Labelled Vacuoles. MDC is an autofluorescent weak base that accumulates in acidic lysosomal vacuoles and autophagolysosomes [21]. Cells attached to glass coverslips were incubated with 0.05 mM MDC (Sigma-Aldrich) in PBS at 37°C for 10 min. After incubation, cells were washed three times with PBS and immediately analyzed with a fluorescence microscope (ZEISS, Axio Imager A1) equipped with Axio Vision Software (v. 4.6.3). Images were captured with a CCD camera and imported into Photoshop. Quantification of cell fluorescence was conducted using ImageJ 1.48v Software.

2.5. Immunofluorescence and Microscopy Analysis. Immunofluorescence staining was performed as previously described [22]. Essentially, cells cultured on glass coverslips were washed with PBS, fixed with cold methanol, and permeabilized with 0.2% Triton X-100 in PBS. The coverslips were then incubated with the indicated primary antibodies in PBS contained 0.1% Triton X-100 and 4% FBS overnight at 4°C and thereafter incubated with the appropriate secondary antibodies for 1 hr at room temperature. Nuclei were stained with DAPI. Coverslips were mounted on microscope slides and monitored under a ZEISS fluorescence microscope (Axio Imager A1) equipped with Axio Vision Software (v. 4.6.3). Images were captured with a CCD camera and imported into Photoshop. Pearson correlation coefficients were calculated using ImageJ 1.48v Software to assess the degree of colocalization of protein markers of autophagy.

2.6. Immunoblotting. The cell monolayer was washed with ice-cold PBS and lysed in Extraction Buffer (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 5 mmol/L EDTA, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 5 mmol/L benzamidine, 1 mmol/L PMSF, 10 mg/mL aprotinin, 10 mg/mL Leupeptin, and 1 mg/mL Pepstatin A) at 4°C for 20 min. Cell extracts were obtained after centrifugation at 12,000 g at 4°C for 30 min and cellular proteins were quantified using Peterson's method [23]. Equal amounts of protein homogenates were loaded, separated by SDS-PAGE (concentration of acrylamide varied depending on the size of the protein to be detected), and transferred onto PVDF membrane (Millipore Corp., Billerica, MA, USA). The membranes were blocked with 5% nonfat dried milk in PBS containing 0.05% Tween-20 and probed with the indicated primary and secondary (horseradish peroxidase-conjugated) antibodies. β-Actin was used for protein loading control. The bands were visualized using the enhanced chemiluminescence reagent (Millipore Corp.). Band densitometry quantification was performed using the Gel-Pro analyzer (Version 32).

2.7. Small Interfering RNA Transfection. BECLIN 1 silencing was achieved using RNA interference as previously described [24]. Cells were transfected using Oligofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. Protein knockdown was determined by immunoblotting 48 hr after transfection. As control (sham transfection), a nonspecific scramble sequence siRNA was used.

2.8. Statistics. In each individual experiment triplicate wells were used for each treatment and control. All experiments were repeated at least three times. Statistical analyses were performed using Student's t-test. Results were expressed as means ± SD. Significance was established when the P value was less than 0.05.

3. Results

3.1. SKOV3 Cells Retain Viability and Proliferative Potential under Long-Term Arginine Deprivation In Vitro. Tumour cell lines in monolayer cell culture substantially differ in

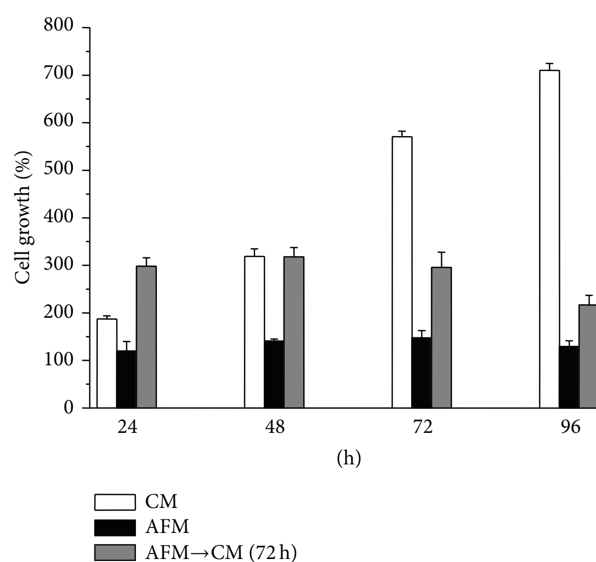


Figure 1: Effect of arginine deprivation on SKOV3 cell proliferation. The growth of SKOV3 cells in arginine-sufficient complete medium (CM), in arginine-free medium (AFM), and the ability of SKOV3 cells to rescue cell proliferation after different periods of arginine starvation was assessed by counting only the viable cells. After the indicated periods of incubation in AFM, cells were shifted to a fresh CM and allowed to proliferate for additional 72 h (AFM → CM (72 h)). Viable cells were determined by the trypan blue exclusion test. The initial number of cells (time-point zero) was considered as 100%. Data from three independent experiments in triplicate.

their sensitivity to a single amino acid withdrawal [18]. Therefore, we first analyzed whether and to what extent arginine deprivation affects the viability and the proliferative potential of SKOV3 cells. Upon shifting to a defined arginine-deficient medium (AFM), growth arrest and reduction in the proportion of viable cells were observed. It is to be noted that, even after 4 days of arginine starvation, SKOV3 cells were still able to resume cell proliferation in response to arginine resupplementation, though regrowth potential progressively declined in the course of incubation in AFM (Figure 1). No signs of PARP fragmentation as a reporter of apoptosis in arginine-starved SKOV3 cells were observed (data not shown). This observation suggested that a substantial fraction of SKOV3 cells remained viable even after the prolonged arginine withdrawal indicating that these cells are rather resistant to this metabolic stress. For comparison, hepatocellular carcinoma HepG2 cells lose their proliferative potential already after 2 days of arginine starvation and exhibit concomitant apoptosis [18].

3.2. Arginine Is an Essential Amino Acid for Cultured SKOV3 Cells. The effect on growth arrest in AFM (Figure 1) suggested that arginine is an essential amino acid for SKOV3 cells. The RT-PCR analysis of arginine key anabolic enzymes of the urea cycle revealed that SKOV3 cells incubated in complete medium do not express mitochondrial arginine biosynthetic enzyme ornithine transcarbamylase (OTC; it converts ornithine to citrulline) but do express cytosolic

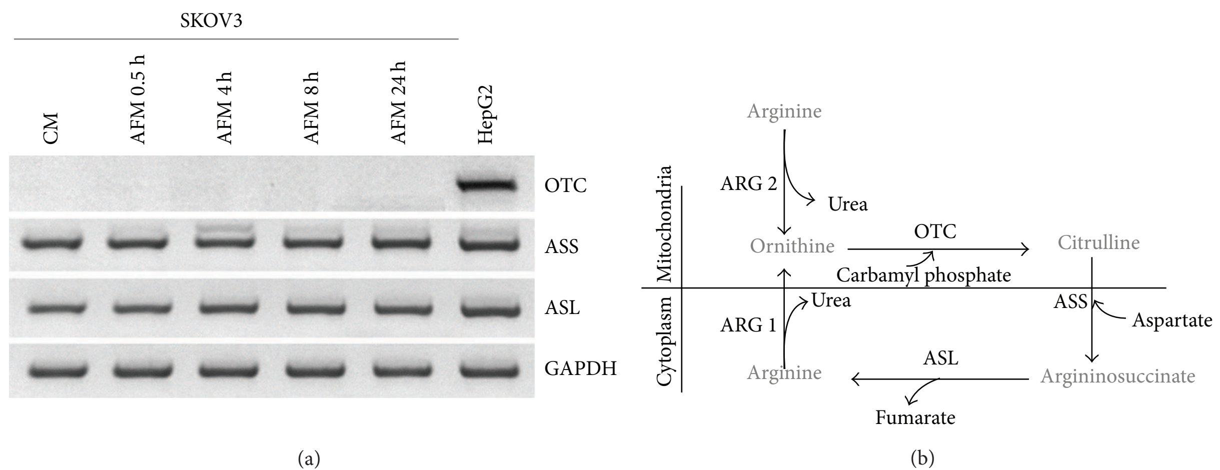


Figure 2: Expression of the key genes of arginine anabolism in SKOV3 cells. (a) Specific mRNA levels determined by RT-PCR analysis as described in Section 2. Cells were cultured in arginine-free medium (AFM) for 24 h or in arginine-sufficient complete medium (CM). Human hepatocarcinoma HepG2 cells (which express urea cycle enzymes) were used as a positive control. GAPDH expression was used as an internal loading control. (b) Scheme of arginine biosynthesis in the urea cycle. ARG1: arginase I, ARG2: arginase II, OTC: ornithine transcarbamylase, ASS: argininosuccinate synthetase, and ASL: argininosuccinate lyase.

argininosuccinate synthetase (ASS; it converts citrulline to argininosuccinate) and argininosuccinate lyase (ASL; it converts argininosuccinate to arginine) (Figures 2(a) and 2(b)). Arginine deprivation did not trigger an upregulation of ASS or ASL, often observed in other tumour cell lines, or induction of OTC transcription (Figure 2(a)). Human hepatocellular carcinoma HepG2 cells were used as a positive control [18]. OTC deficiency in cultured SKOV3 cells implies that they are deficient in endogenous arginine anabolism, and arginine can only be synthesized via ASS-mediated conversion of exogenously supplied citrulline (which is absent in standard DMEM medium). Accordingly, exogenous ornithine did not support proliferation of SKOV3 cells in AFM (data not shown). Therefore, we can assume that incubation in AFM surely induces arginine starvation in SKOV3 cells.

3.3. Arginine Deprivation in SKOV3 Cells Triggers Profound Autophagic Response. One of the prosurvival responses of tumour cells triggered upon amino acids limitation is the elevated intracellular protein recycling via autophagy [19]. In particular, arginine deprivation has been shown to induce autophagy in melanomas [25]. However, it is known that different tumour cells exhibit varying basal and stimulus-dependent inducible autophagic proficiency [26]. We addressed the question whether low sensitivity (in terms of cell survival and proliferation) of SKOV3 cells to arginine withdrawal as an essential amino acid (Figure 1) was associated with, or causally linked to, induction of autophagy. To monitor autophagy, we first employed a classical staining of acidic vacuoles, which includes lysosomes and autophagolysosomes, with the vital fluorescent dye MDC [21]. Arginine deprivation rapidly and profoundly induced the expansion of the autophagolysosomal compartment in SKOV3 cells (Figure 3). We observed MDC-labelled intracellular vacuoles in SKOV3 cells already after 30 min of arginine starvation. Treatment of the starved cells with 3-methyladenine (3MA, 10 mM), a classic inhibitor of autophagy that inhibits the formation of autophagosomes [22], strongly diminished the fluorescence signal (Figure 3). Upon treatment with chloroquine (CQ, 25 μM), another known inhibitor of autophagy [21], MDC staining of the cells further increased (Figure 3). This effect was expected, as CQ impairs the late stages of autophagy and leads to the accumulation of autophagosomes and of autophagolysosomes [21]. By contrast, a partial decrease in MDC staining in arginine-deprived SKOV3 cells was observed upon concomitant treatment with an excess of asparagine (Asn, 50 mM), which is known to downregulate autophagy through the stimulation of mTOR, a negative regulator of autophagy [21]. Quantification of cell fluorescence under arginine starvation and upon cotreatment with inhibitors using ImageJ 1.48v Software revealed an approximately four-time decrease in fluorescence between control arginine-starved and CQ-treated cells versus those treated with 3MA and Asn at 4 hours of incubation (data not shown). Taken together, the above data indicate that MDC staining is indeed mirroring the induction of autophagolysosomes in arginine-deprived SKOV3 cells. To definitively demonstrate the induction of autophagy, in a parallel experiment the cells cultured on coverslips were immunostained for LC3 (a hallmark of autophagosomes) and LAMP1 (a marker of lysosomes), as well as for BECLIN 1 (a component of the autophagy interactome) and Golgin97 (which labels the Golgi complex). Colocalization of LC3 and LAMP1 is a reliable indicator for the formation of autophagolysosomes, while formation of BECLIN 1 aggregates in the Golgi area is indicative of activation of the autophagy process [24].

We observed the colocalization of such signals (yellow fluorescence) already at 30 min of arginine deprivation (Figures 4(a) and 4(b)), indicating a fast upregulation of the autophagy process. Colocalization of autophagosome-

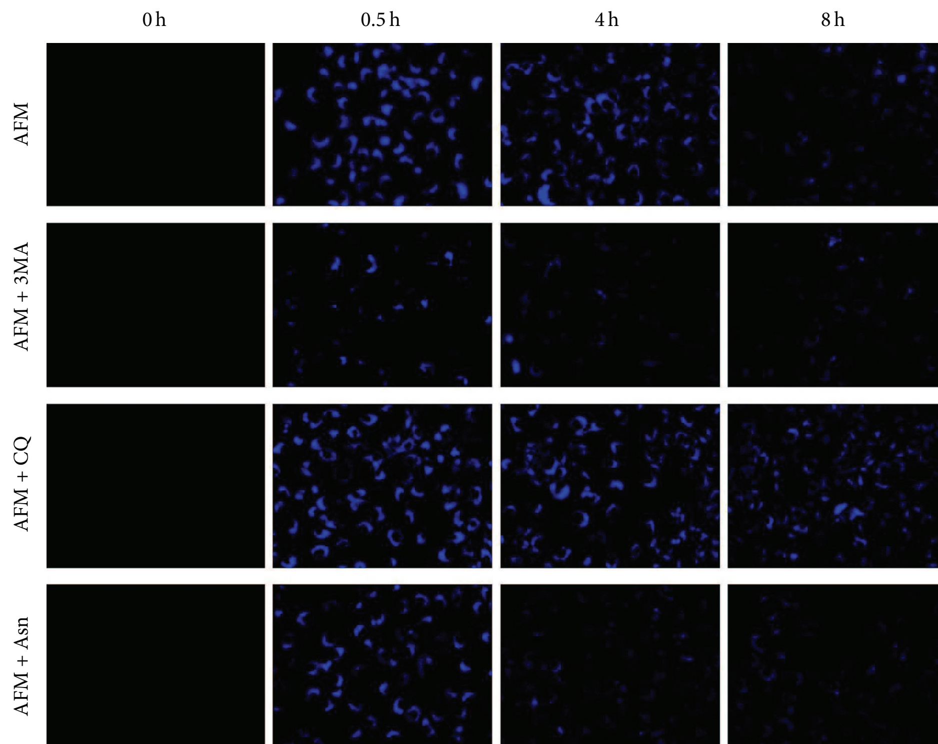
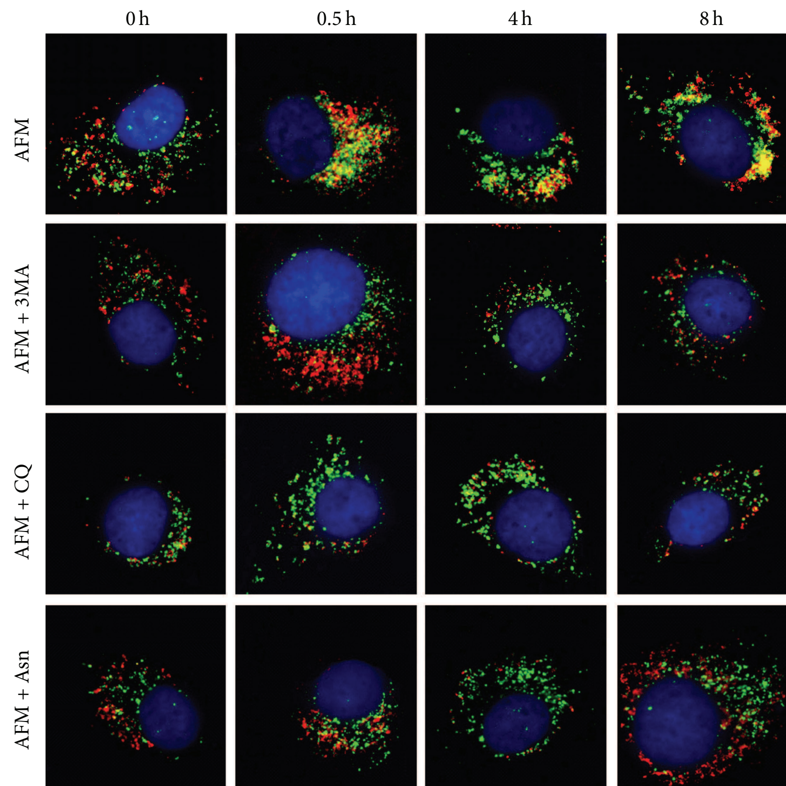


Figure 3: MDC staining of acidic vacuoles in ovarian carcinoma SKOV3 cells subjected to arginine deprivation (AFM). The cells were labelled with MDC as described in Section 2 at zero point and after 0.5, 4, and 8 hours of arginine withdrawal and immediately monitored under a fluorescence microscope. Magnification 400x.

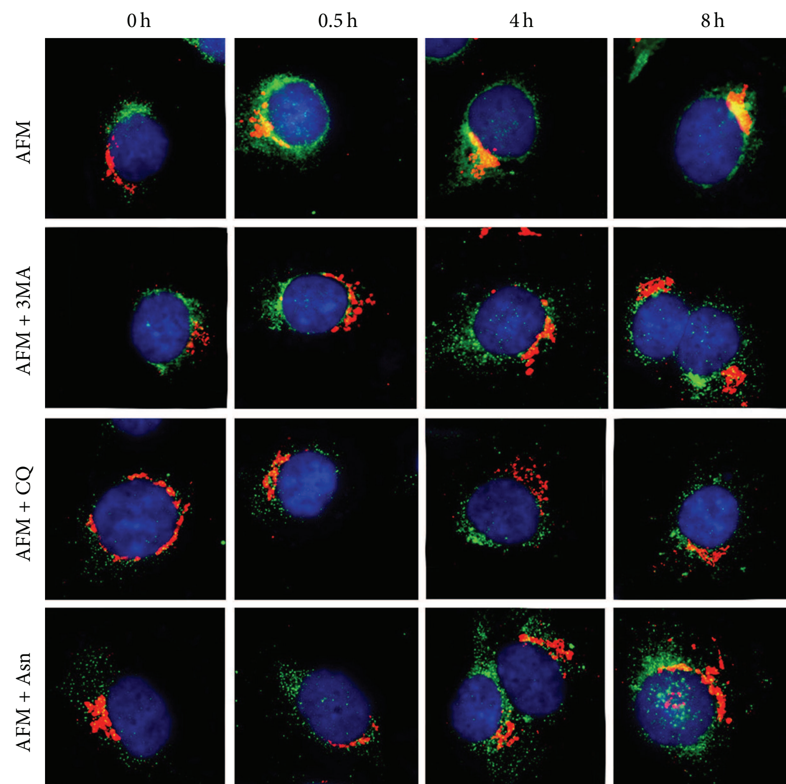
and lysosome-associated proteins was evident during the whole time course of our analysis (Figures 4(a) and 4(b)). Importantly, in agreement with the MDC data (Figure 3), 3MA and Asn reduced while CQ increased the number and the size of LC3-positive vacuoles (i.e., autophagosomes) and also the aggregation of BECLIN 1 in the Golgi area (Figures 4(a) and 4(b)). The calculated values of Pearson correlation coefficient for the pairs LC3/LAMP1 and BECLIN 1/Golgin97 were ≥0.5 for arginine-starved cells, whereas for cells treated with inhibitors (3MA, CQ, and Asn) the coefficients values were 0 to ≤0.2. From these data we conclude that arginine deprivation strongly induces an autophagic response in ovarian carcinoma SKOV3 cells.

3.4. Effects of Arginine Deprivation and Autophagy Impairment on mTOR-Dependent Biosynthesis Pathway. Amino acid starvation is known to inhibit the biosynthetic pathway and, in parallel, to induce the autophagy degradation of redundant protein as an attempt to rescue the amino acids needed for the synthesis of vital proteins. The mTOR kinase is placed at the cross-point and is a master regulator of both these pathways [27, 28]. To get an insight into the relationship between the induction of autophagy and the biosynthetic pathway under arginine-deprivation conditions, we assayed the activation status of 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) and of p70-S6K (ribosomal p70 S6 kinase), two downstream substrates of mTOR that direct protein synthesis [29], in the presence of CQ, that hampers the last step of autophagy. In fact, in the presence of CQ the consumption of autophagosomes is interrupted as witnessed by the induced accumulation of LC3II both in CM and in AFM conditions, and this accumulation increases with time of incubation (Figure 5). However, in the cells subjected to arginine deprivation the level of LC3I decreases with time of incubation despite the fact that no further increase in LC3II is observed, indicating a rapid autophagy flux and consumption of autophagosomes. When these cells were concomitantly exposed to CQ the autophagosomes in fact accumulated with time (as indicated by increased LC3II at 8 versus 2 h). Next we looked at the translational activity in SKOV3 cells cultivated under these conditions. While phosphorylation of 4E-BP1 elicits its inhibition and therefore reliefs the inhibitory action of 4E-BP1 on protein synthesis, the phosphorylation of p70-S6K elicits the activation of the translational process [29]. Thus, the phosphorylation of both 4E-BP1 and p70S6k converges on triggering the initiation of protein synthesis. Both 4EBP and p70-S6K were highly phosphorylated in the cells cultivated in CM in the presence of CQ, while their activation status was greatly reduced under AFM culture condition regardless of whether CQ was or was not present (Figure 5).

3.5. Effect of Autophagy Inhibition by Transcriptional Silencing of BECLIN 1 or with CQ on the Sensitivity of SKOV3 Cells to Arginine Starvation. To elucidate the role of autophagy in maintaining SKOV3 cell viability under arginine deprivation, we manipulated the autophagy pathway by genetic and pharmacologic approaches. Transient transfection with a specific



(a)



(b)

FIGURE 4: (a) Immunofluorescence staining of the autophagosomal protein LC3 (green fluorescence) and of the lysosomal protein LAMP1 (red fluorescence) and (b) of BECLIN 1 (green fluorescence) and Golgi-associated Golgin97 (red fluorescence) in SKOV3 cells subjected to arginine starvation. Nuclei were labelled with DAPI. Images were captured with ZEISS fluorescence microscope (Axio Imager A1) equipped with Axio Vision Software v. 4.6.3. Magnification 1000x.

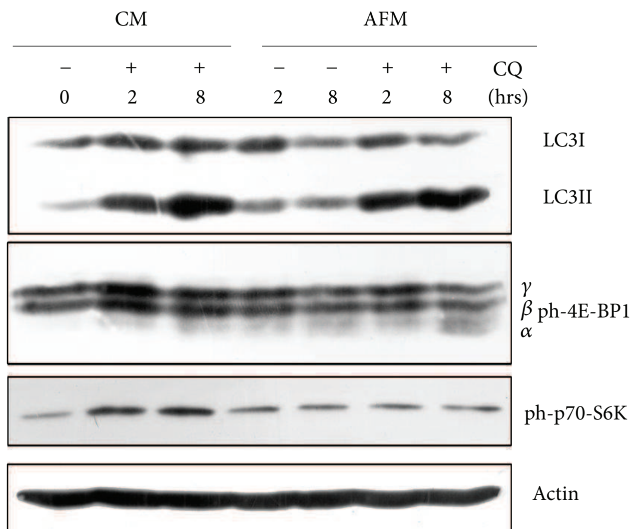


Figure 5: Effect of arginine deprivation and CQ treatment on accumulation of autophagosomal protein LC3II and phosphorylation of mTOR substrates. After indicated periods of treatment, the cells were washed and harvested for WB analysis. 50 μg of total sample protein was loaded per lane. β-Actin served as the loading standard.

siRNA elicited the transcriptional silencing (approximately 54%) of the autophagy protein BECLIN 1 (Figure 6(a)). After three days of incubation in AFM, the number of viable cells in siRNA-BECLIN 1 transfected culture decreased by approximately 40% as compared to the sham-transfected culture (Figure 6(b)). Arginine-deprived BECLIN 1-silenced cells also exhibited a decrease in their proliferative potential upon shift to the fresh CM relative to control cells (Figure 6(b)), thus suggesting a significant prosurvival role of autophagy in the cell response to arginine starvation.

Next, we addressed the question whether cotreatment with CQ, which impairs the late steps of the autophagy pathway, affects the survival of SKOV3 cells under arginine deprivation in a similar manner as observed with the silencing of BECLIN 1 expression. To this end, we monitored cell viability under the combined treatment and cell proliferation upon arginine resupplementation. The treatment with CQ (25 μM) dramatically decreased cell viability and the proliferative potential under arginine starvation (Figure 7). Importantly, CQ treatment rendered arginine-starved SKOV3 cells unable to resume proliferation in fresh CM already after two days of the combined treatment (Figure 7; cf. Figure 1). It is to be noted that a prolonged incubation with this same concentration of CQ was cytotoxic for SKOV3 cells also in CM medium (data not shown). No signs of apoptosis (as tested by western blotting of PARP and Annexin V staining) and no signs of senescence (as tested by positivity for beta-galactosidase staining) were detected in AFM plus CQ cotreated cells up to 144 h. However, SKOV3 cells double staining with ethidium bromide and Hoechst 33342 revealed that under this culture condition the majority of cells died via necrosis (not shown).

4. Discussion

Autophagy, a cytosol-to-lysosome membrane-trafficking process of degradation of cellular constituents, is a housekeeping homeostatic pathway and also plays a fundamental role to preserve cell viability upon different stress conditions [19]. One of such stresses is nutrient limitations, in particular amino acid restriction [30]. Autophagy is known to have dual function in cancerogenesis, playing both negative and positive roles in cancer progression and being implicated in chemoresistance of certain tumour types (for review: [26, 31–33]). It was also established that even single amino acid starvation triggers autophagic response in tumour cells [11, 34]. Previously reported [35] and our unpublished data suggest that the most profound autophagic response in tumour cells is triggered by starvation for arginine, methionine, lysine, and leucine relative to other amino acids. It remains to be elucidated whether specific regulation by this set of amino acids involves the mTORC1 complex or other mechanisms. Our data indicate that single arginine deprivation early affects the mTOR-dependent biosynthetic pathway.

Arginine, besides being required for protein biosynthesis, has other versatile functions in the cell as a precursor of nitric oxide, agmatine, and polyamines and as a regulatory molecule (for review: [17]). For cultured tumour cells arginine is an essential amino acid due to their deficiency in arginine biosynthesis *de novo* [18]. Ovarian carcinomas were recently added to the growing list of tumour types potentially sensitive to the treatment with recombinant arginine-degrading enzymes. In fact, it was demonstrated that relapses of ovarian carcinomas resistant to cisplatinum treatment concomitantly become deficient in argininosuccinate synthetase, a rate limiting enzyme of arginine biosynthesis, and thus potentially sensitive to arginine-degrading enzymes [14]. In this work we investigated how modulation of autophagy affects ovarian cancer cells viability under arginine deprivation. Human ovarian carcinoma SKOV3 cells were used as an experimental model. We found that SKOV3 cells exhibit high resistance to the stress exerted by arginine deprivation (Figure 1). This fact potentially allows studying the physiological role of autophagy under arginine withdrawal without interfering with processes of programmed cell death (apoptosis) that are often triggered to a different extent in cancer cells under such conditions [18]. Although SKOV3 cells exhibit high expression of argininosuccinate synthetase (as we show in Figure 2), they still are a suitable informative model for *in vitro* studies since SKOV3 cells are fully dependent on exogenous arginine supply due to the deficiency in ornithine transcarbamylase (OTC), an upstream enzyme of arginine anabolism (Figure 2). In this report we demonstrate that arginine withdrawal rapidly and markedly induces autophagy in SKOV3 cells (Figures 3 and 4). Under starvation, the biosynthetic pathway is impaired while basal autophagy rises up, and both these pathways are controlled by mTOR [27, 36]. Autophagy fuels the cytoplasm with the amino acids deriving from proteolysis and CQ is expected to interrupt this process by impairing the formation of autophagolysosomes and by inhibiting the acid-dependent proteolysis mediated

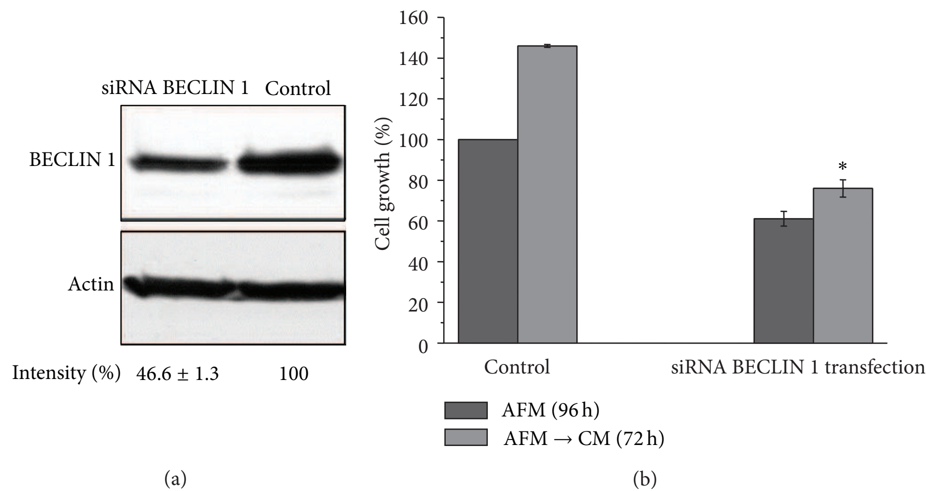


Figure 6: Transcriptional silencing of BECLIN 1 expression leads to decreased viability of SKOV3 cells under arginine starvation. (a) Western blot analysis of BECLIN 1 protein in SKOV3 cells transfected with BECLIN 1 siRNA or with nonsilencing control siRNA. β-Actin served as the loading standard. Densitometry quantification and the calculation of the relative band intensity were performed as described in Section 2. Intensity represents the mean value of three independent experiments and is expressed as means ± SD; (b) histogram represents the effect of BECLIN 1 silencing on the viability of arginine-starved cells and their proliferative potential after arginine resupplementation. $^{*}P < 0.05$.

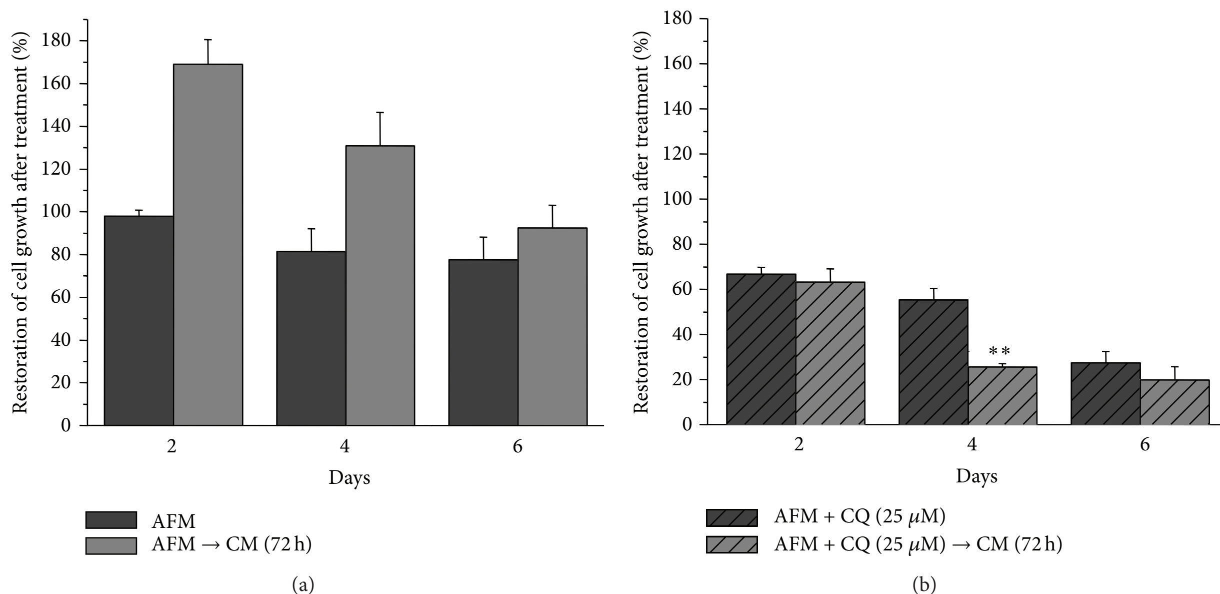


Figure 7: Effect of the autophagy inhibitor chloroquine (CQ) on SKOV3 cells viability and proliferative potential. Histograms showing cell survival and ability to resume proliferation after arginine resupplementation in cells deprived of arginine (AFM) (a) or cotreated with CQ (b). After the indicated periods of the treatment, the medium was changed to CM and cells were allowed to grow for additional 72 h. Viable cell numbers were determined by the trypan blue exclusion test. 100% is the number of cells at zero time point. $^{**}P < 0.01$.

by lysosomal cathepsins. The mTORC1 complex senses the availability of amino acids and phosphorylates downstream substrates in order to switch on and off the pathways for autophagy degradation or for protein synthesis accordingly [28]. Arginine deprivation in fact depressed the activation of the signalling kinases 4E-BP1 and p70-S6k that govern the protein synthesis pathway (Figure 5). These kinases are downstream of mTOR [29], which also negatively controls autophagy. CQ, which further reduces the availability of autophagy-derived amino acids, affected the signalling that governs the biosynthetic pathway in the cells cultivated in CM, indicating that despite the presence of amino acids in the culture medium the block of the autophagy degradation imposed by chloroquine was sensed by mTOR. By contrast, in AFM the mTOR pathway was inactive since the first 2 h of incubation and CQ did not reduce further the level of phosphorylation of 4E-BP1 and p70-S6k, indicating that arginine deprivation *per se* was sufficient to limit or inhibit the

activation of the protein synthesis pathway. We also demonstrate that autophagy process is important for maintaining cell viability under arginine deprivation. This conclusion is supported by the significant drop in viability of arginine-starved SKOV3 cells in which autophagy is inhibited. In this respect, either coadministration of CQ or transcriptional silencing of the essential autophagy protein BECLIN 1 produced similar effects (Figures 6 and 7). Strikingly, in the case of BECLIN 1 siRNA silencing, the observed decrease in viability and proliferative potential was roughly proportional to the remaining level of BECLIN 1 protein in the transfected culture (Figure 6). Preliminary data from our laboratories indicate that coapplication of taxane (taxol) at low doses may further decrease viability of ovarian carcinoma SKOV3 cells under arginine deprivation (to be published elsewhere). In this context, it is to be noted that taxol is a disruptor of the cytoskeleton and negatively impacts on the autophagosome-lysosome fusion step. In conclusion, our data support the conception that combinational treatment based on arginine deprivation and an autophagy inhibitor (e.g., chloroquine, a known nontoxic antimalarial drug) can potentially be applied as a second line treatment for a subset of ovarian carcinomas deficient in ASS.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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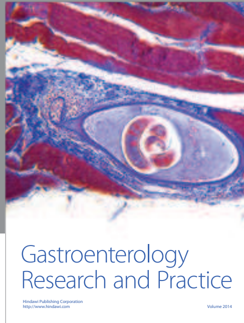

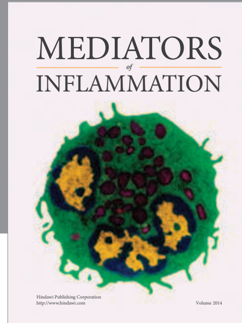

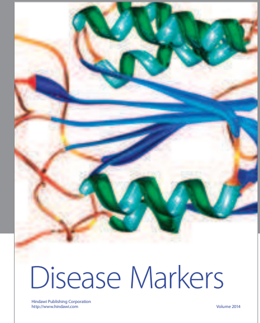

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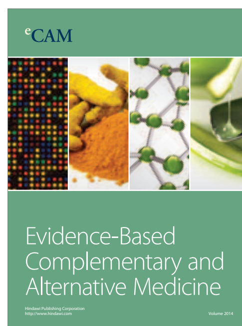

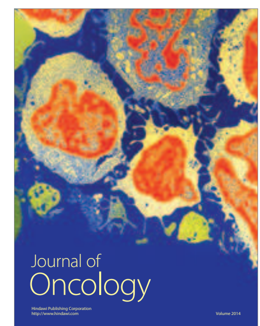

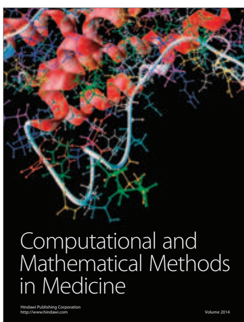

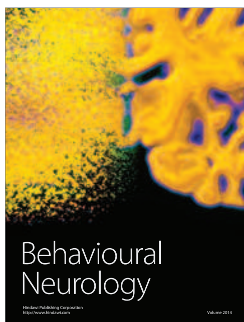

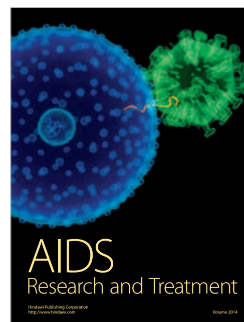

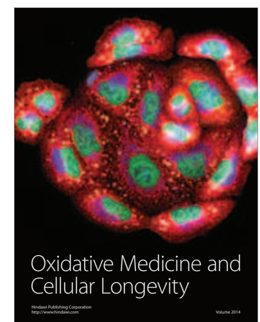

THE ONCOSUPPRESSOR ROLE OF BECLIN 1 IN OVARIAN AN BREAST CANCERS.

3. Expression and Clinical Significance of Autophagy Proteins BECLIN 1 and LC3 in Ovarian Cancer

Guido Valente, Federica Morani, Giuseppina Nicotra, Nicola Fusco, Claudia Peracchio, Rossella Titone, Oscar Alabiso, Riccardo Arisio, Dyonissios Katsaros, Chiara Benedetto and Ciro Isidoro

Epithelial ovary cancers (EOCs) represent the vast majority (approximately 90%) of all ovary tumors. Ovarian cancer ranks as the sixth to eighth most frequent cancer in developed countries and the most lethal among all the gynaecologic malignancies, with a 5-year survival of less than 30%. This retrospective study included 61 cases of ovary carcinomas of various histologic types selected from our archived materials. The following information was available: clinical stage at first diagnosis, histologic type, objective response to chemotherapy, and clinical outcome. Immunohistochemistry and immunofluorescence were performed in deparaffinized tissue and the expression of two important autophagy proteins was analyzed; BECLIN 1 and LC3. Consistently, high level of expression of BECLIN 1 and LC3 in tumors is well correlated with the overall survival of the patients.

Personal Contribution

In this paper, I contributed to the assessment of autophagy markers (BECLIN 1 and LC3) and expression of BCL2 by western blotting, and data interpretation.

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Research Article

Expression and Clinical Significance of the Autophagy Proteins BECLIN 1 and LC3 in Ovarian Cancer

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Autophagy is dysregulated in cancer and might be involved in ovarian carcinogenesis. BECLIN-1, a protein that interacts with either BCL-2 or PI3k class III, plays a critical role in the regulation of both autophagy and cell death. Induction of autophagy is associated with the presence of vacuoles characteristically labelled with the protein LC3. We have studied the biological and clinical significance of BECLIN 1 and LC3 in ovary tumours of different histological types. The positive expression of BECLIN 1 was well correlated with the presence of LC3-positive autophagic vacuoles and was inversely correlated with the expression of BCL-2. The latter inhibits the autophagy function of BECLIN 1. We found that type I tumours, which are less aggressive than type II, were more frequently expressing high level of BECLIN 1. Of note, tumours of histologic grade III expressed low level of BECLIN 1. Consistently, high level of expression of BECLIN 1 and LC3 in tumours is well correlated with the overall survival of the patients. The present data are compatible with the hypotheses that a low level of autophagy favours cancer progression and that ovary cancer with upregulated autophagy has a less aggressive behaviour and is more responsive to chemotherapy.

1. Introduction

Epithelial ovary cancers (EOCs) represent the vast majority (approximately 90%) of all ovary tumours. Based on morphological criteria, EOCs are classified as serous (of low and high grade), clear cell, endometrioid, mucinous transitional (Brenner type), mixed mesodermal, and undifferentiated histologic subtypes [1]. The histogenesis of EOC is still debated. Very recently, the traditional view that EOCs arise from the metaplastic transformation of the mesothelium overlying the ovaries has been challenged by a new paradigm suggesting that these carcinomas indeed arise in extraovarian sites and involve the ovaries secondarily [1]. Based on genetic and clinical features, ovarian carcinomas are classified as type I that comprise the low-grade serous, low-grade endometrioid, clear cell, mucinous, and transitional (Brenner) histologic types and as type II that comprise the high-grade serous, high-grade endometrioid, undifferentiated, and mixed mesodermal histologic types [1]. Type I ovarian carcinomas are genetically more stable and clinically indolent and less aggressive than type II ovarian carcinomas [1].

Ovarian cancer ranks as the sixth to eighth most frequent cancer in developed countries [2] and, in spite of the recent progresses made in understanding the genetic and biologic

bases [3, 4], it remains the most lethal among all the gynaecologic malignancies, with a 5-year survival of less than 30% [5]. Bad prognosis is essentially due to the fact that diagnosis of ovarian cancers often occurs at a late stage (because of the lack of precocious alarming symptoms) and also due to the recurrence of chemoresistant tumours. Therefore, new biomarkers for early detection and for monitoring the progression of ovarian cancers [6], as well as new therapeutic strategies that could specifically target the chemoresistant clones [3, 4], are needed.

Autophagy, a lysosomal-dependent pathway for the degradation of redundant or damaged cell components, has recently been suggested to play a role in ovarian carcinogenesis and to be a potential therapeutic target to combat this cancer [7]. Autophagy begins with the production of double-membrane vacuoles (named autophagosomes) that entrap the material to be degraded and eventually fuse with lysosomes (reviewed in [7]). The autophagosomes are characteristically marked by the presence of protein LC3 (deriving from posttranslational modifications of a microtubule-associated protein precursor) on their membranes [8]. Among the many proteins that directly or indirectly regulate the autophagy process, BECLIN 1 seems to be of particular relevance in ovarian carcinogenesis. BECLIN 1 was initially isolated as an interactor of the oncogenic antiapoptotic protein BCL-2, and it was reported to be deleted in up to 75% of human ovarian cancers [9, 10]. The monoallelic deletion of BECLIN 1 in mice caused the spontaneous development of tumours, including ovarian cancer, in association with reduced autophagy [11].

To trigger autophagy, BECLIN 1 must release BCL-2 and form dimers which interact with PI3-kinase class III (or Vps34), thus forming an oligomeric complex that can be evidenced by immunohistochemistry or immunofluorescence as definite spots in the cytoplasm [12, 13]. Autophagy-active BECLIN 1 has been proposed as a potential prognostic biomarker in several tumours [13–15]. However, the prognostic significance of BECLIN 1 expression in ovarian carcinomas appears controversial. Shen et al. [16] found that BECLIN 1 expression was significantly higher in benign and borderline ovarian tumours than in malignant EOC, which was consistent with the view that a decreased capacity of autophagy could favour tumorigenesis in the ovary. Recently, this same group confirmed this observation in a larger cohort of patients and also found that low expression of BECLIN 1 and high level of expression of BCL-2 were associated with advanced clinical stage at diagnosis and poor prognosis [17]. In contrast, another study found that BECLIN 1 expression was increased in malignant versus benign ovary tissues and that such high expression was associated with worse prognosis [18]. Increased expression of BECLIN 1 was found also to be associated with the most aggressive endometrioid adenocarcinomas and poor 5-year overall survival, probably because of concomitant tumour hypoxia [19]. In this same line, it was reported that the high expression of LC3A, the marker of autophagosomes, was associated with hypoxia and poor prognosis in clear cell, but not other examined subtypes, ovarian cancers [20].

In this work, we assessed by immunohistochemistry and immunofluorescence the expression of BECLIN 1 and of LC3 in various histologic subtypes of ovarian cancer. The ratio of BECLIN 1 and BCL-2 expression was also determined by western blotting in some selected cases. We noted that type I ovarian carcinomas that are clinically less aggressive than type II were more frequently expressing high level of BECLIN 1. Conversely, low level of BECLIN 1 expression correlated with histologic grade III tumours. No statistically significant association with patient survival was found in the cases judged negative for BECLIN 1 expression. On the other hand, granular-like positivity of BECLIN 1 and LC3, which is indicative of ongoing autophagy, was more frequently observed in tumours from patients with a better survival. These data suggest that ovarian cancer progression is facilitated by low level of intrinsic autophagy and that ovarian cancers with upregulated autophagy are more likely to respond to therapeutic treatments and to progress more slowly.

2. Materials and Methods

2.1. Patients, Therapeutic Treatments, and Tissue Collection. The present retrospective study includes 61 cases of ovarian carcinomas selected in the years 1999–2004 from the archived materials of the Department of Gynecology of Università di Torino (Italy). All cases were classified according to the current WHO Classification of Neoplasms. All patients underwent surgery. With the exception of those staged as pT1/G1 (for whom no further treatment was required), all patients were thereafter subjected to a standard chemotherapy regimen which included Carboplatin AUC 5/6 and Paclitaxel 175 mg/mq every 3 weeks for 6 cycles, outside of clinical trials. Follow-up ended in 2009. Biopsies were obtained at the time of the first surgery. Formalin-fixed paraffin-embedded tissue sections were prepared and used for diagnostic purposes and for the present investigation. No oral or written informed consent was obtained from the patients for the use of these retrospective samples, since it was not deemed necessary by the local ethics committee. All samples were treated anonymously.

2.2. Tissue Immunoreactivity for BECLIN 1, LC3, and BCL-2. Immunohistochemistry and immunofluorescence were performed in deparaffinized tissue sections following our published protocol [13]. Proteins of interest were revealed by subsequent incubation of the tissue with a primary (first step) and a secondary (second step) antibody. In the first step the following primary antibodies, either alone or in appropriate combination, were used: (a) anti-BECLIN 1 mouse monoclonal (BD Pharmingen, San Diego, CA; dilution 1 : 100) or anti-BECLIN 1 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1 : 100); (b) anti-LC3 rabbit polyclonal (Novus Biological, Littleton, Colorado; dilution 1 : 500); (c) anti-BCL-2 mouse monoclonal (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1 : 100). Appropriate secondary antibodies, goat-anti-mouse IgG or goat-anti-rabbit IgG (Sigma-Aldrich Inc., St. Louis, MO; dilution

1 : 200), labelled with horse-radish-peroxidase (for immunohistochemistry) or with FITC or Texas Red fluorescent dye (for immunofluorescence), were used in the second step. The section subjected to immunofluorescence were also stained with the fluorescent dye 4-6-diamidino-2-phenylindole-dihydrochloride (DAPI, 1 : 500 from a stock solution 20 mg/mL; 1 h) to evidence the nucleus. The sections were mounted with Slow-FAD (Light AntiFADE kit, Molecular Probes Invitrogen, Carlsband, CA, USA), observed under a fluorescence microscope (Leica DM1600, Leica Microsystem, Heidelberg, Germany) and representative areas were imaged with a digital camera.

2.3. Evaluation of Tissue Positivity for Autophagy Proteins. To assess the immunoreactivity for BECLIN 1 and for LC3 proteins, at least five fields randomly chosen (approximately 5000 cells) per section were evaluated independently by three investigators (GV and CI for IHC; GN and CI for IF). The sample was considered positive only when the immunoreaction presented with a granular-like pattern. For this purpose, high magnification images were used. Only neoplastic cells were counted. The proportion of positive cells over the total number of cells present in the imaged areas were expressed as percentage. A final hybrid score (H) was assigned to each sample, based on the product of a 0–3 scale of staining intensity and of the percentage of positive cells (0–100%), with a possible range of results from 0 to 300. Each biopsy was tested at least two times.

2.4. Tissue Western Blotting of BECLIN 1 and of BCL-2. For some samples a frozen biopsy was also available and used for western blotting detection of BECLIN 1 and of BCL-2, following our published protocol [13]. Essentially, a piece of frozen biopsy was homogenized by several cycles of freeze-thawing and sonication in a phosphate buffer containing detergents and protease inhibitors. A 30 μg of protein homogenate was resolved by SDS-polyacrylamide gel electrophoresis and thereafter electrotransferred into a nitrocellulose membrane. Standard procedure for western blotting was used [21] to detect BECLIN 1 and BCL-2, respectively, with a monoclonal antibody (BD Pharmingen; dilution 1 : 250) and a rabbit polyclonal antiserum (Santa Cruz Biotechnology; dilution 1 : 100). After stripping, the filter was reprobed to detect actin, used as a loading marker. Appropriate peroxidase-conjugated secondary antibodies (purchased from Sigma-Aldrich; dilution 1 : 20.000) were used to reveal the immunocomplexes through peroxidase-induced chemiluminescence reaction (Biorad, Hercules, CA, USA).

2.5. Statistical Analysis. BECLIN 1 and LC3 granular-like positivity as assessed by IHC and/or IF was correlated to the clinical outcome referred to as complete remission (CR) and overall survival (OS) at 5 years. The odds ratio, the relative risk, and the Chi-square were calculated using the Microsoft Excel XLStat 2010 software. The Fisher's exact test was also employed for pairwise comparison of distributions of categorized groups. A P value lower than 0.05 was taken to indicate data statistically significant.

3. Results

3.1. Histologic Type and Main Clinical Characteristics of Ovary Carcinomas Included in the Study. This retrospective study included 61 cases of ovary carcinomas of various histologic types selected from our archived materials. The tumours were grouped as type I and type II [1]. All patients were subjected to surgery and chemotherapy, following standard criteria based on clinical stage and patient performance status. The following information was available: clinical stage at first diagnosis, histologic type, objective response to chemotherapy, and clinical outcome. Response to therapy regimen was evaluated according to the international guidelines. Clinical outcomes were classified as complete remission (CR) or alternatively as not evidence of disease (NED), that is, disappearance of any evidence of disease during the follow-up or for at least four years), partial remission (PR, $\geq$ 50% decrease of tumour lesions for at least 24 months), and DOD (dead of disease). Overall survival (OS) was calculated from the time of first diagnosis to the end of the follow-up, which terminated in 2009. The database with the histologic, clinical, and patients' main information of the cases included in the present study is reported in Supplementary Table ST1 (see Supplementary Table ST1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/462658).

3.2. Immunodetection of BECLIN 1 in Ovary Cancer Tissues. The presence and the cytoplasmic distribution of BECLIN 1 were first analysed by immunohistochemistry (IHC) in paraffin-embedded tissue sections of ovary carcinomas. BECLIN 1 immunoreactivity in tumour cells presented as a faintly detectable staining diffused in the cytoplasm or as discrete stained puncta (referred to as granular-type) clearly evident in the vicinity of the nucleus. The former immunoreactivity pattern was considered as negative, whereas the latter was considered as positive in terms of BECLIN 1 macroaggregates and indicative of active autophagy. A parallel analysis of BECLIN 1 expression was conducted by immunofluorescence (IF) in the same sections. Results from both techniques overlapped, though some cases judged negative on IHC appeared faintly positive on IF, owing to the highest sensitivity of the latter technique. Representative images of BECLIN 1 expression and cellular distribution, as assessed by IHC and IF in selected cases, are shown in Figures 1 and 2, respectively.

3.3. Correlation of BECLIN 1 Expression with Histologic Type. Based on the proportion of BECLIN 1-positive cells within the tumour tissue, the samples were initially stratified into four ranges of positivity: <10%; 10–20%; 20–40%; >40%. Based on the intensity (on a 0 to 3 scale) and on the proportion of the cells positive for BECLIN 1 as assessed by IHC, hybrid score (H) was assigned to each section independently by two pathologists (GV and CI). To indicate positivity for BECLIN 1 expression the final threshold was set at $\geq$20% of cells showing a granular-like staining of intensity $\geq$2 (H $\geq$ 40). A high proportion of BECLIN 1-positive cells was reported in 41 out of 61 tumours. Of note, while type II tumours showed an approximately equal distribution of BECLIN 1 positivity (11

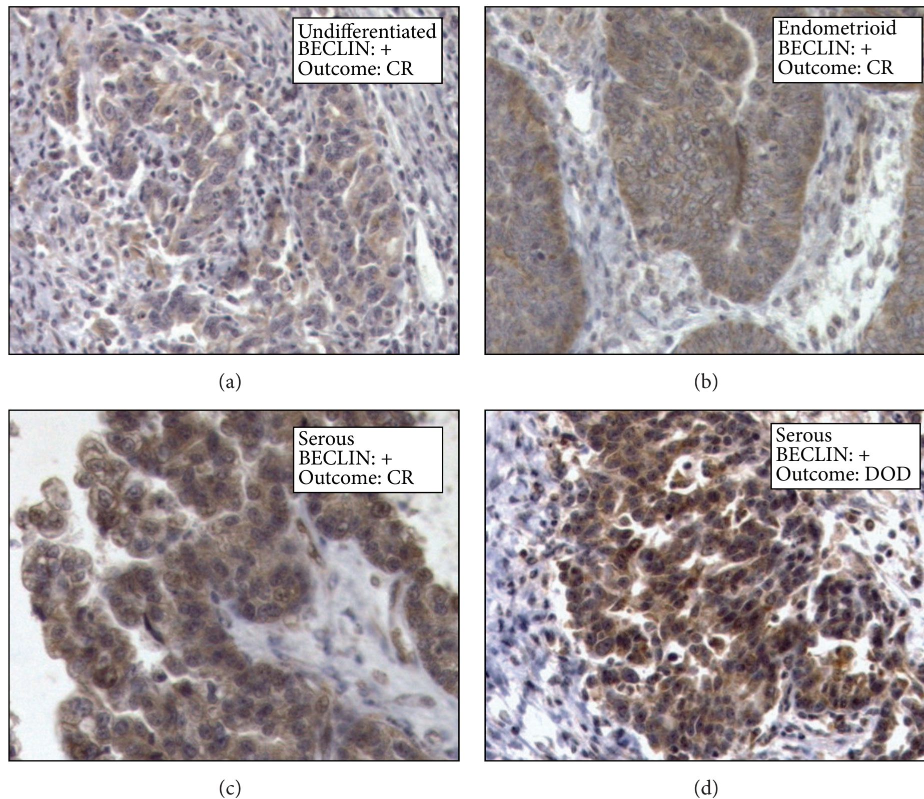

Figure 1: Immunohistochemical detection of BECLIN 1. Selection of representative cases. The histologic type and the clinical outcome (CR: complete remission; DOD: dead of disease) are indicated. Magnification 220x.

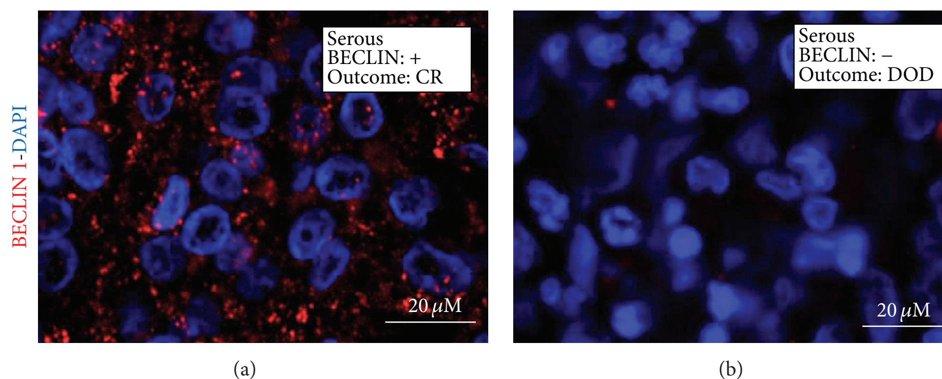

Figure 2: Immunofluorescence detection of BECLIN 1. Selection of representative cases. The histologic type and the clinical outcome (CR: complete remission; DOD: dead of disease) are indicated. The nuclei are evidenced by DAPI staining.

positive and 8 negative), as many as 20 out of 23 type I tumours were found highly expressing BECLIN 1. More in detail, >20% BECLIN 1-positive cells (>40 H) were found in the majority of endometrioid adenocarcinomas (11/13) and of serous cystadenocarcinomas (19/27). However, there was no statistically significant association between the extent of BECLIN 1-positive cells and a particular histologic type of ovarian cancer (Table 1).

3.4. BECLIN 1 Expression Correlates with Histologic Grading but Not with Pathological Staging at Diagnosis. Next, we looked for any correlation between the extent of BECLIN 1 expression and the aggressiveness of ovarian cancers as mirrored by the histologic grading and the pathological stage at diagnosis. It was found that while tumours with a high expression of BECLIN 1 were equally distributed in I-II and III grade, tumours negative or low expressing BECLIN 1 more frequently (18 out of 20) belonged to grade III (Table 2(a)). This correlation was statistically significant ($P = 0.004$). With regard to the pathological staging, it was found that of the 20 carcinomas with <20% of BECLIN 1-positive cells, 10 were classified as I-II stage and 10 as III-IV stage; of the 41

Table 1: Distribution of BECLIN 1 positivity (in terms of H ≥ 40) among ovarian carcinoma histologic types.

BECLIN 1 positive	Yes	No	Number of cases
Histologic type I			
Serous (low grade)	8	0	8
Endometrioid (low grade)	8	0	8
Clear cell	2	2	4
Mucinous	2	1	3
Transitional (Brenner)	0	1	1
Histologic type II			
Serous (high grade)	11	8	19
Endometrioid (high grade)	3	2	5
Undifferentiated	6	5	11
Mixed mesodermal	1	1	2
Total	**41**	**20**	**61**

Table 2: Correlation of BECLIN 1 positivity (in terms of H ≥ 40) with clinical-pathological characteristics. (a) Statistical correlation with histologic grade; (b) statistical correlation with pathologic stage at diagnosis.

(a)

Grade BECLIN 1	I-II	III	Number of cases
+	21	20	41
−	2	18	20
Total	**23**	**38**	**61**

Chi-square = 8.05
DF = 1
$P = 0.0046$
Fischer's test $P = 0.002$.

(b)

Stage BECLIN 1	I-II	III-IV	Number of cases
+	24	17	41
−	10	10	20
Total	**34**	**27**	**61**

Chi-square = 0.13
DF = 1
$P = 0.7$
Fischer's test $P = 0.59$.

carcinomas with ≥20% BECLIN 1-positive cells, 24 were of I-II stage and 17 of III-IV stage (Table 2(b)). No significant correlation was found between the positivity for BECLIN 1 and the pathological stage ($P = 0.7$). On the whole, these findings indicate that the absence of BECLIN 1 expression, which likely determines defective autophagy, favours a more malignant phenotype of the tumour, though other factors, independent of the intrinsic autophagy capacity, influence the evolution of the disease and the accompanying general symptoms that lead to the first diagnosis.

3.5. Ovarian Carcinomas Highly Expressing BECLIN 1 Associate with Better Patient's Clinical Outcome. We asked about the clinical significance of BECLIN 1 expression in terms of the impact on the posttherapy outcome. The patients were all subjected to surgical removal of the ovaries and annexes, followed by a standard chemotherapeutic treatment protocol. Chemotherapeutics included Carboplatin and Paclitaxel. For seven patients, staged as pT1 and bearing a G1 tumour, no adjuvant chemotherapy was administered. We first correlated the expression of BECLIN 1 with the patient's overall survival (OS) at 5 years. Patients bearing a tumour with a low expression of BECLIN 1 (H < 40) showed no differences in terms of OS, with 9 being dead and 11 still alive at the time of the end of the follow-up (Table 3(a)). By contrast, a statistically significant correlation was found between the high expression of BECLIN 1 (i.e., tumours with ≥20% of positive cells) and patient's OS. In particular, of the 41 patients bearing a tumour highly expressing BECLIN 1, 34 (~83%) were still alive at the end-point of the study and only 7 (17%) died during the observation period. These correlations were statistically significant ($P < 0.03$). We then considered the clinical outcome separately as CR (or NED), PR, and DOD to see any correlation with the expression of BECLIN 1. Amongst the 61 cases, 32 patients (52%) underwent CR. Of these, as many as 24 (75%) were bearing an ovary cancer with ≥20% BECLIN 1-positive cells. Conversely, only 8 out of 32 (25%) patients in CR were bearing a cancer with a ≤20% of BECLIN 1-positive cells (Table 3(b)). PR was more frequently observed in the group of patients bearing a cancer with a high proportion of BECLIN 1-positive tumour cells than in the group of patients bearing a BECLIN-negative cancer (25% versus 15%), and DOD was also less frequent in the former than in the latter group of patients (17% versus 45%). These correlations were, however, not statistically significant ($P < 0.06$). Altogether, these observations support the content that the high expression of BECLIN 1 in ovarian carcinomas associates with a better prognosis. However, no correlation with the clinical outcome was found in the group of patients bearing a tumour negative or low expressing BECLIN 1. We have also performed the analysis of the overall survival probability of the patients by the Kaplan-Meier method (Supplementary Figure 1, SF1). Log-rank test indicated that the association of high expression of BECLIN 1 in the tumour with a good prognosis was statistically significant. Yet, a larger number of patients should be studied in order to substantiate the above finding.

3.6. BECLIN 1 and LC3 Double Positivity Predicts a Favourable Prognosis in Ovarian Cancer. In autophagy active cells, the microtubule-associated LC3 protein is posttranslationally translocated into the membranes of autophagosomes [8]. Therefore, the detection of a granular-like staining of LC3 can be assumed *bona fide* as the proof of the presence of autophagic vacuoles (either autophagosomes or autophagolysosomes) in the cell. We analysed by immunofluorescence the expression of LC3 in selected BECLIN 1-positive ($n = 30$) and BECLIN 1-negative cases ($n = 12$). Cells were considered positive for ongoing autophagy when showing a granular-like staining for LC3 and the tumour was considered autophagy-active when ≥20% of the cells were LC3 positive.

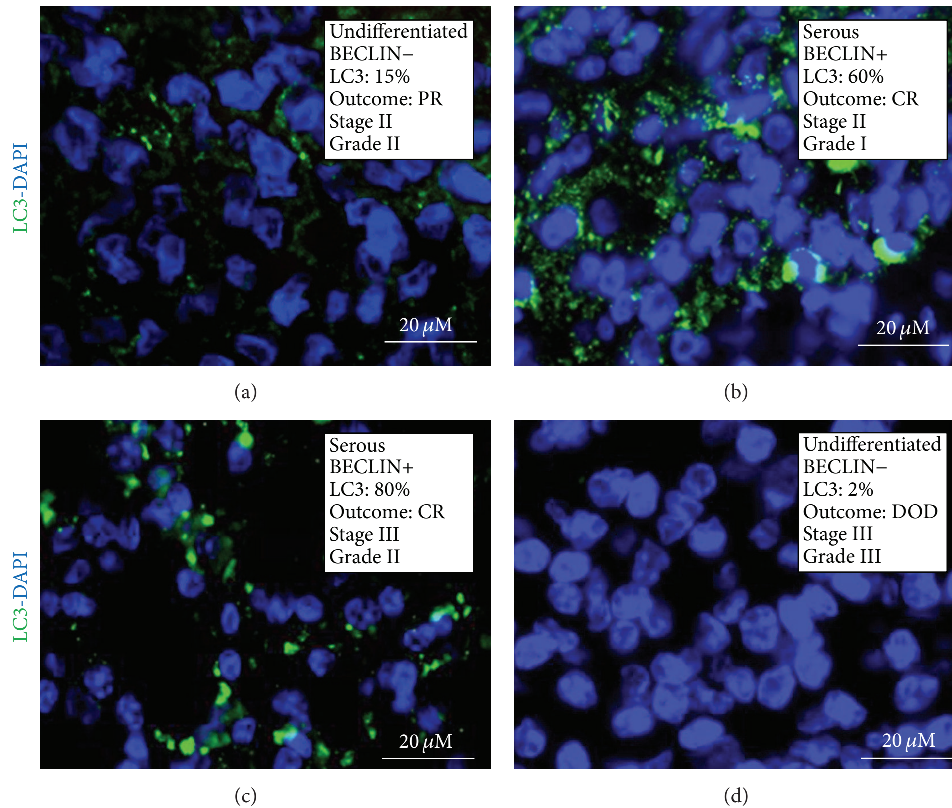

Figure 3: Immunofluorescence staining of LC3 in ovarian cancer tissue sections. Selection of representative cases. The histologic type, the positivity for BECLIN 1 aggregates, the percentage of cells positive for vacuolar LC3, the clinical outcome (CR: complete remission; PR: partial remission; DOD: dead of disease), the pathological stage, and the histologic grade are reported. The nuclei are evidenced by DAPI staining.

Table 3: Correlation of BECLIN 1 expression with clinical outcome in patients.

(a)

Clinical outcome BECLIN 1	Survivors	DOD	Number of cases
+	34	7	41
−	11	9	20
Total	**45**	**16**	**61**

Chi-square = 4.07
DF = 1
$P = 0.04$
Fischer's test
$P = 0.03$.

(b)

Clinical outcome BECLIN 1	CR	PR	DOD	Number of cases
+	24	10	7	41
−	8	3	9	20
Total	**32**	**13**	**16**	**61**

Chi-square = 5.4
DF = 2
$P = 0.066$.

Examples of LC3 staining in BECLIN 1-positive tumour cells are shown in Figure 3. With a few exceptions, cases judged positive for BECLIN 1 were highly positive also for LC3. On the whole, we found a concordance of 70% between the expression of both BECLIN 1 and LC3.

To further substantiate the involvement of autophagy in the progression and chemotherapeutic response of ovarian carcinomas, we correlated the expression of LC3 with the clinical outcome. When restricted to the group of BECLIN 1-positive tumours, it was found that 20 out of 21 patients bearing a tumour also positive for LC3 were still alive, while 6 out of 9 of those patients bearing a tumour negative for LC3 were DOD, at 5 years after diagnosis (Table 4(a)). These correlations were statistically significant ($P < 0.0002$). Statistics was then applied to the whole group of tumours analyzed for LC3 positivity, including both the BECLIN 1 positive and BECLIN 1 negative. On the whole, 23 out of 24 patients with an LC3-positive tumour were still alive, while 11 out 18 patients with an LC3-negative tumour were DOD, at 5 years after diagnosis (Table 4(b)). Of note, in this case the correlations were even more significant ($P < 0.0002$).

3.7. Coexpression of BECLIN 1 and BCL-2 in relation to Autophagy in Ovarian Cancers. The interaction of BECLIN 1 with BCL-2 abrogates the induction of autophagy [22]. On the other hand, high expression of BCL-2 inhibits not only

Table 4: Correlation of LC3 expression with patients overall survival. (a) Group of BECLIN 1 positive tumours; (b) group of BECLIN 1 positive and negative tumours.

(a)

% LC3 positive	Survivors 5 y	DOD	Number of cases
<20%	3	6	9
≥20%	20	1	21
Total	**23**	**7**	**30**

Chi-square = 13.5
DF = 1
$P = 0.0002$.

(b)

% LC3 positive	Survivors 5 y	DOD	Number of cases
<20%	7	11	18
≥20%	23	1	24
Total	**30**	**12**	**42**

Chi-square = 16.34
DF = 1
$P = 0.0001$.

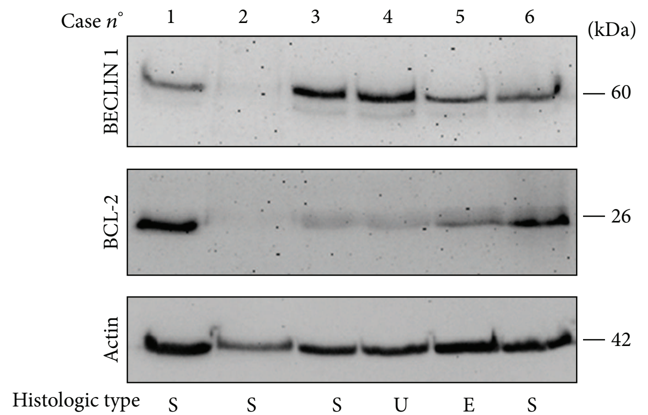

Figure 4: Western blotting analysis of the expression of BECLIN 1 and of BCL-2 proteins in ovarian carcinomas. Selection of representative cases. Tissue homogenates were subsequently probed for BECLIN 1, BCL-2, and actin (the latter was used as reference of homogenate protein loading). The molecular weight of proteins detected with the specific antibodies is indicated. Histologic type: S: serous; U: undifferentiated; E: endometrioid.

autophagy but also apoptosis, thus influencing the cytotoxic response of ovarian cancer cells to chemotherapeutics [17, 21]. Thus, evaluating the level of expression of BECLIN 1 may not be sufficient to draw conclusions about the capacity of the cell to activate autophagy. We have analysed by western blotting the expression of BECLIN 1 and of BCL-2 in a small subset of carcinomas for which the frozen biopsy was available (representative cases are shown in Figure 4). In general, the expression of these proteins was inversely related. To seek for a functional relationship between the two proteins, we performed the immunostaining of BECLIN 1, BCL-2, and LC3 in two paradigmatic situations among the cases analysed by western blotting. In case 1, the expression of BCL-2 was quite high, which could account for inhibition of BECLIN 1 proautophagic activity, and in fact this tumour was negative for LC3 staining (Figure 5(a)). On the opposite, BCL-2 and BECLIN 1 were not detectable (by western blotting) in the tumour case 2, and in spite of this the tumour was intensely LC3 positive (Figure 5(b)), which possibly was associated with BECLIN 1-independent autophagy.

4. Discussion

Autophagy, a cell homeostatic process for the lysosome-driven degradation of aged, damaged, and redundant self-constituents, may either suppress or facilitate carcinogenesis [7, 23]. The heterozygous deletion of the autophagy gene *BECLIN 1* in transgenic mice predisposes to the development of spontaneous tumours, including ovarian cancers [11, 24]. Accordingly, the expression of the BECLIN 1 protein and also of the autophagosome protein LC3 was found much lower in malignant ovarian cancers compared to benign ovary epithelial tissues [16, 17]. In our series, we also have found that 18 out of 20 ovarian cancers of histologic grade III were negative or low expressing BECLIN 1. This is consistent with the view that defective autophagy might favour cancer progression. In this same line, a decreased level of BECLIN 1 expression, especially in conjunction with increased expression of BCL-xL, was correlated with poor prognosis in ovarian cancer bearing patients [17]. Here we have analysed the tissue expression of BECLIN 1 in a series of 61 cases of ovarian carcinomas of various histologic types. BECLIN 1 staining presented with either a cytoplasmic diffused pattern (regarded as negative) or a granular-like pattern (regarded as positive). The latter likely reflected the engagement of BECLIN 1 in the oligomeric interactome with PI3-kinase class III [12], which preludes to the initiation of autophagy [22]. Fourteen (of the 61) cancers examined showed positive for BECLIN 1 in a percentage of cells ranging from 20% to 90%. The expression of BECLIN 1 was not correlated with patient's age at the time of diagnosis, nor was it correlated with a particular histologic type. It is to be noted, however, that in our series some histologic types were underrepresented so that no conclusion could be drawn with regard to the association between autophagy and histotypes. On the other hand, being autophagy, an evolutionary conserved and ubiquitous process, it is conceivable that it is not restricted to a particular subtype of cancer. Setting the cut-off at 20% of positive cells (in terms of BECLIN 1 macroaggregates), a positive correlation was found between negative expression and high histologic grade. In general, the clinically indolent type I tumours were more frequently expressing BECLIN 1 at high level.

However, no statistically significant correlation was found between the positive expression and the pathological stage at diagnosis. Thus, while defective autophagy likely favours the emergence of highly malignant clones, other factors influence the general evolution of the disease in the patient.

Tumours negative for BECLIN 1 showed no correlation with prognosis (11 survivors and 9 DOD), whereas of the 41

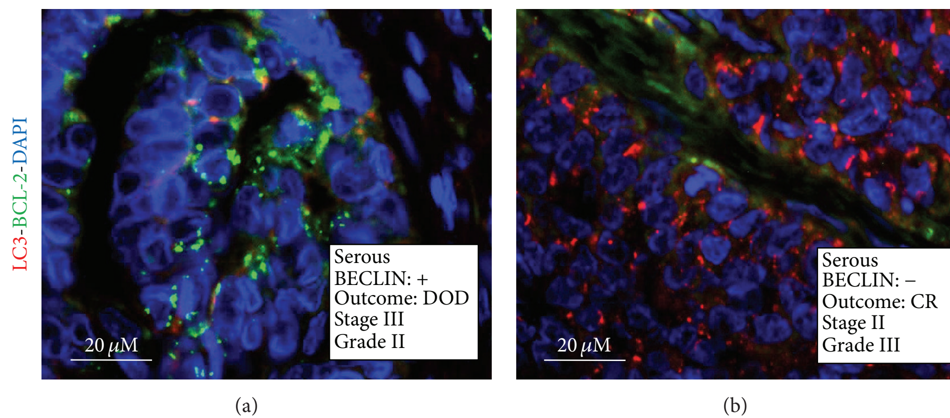

Figure 5: Immunofluorescence staining of LC3 and BCL-2. Selection of representative cases. The histologic type, the positivity for BECLIN 1 aggregates, the clinical outcome (CR: complete remission; DOD: dead of disease), the pathological stage, and the histologic grade are reported. The nuclei are evidenced by DAPI staining.

patients bearing a BECLIN 1-positive tumours as many as 34 showed a favourable prognosis (24 CR and 10 PR). Seen from a different point, of the 32 patients that underwent CR as many as 24 were bearing a BECLIN 1-positive cancer and only 8 were bearing a BECLIN 1-negative cancer. These correlations were statistically significant. While our data seem to be consistent with the findings reported by Shen et al. [16] and Lin et al. [17], other authors have reported opposite findings. In one study [18], the expression of BECLIN 1 was inversely correlated with the histologic grade of differentiation of ovarian carcinomas and the high level of BECLIN 1 expression was associated with a lower relapse-free survival rate of the patients. High level of BECLIN 1 was also found associated with invasive endometrioid cancers and poor 5-year survival [19]. However, both in these studies BECLIN 1 was not an independent prognostic factor. In our series, we have indeed observed that seven patients bearing a cancer with >20% of BECLIN1-positive cells deceased within the follow-up period. Assuming that BECLIN 1 main function was to drive autophagy and that autophagy was playing a positive role in the response to chemotherapy treatments, we considered the possibility that failure in the chemotherapy response in those patients could arise from impaired (or insufficient) induction of autophagy in the tumour cells expressing BECLIN 1. To better detect autophagy active cells in the tumour, we stained the cells for LC3, an autophagosomal protein considered to be hallmark of ongoing autophagy [8]. In general, a high concordance between BECLIN 1 and LC3 positivity was observed in the large majority of the cases. In some cases, LC3 was negative in spite of the positivity for BECLIN 1. This fact was likely due to the concomitant high expression of BCL-2, which is known to nullify the autophagy function of BECLIN 1 [22], as was proven in at least some of the cases. We found that the BECLIN 1-positive cancers associated with the patients deceased during the study were indeed negative for vacuolar LC3 staining and highly expressing BCL-2. Though not statistically relevant because of the small number of cases, indirectly our finding agrees with that reported by Lin et al. [18], who showed that low expression of BECLIN 1 in combination with high expression of BCL-xL predicts a poor survival in ovarian cancer patients. Of note, also LC3 positivity significantly correlated with patient's overall survival at 5 years after diagnosis, thus supporting the contention that the patients bearing a tumour with a high proportion of autophagy-active cells had a better prognosis. In this regard, it is to be mentioned that, in clear cell ovarian cancer histotypes, but not in other examined histotypes, the high expression of LC3A was found to significantly correlate with hypoxia and poor prognosis [20]. We could not compare with this study, as in our series we had only 4 cases of clear cell carcinomas, 2 each either BECLIN 1 positive or BECLIN 1 negative.

It remains to be explained through which molecular pathway the ongoing autophagy in cancer cells could turn of benefit in the chemotherapeutic response so that the patient experiences a better prognosis. The two-hit model predicts a synergistic death effect of two proautophagic stimuli [25]. In fact, although autophagy is in principle a prosurvival pathway, it might also lead to cell death if dysregulated [12, 23, 26]. In particular, cells in which autophagy is basally upregulated may undergo apoptosis if subjected to an additional metabolic or genotoxic stress that hyperinduces autophagy [25]. We hypothesize that autophagy-active cancer cells may succumb in response to drugs that hyperstimulate autophagy. This is the rationale for the use of mTOR inhibitors in ongoing clinical trials for the treatment of ovarian cancers [7]. With relevance to our chemotherapy protocol, it has been reported that the transgenic overexpression of BECLIN 1 sensitizes cervical cancer cells to carboplatin and to paclitaxel by promoting apoptosis and autophagic cell death [27, 28]. BECLIN 1 and BCL-2 occupy a central role in the complex cross-talk between autophagy and apoptosis [29], and chemotherapeutic drugs could be more effective in those cells with an altered ratio between these two proteins. Consistent with our hypothesis, it was recently shown that the Src/Abl kinases inhibitor Dasatinib arrested the growth of ovarian cancer xenograft by inducing BECLIN 1-dependent autophagic cell death, and hyperstimulation of autophagy was associated with downregulation of BCL-2 expression [30].

This could also explain the poor survival reported in women bearing an ovarian cancer expressing low level of BECLIN 1 and high level of BCL-xL [17]. Besides, the high expression of BECLIN 1 could enhance the cytotoxic response to a chemotherapeutic drug in ovarian cancer cells also via an autophagy-independent mechanism [31]. Additionally, the hypothesis that tumour with intrinsic high level of basal autophagy may have a better prognosis even without chemotherapy cannot be excluded. Though we could not test directly this hypothesis, we note that of the 7 patients for whom chemotherapy was not deemed (because they were staged as pT1 and the tumour was of grade 1) 6 were bearing a BECLIN 1-positive tumour and underwent CR, whereas 1 was bearing a BECLIN 1-negative tumour and was DOD.

In conclusion, while on one hand the upregulation of basal autophagy associated with a higher ratio of BECLIN 1 versus BCL-2 proteins expression enables the cancer cells to overcome the metabolic stresses caused by the lack of oxygen and nutrients, it on the other hand also renders these cells more susceptible to chemotherapeutic drugs that overstimulate autophagy. Given the role of mitochondria in the apoptotic response to chemotherapeutics [32], we suspect that in the latter case apoptosis ensues because of the exaggerated mitophagy. Thus, to improve the chance to cure ovarian carcinomas, one should carefully consider whether to employ autophagy inhibitors or autophagy-enhancer drugs in the chemotherapy cocktail depending on the ratio of BECLIN 1 and BCL-2 expression and the actual level of autophagy in the cancer cells.

Conflict of Interests

The authors declare that no conflict of interests exists.

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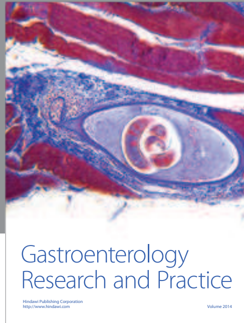

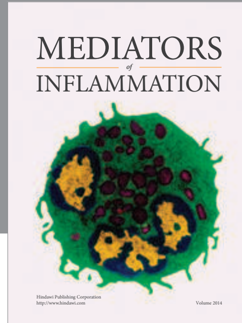

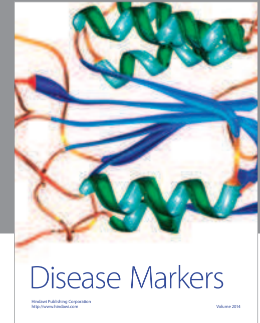

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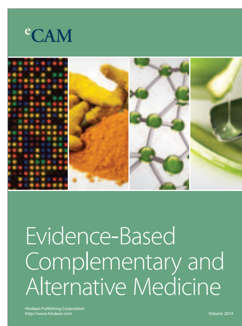

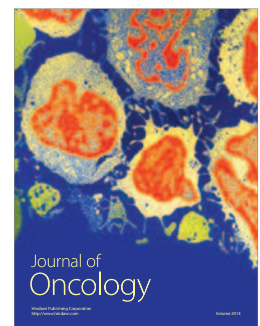

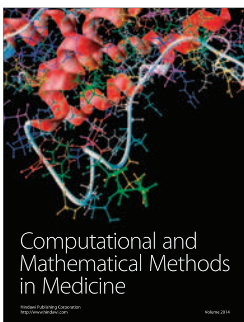

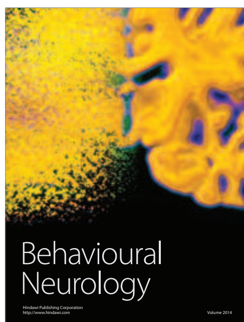

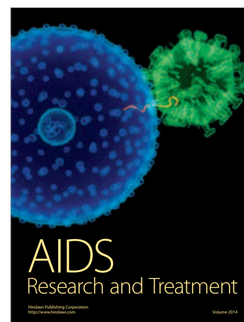

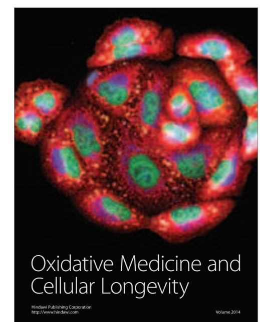

4. Decreased BECN1 mRNA Expression in Human Breast Cancer is Associated with Estrogen Receptor-Negative Subtypes and Poor Prognosis

Hao Tang, Salwa Sebti, Rossella Titone, Yunyun Zhou, Ciro Isidoro, Theodora S. Ross, Hanina Hibshoosh, Guanghua Xiao, Milton Packer, Yang Xie and Beth Levine

The essential autophagy gene BECLIN 1 (BECN1) is a haploinsufficient tumor suppressor that is also located on the breast cancer tumor susceptibility chromosomal locus 17q21, ~150kb centromeric to BRCA1. Monoallelic loss of BECN1 has been observed in about 40% of human breast cancers. We interrogated two large independent publicly available breast cancer datasets: The Cancer Genome Atlas Project (TCGA) in the United States and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) in the United Kingdom and Canada. Our data suggest that decreased mRNA expression of the autophagy gene BECN1 (but not low BRCA1 mRNA) may contribute to the pathogenesis and progression of HER2-enriched, basal-like, and TP53 mutant breast cancers.
These observation could be important to predict the response to specific chemotherapeutic regimens or whether strategies that increase BECN1 function might be therapeutic in patients with low BECN1 expression.

Personal Contribution

In this paper, I contributed to the study design and have performed the extensive analysis of data sets.

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Decreased *BECN1* mRNA Expression in Human Breast Cancer is Associated With Estrogen Receptor-Negative Subtypes and Poor Prognosis

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ABSTRACT

Both *BRCA1* and *Beclin 1* (*BECN1*) are tumor suppressor genes, which are in close proximity on the human chromosome 17q21 breast cancer tumor susceptibility locus and are often concurrently deleted. However, their importance in sporadic human breast cancer is not known. To interrogate the effects of *BECN1* and *BRCA1* in breast cancer, we studied their mRNA expression patterns in breast cancer patients from two large datasets: The Cancer Genome Atlas (TCGA) (n = 1067) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (n = 1992). In both datasets, low expression of *BECN1* was more common in HER2-enriched and basal-like (mostly triple-negative) breast cancers compared to luminal A/B intrinsic tumor subtypes, and was also strongly associated with *TP53* mutations and advanced tumor grade. In contrast, there was no significant association between low *BRCA1* expression and HER2-enriched or basal-like subtypes, *TP53* mutations or tumor grade. In addition, low expression of *BECN1* (but not low *BRCA1*) was associated with poor prognosis, and *BECN1* (but not *BRCA1*) expression was an independent predictor of survival. These findings suggest that decreased mRNA expression of the autophagy gene *BECN1* may contribute to the pathogenesis and progression of HER2-enriched, basal-like, and *TP53* mutant breast cancers.
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1. Introduction

Estrogen-receptor (ER) negative breast cancer comprises 25–30% of all sporadic breast cancer and is characterized by advanced histological grade, aggressive clinical behavior, a high rate of metastasis to the brain and lung, and resistance to hormone deprivation therapy (Yersal and Barutca, 2014; Sorlie et al., 2001; Rakha et al., 2008a). Based on molecular profiling (Yersal and Barutca, 2014; Sotiriou and Pusztai, 2009), these cancers generally fall into two subtypes: (1) HER2-enriched tumors (those with overexpression or amplification of human epidermal growth factor receptor 2 [HER2]) and (2) basal-like tumors (which generally do not express estrogen or progesterone receptors or HER2/neu, but have high levels of basal markers and/or epidermal growth factor receptor expression and a high rate of *TP53* mutations) (Sorlie et al., 2001; Perou et al., 2000).

The pathogenesis of the basal-like subtype has not been defined, but some studies have suggested an association with dysfunction of the DNA repair *BRCA1* pathway (Turner et al., 2004, 2007; Mueller and Roskelley, 2003; Valentin et al., 2012). The basal-like subtype is frequent in women with *BRCA1* germline mutations (Foulkes et al., 2003) who are at markedly increased risk of breast cancer. Levels of *BRCA1* expression have also been reported to be low in women with sporadic breast cancers that have basal-like features (Turner et al., 2007; Mueller and Roskelley, 2003), and may be related to the frequent loss of

Abbreviations: BECN1, beclin 1 autophagy related gene; *BRCA1*, breast cancer 1, early onset gene; TCGA, The Cancer Genome Atlas; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; HER2, human epidermal growth factor receptor 2; *TP53*, tumor protein p53 gene; ER, estrogen receptor; GISTIC, genomic identification of significant targets in cancer; PR, progesterone receptor; PAM50, 50-gene prediction analysis of microarray; ATG5, autophagy related 5 gene; BCL-2, B-cell CLL/lymphoma 2; EGFR, epidermal growth factor receptor; OR, odds ratio; CI, confidence interval; CNV, copy-number variation; LQ, low quartile; HQ, high quartile; NA, not available.

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heterozygosity at the breast cancer tumor susceptibility locus on chromosome 17q21 (Staff et al., 2003) and/or *BRCA1* promoter hypermethylation (Birgisdottir et al., 2006) or increased expression of negative regulatory factors (Turner et al., 2007; Garcia et al., 2011; Z.Q. Wu et al., 2012).

The essential autophagy gene *beclin 1* (*BECN1*) is a haploinsufficient tumor suppressor (Liang et al., 1999; Qu et al., 2003; Yue et al., 2003) that is also located on the breast cancer tumor susceptibility chromosomal locus 17q21, ~150 kb centromeric to *BRCA1* (Aita et al., 1999). Monoallelic loss of *BECN1* has been observed in about 40% of human breast cancers (Aita et al., 1999; Li et al., 2010), and enforced expression of *BECN1* in breast cancer cells with allelic loss of 17q21 inhibits proliferation and tumorigenesis (Liang et al., 1999). Heterozygous deletion of *BECN1* in mice leads to an increased incidence of spontaneous carcinomas (Qu et al., 2003; Yue et al., 2003), including breast carcinoma with basal-like features (Cicchini et al., 2014).

Given the likely roles of both *BRCA1* and *BECN1* in the development of mammary malignancy and the close proximity of *BRCA1* and *BECN1* genes on chromosome 17q21, large genomic deletions of the 17q21 locus could increase the risk of sporadic breast cancer through loss of expression of both genes, or alternatively, through the loss of only one gene, with loss of the other representing a bystander effect (Laddha et al., 2014). Therefore, we sought to determine the importance of loss of *BECN1* and of *BRCA1* expression in women with ER-negative subtypes of breast cancer.

2. Methods

2.1. Genetic Profiling in Two Breast Cancer Datasets

We interrogated two large independent publicly available breast cancer datasets: The Cancer Genome Atlas Project (TCGA) in the United States (Anon., 2012) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) in the United Kingdom and Canada (Curtis et al., 2012). The patient characteristics in each dataset are shown in Supplementary Table 1.

TCGA breast cancer mRNA gene expression, copy number alteration, and clinical data were downloaded from UCSC cancer browser at https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/ (data processed in August 2014). TCGA gene expression profile was measured using the Illumina HiSeq 2000 RNA Sequencing platform. RSEM (RNA-Seq by Expectation-Maximization) normalized count was used as gene-level expression estimates in this study. TCGA copy number profile was measured using genome-wide SNP6 array. Gene-level somatic copy number alterations were estimated from TCGA FIREHOSE pipeline (https://confluence.broadinstitute.org/display/GDAC/Home) using the GISTIC2.0 (Mermel et al., 2011) method. The GISTIC2.0 summarized the copy number of each gene into −2, −1, 0, 1, 2, representing homozygous deletion, heterozygous deletion, diploid normal copy, low-level amplification, or high-level amplification. For analysis, the homozygous deletion and heterozygous deletion groups were combined. *TP53* mutation status was also obtained from the TCGA FIREHOSE pipeline using MutSig method (Lawrence et al., 2013). TCGA tumor grade information was manually extracted from the pathologic reports provided by the cBio portal. ER, progesterone receptor (PR), and HER2 status was defined by protein expression (measured by immunohistochemistry), as provided in the original TCGA publication; (Anon., 2012) receptor status was classified as positive, negative, or equivocal, with less than 10 samples classified as equivocal.

In the METABRIC dataset, mRNA expression was measured using the Illumina HT-12 platform. Normalized gene-level expression and copy number segment files from METABRIC were downloaded from the European Genome-phenome Archive (EGA) with accession number EGAS00000000083. The copy number profile of METABRIC data was measured using the Affymetrix SNP6 array. As for the TCGA, the copy number data of the METABRIC cohort was processed using the GISTIC2.0 (Mermel et al., 2011) algorithm to identify homozygous deletion, heterozygous deletion, diploid normal copy, low-level amplification or high-level amplification for each gene for each sample. Clinical variables were obtained from Supplementary Tables 2 and 3 of the original METABRIC publication (Curtis et al., 2012). ER, PR and HER2 status was determined by mRNA expression as positive or negative, as defined in the original METABRIC publication (Curtis et al., 2012).

Intrinsic subtyping was performed using the research-based 50-gene prediction analysis of microarray (PAM50) subtype predictor (Parker et al., 2009), which classifies tumors into the following groups: Luminal A, Luminal B, HER2-enriched, basal-like and normal-like. Samples without PAM50 data or those identified as normal-like (which often represent inadequate tumor cellularity) were excluded from the analysis. For the TCGA dataset, we used subtype calls downloaded from the database that were based on RNA-Seq measurements. For the METABRIC dataset, we used the PAM50 subtypes provided in the database; basal-like cancers were further refined into two sub-categories (IntClust categories 4 and 10) based on the clustering analysis of expression profiles as provided in the original METABRIC publication (Curtis et al., 2012).

2.2. Statistical Analyses

Our final analysis focused on 1067 and 1992 primary breast cancers in the TCGA and METABRIC datasets, respectively. Low versus high-expression patient groups were defined relative to the median expression level of all patients in each data set. Chi-square and Fisher's Exact tests were used to investigate the relationship between dichotomized *BECN1* or *BRCA1* expressions and PAM50 intrinsic tumor subtypes, *TP53* mutation status, advanced tumor grade, and the groups defined by ER, PR, and HER2 status. To reduce potential bias from dichotomization, the expression levels of *BECN1* and *BRCA1* were also displayed as a continuous variable and were compared across different PAM50 subtypes, *TP53* mutation status and tumor grades using a *t*-test. All cut-off values were set before analysis, and all tests were two-tailed.

Survival analysis was performed only in the METABRIC dataset because of the long median duration of follow-up (7.3 years in METABRIC and <2 years in TCGA), using the survival R package. Patients were grouped based on the mRNA expression of *BECN1* or *BRCA1* genes, with the upper 25%, 25–75% and lower 25% representing the high, intermediate and low expression groups, respectively. Survival curves of the three groups were estimated by the Kaplan–Meier method and compared using the Cox regression model assuming an ordered trend for the three groups as previously described (Cheng et al., 2013; Shedden et al., 2008) and the log-rank test was used to compare the overall survival curves among three groups. Only deaths related to breast cancer (disease-specific deaths) were considered in the analysis. Multivariate survival analysis using the Cox regression model was performed to assess the relative contribution of *BECN1* or *BRCA1* mRNA expression, after adjusting for age, tumor grade, size, stage, molecular subtype, *TP53* mutation and perioperative therapy.

3. Results

3.1. Correlation of BECN1 and BRCA1 Deletions

BECN1 and *BRCA1* were each deleted in approximately one-third of the breast tumors in both the TCGA and METABRIC datasets (*BECN1* deletion in 34% in TCGA and 33% in METABRIC; *BRCA1* deletion in 35% in TCGA and 27% in METABRIC) (Supplementary Table 2). The vast majority of these deletions represented heterozygous loss (350 of 354 for *BECN1* and 353 of 361 for *BRCA1* in the TCGA dataset; 493 of 643 for *BECN1* and 501 of 522 for *BRCA1* in the METABRIC dataset). As expected due to the close proximity of these two genes on chromosome 17q21,

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co-occurrence analysis of copy number alterations showed that the two events were highly correlated (Supplementary Table 2).

In contrast to a previous report by Laddha et al. (2014), our analyses of TCGA (n = 1033 samples) did not reveal a significant difference between the number of *BECN1* alone (n = 3) versus *BRCA1* alone deletions (n = 10) (P = 0.095). Moreover, in the METABRIC dataset (n = 1929 samples), *BECN1* alone deletions (without *BRCA1* deletions) (n = 153) were significantly more common than *BRCA1* alone deletions (without *BECN1* deletions) (n = 32) (P = 1.5E−19). Therefore, when discordant, *BECN1* deletions were more common than *BRCA1* deletions.

Nonetheless, since the majority of breast cancer cases with *BRCA1* or *BECN1* copy number alteration contain concurrent deletions of both *BRCA1* and *BECN1*, it is difficult to use copy number alterations as a parameter for distinguishing the effects of these two genes in breast cancer. Genes with a high correlation between their copy number and mRNA expression are more likely to be driver genes and regulate tumorigenesis, since gene expression rather than copy number better defines phenotype (Akavia et al., 2010). Notably, the relationship between copy number loss and mRNA expression was more significant for *BECN1* than for *BRCA1* in both the TCGA dataset (P = 2.77E−88 and P = 4.12E−10, respectively) (Fig. 1A–B) and the METABRIC dataset (P = 6.87E−31 and P = 5.02E−8, respectively) (Fig. 2A–B).

3.2. Association of low BECN1 mRNA Expression with HER2-Enriched and Basal-Like Tumor Subtypes, TP53 Mutations, and Advanced Tumor Grade

In TCGA dataset, the mRNA expression of *BECN1*, but not *BRCA1*, was associated with ER-negative intrinsic subtypes and aggressive features (Table 1). As compared with a high level of *BECN1* mRNA expression, a low level of *BECN1* mRNA expression was strongly associated with HER2-enriched breast tumors (odds ratio 8.5 [95% CI 4.4 to 17.9], P = 8.5E−14); with basal-like breast tumors (odds ratio 35.5 [95% CI 16.4 to 91.8], P = 3.8E−43); with the presence of *TP53* mutations (odds ratio 7.1 [95% CI 5.0 to 10.4], P = 2.6E−32), and with tumor grade III (odds ratio 10.3 [95% CI 5.6 to 19.2], P = 2.4E−17). In contrast, low levels of *BRCA1* mRNA expression were not significantly associated with any of these features. Low *BECN1* (but not *BRCA1*) expression was also associated with HER2-positive and triple-negative tumors identified by immunohistochemical staining (Supplementary Table 3).

In TCGA dataset, among four distinct groups with low *BECN1*/low *BRCA1*, low *BECN1*/high *BRCA1*, high *BECN1*/low *BRCA1*, and high *BECN1*/low *BRCA1* expression, only low *BECN1* expression (regardless of *BRCA1* expression) was related to the frequency of HER2 and basal-like subtypes, *TP53* mutations, and grade III tumors (Supplementary Fig. 1). Basal-like breast tumors were seen in 42.0% of patients who had low *BECN1* but high *BRCA1* expression but in only 0.7% of patients who had high *BECN1* but low *BRCA1* expression (P = 9.05E−17 for the difference between groups) (Supplementary Fig. 1A). Similarly, as compared with the high *BECN1*/low *BRCA1* expression group, tumors with low *BECN1*/high *BRCA1* expression were more likely to be HER2-enriched (14.3% versus 0.7%, P = 4.46E−05); have *TP53* mutations (57.5% versus 9.2%, P = 2.69E−16); and exhibit grade III characteristics (68.5% versus 20.0%, P = 1.61E−17) (Supplementary Fig. 1B–D).

The association between mRNA expression of *BECN1* (but not *BRCA1*) and ER-negative tumors was confirmed in the METABRIC dataset (Table 2). As compared with a high level of *BECN1* mRNA expression, a low level of *BECN1* mRNA expression was strongly associated with HER2-enriched breast tumors (odds ratio 5.5 [95% CI 4.0 to 7.7], P = 1.4E−30); with basal-like breast tumors (odds ratio 10.0 [95% CI 7.3 to 14.1], P = 1.4E−61); with *TP53* mutations (odds ratio 3.0 [95% CI 1.9 to 4.8], P = 8.9E−07); and with tumor grade III (odds ratio 2.9 [95% CI 2.0 to 4.1], P = 5.8E−10). Low levels of *BRCA1* mRNA expression were not significantly associated with any of these features, and low *BRCA1* levels were actually inversely associated with tumor grade III (odds ratio 0.4 [95% CI 0.3–0.5], P = 1.1E−08). Low *BECN1* (but

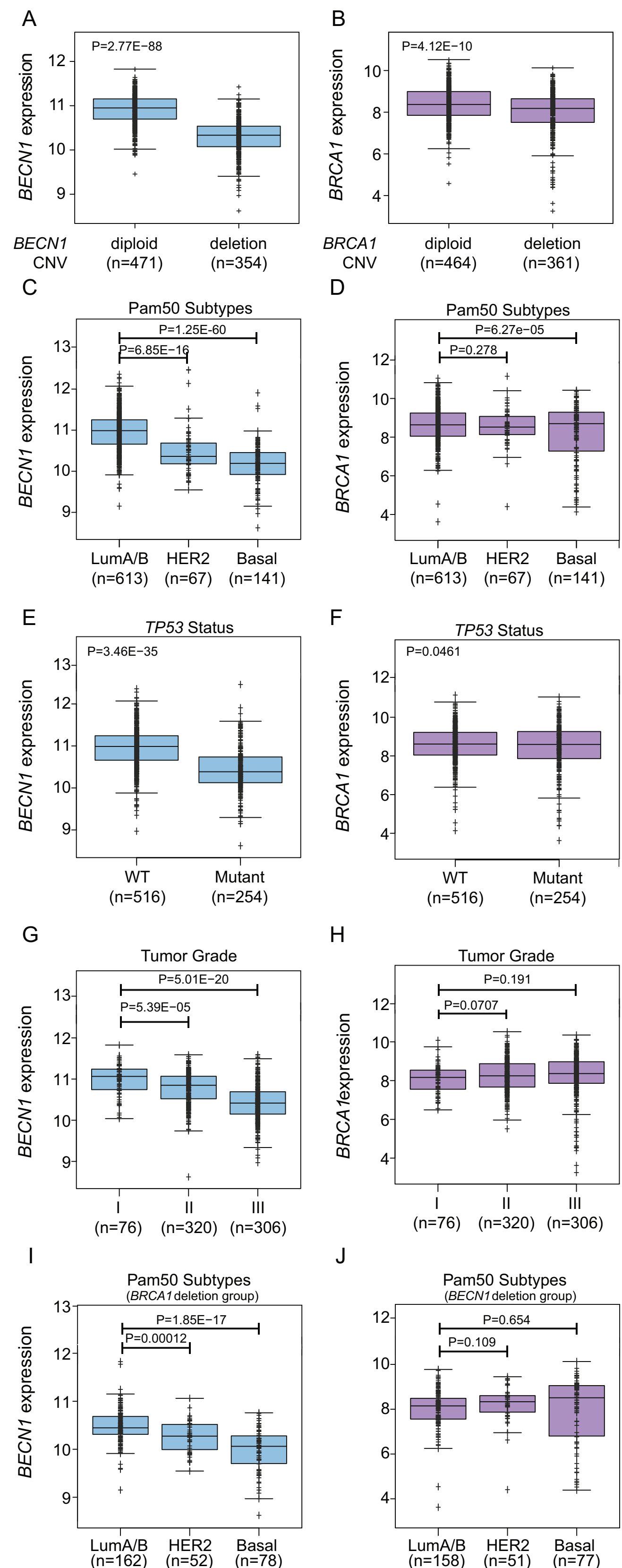

Fig. 1. Boxplot showing the distribution of *BECN1* expression and *BRCA1* expression in TCGA, according to copy number status (panels A and B), PAM50 subtypes (panels C and D), *TP53* mutation status (panels E and F), tumor grade (panels G and H), and PAM50 subtypes in copy number loss subgroups (panels I and J). The boxes represent the median (black middle line) and the 25th–75th percentiles (lower and upper box borders). Units for gene expression represent $\log_2$ RSEM counts (see Methods).

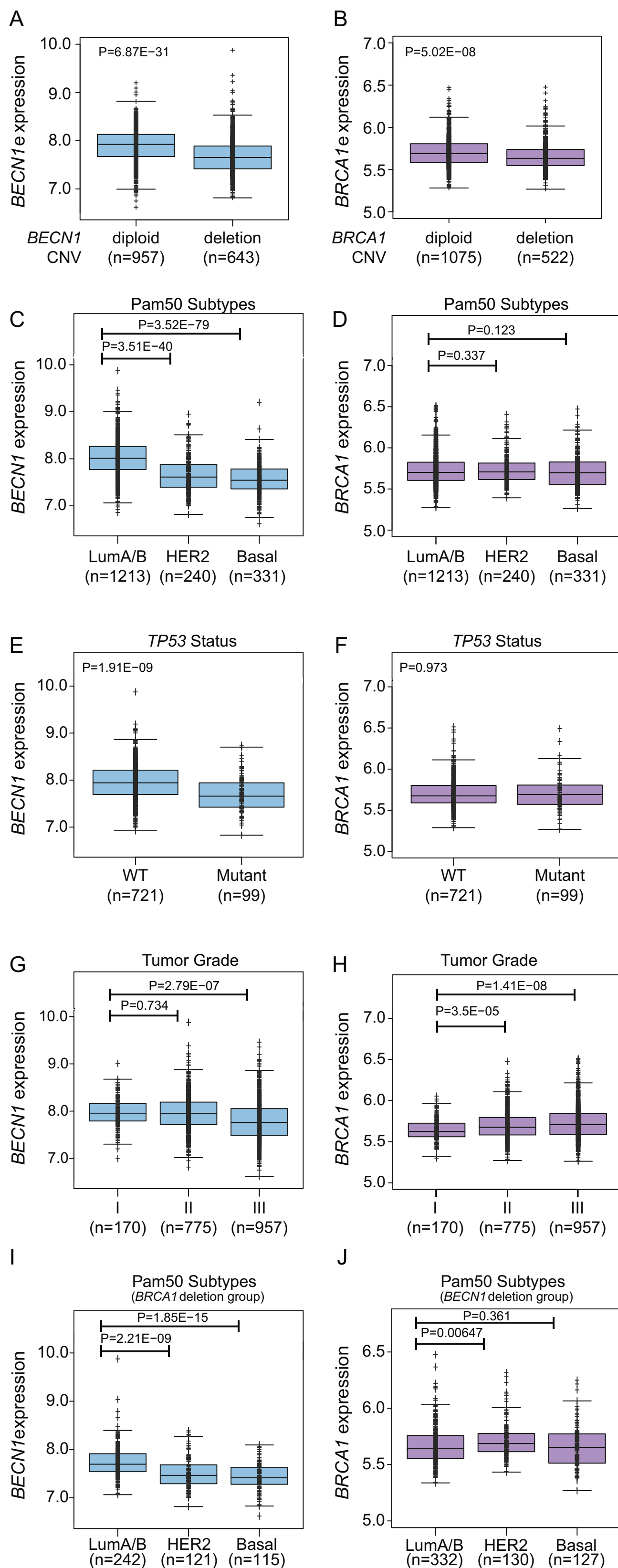

Fig. 2. Boxplot showing the distribution of *BECN1* expression and *BRCA1* expression in METABRIC according to the copy number status (panels A and B), PAM50 subtypes (panels C and D), *TP53* mutation status (panels E and F), tumor grade (panels G and H), and PAM50 subtypes in copy number loss subgroups (panels I and J). The boxes represent the median (black middle line) and the 25th–75th percentiles (lower and upper box borders). Units for gene expression represent $\log_2$ intensities of Illumina array values (see Methods).

not *BRCA1*) expression was also associated with HER2-positive and triple-negative tumors identified by mRNA expression analysis (Supplementary Table 3).

Analysis of the METABRIC dataset also confirmed that low *BECN1* expression, independently of *BRCA1* expression, was associated with an increased frequency of basal-like and HER2-enriched tumors, *TP53* mutations, and tumor grade III. Basal-like breast tumors were seen in 31.0% of patients who had low *BECN1* but high *BRCA1* expression but in only 4.3% of patients who had high *BECN1* but low *BRCA1* expression (P = 3.39E − 24 for the difference between groups) (Supplementary Fig. 1E). Similarly, as compared with the high *BECN1*/low *BRCA1* expression group, tumors with low *BECN1*/high *BRCA1* expression were more likely to be HER2-enriched (21.7% versus 4.6%, P = 2.74E − 13); have *TP53* mutations (22.1% versus 6.7%, P = 4.32E − 05); and exhibit grade III characteristics (65.7% versus 29.8%, P = 1.19E − 24) (Supplementary Fig. 1F–H).

These findings were confirmed when gene expression was compared across different tumor subtypes without dichotomization. In TCGA, *BECN1* (but not *BRCA1*) expression was significantly lower in basal-like (P = 1.25E − 60) and HER2-enriched tumors (P = 6.85E − 16), tumors with *TP53* mutations (P = 3.46E − 35), and tumors with an advanced histological grade (P = 5.39E − 05 for grade II, P = 5.01E − 20 for grade III) (Fig. 1C–H). Similarly, in the METABRIC cohort, *BECN1* (but not *BRCA1*) expression was also significantly lower in basal-like (P = 3.52E − 79) and HER2-enriched tumors (P = 3.51E − 40), tumors with *TP53* mutations (P = 1.91E − 09), and tumors with an advanced histological grade (P = 2.79E − 07 for grade III) (Fig. 2C–H). In the TCGA (but not in METABRIC) dataset, despite higher median values for *BRCA1* expression in basal-like tumors, a small proportion had very low levels of *BRCA1* expression (Fig. 1D).

Similar results confirmed the association of low *BECN1* expression with ER-negative tumor subtypes when the analyses were confined to tumors with *BRCA1* deletions. In TCGA dataset (Supplementary Table 4), low *BECN1* expression was associated with basal-like breast tumors (odds ratio 8.3 [95% CI 4.2 to 17.3], P = 3.8E − 12), HER2-enriched breast tumors (odds ratio 3.5 [95% CI 1.7 to 7.1], P = 1.7E − 04), and tumors with *TP53* mutation (odds ratio 3 · 1 [95% CI 1.8 to 5.3], P = 1.0E − 05). In the METABRIC dataset (Supplementary Table 4), low *BECN1* expression was associated with basal-like breast tumors (odds ratio 5.6 [95% CI 3.4 to 9.6], P = 5.5E − 13), HER2-enriched breast tumors (odds ratio 4.1 [95% CI 2.5 to 6.7], P = 8.8E − 10), tumors with *TP53* mutation (odds ratio 2.3 [95% CI 1.1 to 4.9], P = 0.017), and grade III tumors (odds ratio 3.4 [95% CI 1.5–8.5], P = 0.002). In both the TCGA and METABRIC datasets, in the *BECN1* deletion subgroup, low *BRCA1* expression was not associated with ER-negative tumor subtypes, *TP53* mutations or advanced tumor grade; in fact, in METABRIC, low *BRCA1* expression was inversely associated with HER2-enriched (odds ratio 0.5 [95% CI 0.3–0.8], P = 0.0018) and grade III tumors (odds ratio 0.3 [95% CI 0.2–0.6], P5.5E − 04) (Supplementary Table 4). In both TCGA and METABRIC, when gene expression was compared across different tumor types without dichotomization, *BECN1* expression in the *BRCA1* deletion group was significantly lower in HER2-enriched tumors (P = 0.00012 and P = 2.21E–09, respectively) and basal-like tumors (P = 1.85E–17 and P = 1.85E–15, respectively) (Fig. 1I, Fig. 2I). In contrast, there was no association between low *BRCA1* expression and these ER-negative PAM50 subtypes in the *BECN1* deletion group (Fig. 1J, Fig. 2J).

In a subgroup analysis of patients who were diploid for *BECN1* and *BRCA1*, in both the TCGA and METABRIC datasets, we also found that low *BECN1* mRNA expression but not low *BRCA1* mRNA expression was associated with HER2-enriched and basal-like tumor subtypes, *TP53* mutations, and grade III tumors (Supplementary Table 5). Moreover, for both TCGA and METABRIC datasets, low *BECN1* expression was associated with these same features in patients with high *BRCA1* expression (Supplementary Table 6) or low *BRCA1* expression (Supplementary Table 7). In contrast, low *BRCA1* expression was not positively associated with any of these features in high *BECN1* or low

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Table 1
BECN1 and *BRCA1* expression association with clinical features (TCGA cohort).

	BECN1 expression				*BRCA1* expression			
	mRNA expression		Odds ratio (95% confidence interval)	P value	mRNA expression		Odds ratio (95% confidence interval)	P value
	High	Low			High	Low		
PAM50 subtypes								
Luminal A/B	399	214	Reference		348	265	Reference	
HER2-enriched	12	55	8.5 [4.4, 17.9]	8.5E−14	37	30	1.1 [0.6, 1.8]	0.90
Basal-like	7	134	35.5 [16.4, 91.8]	3.8E−43	82	59	0.9 [0.6, 1.4]	0.78
TP53 mutation								
Wild type	337	179	Reference	2.6E−32	300	216	Reference	
Mutant	53	201	7.1 [5.0, 10.4]		138	116	1.1 [0.8, 1.5]	0.49
Tumor grade								
I	55	21	Reference	0.003	25	51	Reference	
II	171	149	2.3 [1.3, 4.2]	2.4E−17	133	187	0.7 [0.4, 1.2]	0.19
III	62	244	10.3 [5.6, 19.2]		145	161	0.5 [0.3, 0.9]	0.03

BECN1 expression subgroups (Supplementary Tables 6 and 7). Low *BRCA1* expression was actually inversely associated with several of these features; however, the only associations which were significant in both the TCGA and METABRIC datasets were between low *BRCA1* expression and a reduced odds of grade III tumors. This was observed in both patients with high *BECN1* expression (Supplementary Table 6) or low *BECN1* expression (Supplementary Table 7).

3.3. Association Between Low BECN1 mRNA Expression and Worse Patient Survival

Patients whose tumors had the lowest levels of *BECN1* expression had the worst prognosis (P = 2.15E−11) (Fig. 3A). In contrast, the level of *BRCA1* expression was not associated with survival (P = 0.164). Similar results were observed when the analyses were restricted to ER-negative intrinsic subtypes. Overall, the level of *BECN1* expression was directly associated with length of survival in patients with HER2-enriched tumors (P = 3.79E−04) (Fig. 3C), basal-like with IntClust 4 (P = 5.29E−04) (Fig. 3E) and basal-like with IntClust 10 (P = 0.036) (Fig. 3G). In contrast, *BRCA1* expression was not associated with survival in HER2-enriched tumors or in the two basal-like subgroups (Fig. 3D, F, and H).

By multivariate analysis, low *BECN1* expression was significantly associated with shortened survival, even after adjustment for *BRCA1* expression, age, tumor grade, tumor size, stage, intrinsic subtypes, *TP53* mutation and treatment (hazard ratio 0.6 [0.4–0.9], P = 0.02) (Table 3). Furthermore, in the *BRCA1* deletion subgroup, patients with low *BECN1* expression had a significantly worse survival than those with high *BECN1* expression (P = 0.00589) (Fig. 3I), whereas in the *BECN1* deletion subgroup, there was no significant relationship between high and low levels of *BRCA1* expression and survival (Fig. 3J).

4. Discussion

As expected due to their close proximity on chromosome 17q21, *BECN1* and *BRCA1* are often concordantly deleted or amplified in breast cancers. However, our findings indicate that decreased *BECN1* (but not decreased *BRCA1*) expression characterizes breast cancers that have aggressive molecular and clinical characteristics. When compared with tumors with high levels of expression, tumors with low *BECN1* expression were more likely to have a higher histological grade, *TP53* mutations, HER2-enriched or basal-like intrinsic subtypes, triple-negative status, and worse survival. In contrast, the levels of *BRCA1* expression did not distinguish tumors with these aggressive characteristics or unfavorable prognosis. Furthermore, in tumors with deletion of *BRCA1*, levels of *BECN1* expression provided important additional discriminatory information; however, in tumors with deletion of *BECN1*, levels of *BRCA1* expression did not distinguish the molecular and clinical features of tumors. Importantly, these relationships were observed across two independent regional databases with different expression analysis platforms (RNA-seq and microarray), suggesting that our results cannot be explained by population differences or idiosyncrasies in the characterization of tumors.

Our findings are consistent with earlier studies of *BECN1* in small cohorts of patients with breast cancer. Levels of *BECN1* mRNA expression have been reported to be reduced in breast cancer (Li et al., 2010; T. Wu et al., 2012) and have been associated with poor differentiation, and increased tumor size, proliferation and risk of metastasis (T. Wu et al., 2012; Yao et al., 2011). In small datasets, low *BECN1* mRNA expression was associated with triple-negative breast cancer (Cicchini et al., 2014) and with worse prognosis regardless of ER status (Perou et al., 2000; Dong et al., 2013). In addition, *BECN1* DNA copy number loss has been reported to be associated with HER2 amplification and *TP53* mutations (Negri et al., 2010).

Table 2
BECN1 and *BRCA1* expression association with clinical features (METABRIC cohort).

	BECN1 expression				*BRCA1* expression			
	mRNA expression		Odds ratio (95% confidence interval)	P value	mRNA expression		Odds ratio (95% confidence interval)	P value
	High	Low			High	Low		
PAM50 subtypes								
Luminal A/B	804	409	Reference		656	557	Reference	
HER2-enriched	63	177	5.5 [4.0, 7.7]	1.4E−30	136	104	0.9 [0.7, 1.2]	0.48
Basal-like	54	277	10.0 [7.3, 14.1]	1.4E−61	168	163	1.1 [0.9, 1.5]	0.29
TP53 mutation								
Wild type	423	298	Reference		345	376	Reference	0.52
Mutant	32	67	3.0 [1.9, 4.8]	8.9E−07	51	48	0.9 [0.6, 1.3]	
Tumor grade								
I	111	59	Reference		53	117	Reference	
II	462	313	1.3 [0.9, 1.8]	0.194	371	404	0.5 [0.3, 0.7]	8.3E−05
III	379	578	2.9 [2.0, 4.1]	5.8E−10	526	431	0.4 [0.3, 0.5]	1.1E−08

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One previous analysis of TCGA dataset by Laddha et al. (2014) reported deletions of *BRCA1* alone but not *BECN1* alone in human breast cancer. That study, however, used an ad hoc heuristic approach for identifying deletions; our analyses of copy number variations based on the more rigorous GISTIC method could not confirm this earlier report. In fact, in METABRIC, *BECN1* alone deletions were more common

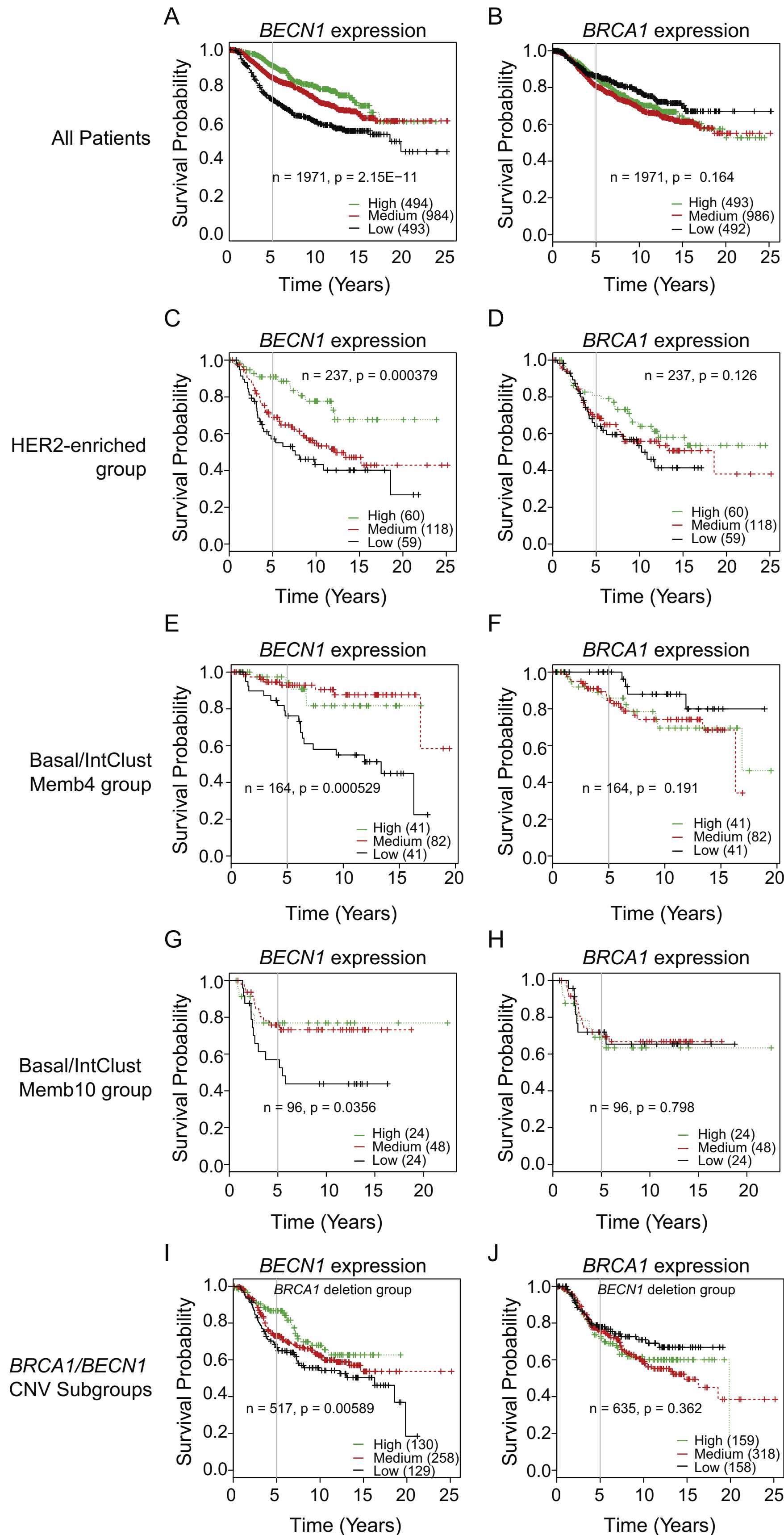

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Table 3
Multivariate survival analysis.*

	Hazards ratio (95% confidence interval)	P value
BRCA1 expression	1.0 (0.4, 2.2)	0.95
BECN1 expression	0.6 (0.4, 0.9)	0.02
Age at diagnosis	1.02 (1.00, 1.03)	0.03
Tumor grade		
I	Reference	
II	1.0 (0.5, 2.2)	0.94
III	1.4 (0.7, 3.1)	0.37
Tumor size		
<= 20 (T1)	Reference	
20–50 (T2)	1.6 (1.1, 2.2)	0.01
>50 (T3)	1.5 (0.7, 3.3)	0.27
Tumor stage		
Stage 0	Reference	
Stage 1	0.8 (0.5, 1.2)	0.31
Stage 2	0.5 (0.3, 0.8)	2.8E−03
Stage 3	1.8 (1.0, 3.2)	0.06
Stage 4	1.7 (0.4, 7.4)	0.46
PAM50 subtype		
Luminal A/B	Reference	
HER2-enriched	0.9 (0.5, 1.6)	0.68
Basal-like	0.6 (0.3, 1.1)	0.07
TP53 mutation status		
Wild type	Reference	
Mutant	2.1 (1.5, 3.0)	7.4E−05
Treatment		
Radiation therapy	Reference	
Hormonal therapy	1.0 (0.5, 2.0)	0.94
Hormonal/radiation therapy	1.5 (0.8, 2.9)	0.20
Chemotherapy	1.5 (0.8, 2.8)	0.17
Chemotherapy/radiation therapy	6.8 (2.6, 17.8)	8.1E−05
Chemotherapy/hormonal therapy	5.4 (2.3, 12.6)	8.7E−05
Chemotherapy/hormonal/radiation therapy	2.2 (0.5, 10.4)	0.32
Radiation therapy	2.3 (1.1, 4.9)	0.04

* Multivariate Cox regression model was performed to assess the relative contribution of *BECN1* or *BRCA1* mRNA expression in predicting prognosis, after adjusting for other clinical factors listed in the table. To reduce potential bias from dichotomization, continuous gene expression values were used.

than *BRCA1* deletions, indicating a further lack of confirmation of the findings of Laddha et al. In addition, Laddha et al. reported that there were no changes in the mean level of *BECN1* mRNA expression in breast tumor samples versus normal tissue. However, the validity of this comparison is difficult to assess, since epithelial cells (which have very high levels of *BECN1* expression) comprise the majority of cells in tumor samples but only a small proportion of cells in normal breast tissue. Most importantly, Laddha et al. considered human breast cancer to be a homogenous disease and did not analyze the relationship between *BECN1* mRNA expression and specific clinical and pathological features of breast cancer. Our analyses of two large datasets, TCGA and METABRIC, revealed a marked association between low *BECN1* expression and ER-negative breast cancers subtypes with aggressive clinical features.

Our finding that low *BRCA1* expression was not associated with basal-like subtype or worse survival is consistent with the lack of evidence that somatic loss of *BRCA1* contributes meaningfully to sporadic breast cancer. Only homozygous, not heterozygous, *Brca1* knockout mice develop breast cancers (Evers and Jonkers, 2006), whereas breast (and other) cancers develop in *Becn1* heterozygous knockout mice (Qu et al., 2003; Yue et al., 2003; Cicchini et al., 2014). Moreover, loss of *BRCA1* heterozygosity in humans with germline *BRCA1* mutations is necessary for the development of BRCA1 mutant-associated breast cancers (Futreal et al., 1994). This is likely because haploinsufficient *BRCA1* expression is sufficient for full DNA repair (Latimer et al., 2005). Thus, given the rare frequency of somatic *BRCA1* mutations (despite the high prevalence of *BRCA1* heterozygous loss) (Futreal et al., 1994), a role for *BRCA1* deficiency in sporadic breast cancer is not established.

Nonetheless, previous studies have shown similarities between the clinical and molecular features of sporadic basal-like tumors and familial *BRCA1*-mutated tumors, resulting in the model that basal-like tumors may be associated with *BRCA1* dysfunction (Turner et al., 2004, 2007; Valentin et al., 2012; Turner and Reis-Filho, 2006). Low *BRCA1* expression and/or *BRCA1* promoter methylation has been associated with basal-like sporadic breast cancers in some reports (Turner et al., 2007; Joosse et al., 2011; Lee et al., 2010; Rakha et al., 2008b), but not others (Matros et al., 2005; Richardson et al., 2006). Regardless of their findings, these studies generally analyzed small numbers of patients; did not identify tumor subtypes by molecular profiling; and identified low *BRCA1* samples using immunohistochemical staining for protein expression or quantitative PCR for mRNA expression, which are both subject to difficulties in standardization and reproducibility. Our study is the first to apply current state-of-the-art methods for *BRCA1* mRNA quantification to a large number of samples characterized by intrinsic molecular subtypes. Our inability to find a relation between *BRCA1* expression and basal-like breast cancers supports the concept that the phenotypic similarities of sporadic basal-like breast tumors and hereditary *BRCA1* mutated tumors may be explained by factors other than *BRCA1* dysfunction (Matros et al., 2005). Alternatively, our data (Fig. 1D) suggests that low *BRCA1* expression may characterize only a small subgroup of basal-like tumors, whose specific features are yet to be defined. Another possible explanation is that other factors, besides somatic mutations or decreased mRNA expression (either as a result of copy number variation or epigenetic regulation), act to impair *BRCA1* function in sporadic breast cancer. Thus, although our results consistently show a lack of relationship between decreased *BRCA1* expression and basal-like breast cancer, they cannot definitively exclude a role for *BRCA1* dysfunction in sporadic basal-like breast cancer.

We propose that the decreased expression of *BECN1* (another tumor suppressor gene located near *BRCA1*) in sporadic basal-like breast tumors may partly explain the phenotypic overlap of this disease with hereditary *BRCA1* breast cancer. Patients with germline mutations in *BRCA1* usually have somatic deletion of wild-type chromosome 17q21 in their breast tumors; (Turner et al., 2004; Palacios et al., 2008) thus, the co-deletion of *BECN1* in such cases may contribute to the development of basal-like features. Independently of whether the co-deletion of *BECN1* plays a role in hereditary *BRCA1* breast cancer, decreased *BECN1* expression — which results in reduced levels of autophagy (Qu et al., 2003) — may exert effects on the DNA damage repair pathway in sporadic breast cancer similar to those produced by a *BRCA1* mutation and loss of heterozygosity in hereditary breast cancer. In support of this theory, knockdown of another essential autophagy gene, *ATG5*, suppresses the expression of RAD51, a key protein that functions in homologous recombination and repair of DNA double-stranded breaks (Mo et al., 2014).

Taken together, our findings suggest that decreased *BECN1* expression may contribute to the pathogenesis and/or progression of certain breast cancers, especially the ER-negative subtypes. A deficiency of *BECN1* leads to defects in autophagy (Qu et al., 2003), a lysosomal

Fig. 3. *BECN1* expression but not *BRCA1* expression is associated with disease-specific survival. Panels A and B: Kaplan–Meier curves for all patients for *BECN1* expression (panel A) or *BRCA1* (panel B) expression. Panels C through J: Kaplan–Meier curves within HER2-enriched group (panels C and D), Basal/IntClust Memb4 (panels E and F), Basal/IntClust Memb10 (panels G and H), and in copy number loss subgroups (panels I and J). Green, red and black lines indicate high (1st quartile), medium (2nd and 3rd quartiles), and low (4th quartile) expression level groups, respectively. + denotes censored observations. P values were obtained by the Cox regression model assuming an ordered trend for the three expression groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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degradation "housekeeping" pathway that prevents chromosomal instability and DNA damage and inhibits cellular proliferation; (Levine and Kroemer, 2008) alternatively, loss of other functions of *BECN1* (e.g., receptor endocytosis) (Funderburk et al., 2010) may play a role in carcinogenesis. Future clinical trials should evaluate whether the level of *BECN1* expression predicts the response to specific chemotherapeutic regimens or whether strategies that increase *BECN1* function might be therapeutic in patients with low *BECN1* expression. Of note, the autophagy activity of Beclin 1 is inhibited by interaction with BCL-2 family members (Pattingre et al., 2005; Maiuri et al., 2007), by oncogenic kinase AKT and EGFR-mediated Beclin 1 post-translational modifications (Wang et al., 2012; Wei et al., 2013), and by interactions with HER2 (Han et al., 2013). Thus, currently available Beclin 1/BCL-2 binding inhibitors, AKT inhibitors, EGFR inhibitors and HER2 inhibitors may act to increase Beclin 1 function in tumors with low *BECN1* expression, and thereby, improve clinical outcomes.

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Author Contributions

HT, SS, MP, YX, and BL contributed to the study design, data analysis, data interpretation and writing of the report. RT contributed to the study design and data analysis. YZ and GX contributed to data analysis. CI and HH contributed to the study design and data interpretation. TR contributed to data interpretation and writing of the report.

Declaration of Interests

Beth Levine has received consulting fees from Novus Biologicals.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.01.008.

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INVOLVEMENT OF AUTOPHAGY IN THYROID AND SKIN CANCERS: THE ROLE OFONCOSUPPRESSORS PTEN AND P53

5. Autophagy and Thyroid Carcinogenesis: Genetic and Epigenetic Links

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Thyroid cancer accounts for almost 90% of all endocrine related cancers, and is responsible for the majority of deaths from endocrine malignancies. Autophagy plays an important role in cancer. In thyroid cancer is involved in the cytotoxic response to chemotherapeutics. In this review we report our preliminary observation on the detection of autophagy markers. We detected autophagy markers in tissue biopsies and in cultured cells of thyroid cancer origin by western blotting and immunofluorescence. We highlight the genetic and epigenetic factors that mechanistically link thyroid carcinogenesis and autophagy, thus substantiating the rationale for an autophagy-targeted therapy of aggressive and radio-chemo-resistant thyroid cancers.

Personal Contribution

In this paper, I contributed to the literature search and discussion of data.

Autophagy and thyroid carcinogenesis: genetic and epigenetic links

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Abstract

Thyroid cancer is the most common cancer of the endocrine system and is responsible for the majority of deaths from endocrine malignancies. Although a large proportion of thyroid cancers belong to well differentiated histologic subtypes, which in general show a good prognosis after surgery and radioiodine ablation, the treatment of radio-resistant papillary-type, of undifferentiated anaplastic, and of medullary-type thyroid cancers remains unsatisfactory. Autophagy is a vesicular process for the lysosomal degradation of protein aggregates and of damaged or redundant organelles. Autophagy plays an important role in cell homeostasis, and there is evidence that this process is dysregulated in cancer cells. Recent *in vitro* preclinical studies have indicated that autophagy is involved in the cytotoxic response to chemotherapeutics in thyroid cancer cells. Indeed, several oncogenes and oncosuppressor genes implicated in thyroid carcinogenesis also play a role in the regulation of autophagy. In addition, some epigenetic modulators involved in thyroid carcinogenesis also influence autophagy. In this review, we highlight the genetic and epigenetic factors that mechanistically link thyroid carcinogenesis and autophagy, thus substantiating the rationale for an autophagy-targeted therapy of aggressive and radio-chemo-resistant thyroid cancers.

Key Words

- autophagy
- thyroid cancer
- oncogenes
- epigenetics
- microRNA

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Introduction

Thyroid cancer accounts for almost 90% of all endocrine-related cancers, and is responsible for the majority of deaths from endocrine malignancies (Siegel *et al.* 2013). Thyroid cancers may arise from either the follicular (thyroid hormone-producing) or the parafollicular (calcitonin-producing) cells (Fig. 1). The large majority of follicular cell-derived thyroid cancers are well differentiated and are classified as papillary (about 80% of all thyroid cancers) or follicular thyroid cancer (PTC and FTC, respectively), and a minor portion show a poorly differentiated or undifferentiated (anaplastic) phenotype (named as poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC), respectively). Thyroid cancers arising from parafollicular cells, named medullary thyroid cancers (MTC), account for 3–5% of all thyroid cancers. PTC is generally associated with favorable outcomes after surgery and radioactive iodine therapy, although 5% of these tumors show radio- and chemo-resistance (Fassnacht *et al.* 2009, Grodski & Delbridge 2009). On the other hand, ATC is extremely aggressive and soon leads the patient to death (Ain 1998, Smallridge 2012, Smallridge *et al.* 2012). MTC also have a generally

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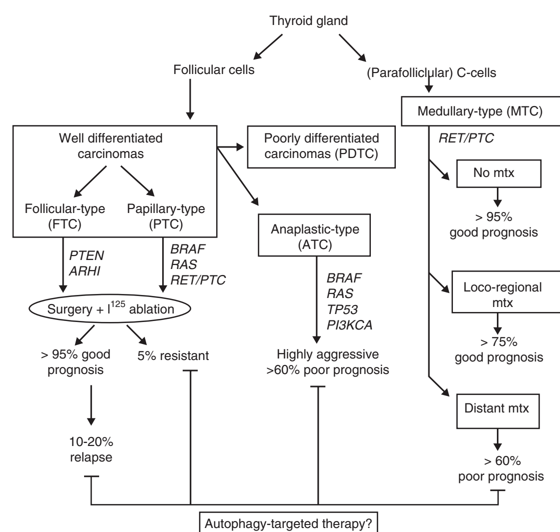

Figure 1
Autophagy-targeted opportunities for the therapy of thyroid cancers. Origin and aggressive phenotypes of thyroid cancers and opportunities for autophagy-targeted therapies. The main alterations in oncogenes and oncosuppressor genes in thyroid cancer histotypes are indicated in the boxes. C-cell, calcitonin-producing parafollicular cell; mtx, metastases.

good prognosis, with an overall survival rate at 10 years of ~95% if the tumor is confined to the thyroid gland, which drops to ~40% in the presence of metastases (Roman *et al*. 2006). Patients with an unresectable tumor or with distant metastases can be treated with chemotherapeutics (e.g., doxorubicin, 5-fluorouracil, cisplatin), yet the response rate is very low. Molecular therapy with inhibitors of mitogenic kinases has been disappointing, because it has not substantially improved the survival of patients with aggressive thyroid cancers, while showing an adverse side-effect profile (Gild *et al*. 2011). For instance, the multi-kinase inhibitor vandetanib, which has been approved for the treatment of inoperable or metastatic MTC (Thornton *et al*. 2012), has shown a modest efficacy toward MTC progression, but an extremely toxic profile that includes gastrointestinal, cardiovascular, and neurological disorders (Chau & Haddad 2013). Similarly, the results of a phase II clinical trials on the efficacy of the mammalian target of rapamycin (mTOR) inhibitor everolimus for the treatment of locally advanced or metastatic thyroid cancer have been disappointing (Lim *et al*. 2013). Thus, the lack of efficacious and safe treatment options provokes the search for novel molecular targeted drugs for the cure of such highly malignant thyroid cancers.

Recently, autophagy has emerged as a potential target for the therapy of hematologic and epithelial malignancies (Chen & Karantza 2011, Gundara *et al*. 2012, Wu *et al*. 2012). *In vitro* pre-clinical studies support the possibility of harnessing autophagy for the therapy of thyroid cancers (Lin *et al*. 2009, 2010, 2012*a*,*b*, Lu *et al*. 2012, Jin *et al*. 2013).

Autophagy (literally, 'self-eating') is the process through which damaged or redundant cytoplasmic constituents are degraded within lysosomes. Several oncogenes and oncosuppressor genes regulate the induction of autophagy. Autophagy is also epigenetically regulated through the methylation of autophagy regulatory genes, the activity of histone deacetylases (HDAC), and the expression of microRNAs (miRNAs). Here, we examine the genetic and epigenetic links between autophagy and thyroid carcinogenesis. A better understanding of such mechanistic connections could help to identify new targets for a more accurate diagnostic, prognostic, and therapeutic management of thyroid cancers.

The autophagy machinery

Macroautophagy is a vesicular-driven process through which protein macroaggregates, large portions of membranes, and entire organelles can be delivered to lysosomes for complete degradation. This process is distinct from chaperon-mediated autophagy and microautophagy in which, respectively, only a single protein at a time or a small amount of cytoplasmic material is internalized in the lysosome. Readers may refer to the many excellent reviews in which the morphological and biochemical features of these processes are described in detail (Orenstein & Cuervo 2010, Yang & Klionsky 2010, Mizushima & Komatsu 2011, Sahu *et al*. 2011). Here, we will focus on macroautophagy (from now on simply named autophagy), as this is the major pathway contributing to the macromolecular turnover and cell homeostasis. In the following paragraphs, we briefly detail the key morphological and regulatory steps of autophagy.

Morphological features and physiological significance

Autophagy comprises the following principal steps: i) the formation of a vacuole, named the autophagosome, that entraps the cargo to be degraded; ii) the fusion of the autophagosome with endosomes and lysosomes that leads to the formation of an autophagolysosome; and iii) the degradation of the autophagy cargo (Fig. 2). The autophagosome is a double-layered vesicle that forms in the proximity of the trans-Golgi network. The progenitor membrane donor of the autophagosome is the smooth

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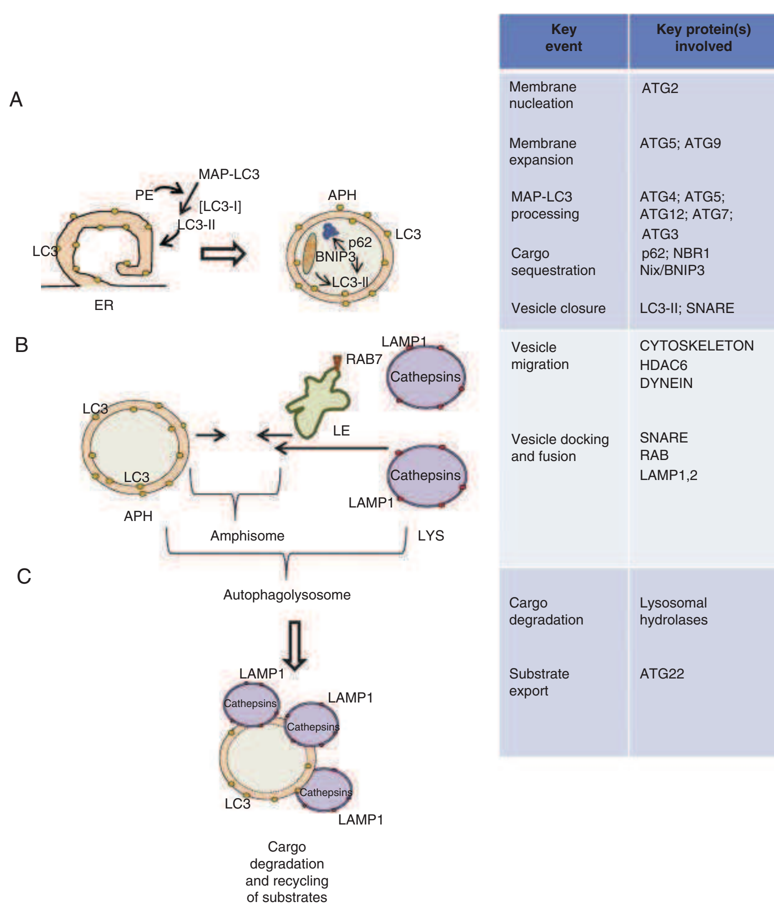

Figure 2

Morphological features of the (macro)autophagy process. The process can be dissected in three main steps. The key events and the relative protagonists of each step are indicated. (A) Formation of the autophagosomes (APH). Vesicle nucleation and membrane expansion start from the endoplasmic reticulum (ER). The autophagosome is marked by the presence on its internal and external membranes of LC3-PE, arising from MAP-LC3 (MAP-LC3, microtubule associate protein light chain 3; PE, phosphatydylethanolamine). The autophagy cargo includes portions of cytoplasm, protein aggregates, and mitochondrion, which are targeted by specific proteins such as p62, neighbor of BRCA1 (NBR1), and Bcl2/adenovirus E1B 19-kDa interacting protein (BNIP3). (B) Fusion of the autophagosome with late endosomes (LE, identified by RAB7) to form an amphisome, and with lysosomes (LYS, identified by LAMP1) to form an autophagolysosome. This step requires HDAC6-mediated deacetylation of tubulin and the activity of dynein. Tethering of autophagosomes and lysosomes relies on soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins. (C) Degradation of autophagy cargo and recycling of substrates. In the autophagolysosome, the inner membrane of the autophagosome along with its cargo is degraded by lysosomal acid enzymes (essentially the cathepsins). This process is marked by the consumption of LC3 present in the inner membrane of the autophagosome. Substrates are then exported in the cytoplasm and recycled in the biosynthetic pathway. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0271.

endoplasmic reticulum, though membranes from other sources, including the plasma membrane and the outer mitochondrial membrane, are subsequently recruited and contribute to the expansion of this vesicle (Ravikumar *et al.* 2010, Tooze & Yoshimori 2010, Rubinsztein *et al.* 2012, Hamasaki *et al.* 2013). During this process, the lipidated form of light chain 3 (LC3, also known as ATG8) is post-translationally inserted into the expanding autophagosomal membrane through the intervention of several autophagy-related (ATG) proteins (Mizushima *et al.* 2011; box in Fig. 2). The synthesis and membrane translocation of LC3II is considered an hallmark of autophagosome biogenesis (Klionsky *et al.* 2012). Autophagy substrates are specifically sequestered in the lumen of the nascent autophagosome through the intervention of proteins that bridge the substrate to LC3 in the internal membrane (Noda *et al.* 2010; box in Fig. 2). The autophagy process proceeds with the fusion of the autophagosome with several endosomes and lysosomes (at the end an autophagolysosomal vacuole is formed) and the subsequent full

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degradation of the autophagy substrates is conducted by lysosomal acid hydrolases (Eskelinen 2005). Finally, fully degraded substrates are exported to the cytoplasm and reutilized in biosynthetic pathways. It is to be stressed that autophagy has to proceed to completion to exert its prosurvival effects. The production of autophagosomes that do not completely fuse with lysosomes is of no benefit to the cell, because the autophagy substrates are not fully degraded and recycled, and may eventually become toxic. Therefore, to understand the pathophysiological outcome of autophagy, it is important to clearly determine the formation of autophagosome vs the autophagy flux (see section Detection of autophagy in thyroid cancer cells).

Biochemical regulation of autophagy at a glance

The induction and progression of autophagy are controlled by a complex network of signaling pathways that involve a number of protein- and lipid-kinases, protein- and lipid-phosphatases, and monomeric and trimeric GTPases (Mehrpour *et al*. 2010, Chen & Klionsky 2011). The pathways illustrated in Fig. 3 represent an obvious, though efficacious, oversimplification. The master signal that triggers autophagy comes from the Unc51-like kinase 1 (ULK1, homolog of yeast Atg1) complex (Wong *et al*. 2013). Two upstream kinases, namely the mTOR-raptor complex 1 (mTORC1) and AMPk, control the activation of ULK1, the former acting as a repressor (Ganley *et al*. 2009, Jung *et al*. 2009) and the latter acting as an activator (Egan *et al*. 2011). mTORC1 integrates the signals from: i) the phosphatydilinositol-3-kinase (PI3k) class I/AKT pathway, which senses the presence of growth factors; ii) the AMPk pathway, which senses the lack of energy; and iii) the Rag A/B (a Ras-related GTPase) complex, which senses the availability of amino acids. In the presence of growth factors, the PI3k/AKT pathway negatively regulates autophagy through a tonic activation of mTORC1 via the tuberous sclerosis complex (TSC) and Rheb (Ras homolog enriched in the brain) (Petiot *et al*. 2000, Arico *et al*. 2001, Inoki *et al*. 2002). The lipid phosphatase activity of phosphatase and tensin homolog (PTEN) shuts down this pathway, thus abolishing the mTOR-mediated repression of ULK1. When amino acids are abundant, the RagA/B complex activates mTORC1 (Sancak *et al*. 2010), while the lack of amino acids is sensed by the RAS–BRAF–ERK1/2 pathway that triggers autophagy through the stimulation of heterotrimeric GTP proteins (Ogier-Denis *et al*. 1995, 2000). Glucose depletion and other metabolic stresses that reduce the production of ATP or provoke the production of reactive oxygen species (ROS) activates the AMPk pathway, that in turn represses mTORC1 and activates ULK1 (Alers *et al*. 2012), thus initiating autophagy (Alexander *et al*. 2010, Castino *et al*. 2011, Janda *et al*. 2012, Wong *et al*. 2013). Downstream to ULK1, class III PI3k (also known as Vps34) produces phosphatydil-3-phosphate (PI3P), the starting platform for the biogenesis of the autophagosome (Noda *et al*. 2010). Vps34 is activated through its interaction with Beclin1 (also known as ATG6 or Vps30) and p150 (homolog of Vps15), besides other regulating proteins (He & Levine 2010). This pathway is impaired when Beclin1 is sequestered through the binding with BCL2 (Pattingre *et al*. 2005).

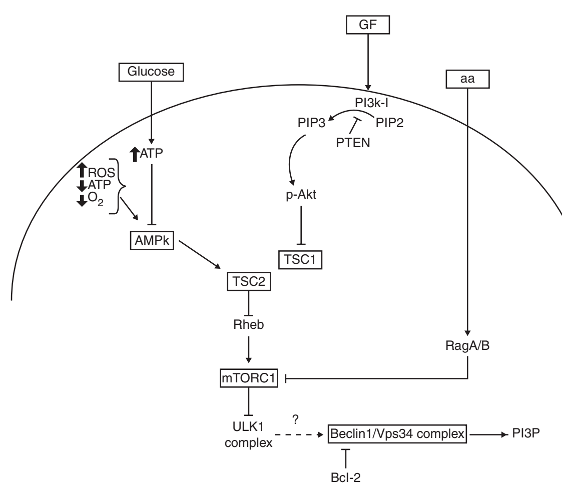

Figure 3
Biochemical regulation of the autophagy process. The scheme illustrates the main signaling pathways that control the induction of autophagy. Growth factors (GF) stimulate the activity of class I PI3k, which produces PIP3 and leads to activation of the AKT–mTOR axis. PTEN reduces the availability of PIP3, and therefore prevents the activation of the AKT–mTOR axis. Amino acids signal through the RagA/B pathway to keep mTOR active. The activation of mTORC1 negatively impinges on the ULK complex, thus inhibiting the induction of autophagy. In the absence of growth factors and of amino acids (starvation) this pathway is switched off, and mTOR is inactive, thus allowing the rise in the level of basal autophagy. The lack of glucose and of oxygen, as well as the presence of pro-oxidant species (e.g., ROS), activate the AMPk pathway, which results in the inhibition of mTOR and direct activation of the ULK complex and ultimately in the rise of autophagy. Largely unknown is the link between the ULK complex and the BECLIN1–Vps34 complex. The latter is inhibited when BCL2 binds to BECLIN1. Once activated, Vps34 produces PI3P.

Role of autophagy in cancer development and progression

In quiescent and appropriately fed cells, autophagy runs at a constant basal level that ensures the homeostatic macromolecular turnover (Ravikumar *et al*. 2010), and it is

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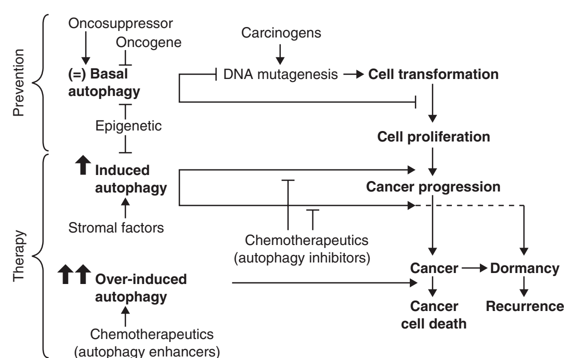

Figure 4
Double-face role of autophagy in the carcinogenic process and role of genetics and epigenetics in its regulation. Basal autophagy exerts a protective action in cells by eliminating molecules that could lead to DNA mutagenesis and cell transformation. Primitive mutations in certain oncogenes or oncosuppressors, as well as epigenetic regulation of certain autophagy-related genes, could limit the benefit of autophagy at this preventive step. Once cancer is established, autophagy may confer the advantage to cancer cells to face metabolic stresses (such as nutrient depletion, hypoxia, and chemotherapy-induced damages) and possibly to survive in a dormant state. At this stage, a chemotherapeutic regimen including autophagy-inhibiting drugs could elicit cancer cell death. On the other hand, chemotherapeutics that increases autophagy to a level beyond the point-of-no-return could also elicit cell death.

upregulated above the baseline when the lack of nutrients or of energy imposes the degradation of redundant self-constituents to recover substrates and energy necessary for cell survival, or when it is necessary to eliminate a cell component that has been damaged by an extracellular insult (a toxic drug, radiation, or oxidative stress) (Kroemer *et al*. 2010, Ravikumar *et al*. 2010). Given these housekeeping functions, it is logical to suspect that autophagy is deregulated in cancer or, conversely, that deregulation of autophagy can promote carcinogenesis. However, the role of autophagy in cancer is not unequivocal, because, paradoxically, it may either prevent cell transformation or favor the survival of cancer cells, depending on how autophagy is regulated in the various steps of the carcinogenic process (White & Di Paola 2009; Fig. 4). On one hand, autophagy opposes cell transformation by helping to get rid of mutagenic pro-oxidant molecules and by cooperating with the DNA repair system (Robert *et al*. 2011, Rodriguez-Rocha *et al*. 2011). Yet, this same process can turn to the advantage of cancer cells subjected to the genotoxic stress imposed by radiotherapy and chemotherapy. In addition, in growing tumors with defective vascularization there are areas in which the supply of nutrients and of oxygen is insufficient, and here autophagy is upregulated allowing cancer cells to survive despite the prohibitive metabolic conditions (Degenhardt *et al*. 2006), possibly in a dormant state (Lu *et al*. 2008). These cells resist to chemo- and radio-therapeutic treatments, and eventually give rise to cancer relapse. Further, a transient upregulation of autophagy is observed during the epithelial–mesenchymal transition (EMT; Akalay *et al*. 2013), and this function prevents the cell death by anoikis of cancer cells which have detached from the basement membrane to invade the extracellular matrix (Fung *et al*. 2008). Thus, autophagy is differently regulated during the carcinogenic process and its actual level in the cell could vary in subclones, depending on the acquisition of new oncogenic assets. The situation is far more complicated considering that autophagy is influenced by epigenetic factors (see below) and by extracellular factors such as oxygen, glucose, nutrients, growth factors, hormones, and cytokines.

From the above considerations it appears clear that autophagy has a great impact on the progression of tumors and on the response to therapeutic treatments, and therefore influences the prognosis. Consistently, certain autophagy-related proteins have been shown to be of prognostic value. For instance, the hyperexpression of BECLIN1 and of LC3 in general associates with a better prognosis in patients with glioblastoma (Pirtoli *et al*. 2009), colorectal cancer (Li *et al*. 2009, Koukourakis *et al*. 2010), lymphomas (Nicotra *et al*. 2010, Huang *et al*. 2011), or duodenal adenocarcinoma (Wu *et al*. 2013). Conversely, low expression of BECLIN1 or of LC3 associates with poor prognosis in patients with hepatocarcinoma (Ding *et al*. 2008), glioblastoma (Huang *et al*. 2010), colorectal cancer (Koukourakis *et al*. 2010), lymphoma (Nicotra *et al*. 2010), or lung carcinoma (Won *et al*. 2012).

Autophagy and thyroid cancer

A systematic study addressing the prognostic value of autophagy in thyroid cancers has not yet been performed. Still, some *in vitro* studies have proven the involvement of autophagy in the cytotoxic response of thyroid cancer cells to anti-tumor drugs (Table 1). In this section, we provide some technical tips for assessing the presence of autophagy in thyroid cancer biopsies and discuss the mechanistic links between autophagy and thyroid carcinogenesis.

Detection of autophagy in thyroid cancer cells

The expression of autophagy protein markers can be demonstrated in *ex-vivo* thyroid tumor tissue and in

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Table 1 Studies involving autophagy in thyroid cancer cells

Thyroid cancer histotype	Anti-tumor treatment	Autophagy manipulation	Autophagy involvement	Conclusions	Reference
Papillary (TPC1 and 8505-C cells)	Doxorubucin (anthracycline antibiotic); radiation	3-MA inhibition of PI3K	Inhibition of autophagy promotes resistance to doxorubicin and radiation	Autophagy may be useful for the treatment of refractory papillary thyroid cancer	Lin *et al.* (2009)
Papillary (TPC1 and 8505-C cells)	RAD001 (rapamycin analogue, also known as everolimus or afinitor)	RNAi knockdown of ATG5; transfection with EGFP-LC3 plasmid; 3-MA inhibition of PI3K	ATG5 RNAi knockdown abrogates the effects of RAD001. Autophagic activation resulted in Src phosphorylation and Met dephosphorylation	RAD001 induces autophagy that enhances the therapeutic response to doxorubicin and external beam radiation	Lin *et al.* (2010)
	Other treatments: doxorubicin, desatinib, PHA665752		Src inhibition did not reverse the effects of RAD001, whereas MET inhibition reversed the effects of autophagy blockade on chemosensitivity		
Follicular (WRO cells)	Reversine (2,6-disubstituted purine ATP-analog)	Transfection with pEGFPC1-LC3; 3-MA inhibition of PI3K	Autophagy promotes anti-cancer activity through cell-cycle arrest and apoptosis	Reversine is effective to induce autophagy (autophagosome formation) and reduce the activation of Akt/mTOR pathway	Lu *et al.* (2012)
Medullary (MTC1.1 and TT cells)	Sunitinib, sorafenib (RET inhibitors)	Transfection with ATG5 siRNA	Silencing of ATG5 diminishes the antiproliferative effects of sunitinib and sorafenib, and abrogates both everolimus and trehalose-induced increases in tyrosine kinase inhibitor efficacy	Activation of autophagy potentiates the anti-cancer effect of sunitinib and sorafenib	Lin *et al.* (2012*a*)
	Everolimus, trehalose (autophagy activators)			Autophagy activators should be combined with targeted RET protooncogene therapy in MTC	
Papillary (TPC1 cells) and anaplastic (FRO cells)	Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)	Transfection with ATG7 siRNA	Inhibition of autophagy renders TPC1 cells resistant to the effect of TRAIL, while it sensitizes FRO cells to TRAIL-induced apoptosis	Modulation of autophagy could be combined with TRAIL for the treatment of thyroid cancer	Jin *et al.* (2013)

3-MA, 3-methyladenine.

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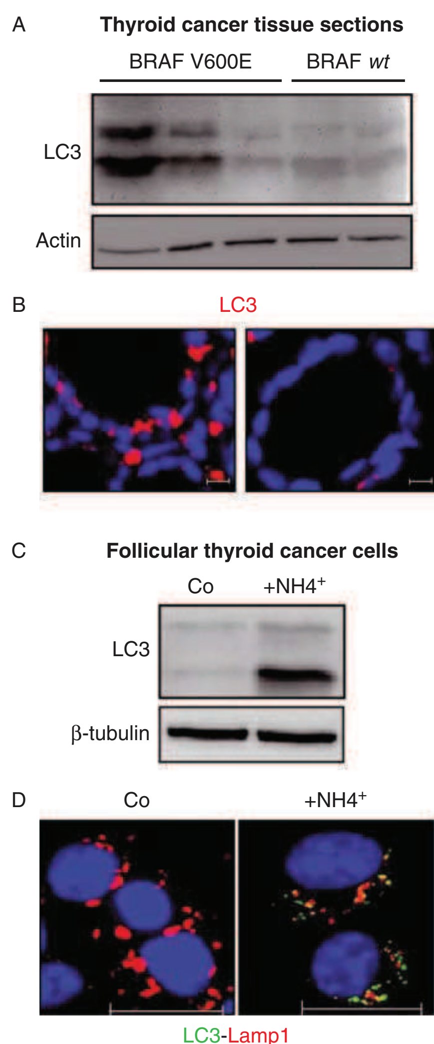

Figure 5
Detection of autophagy in thyroid cancer. Representative images showing the detection of markers of autophagy in thyroid cancer tissues and cells. (A and B) Analysis of the expression and accumulation of LC3 in thyroid cancer tissues. (A) Western blotting of LC3 in homogenates of frozen biopsies from BRAF-mutated and BRAF wt thyroid cancers. The presence of the LC3II isoform (lower band) is indicative of autophagy. In a preliminary study conducted on 19 cases (12 of which with mutated BRAF), we have not found a statistically significant correlation between the mutation of BRAF and the level of LC3II in thyroid cancer. (B) Immunofluorescence of LC3. The puncta in the cytoplasm of cancer cells are indicative of the presence of autophagosomes. (C and D) Expression of LC3 in cultured thyroid cancer cells (FTC133 cell line) incubated or not for 24 h with ammonium chloride (NH_4^+). (C) Western blotting; (D) immunofluorescence co-staining of LC3 and LAMP1. It is evident the accumulation of LC3 in the cells incubated with ammonium chloride which in fact prevented the fusion of autophagosomes with lysosomes (as can be appreciated in panel D) and the degradation of LC3II (as can be appreciated in panel (C). Microscope magnification: (B) 40×; (D) 63×. Scale bars: (B) 10 μm; (D) 20 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0271.

cultured thyroid cancer cells. Paraffin-embedded and cryostatic tissue sections can be processed for immunohistochemistry and immunofluorescent detection of autophagy markers, while western blotting analysis is better performed in freshly isolated or frozen biopsy tissues. Both these techniques are complementary and should be employed.

As an example, we report our preliminary observations on the detection of autophagy markers in tissue biopsies and in cultured cells of thyroid cancer origin. Western blotting of LC3 reveals the presence of the autophagosome-bound LC3II isoform, which arises from LC3I (Fig. 5A and C). One important caveat is that infiltrating fibroblasts, macrophages, and mastocytes might also express autophagy markers. This should be taken into consideration when performing a western blot with the whole homogenate of a biopsy, unless cancer cells are isolated by laser microdissection. In immunofluorescence, nontumor cells can be discriminated by using appropriate markers for stromal cells. A faintly detectable diffuse cytoplasmic signal of LC3 is considered as negative background, while a pattern of many LC3-positive puncta (in general, more than 10 per cell) is indicative of ongoing autophagy (Fig. 5B and D). Immunocostaining with multiple markers (e.g., LC3, p62, BECLIN1, lysosome-associated membrane protein (LAMP1)) is recommended for a better assessment of the autophagy flux and of the signaling molecules involved. The true level of ongoing autophagy is much easier assayed in cultured cells through the pharmacologic or genetic manipulation of the autophagy flux. For instance, in the presence of drugs that alkalinize the lysosome pH (e.g. ammonium chloride) all the autophagosomes produced during the incubation time accumulate in the cell as they do not fuse with lysosomes (Kawai *et al*. 2007, Klionsky *et al*. 2012), and this reflects in the accumulation of LC3II protein (Fig. 5C and D).

The genetic connection: oncogenes and oncosuppressor genes

Numerous oncogenes and tumor suppressor genes regulate autophagy (Maiuri *et al*. 2009). In general, oncogenic proteins exert a negative activity and oncosuppressor proteins exert a positive activity on autophagy induction and progression. Several oncogenes and oncosuppressors implicated in thyroid carcinogenesis also play a role in the regulation of autophagy.

The main signaling pathways that link thyroid carcinogenesis with autophagy deregulation are the

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RAS–RAF–ERK and the class I PI3k–AKT–mTOR pathways. The RAS/RAF/MEK/ERK pathway controls the mTOR-dependent pathway by sensing the absence of amino acids (Ogier-Denis *et al.* 2000). Aberrant signaling through the RAS/RAF/MEK/ERK cascade has been implicated in thyroid tumor initiation and development. For instance, the *RET/PTC* rearrangement, which leads to the RET/PTC fusion oncoprotein, activates the RAS–RAF–MAPK cascade (Knauf *et al.* 2003, Santoro *et al.* 2004). However, the most frequent aberration of this signaling pathway in thyroid cancer is associated with the oncogenic activation of BRAF. The BRAF V600E mutation, which leads to constitutive activation of BRAF kinase, is frequently found in PTC (Xing 2007) and, though less frequently, also in ATC (Nikiforova *et al.* 2003, Takano *et al.* 2007) and PDTC (Begum *et al.* 2004). In melanomas, oncogenic BRAF has been associated with inhibition of mTOR and upregulation of basal autophagy (Maddodi *et al.* 2010). However, another study showed that in metastatic melanomas oncogenic BRAF opposed the induction of autophagy by chemotherapeutics or rapamycin (Armstrong *et al.* 2011). Another oncogene of this same pathway which is mutated in a large percentage of thyroid cancers is the *RAS* oncogene (Motoi *et al.* 2000, Nikiforova & Nikiforov 2009). Activating mutations of RAS are associated with aggressive phenotypes of thyroid cancer and poor prognosis (Garcia-Rostan *et al.* 2003). Remarkably, the oncogenic mutants Ha-RAS and K-RAS have been shown to confer a metabolic advantage to cancer cells through the upregulation of basal autophagy (Guo *et al.* 2011, Kim *et al.* 2011). The above findings on the effects of active BRAF and Ha-RAS on autophagy seem to contradict the general rule that oncogenes signal to downregulate autophagy. Besides the fact that basal and stress-induced autophagy should be distinguished, it is likely that the true effect of oncogenic BRAF and RAS on autophagy regulation is cell context dependent and also reliant on the extracellular trigger. In addition, it should be considered that oncogenic RAS can signal through either the RAF–MEK–ERK1/2 pathway or the PI3K/AKT pathway, with a different impact on the regulation of autophagy (see below). Therefore, the final outcome on autophagy regulation by oncogenic RAS will depend on which downstream pathway will predominate.

The class I PI3k-(PTEN)–AKT–mTOR pathway is the other oncogenic pathway aberrantly hyperactive in thyroid cancer cells and is also known to regulate autophagy (see Fig. 3). Growth factors activate class I PI3k, which then phosphorylates phosphatidylinositol-3,4-diphosphate into phosphatidylinositol-3,4,5-triphosphate (PIP3), the phosphate donor needed for the phosphorylation of AKT. Active AKT then phosphorylates a number of downstream targets that ultimately regulate various cellular functions, including cell survival, proliferation, autophagy, protein synthesis, angiogenesis, and migration. Genetic alterations in the PI3k/AKT signaling pathway have been linked to thyroid cancers (Garcia-Rostan *et al.* 2005, Shinohara *et al.* 2007, Wang *et al.* 2007). The PIK3CA gene (encoding the catalytic subunit of p110α of class I PI3k) has been found amplified or mutated in thyroid carcinomas (Wu *et al.* 2005, Wang *et al.* 2007). Increased AKT activity has been associated with the aggressive behavior of FTCs and PTCs (Ringel *et al.* 2001, Shinohara *et al.* 2007). AKT negatively regulates autophagy through the mTOR pathway (Arico *et al.* 2001, Castino *et al.* 2008) and, directly, through phosphorylation of BELCIN1 (Wang *et al.* 2012). The class I PI3k/AKT pathway may be abnormally upregulated as a consequence of PTEN loss-of-function. The lipid phosphatase activity of PTEN removes the phosphate in position 3 from PIP3, thus limiting the availability of PIP3 needed for the activation of AKT. By shutting down the activation of AKT, PTEN relieves the AKT–mTOR block on autophagy (Arico *et al.* 2001). It is worth noting that mutations or deletions of the tumor suppressor gene *PTEN* have been recognized as an important step in the development of thyroid gland carcinomas (Dahia *et al.* 1997, Eng 2002).

Another oncogene that might link autophagy deregulation with thyroid carcinogenesis is c-MET. This proto-oncogene encodes a membrane tyrosine kinase receptor for the hepatocyte growth factor (HGF), which is a potent mitogen for epithelial cells and promotes cell motility and invasion in carcinoma cells (Stella *et al.* 2010). A large cohort study revealed that about 50% of PTCs are characterized by MET overexpression (Di Renzo *et al.* 1992), and this represents a sign of more aggressive disease (Ramirez *et al.* 2000, Mineo *et al.* 2004). Very recently, it has been shown that c-MET overexpression or its activation by HGF negatively regulates autophagy in A549 carcinoma cells (Liu *et al.* 2012). In the context of thyroid cancer, it has been reported that induction of autophagy by RAD001-mediated inhibition of mTOR sensitized PTC cells to chemo- and radio-therapy through the inhibition of c-MET (Lin *et al.* 2010).

What about other tumor suppressors, besides PTEN, that are deleted or mutated in thyroid cancers and might play a role in the regulation of autophagy?

The haplo-insufficient tumor suppressor gene *BECLIN1* was the first oncosuppressor gene that proved the link between autophagy and cancer susceptibility

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(Liang *et al*. 1999, Qu *et al*. 2003, Yue *et al*. 2003). *BELCIN1* has been found mutated or monoallelically deleted in a large proportion of a variety of epithelial cancers, including breast and ovary carcinomas (Qu *et al*. 2003, Yue *et al*. 2003). Predictably, similar gene alterations could also be found in thyroid cancers. However, as yet no studies have addressed this issue.

The other tumor suppressor that may link autophagy and thyroid cancer progression is p53. Contrary to what is seen in many other cancers, TP53 loss-of-function mutations occur late in thyroid tumorigenesis. TP53 mutations are practically absent in differentiated thyroid cancers, while their prevalence is high (17–38%) in PDTC and is even higher (55–88%) in ATC (Fagin & Mitsiades 2008, Smallridge *et al*. 2009). The regulatory activity of p53 in the autophagy pathway is quite ambiguous (Maiuri *et al*. 2010). As a transcription factor, wild-type p53 promotes autophagy by directing the transcription of certain autophagy genes, including damage-regulated autophagy modulator (DRAM) and ULK1/2 (Crighton *et al*. 2006, Gao *et al*. 2011). However, in many different cell types the lack of p53 has been shown to stimulate the autophagic flux, suggesting an inhibitory function of this tumor suppressor. In line with this finding, certain DNA-binding deficient p53 mutants that fail to relocate to the nucleus, and instead reside in the cytoplasm, have been shown to repress the induction of autophagy by metabolic stresses.

The ras-homolog GTPase aplasia Ras homolog member I (*ARHI*) (or *DIRAS3*) is another oncosuppressor that potentially links autophagy to thyroid carcinogenesis. ARHI positively regulates the autophagy-mediated dormancy of tumor cells (Lu *et al*. 2008). Of interest, ARHI is found underexpressed in FTCs (Weber *et al*. 2005).

The epigenetic liaisons: the role of DNA methylation, histone deacetylates, and miRNAs

Epigenetics refers to heritable (i.e., transmitted via meiosis or mitosis) changes in the expression of a gene or a set of genes not dependent on variations in the primary DNA sequence. Given that all cells in the same organism bear the same genome, epigenetics explains the different cellular phenotypes in the body as a result of the silencing of different subsets of genes. It is now clear that epigenetics plays a role in cancer, as cancer cells have their own epigenome. During cell proliferation, the epigenome is transmitted to daughter cells, although novel epigenetic signatures may emerge in the progeny as a consequence of (micro)environmental interference, thus explaining the appearance of clones with different behavior in the context of the tumor (Timp & Feinberg 2013). There are four epigenetic mechanisms known to regulate gene expression: DNA methylation in correspondence to the promoter region, histone conformation changes (essentially dictated by acetylation and deacetylation of certain lysine residues), chromatin remodeling (both histone conformation changes and chromatin remodelling affect DNA accessibility), and miRNAs (short, noncoding mRNAs that impair RNA translation by hybridizing to specific domains of the UTR of target mRNAs).

In recent years, a large body of evidence has accumulated showing the pivotal role of epigenetic changes in thyroid tumorigenesis driven by DNA methyl transferases (DNMTs), HDAC, and miRNAs (reviewed in Pallante *et al*. (2010), Braun & Hüttelmaier (2011), Russo *et al*. (2011) and Catalano *et al*. (2012*a*)). In this section, we provide a brief overview of the proteins and of the mechanisms involved in the epigenetic regulation in thyroid cancer that also have an impact on the regulation of autophagy.

For instance, the hypermethylation of the *PTEN* promoter region sporadic thyroid cancers has been reported (Alvarez-Nuñez *et al*. 2006). The lack of PTEN expression maintains active AKT in thyroid cancer cells, and consequently autophagy in these cells will be repressed.

The promoter of death-associated protein kinase (DAPk) has also been found to be hypermethylated in a large proportion of thyroid cancers (Hoque *et al*. 2005). This kinase induces autophagy by disrupting the BECLIN1–BCL2 complex (Zalckvar *et al*. 2009), and its epigenetic silencing in thyroid cancer cells might impair the induction of autophagy under stress conditions.

An intriguing epigenetic liaison between autophagy and thyroid carcinogenesis could involve the tumor suppressor ARHI, a Ras homolog that was recently shown to induce autophagy and autophagy-mediated dormancy in ovarian cancer cells (Lu *et al*. 2008). *ARHI* is generally monoallelically expressed (from the paternal allele), as the maternal allele is inherited in the hypermethylated (and therefore silenced) state. *ARHI* maps to 1p31, a region that is frequently deleted in thyroid cancers, particularly in FTCs (Weber *et al*. 2005). It has been hypothesized that silencing of ARHI expression, as a result of combined hypermethylation of the maternal allele and deletion of the paternal allele, is pivotal to thyroid carcinogenesis (Weber *et al*. 2005).

Histone deacetylation promoted by HDACs negatively affects gene transcription. There is evidence that certain HDACs (namely SIRT1, HDAC1, HDAC2, HDAC6) can

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regulate the autophagy process at the level of gene transcription and of protein function (Moresi *et al*. 2012, True & Matthias 2012, Füllgrabe *et al*. 2013). As histone deacetylation decreases DNA accessibility, the inhibitors of HDACs promote gene transcription. Indeed, the treatment with the HDAC inhibitor suberoylanide hydroxamic acid was shown to induce autophagy in several cell types (Shao *et al*. 2004, Gammoh *et al*. 2012). There is promising evidence that HDAC inhibitors can be used in combination with other chemotherapeutics to improve their effectiveness in the treatment of thyroid cancers. HDAC inhibition was shown to increase the expression of E-cadherin, thus promoting cell–cell adhesion and reducing the migration of *in vitro* cultured thyroid cancer cells (Catalano *et al*. 2012*b*). E-cadherin is downregulated during the EMT process that precedes the metastasization of cancer cells, and, it is worth noting, the actual level of autophagy in cancer cells increases at this step in concomitance with E-cadherin silencing (Akalay *et al*. 2013). Whether the reexpression of E-cadherin following HDAC inhibition also associates with repression of autophagy in thyroid cancer cells has yet to be proven. Besides histones, some HDACs also deacetylate target cytoplasmic proteins, among which are also some proteins involved in the initiation and progression of autophagy (Lee *et al*. 2008, 2010, Lin *et al*. 2012*a*,*b*, Yi *et al*. 2012). For instance, SIRT1 deacetylates ATG5, ATG7, and ATG8 (Lee *et al*. 2008), which are essential for the formation of autophagosomes, while HDAC6 deacetylates the cytoskeletal protein tubulin, thus favoring the fusion of autophagosome with lysosomes (Lee *et al*. 2010).

The third epigenetic mechanism that potentially links autophagy and thyroid cancer are the miRNAs, a class of noncoding RNAs of 20–24 nucleotides that control gene expression at the post-transcriptional level (Ghildiyal & Zamore 2009). The link between miRNAs and epigenetics is bidirectional, as on one hand the expression of miRNAs is dictated by the methylation and acetylation status of DNA, and on the other hand miRNAs themselves regulate the expression of DNMTs and HDACs (Iorio & Croce 2009). For simplicity, we will only refer to those miRNAs that interfere with the expression of proteins involved in the regulation of autophagy and that are abnormally expressed in thyroid cancers.

Several miRNA profiling studies have identified changes in miRNA patterns occurring during thyroid cancer development and progression, opening a new field for the understanding of this disease and providing improved diagnostic, prognostic, and therapeutic approaches (reviewed in Pallante *et al*. (2010), Braun & Hüttelmaier (2011), De la Chapelle & Jazdzewski (2011) and Gundara *et al*. (2012)). Autophagy in itself, as a metabolic process regulated by an intricate network of proteins, is regulated by miRNAs at various levels (Gundara *et al*. 2011, Frankel & Lund 2012, Zhai *et al*. 2013). In principle, any miRNA targeting a signaling pathway involved in the control of autophagy would exert its regulatory activity on this pathway. For instance, miR221/222 and miR21, which target PTEN and consequently sustain the hyperactivation of AKT (Garofalo *et al*. 2009, Chun-Zhi *et al*. 2010), indirectly have a negative impact on induction of mTOR-dependent autophagy. With relevance to the present context, miR21 was found upregulated in PTCs and ATCs (Braun *et al*. 2010, Frezzetti *et al*. 2011), and miR221/222 cluster was found to be upregulated in PTCs (He *et al*. 2005, Pallante *et al*. 2006, Visone *et al*. 2007, Sheu *et al*. 2010). The loss of miR200 expression marks the progression of thyroid carcinomas culminating in tumor growth factor beta (TGFβ) dependent EMT and elevated invasiveness (Braun *et al*. 2010). Members of the miR200 family (comprising miR141, 200a, 200b, 200c, and 429) are potent suppressors of EMT through the downregulation of the expression of EMT-promoting factors like ZEB1, ZEB2, SNAI2, SMAD2, TGFβR1, and TGFβ2, and by antagonizing the transcriptional repression of E-cadherin (Gregory *et al*. 2008, Park *et al*. 2008, Braun *et al*. 2010). Of note, the actual level of autophagy in cancer cells was found to increase during EMT in concomitance with E-cadherin downregulation (Akalay *et al*. 2013). Thus, one can hypothesize that in thyroid cancer cells with low-levels of miR200, autophagy is upregulated to oppose anoikis in invading cells.

More recently, miRNAs specifically targeting the mRNA of autophagy proteins are being identified. Here, we will mention only those miRNAs involved in both the regulation of autophagy and the progression of thyroid cancers. The first miRNA described in this field is miR30a, which downregulates the expression of Beclin1 (Zhu *et al*. 2009). In the same family, miR30d was shown to target the mRNA of various autophagy proteins including BECLIN1, Bcl2/adenovirus E1B 19-kDa interacting protein (BNIP3L, which plays a role in mitophagy), ATG2, ATG5, and ATG12 (Yang *et al*. 2013). miR30a and miR30d were found to be expressed at a low-level in PTCs (Tetzlaff *et al*. 2007) and at a very low-level in ATCs (Schwertheim *et al*. 2009), respectively. It is intriguing to note that the miR30 family also antagonizes TGFβ-induced EMT and *in vitro* invasiveness of ATC-derived cells (Braun *et al*. 2010). miR130a, one of the most significantly downregulated miRNAs in thyroid cancer cells with BRAF mutation (Cahill *et al*. 2007),

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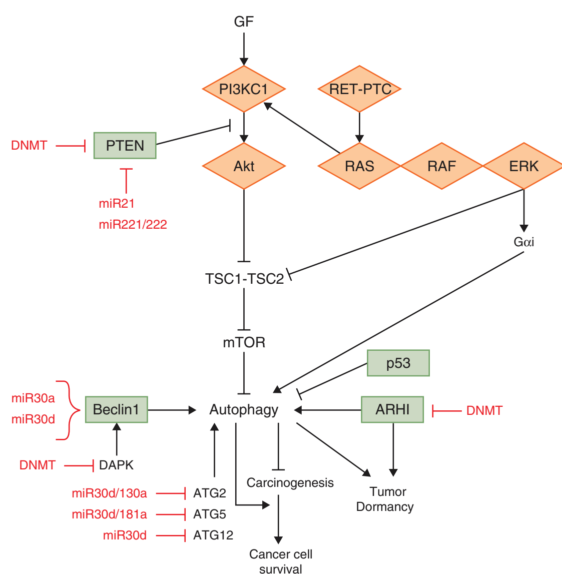

Figure 6
Genetic and epigenetic links between thyroid carcinogenesis and autophagy regulators. The scheme summarizes the potential links between autophagy and thyroid cancer progression at both genetic and epigenetic levels. Oncogenes are represented by rhombus and oncosuppressor genes by rectangles. Epigenetic regulations by DNMTs and miRNAs are indicated. GF, growth factor. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0271

is known to downregulate the expression of ATG2B (Kovaleva *et al*. 2012), while miR181a, which is expressed at a very low level in the presence of *RET/PTC1* rearrangement (Cahill *et al*. 2006), is known to down-regulate ATG5 (Huang *et al*. 2012, Tekirdag *et al*. 2013). Finally, miR183 was recently shown to negatively regulate the expression of LC3 in MTC cells (Abraham *et al*. 2011).

The scheme in Fig. 6 summarizes the potential links between autophagy and thyroid cancer progression at both genetic and epigenetic levels.

Can we exploit autophagy for the therapy of thyroid cancers?

Autophagy-targeted therapy is nowadays considered a valuable strategy to combat radio- and chemo-resistant cancers (Chen & Karantza 2011, Yang *et al*. 2011, Gundara *et al*. 2012). Accumulating experimental data suggest that the efficacy of such therapies is strictly dependent on the actual level of ongoing autophagy in tumor cells, which is dictated by both genetic mutations and epigenetic phenomena, besides the dynamic influence of the tumor microenvironment.

The first report suggesting the involvement of autophagy in the response to therapeutic treatments of thyroid cancer dates back only a few years. Lin *et al*. (2009) found that both doxorubicin and radiation induced autophagy and cell death in cultured PTC cells. As the inhibition of Vps34-mediated autophagy by 3-methyl adenine increased the resistance toward these treatments, these authors concluded that autophagy was instrumental to cell toxicity in both treatments.

With regard to the therapy of thyroid cancer, besides radiation, new molecular therapies are emerging which employ kinase inhibitors, proteasome inhibitors, and epigenetic modulators (Catalano *et al*. 2010), and some of these drugs have been shown to also affect the autophagy process. For instance, sorafenib and sunitinib, two small molecules that inhibit the activity of RET kinase and that are commonly used for the treatment of MTCs, were shown to induce autophagosome accumulation in MTC cultured cells (Lin *et al*. 2012*a*,*b*). Apoptosis of MTC cells induced by these two drugs was abrogated when the essential autophagy protein ATG5 was silenced, and conversely it was enhanced when autophagy was concomitantly upregulated by co-treating with the mTOR inhibitor, everolimus (Lin *et al*. 2012*a*,*b*). Everolimus and other mTOR inhibitors are being tested for their potential efficacy against thyroid cancers (Gild *et al*. 2013, Lim *et al*. 2013). Everolimus is a rapamycin analog and it was introduced to the clinical management of epithelial cancers because of its ability to halt the biosynthetic and proliferative pathways downstream of the AKT–mTOR axis, but it is now recognized that rapamycin analogs may also elicit their beneficial effects via induction of autophagy. Consistently, sensitization of PTC cells to doxorubicin and radiotherapy by everolimus (known also as RAD001) was shown to strictly depend on autophagy (Lin *et al*. 2010).

Statins, when used for the treatment of thyroid cancers, may also act through the induction of autophagy. In a study, a xenograft of ATC in mice was successfully treated by combining paclitaxel, a cytoskeleton disrupting agent, with combretastatin A4 phosphate, a vascular-inhibiting drug, and it was found that the latter drug induced autophagy in cancer cells, besides inducing apoptosis of endothelial cells (Yeung *et al*. 2007). In addition, rosuvastatin, a statin with antiproliferative activity, was shown to induce autophagy and to promote the switch from pro-survival to pro-death autophagy in PTC cells (Zeybek *et al*. 2011).

Further, reversine, a synthetic purine analog with promising therapeutic potential, has recently been shown to induce growth arrest and apoptosis in FTC cells through the induction of autophagy (Lu *et al*. 2012).

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More recently, autophagy was shown to mediate the resistance to apoptosis induced by tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in PTC and ATC cells (Jin *et al*. 2013).

As for epigenetic modulators, the use of HDAC inhibitors for autophagy therapy in cancer has been proposed (Yang *et al*. 2011). The HDAC inhibitor valproic acid (VPA) has been successfully employed in a combination therapy in ATC (Noguchi *et al*. 2009). VPA sensitized ATC cells to paclitaxel (Catalano *et al*. 2007) and to doxorubicin (Catalano *et al*. 2006). The small molecule lithium chloride was shown to synergize with VPA to inhibit the growth of *in vitro* cultured MTC cells (Adler *et al*. 2010). It is interesting to note, in this context, that lithium chloride is an inducer of mTOR-independent autophagy (Fornai *et al*. 2008), and that VPA, by inhibiting HDAC6, negatively interferes with the formation of the autophagolysosome (Lee *et al*. 2010).

Demethylating agents could also work in this manner. For instance, decitabine (2′-deoxy-5-azacytidine) has been shown to favor apoptosis by conventional chemotherapeutics in leukemic cells through the hyperinduction of autophagy (Schnekenburger *et al*. 2011).

Finally, in the near future we might be able to exploit miRNA-targeting autophagy for the cure of thyroid cancers (Gundara *et al*. 2012).

Concluding remarks

The paradoxical role of autophagy in cancer development and progression has important clinical implications in therapy, because it suggests that autophagy-inducer drugs may have benefits in preventing the development and growth of cancer cells while autophagy-inhibitor drugs should improve the efficacy of anti-cancer therapies in developed and metastatic cancers. Indeed, both pro- and anti-autophagy therapeutic drugs have shown their efficacy in *in vitro* and *in vivo* models of cancer, as well as in clinical trials (Chen & Karantza 2011, Gundara *et al*. 2012, Wu *et al*. 2012). These contradictory results could be explained by considering that the actual level of autophagy in cancer cells was likely to be different in the different experimental models because of differences in the genetic and epigenetic background, and also because of the influence of stromal factors (e.g., vascularization, presence of cytokines, interactions with fibroblasts and macrophages).

Whether autophagy can represent a valuable target for the therapy of thyroid cancers remains speculative. A deeper knowledge of the mechanistic links between autophagy and thyroid carcinogenesis is expected to clarify the diagnostic and prognostic potential of autophagic-related biomarkers and could greatly contribute to a more rational use of novel therapeutic approaches based on the modulation of autophagy to cure thyroid cancers.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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6. PTEN Deficiency and Mutant p53 confer Glucose-addiction to Thyroid Cancer Cells: Impact of Glucose Depletion on Cell Proliferation, Cell Survival, Autophagy and Cell Migration

Federica Morani, Suratchanee Phadngam, Carlo Follo, Rossella Titone, Visa Thongrakard, Alessandra Galetto, Oscar Alabiso and Ciro Isidoro

PTEN and TP53 are the most common deleted or mutated oncosuppressors found in human carcinomas. These two oncosuppressors are key regulators of the glucose metabolism and of autophagy. Either PTEN deficiency or p53 mutation has been shown to confer the ability to cancer cells to overcome the metabolic stress caused by hypoxia and nutrients (including glucose) depletion, and to be the driving force for cell proliferation and cell migration. Glucose is an essential precursor for the synthesis of various macromolecules and the main source of the energy needed in the survival and biosynthetic pathways. Here, we have tested whether combined PTEN deficiency and p53 mutation indeed confers a metabolic advantage to cancer cells in response to glucose depletion in terms of cell survival, cell proliferation and cell migration, and of autophagy response. As a cell model, we analyzed the effect of glucose depletion on PTEN and p53 expression in follicular type thyroid cancer cell lines WRO and FTC133. WRO cells express wild-type PTEN and p53, whereas FTC133 cells are PTEN-deficient and express the anti-apoptotic and anti-autophagic p53R273H mutant. We propose that concurrent PTEN deficiency and mutant p53 leads to a glucose addiction state that renders the cancer cell more sensitive to glucose restriction. The present observation substantiates the view that glucose-restriction may be an adjuvant strategy to combat these tumors.

Personal Contribution

In this paper, I contributed with the analysis of cell migration and expression of autophagy markers in migrating cells.

PTEN deficiency and mutant p53 confer glucose-addiction to thyroid cancer cells: impact of glucose depletion on cell proliferation, cell survival, autophagy and cell migration

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ABSTRACT:

Proliferating cancer cells oxidize glucose through the glycolytic pathway. Since this metabolism is less profitable in terms of ATP production, cancer cells consume large quantity of glucose, and those that experience insufficient blood supply become glucose-addicted. We have analyzed the response to glucose depletion in WRO and FTC133 follicular thyroid cancer cells, which differ in the expression of two key regulators of the glucose metabolism. WRO cells, which express wild type p53 and PTEN, showed a higher rate of cell proliferation and were much less sensitive to glucose-depletion than FTC133 cells, which are PTEN null and express mutant p53. Glucose depletion slowed-down the autophagy flux in FTC133 cells, not in WRO cells. In a wound-healing assay, WRO cells were shown to migrate faster than FTC133 cells. Glucose depletion slowed down the cell migration rate, and these effects were more evident in FTC133 cells. Genetic silencing of either wild-type PTEN or p53 in WRO cells resulted in increased uptake of glucose, whereas the ectopic expression of PTEN in FTC133 cells resulted in diminished glucose uptake. In conclusion, compared to WRO, FTC133 cells were higher glucose up-taker and consumer. These data do not support the general contention that cancer cells lacking PTEN or expressing the mutant p53R273H are more aggressive and prone to better face glucose depletion. We propose that concurrent PTEN deficiency and mutant p53 leads to a glucose-addiction state that renders the cancer cell more sensitive to glucose restriction. The present observation substantiates the view that glucose-restriction may be an adjuvant strategy to combat these tumours.

INTRODUCTION

Glucose is an essential precursor for the synthesis of various macromolecules and the main source of the energy needed in the survival and biosynthetic pathways. Normally, in the presence of oxygen, glucose is oxidized through the mitochondrial respiration pathway with the highest rate of production of ATP. However, in highly proliferating cancer cells glucose is preferentially converted into lactate despite the presence of oxygen and functional mitochondria. This aberrant metabolism of glucose, known as the Warburg effect or aerobic glycolysis, is much less convenient in terms of energy gain, and imposes a large consumption of glucose in proliferating cells[1,2]. Vascularization is defective and insufficient in fast growing solid tumours and, consequently, the cells located distant from the blood vessels experience a lack of glucose and oxygen[3]. Under these conditions, cancer cells activate autophagy, a pro-survival lysosomal degradation pathway, and produce

large quantity of lactic acid. Autophagy itself promotes the metabolic switch toward glycolysis, thus facilitating cell survival and cell proliferation[4]. In addition, autophagy and glycolysis are also involved in the 'epithelial-to-mesenchymal' transition phenomenon that precedes the metastasization[5-7]. Therefore, glucose availability and modulation of autophagy have a great impact on the malignant behaviour of cancer cells.

PTEN and TP53 are the most common deleted or mutated oncosuppressors found in human carcinomas. These two oncosuppressors are key regulators of the glucose metabolism and of autophagy[8-11]. Either PTEN deficiency or p53 mutation has been shown to confer the ability to cancer cells to overcome the metabolic stress caused by hypoxia and nutrients (including glucose) depletion, and to be the driving force for cell proliferation and cell migration[8,9,12,13]. Here, we have tested whether combined PTEN deficiency and p53 mutation indeed confers a metabolic advantage to cancer cells in response to glucose depletion in terms of cell survival, cell proliferation and cell migration, and of autophagy response. As a cell model, we employed the follicular type thyroid cancer cell lines WRO and FTC-133. WRO cells express wild-type PTEN and p53, whereas FTC133 cells are PTEN-deficient and express the anti-apoptotic and anti-autophagic p53R273H mutant[14,15]. We found that FTC133 cells were more active up-taker and consumer of glucose than WRO cells. Consistently, the facilitative glucose transporter GLUT1 was basally expressed at higher level on the plasmamembrane of FTC133 cells than on that of WRO cells. Through genetic manipulations we could demonstrate that both the membrane translocation of GLUT1 and the uptake of glucose are controlled by PTEN and p53. Unexpectedly, FTC133 cells were revealed to be more sensitive to glucose-depletion in terms of cell growth, survival and migration, and of autophagy completion, than WRO cells. The present data indicate that the synergistic effects of combined PTEN deficiency and p53 mutation render the cancer cells glucose-addicted and therefore more sensitive to glucose deprivation.

RESULTS

Effect of glucose depletion on PTEN and p53 in WRO and FTC133 cells

The effect of glucose depletion on PTEN and p53 expression was analyzed in WRO and FTC133 thyroid cancer cells. To this end, the cells were cultivated in glucose-containing (2 g/L for RPMI and 4.5 g/L for DMEM, respectively for WRO and FTC133 cells) or in glucose-free standard medium for 24 h and then analyzed by western blotting. Both these cell types present no mutations in the *RAS* and *PI3kCA* genes, while FTC133 cells present the following unique mutations: the R273H P53 mutation and the R130STOP PTEN mutation[14]. FTC133 cells have also been reported to bear a monoallelic deletion of PTEN[15].

As a consequence of the mutations, PTEN protein was not detectable in FTC133 cells (Figure 1A). In WRO cells, PTEN was expressed at high level and its expression was not subjected to substantial changes in dependence of glucose availability (Figure 1A). The mutant p53 was highly expressed in FTC133 cells, when compared to the expression of the wild-type p53 in WRO cells (Figure 1B). This finding is consistent with literature data on the abnormal hyper-expression of mutant p53 in tumours. Noteworthy, glucose depletion greatly reduced the protein level of the mutant p53 in FTC133 cells, not that of the wild-type p53 in WRO cells (Figure 1B).

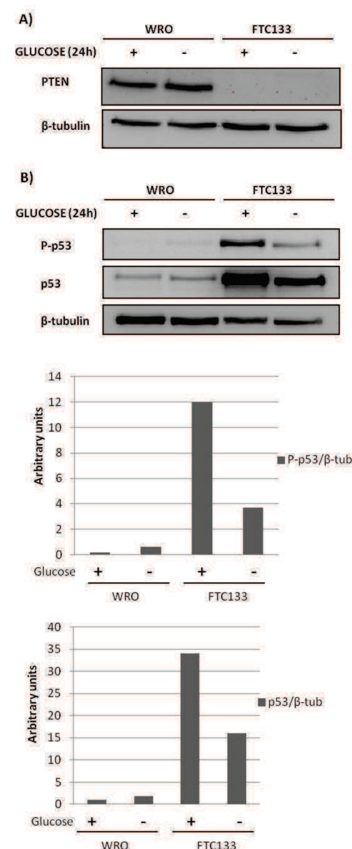

Figure 1: The effect of glucose availability on the expression of PTEN and p53 in WRO and in FTC133 cells. WRO and FTC133 cells were plated and let adhere on Petri dishes and then incubated for 24 h in glucose-rich or in glucose-free standard medium. Cell homogenates were analyzed by western blotting for the expression of PTEN, ser15-phosphorylated p53 and total p53 as indicated, respectively in panel A and B. The filters were stripped and re-probed for β-tubulin as a protein loading marker. Densitometry of p53 bands in panel B is included. The blots here shown are representative of n=3 independent experiments. Glucose-dependent difference in the expression of PTEN in WRO cells (panel A) was not statistically significant.

Table 1: Doubling time of WRO and FTC133 cells after incubation in glucose-containing complete medium or in glucose-free medium.

	24h	48h
WRO +glucose	13.47 ± 1.46	16.33 ± 2.54
WRO -glucose	22.43 ± 3.83	60.09 ± 5.91
FTC133 +glucose	27.08 ± 4.89	26.4 ± 5.05
FTC133 –glucose	95.9 ± 4.47	228.83 ± 0.74

In the presence of glucose the doubling time of FTC133 cells was two-folds longer than that of WRO cells. In glucose depleted culture condition, the doubling time of FTC133 cells increased much more than that of WRO cells, indicating a higher dependence on the availability of glucose for their duplication.

Phosphorylation of p53 at ser15 stabilizes the protein and is indicative of its activation. In fact, wild-type p53 was phosphorylated and its protein level slightly increased in WRO cells cultivated for 24 h in glucose-free medium. Unexpectedly, a large proportion of the mutant p53 in FTC133 cells was phosphorylated, and about one-third of it was degraded upon 24 h glucose depletion (Figure 1B). These data indicate that WRO and FTC133 cells respond differently to glucose depletion in terms of p53 activation and stability.

Glucose depletion differentially affects WRO and FTC133 cell proliferation

To determine the effect of glucose depletion on cell proliferation, WRO and FTC133 cells were plated at the same starting density, let adhere for 24 h in glucose-containing complete medium (cell density at this time was considered as t0), then washed and further cultivated in glucose-containing or glucose-free medium for up to 48 h without medium change. Cell density was evaluated at 24 h and 48 h of incubation and the doubling time (Dt) of the cell population was calculated (Table 1). In the presence of glucose, the rate of proliferation (as mirrored by the Dt) in both cell types remained substantially unaltered, indicating that the consumption of nutrients (glucose, aminoacids) in the first 24 h did not affect much the duplication potential in the subsequent 24 h of incubation. Strikingly, the Dt of FTC133 cells was two folds longer than that of WRO cells, and this in spite of the fact that they were cultured in high-glucose medium. When incubated in the absence of glucose, the Dt increased for both cell types, indicating a strict dependence on the availability of glucose for their duplication. However, in WRO the Dt only increased by 1.5-folds (from ~13.5 h to ~22.5 h) while in FTC133 the Dt increased by 3.5-fold (from 27 h to 96 h), i.e. more than two times. The different dependency from glucose for cell duplication became even more evident when evaluated after 48 h of culture in glucose-free medium. Under this condition, the Dt of FTC133 cells was approximately four-times that of WRO cells (230 h *vs* 60 h).

The difference in the response to glucose availability between the two cell lines was further substantiated by the cell cycle analysis (Figure 2). Between 24 h and 48 h,

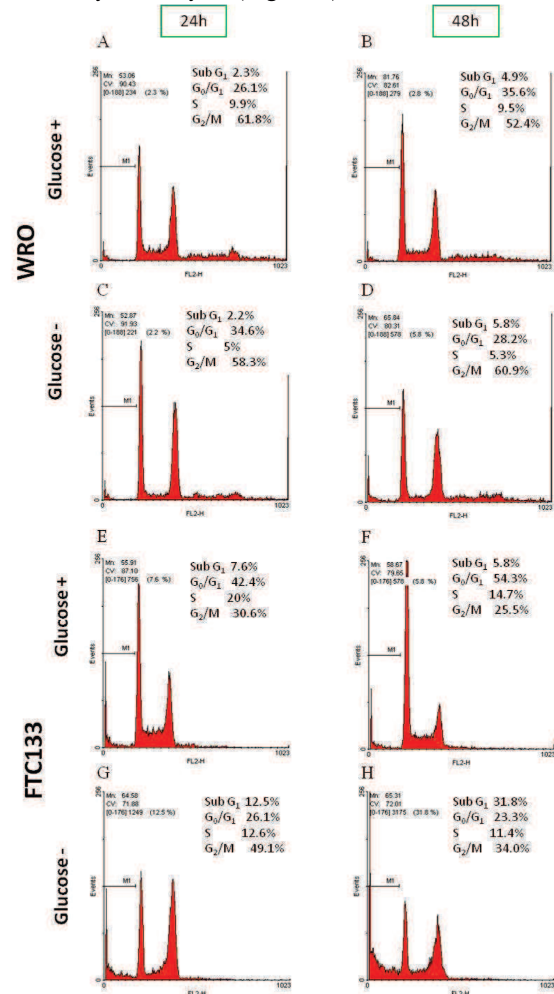

Figure 2: Different growth response to glucose availability between WRO and FTC133 cells. WRO and FTC133 cells were let adhere for 24 h in complete medium and then cultivated in glucose-containing or in glucose-free standard medium for up to 48 h without medium change. At the end, adherent and suspended cells were recovered, fixed in ethanol and labelled with propidium iodide (PI). Finally, the cells were analyzed by cytofluorometry to determine the phases of cell cycle. In panels A-H are shown the percentages of the four cell cycle phases (Sub G1, G0/G1, S, G2/M) obtained using the software Win MDI 2.9. During glucose depletion WRO cells accumulated in the G0/G1 retarding the entry in the S phase (panels C-D *vs* A-B) and only a small fraction of the cells underwent apoptosis (panel D); FTC133 cells in the first 24 h accumulated in G2/M and underwent apoptosis (indicated by subG1 peak, panels G), while in the following 24 h decreased the fraction of cells in G2/M and increased the fraction of subG1 population (panel H *vs* G). Data shown in this Figure have been reproduced independently four times.

WRO cells cultivated in the presence of glucose slightly increased the fraction in G0/G1, while not changing the amount in the S phase (panel B *vs* A). These data are in agreement with the substantial similar Dt calculated at 24 and 48 h (see Table 1). When cultivated in the absence of glucose, WRO reduced the fraction in the S phase and tended to accumulate in the G0/G1, indicating that the lack of glucose retarded the entry in the S phase (panel C *vs* A). In the following 24 h of culture without glucose (panel D *vs* C) no changes were observed, besides the fact that a small fraction (4%) of the WRO cells resting in G0/G1 underwent apoptosis (as indicated by the apparent increase of the subG1 peak).

Between 24 h and 48 h in glucose-containing medium, FTC133 cells reduced their fraction in S and G2/M phases to accumulate in the G0/G1 phase, likely as a consequence of the consumption of nutrients in the first 24 h, but no increase of the SubG1 fraction was recorded (panel F *vs* E). When cultivated for 24 h in the absence of glucose, FTC133 cells reduced their fraction in the G0/G1 and S phases, and tended to accumulate in the G2/M phase and also to undergo apoptosis (as indicated by the increase in the subG1 peak), suggesting that the lack of glucose could have impaired the completion of the mitotic process (panel G *vs* E). In the following 24 h of culture without glucose the above effect was even more evident, as the fraction of FTC133 cells in G2/M further decreased and correspondingly increased the fraction in the subG1 population (panel H *vs* G).

Overall, these data demonstrate that FTC133 are much more sensitive than WRO cells to nutrient shortage, especially to glucose starvation.

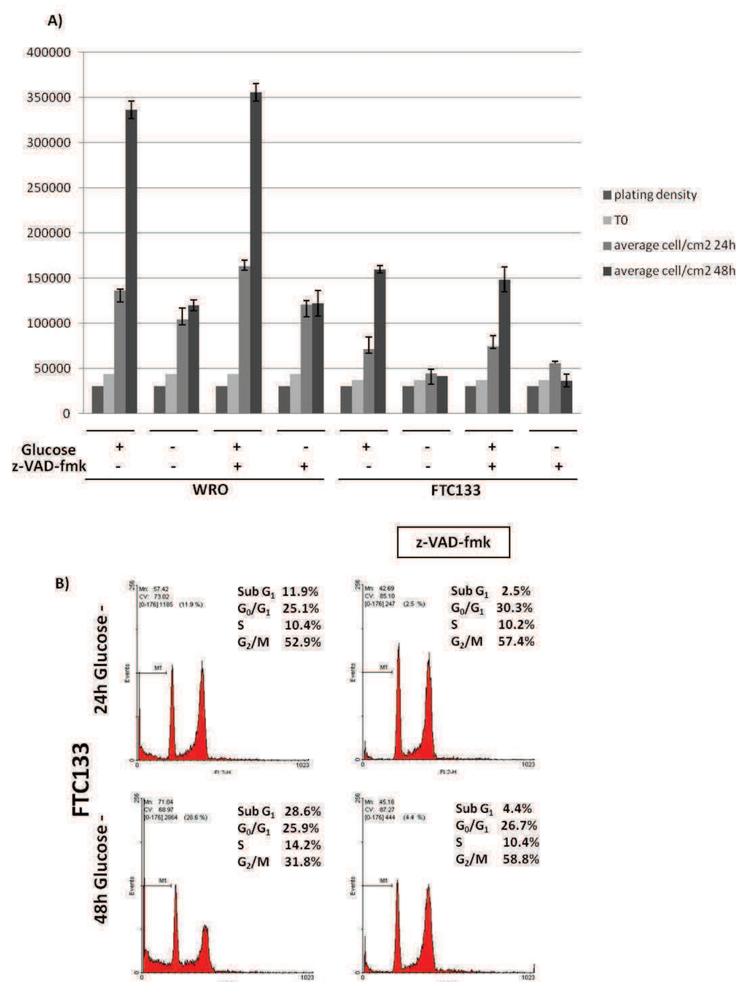

Figure 3: Glucose depletion induces apoptotic cell death in FTC133 cells. A) WRO and FTC133 cells were cultivated in glucose-containing or in glucose-free standard medium for up to 48 h without medium change in the absence or presence of the pan-caspase inhibitor z-VAD-fmk, and then counted. In glucose depletion condition, no apoptosis occurred in WRO cells, while in FTC133 cells apoptotic cell death occurred at 24 h (partially rescued by z-VAD-fmk). B) Cytofluorometry analysis of the cell cycle in FTC133 cells cultivated in glucose-free condition for up to 48 h in the absence or presence of the pan-caspase inhibitor z-VAD-fmk confirmed the induction of apoptotic cell death at 24 h. Z-VAD-fmk prevented apoptosis in the first 24 h and arrested the cell cycle in the G2/M phase, avoiding necrosis, in the following 24 h (48 h). Data shown in this Figure have been reproduced independently four times.

Glucose depletion differentially affects WRO and FTC133 cell survival

To assess whether glucose depletion caused apoptosis to FTC133 cells, we repeated the experiment in the presence of the pan-caspases inhibitor z-VAD-fmk. No apoptosis occurred in WRO cells cultivated under any conditions, while in the case of FTC133 cells cultivated in the absence of glucose a portion of cell loss could be rescued by z-VAD-fmk at 24 h, but not at 48 h (Figure 3A). The latter finding was in apparent contrast with the data shown in Figure 2H. However, a cytofluorometric analysis of the cell cycle confirmed that in fact z-VAD-fmk effectively reduced the subG1 peak (indicative of apoptosis) while increasing the fractions in the G2/M phase (Figure 3B). Consistently, in the presence of z-VAD-fmk the percentage of FTC133 cells positively stained with trypan blue, a dye that monitors necrosis, decreased from approximately 18% to 7% in the 48 h culture in glucose-free medium. Thus, in FTC133 cells cultivated for 48 h in the absence of glucose, z-VAD-fmk prevented apoptosis in the first 24 h, while in the subsequent 24 h it caused a general block of the cell cycle that prevented the onset of necrosis.

Glucose availability differentially impact autophagy in WRO and FTC133 cells

The autophagy-lysosomal degradation pathway is up-regulated under stressful metabolic conditions to provide the cells with anabolic substrates necessary for survival[16]. Nutrients (essentially aminoacids and glucose) shortage, as well as the lack of growth factors, are strong stimuli for rising up basal autophagy to the level needed to overcome the metabolic stress[16]. The altered expression of PTEN and p53 is likely to affect the regulation of autophagy in thyroid cancers[17].

It seemed therefore important to check whether the different susceptibility to glucose deprivation manifested by WRO and FTC133 cells could be associated

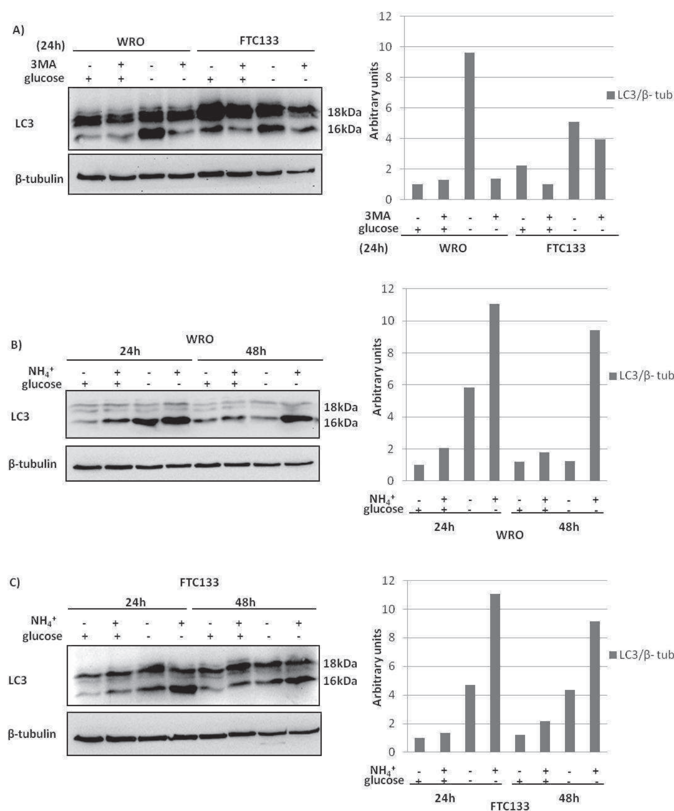

Figure 4: Induction of autophagy in WRO and FTC133 cells by glucose depletion. A) Western blotting analysis of the autophagosome marker LC3 II in WRO and FTC133 cells cultivated for 24 h in glucose-containing or in glucose-free standard medium in the absence or the presence of the PI3k inhibitor 3MA (10mM). The densitometry ratio of LC3 II normalized *versus* β-tubulin is reported. 3MA inhibited glucose-depletion induced autophagy more in WRO cells than in FTC133 cells. B-C) Western blotting analysis showing LC3 II levels in WRO cells (B) and in FTC133 cells (C) cultivated for 24 h and 48 h in glucose-containing or in glucose-free standard medium in the absence or the presence of the weak base ammonium chloride (NH_4Cl, 10mM). The densitometry ratio of LC3 II normalized *vs* β-tubulin is reported. Under glucose depletion autophagy induction appeared similar in the first 24 h for both the cell lines, instead at 48 h the autophagy flux was impaired in FTC133 cells. Data shown in this Figure have been reproduced independently three times.

with an altered activation of the autophagy pawthay. During autophagosome formation, LC3 must be post-translationally conjugated to phosphatydil-ethanolamine in order to be inserted onto the autophagosomal membranes. This processing increases the apparent electrophoretic mobility of LC3, thus making easy in western blotting to distinguish the precursor LC3-I (apparent m.w. of 18 kDa) and the mature isoform LC3-II (apparent m.w. of 16 kDa). In the canonical regulatory pathway of autophagy, PI3k class III (also known as Vps34) provides the essential starting signal for the autophagosome formation. We included in our incubation conditions 3-methyl adenine (3MA), an inhibitor of PI3k. This drug is widely employed as a pharmacological inhibitor of autophagy[18], though it has been reported that depending on the cell line and the dose and time of incubation it might elicit a paradoxic induction of autophagy[19]. Based on the assumption that the cellular level of LC3-II reflects the autophagosomes present in the cells, it appears that in standard culture condition autophagy is basally (2.5-folds) higher in FTC133 than in WRO cells (Figure 4A). 3MA inhibited only partially basal autophagy in FTC133 cells, not in WRO cells. Glucose depletion induced autophagy in both cell lines, yet the response was higher in WRO than in FTC133 cells (the increases of LC3-II in glucose-deprived samples were of 10-folds and of 2.2-folds above the controls, respectively). Of note, 3MA effectively inhibited glucose-deprivation induced autophagy in WRO cells, and only slightly in FTC133 cells. The increase of LC3-II *per se* not necessarily proves the induction of autophagy, since it might also simply represent the accumulation of autophagosomes resulting from the block of their consumption within the lysosomes[18]. To discriminate true autophagosome production from autophagosome accumulation, it is useful to artificially impair the fusion and degradation steps by using a lysosomal pH disruptor such as ammonium chloride[18]. In the following experiments, we assessed the amount of LC3-II in the cells incubated in the presence of ammonium chloride. Under standard (glucose-containing) culture condition, basal autophagy runs at higher level in WRO than in FTC133 cells, as indicated by the higher accumulation of LC3 II in the presence of ammonium chloride (Figure 4B). At 48 h (with no change of medium), only FTC133 cells have increased the production of autophagosomes. In the absence of glucose, autophagy is induced both in WRO and FTC133 cells and the production/consumption rates of autophagosomes (based on LC3 II accumulation in the absence/presence of ammonium chloride) appear similar in the first 24 h. However, at 48 h WRO cells do not produce new autophagosomes, and rather consume all the pre-existing ones, whereas FTC133 cells still accumulate a large part of the pre-existing autophagosomes indicating that the last step of the autophagy flux is impaired. The above data were further corroborated by immunofluorescence staining of LC3-positive vacuoles

Table 2: Percentage of healing in WRO cells (A) and in FTC133 cells (B) in glucose-containing or glucose-free condition at 8 h, 24 h, and 48 h.

A)

	WRO	
	+ Glucose	- Glucose
8h	33.30%	16.60%
24h	50%	27.70%
48h	100%	66.60%

B)

	FTC133	
	+ Glucose	- Glucose
8h	20.00%	10.00%
24h	35%	15.00%
48h	50%	15.00%

Migration was quantified by calculating the area of the wound at the different time points. Cell migration was slower in FTC133 cells than in WRO cells, and in the absence of glucose migration was further reduced.

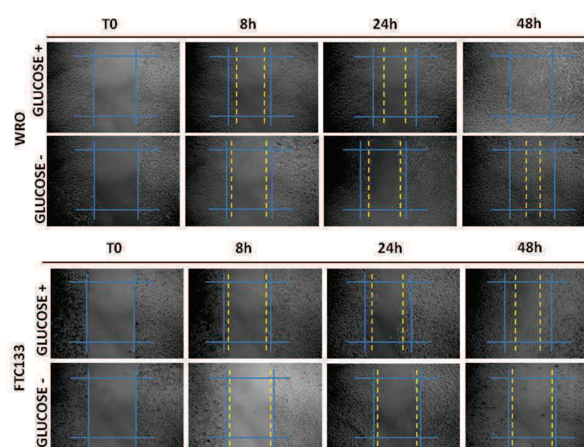

Figure 5: Glucose depletion differentially affect cell migration of WRO and FTC133 cells. WRO and FTC133 cells were plated and let grow to confluence in Petri dishes in glucose-containing standard medium, then a scratch-wound was made using a tip and the cell cultures were switched into glucose-containing or glucose-free conditions and cultured for up to 48 h. Photographs of the wound were taken at time 0 and at 8, 24 and 48 h. The wound area and the percentage of healing were calculated for each time point. WRO cells showed a higher migration rate than FTC133 cells. Data shown in this Figure have been reproduced independently three times.

(not shown).

Glucose depletion differentially affect WRO and FTC133 cell migration

To assay the influence of glucose availability on the migratory potential of thyroid cancer cells, we tested the effect of glucose deprivation in a classical scratch-wound healing assay[20]. WRO cells showed a higher migration rate than FTC133 cells (Figure 5). In glucose-containing medium condition, WRO cells completely healed the wound by 48 h, whereas at this time FTC133 cells only covered a 50 % of the wound area. Under glucose-deprivation condition, cell migration was much slower, yet this effect was particularly evident in FTC133 cells which essentially stopped their migration from 24 h on (Figure 5, Table 2). To get an insight on the functional link between glucose availability and cell migration, we monitored the uptake of glucose in migrating WRO and FTC133 cells in the proximity of the wound. For this purpose, we used 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), a fluorescent analogue of glucose widely used for optical measurements of glucose uptake[21]. The uptake of the fluorescent probe was better appreciated in the cells cultured in glucose-free medium, given that 2-NBDG competes with glucose for the same membrane transporter of the GLUT family. In this condition, the 2-NBDG fluorescent signal was much more intense in FTC133 cells than in WRO cells at any time-point, either in the vicinity of the wound and in the rear (Figure 6A). Thus, despite of the fact that cell proliferation and cell migration were much slower, FTC133 cells appeared more avid than WRO cells in the uptake of glucose. Glucose is internalized in the cells by glucose transporters belonging to the GLUTs family[22]. GLUT1 is the most prevalent isoform in highly aggressive and less-differentiated thyroid cancer histotypes[23-25]. We checked the plasmamembrane expression of GLUT1 in migrating WRO and FTC133 cells cultivated in glucose-

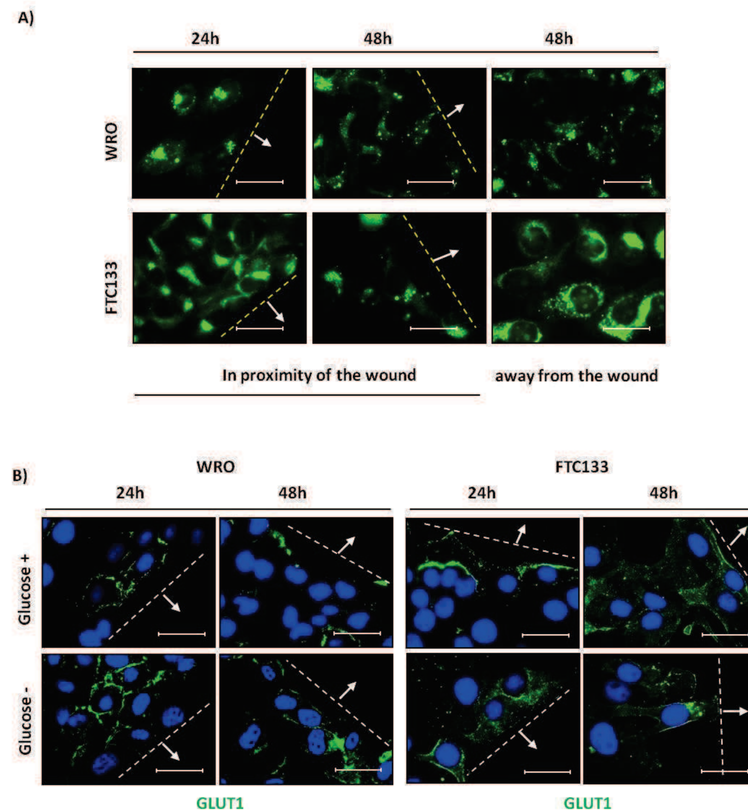

Figure 6: Differential uptake of glucose and plasmamembrane expression of GLUT1 in WRO and FTC133 cells. A) Glucose uptake assay in WRO and FTC133 cell lines. The uptake of the glucose fluorescent analogue 2-NBDG (in green, 50 µM) was monitored during glucose deprivation in proximity of the wound or far from the wound at 24 h and 48 h. Level of 2-NBDG uptake was higher in FTC133 cells than in WRO cells. Scale bar = 20µm. Magnification= 63X. B) Plasmamembrane expression of GLUT1 in proximity of the wound in WRO and FTC133 cell lines. Cells grown on coverslips were cultivated in glucose-containing or glucose-free culture medium for 48 h as per the wound healing assay and immunostained for GLUT1. The expression of GLUT1 in plasmamembrane was higher in FTC133 cells than in WRO cells. In WRO cells the membrane expression of GLUT1 increased under glucose deprivation. Nuclei are stained with DAPI. Scale bar = 20µm. Magnification= 63X. Data shown in this Figure have been reproduced independently three times.

containing or in glucose-free culture medium. Based on immunofluorescence staining, it is apparent that FTC133 cells express GLUT1 on the plasmamembrane at level higher than in WRO cells (Figure 5B). It is to note that in WRO cells the membrane expression of GLUT1 increases upon incubation in glucose-free medium (Figure 6B).

PTEN and p53 control the membrane expression of GLUT1 and the uptake of glucose in thyroid cancer cells

To definitively link PTEN and p53 to the capability of the cancer cells to uptake glucose, we sought to manipulate genetically the expression of these proteins. First, we employed the small interference technology to silence the expression of wild-type PTEN and of wild-type p53 in the WRO cells. After trasnfection, the cells were cultivated for 24 h in the presence or absence of glucose. As shown by immunofluorescence staining in Figure 7A, the transfection with the specific siRNA achieved the effective down-regulation of either PTEN or p53 in the large majority of the cells (it was estimated that >80 % of the cells in the monolayer were negative for the protein considered). It is to be noted, however, that the silencing of p53 was of detriment for cell survival (it was estimated that about 40 % of the transfected WRO cells were detached at the end of the incubation period). This toxic effect was exacerbated in the glucose-deprived culture. Next, we performed in a parallel set of cultures the immunostaining for GLUT1 and for LC3. In control un-transfected and in sham-transfected cells, GLUT1 clustered in a para-golgian area or was relocated at the plasmamembrane depending on whether the cells were cultivated in the presence or the absence of glucose (Figure 7B). To be noted, the cells transfected with either the siRNA specific for PTEN or for p53 basally showed GLUT1 on the plasmamembrane, regardless of the presence or absence of glucose in the culture medium (Figure 7B). LC3-positive autophagic vacuoles were clearly detected in a perinuclear region of the cells cultivated in the absence of glucose, consistent with induction of autophagy by glucose deprivation (see Figure 5B). Of note, LC3-positive vacuoles were also detected in the cells transfected with siRNA p53 cultivated in the presence of glucose.

We then investigated on the functional consequences of the PTEN or p53 knock-down in terms of glucose uptake. The images shown in Figure 7C clearly

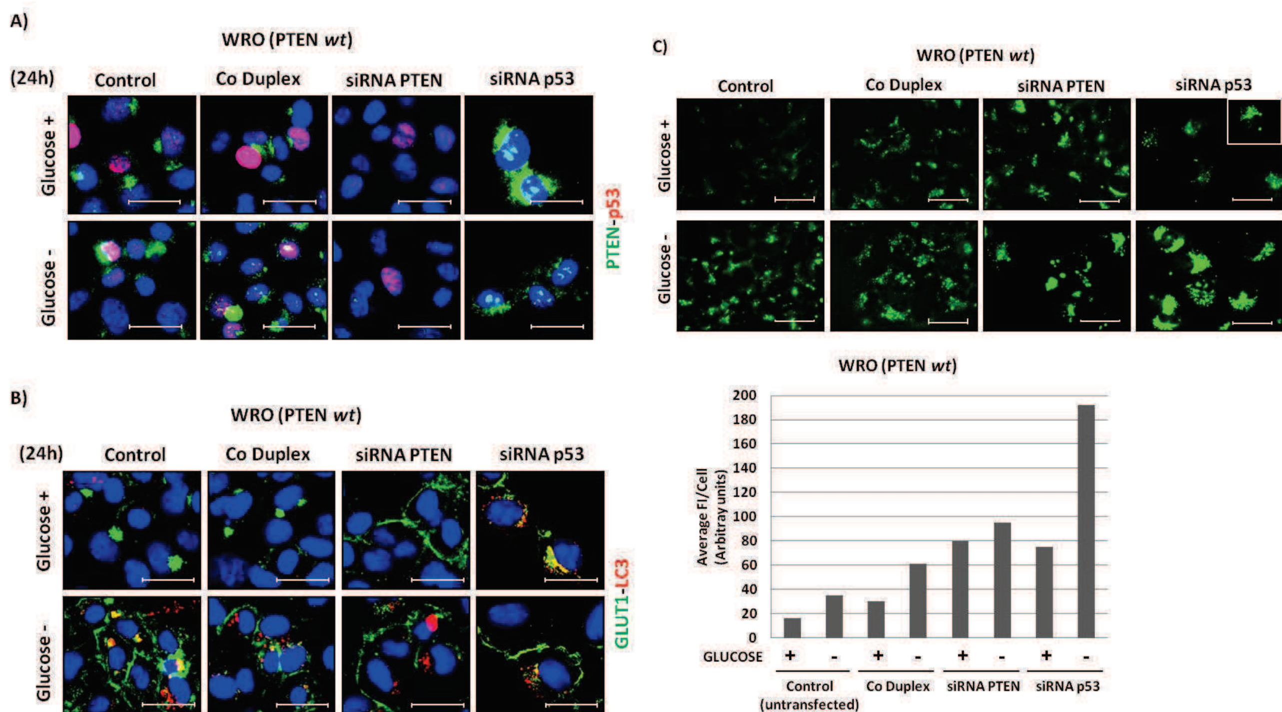

Figure 7: Effect of PTEN and p53 gene knock-down on the cellular expression of GLUT1 and LC3 and on glucose uptake in WRO cells. WRO cells plated on coverslips were transfected with control duplex (sham) or with siRNA specifically targeting PTEN or p53. Control un-transfected cells were also included. The cells were then incubated for 24 h in glucose-containing or glucose-free medium. At the end, the coverslips were processed for immunofluorescence staining. A) co-immunostaining of PTEN and p53; B) co-immunostaining of GLUT1 and LC3. C) A parallel set of cultures was used to assay the uptake of 2-NBDG. ImageJ quantification of cell associated 2-NBDG is included. Data are given as average of Fluorescence Intensity (FI) per cell in the selected fields. These data were reproduced independently two times in double. Scale bar = 20µm. Magnification= 63X.

demonstrate that either the silencing of PTEN or of p53 greatly favoured the uptake of glucose. By ImageJ quantification it was estimated that the uptake of 2-NBDG in siRNA-transfected cells was on average three folds that in the sham-transfected counterpart and five to six folds that in the un-transfected control cells (Figure 7C, lower panel). In siRNA p53-transfected cells cultivated in the absence of glucose the uptake of 2-NBDG was even higher. Note that the stress associated to the transfection *per se* stimulated the uptake of 2-NBDG (compare sham-transfected *vs* un-transfected).

Finally, as a complementary experiment to prove the role of PTEN in glucose uptake, we transgenically expressed wild-type PTEN in the PTEN-null FTC133 cells. After transfection with a plasmid harbouring the PTEN cDNA, the cells were further incubated 24 h in glucose-containing or glucose-free medium. A first set of coverslip was immunostained for PTEN and p53 (Figure 8A). Based on immunofluorescence positivity it was estimated that >60 % of the transfected cells were efficiently expressing PTEN. Of note, the mutant p53 protein localized to the nucleus, especially in the cells cultivated in glucose-free medium (Figure 8A). In control un-transfected and in sham-transfected FTC133 cells GLUT1 was permanently localized on the plasmamembrane, regardless of the availability of glucose (Figure 8B). The ectopic expression of PTEN greatly compromised the membrane localization of GLUT1 (Figure 8B). Yet, when cultivated in the absence of glucose GLUT1 was relocated again on the plasmamembrane (Figure 8A). We then looked at the consequence of PTEN expression on the uptake of glucose in transfected FTC133 cells. Strikingly, the fluorescence associated with the internalization of 2-NBDG was drastically reduced in the PTEN-transfected cultures (Figure 8C). By ImageJ quantification, the uptake of 2-NBDG was halved

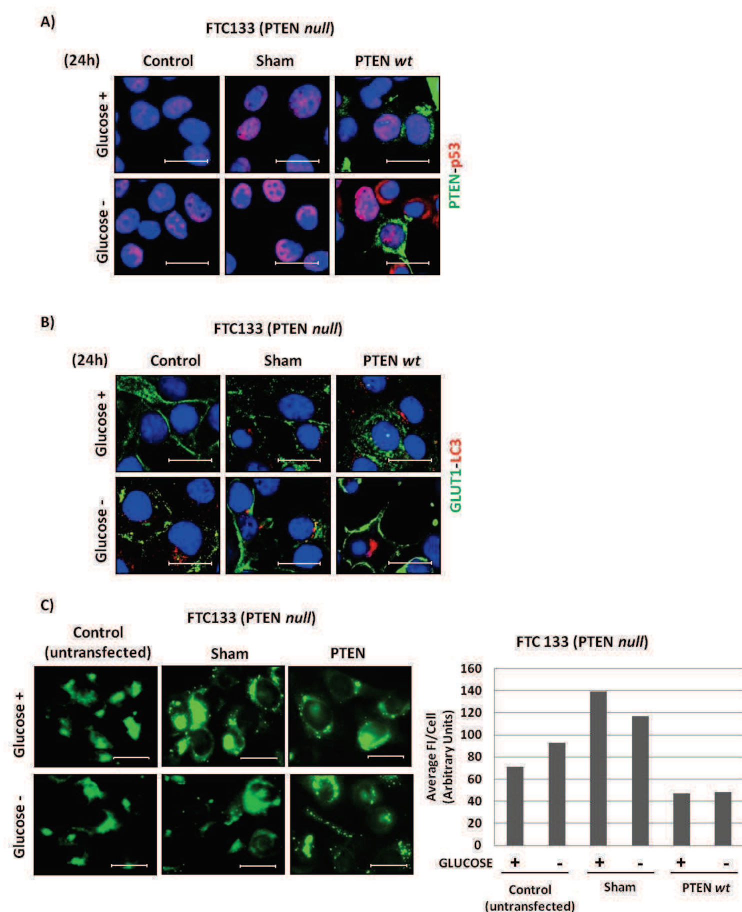

Figure 8: Effect of ectopic expression of PTEN in FTC133 cells on the cellular expression of GLUT1 and LC3 and on glucose uptake. FTC133 cells plated on coverslips were transfected with an empty pcDNA vector (sham) or with the plasmid harbouring the wild-type PTEN cDNA. Control un-transfected cells were also included. The cells were then incubated for 24 h in glucose-containing or glucose-free medium. At the end, the coverslips were processed for immunofluorescence staining. A) co-immunostaining of PTEN and p53; B) co-immunostaining of GLUT1 and LC3. C) A parallel set of cultures was used to assay the uptake of 2-NBDG. ImageJ quantification of cell associated 2-NBDG is included. Data are given as average of Fluorescence Intensity (FI) per cell in the selected fields. These data were reproduced independently three times. Scale bar = 20µm. Magnification= 63X.

compared to that of control un-transfected cells, regardless of whether the cells were cultivated in the presence or the absence of glucose (Figure 8C, right panel). This effect is likely underestimated if one considers that the transfection *per se* stimulated the uptake of the fluorescent probe (compare the sham-transfected with the un-transfected cells) and that not all the transfected cells expressed PTEN at high level.

From these data we can conclude that PTEN and p53 control the membrane expression of GLUT1 and the uptake of glucose in cancer cells.

DISCUSSION

In solid tumours, glucose and oxygen availability decrease in the most inner portion as increases the distance of cancer cells from the peripheral vasculature[3]. Hypoxic tumour cells are more aggressive and metastatic-prone, as they reprogram their metabolism toward aerobic glycolysis through HIF-1α-mediated expression of GLUT proteins and glycolytic enzymes[22,26]. Hypoxia has been shown to induce a metabolic response, mediated by the Hypoxia Inducible Factors (HIF) 1 and 2 alpha, that promotes the migration and spreading of thyroid cancer cells[27,28]. Interestingly, this behaviour was reinforced by the abnormal activation of the PI3k pathway in cells lacking the expression of PTEN[28]. It has also been reported that glycolysis is a main source of energy for malignant thyroid cells[29]. However, no studies have so far analyzed the effect of glucose depletion on the phenotypic behaviour of thyroid cancer cells. Here we have addressed this issue in the thyroid cancer cell lines WRO and FTC133, which differ in the expression of PTEN and p53, two oncosuppressor proteins that are known to play important roles in glucose metabolism. PTEN hyper-expression reduces glucose uptake and favours its mitochondrial oxidation, thus opposing the Warburg effect[8]. Further, PTEN down-regulates the PI3k-AKT-mTOR pathway, that transduces cell proliferation signals[30]. TP53 also reduces the glucose uptake and antagonizes the Warburg effect by controlling the expression of membrane glucose transporters[31] and of glycolytic enzymes[32]. Activation of p53 also leads to cell cycle arrest[33] and apoptosis[34]. Therefore, either loss of PTEN or of p53 is expected to increase glucose uptake and glycolysis, to promote cell proliferation and to impair apoptosis. Indeed, we found that FTC133 cells, which are PTEN deficient and express a mutant p53, are higher up-taker and consumer of glucose respect to WRO cells, which express wild-type PTEN and p53. Experiments of (PTEN and p53) gene silencing and of (PTEN) transgenic expression clearly demonstrated the dominant role of PTEN and p53 in driving the plasmamembrane localization of GLUT1 and the glucose uptake. Worth to note, while the wild-type p53 was stabilized in glucose-depleted WRO cells, the mutant p53 was instead degraded in glucose-depleted FTC133 cells despite it was phosphorylated. Wild-type p53 is mainly degraded through the MDM2-ubiquitin-proteasome system[35], while the mutant p53 is degraded by either the chaperon-mediated autophagy[36] or the macro-autophagy pathway[37]. Also to be noted is that knock-down of wild-type p53 in WRO cells was deleterious for cell survival, while increasing basal autophagy and glucose uptake in the cells. Knock-down of PTEN increased the rate of glucose uptake in WRO cells, and it had no side effects on cell viability and on the accumulation of LC3-vacuoles.

Strikingly, the Dt of FTC133 cells was two-folds that of WRO cells when cultured in glucose-containing medium, and it further increased to four-folds when cultured for up to 48 h in glucose-free medium. Cell cycle analysis data indicated that glucose deprivation mainly affected the Go/G1 to S phase transition in WRO cells, though not toxic, while it dramatically affected the completion of the G2/M phase and eventually provoked apoptotic cell death in FTC133 cells. FTC133 expressed high level of plasmamembrane GLUT1, compared to WRO cells, which is consistent with the loss of function of PTEN and p53. FTC133 cells, besides being PTEN-deficient, express the p53R273H mutant that has been shown to inhibit caspase-dependent apoptosis[38] and autophagy[39] and to promote cell migration and invasion[40]. Therefore, it appears counterintuitive that prolonged glucose depletion could lead FTC133 to caspase-dependent cell death. Not only, FTC133 cells also showed a reduced migration rate compared to WRO cells, and this difference was more evident in glucose-depleted conditions. Autophagy was not inhibited in FTC133, yet chronic glucose-depletion led to impaired consumption of the autophagosomes, likely as a consequence of insufficient energy. Taken together, these facts indicate that FTC133 cells rapidly consume glucose, thus soon becoming glucose-addicted. In this respect, it is to be stressed that FTC133 cells were cultivated in a medium containing much more glucose than that used for culturing WRO cells (4.5 g/L *vs* 2 g/L).

We hypothesize that the synergistic effects of combined PTEN loss-of-function and mutant p53 gain-of-function lead to a metabolic dependence on glucose with unpredicted impact on cell behaviour, that differs from that described for individual oncosuppressor gene alteration. Of particular relevance is the impairment of the autophagy flux in glucose-depleted cells, which may account for loss of protection against the metabolic stress.

It is now clear that the malignant behaviour of cancer cells results from the combination of mutations with gain-of-function or loss-of-function that involve at least five to seven oncogenes and oncosuppressor genes. In addition, epigenetic events contribute to the altered expression of key regulatory proteins. The presence of pro-oxidant molecules and of inflammatory cytokines in the tumour micro-environment further contributes to modify the gene expression in cancer cells. Thus,

cancers that develop spontaneously soon become a very heterogeneous population of subclones each with its unique set of alterations in the expression of oncogenes and oncosuppressors, as well as of key metabolic regulators. This explains why a drug targeting one single pathway, while effective in experimental models (in which only one gene is the dominant driver), often fails when translated into the clinic for the therapy of spontaneous tumours.

Whatever the set of genes altered, for their proliferation and migration the cancer cells need energy and this depends on the availability of oxygen and nutrients such as glucose and aminoacids (among which glutamine is the most important). Thus, targeting the energetic metabolism of cancer cells may be the clue to revolutionize cancer treatment[41-43]. In particular, glucose availability and glycolysis have been shown causally linked to chemoresistance, proliferation and metastasization of tumours[44-46]. Therefore, glucose-restriction is expected to elicit beneficial effect against glucose-addicted cancer cells. Indeed, there are indications in this sense. Reducing the uptake of glucose with the GLUT1 inhibitor Phloretin favoured doxorubicin toxicity in P-glycoprotein expressing chemoresistant cancer cells[47]. In addition, the administration of 2-deoxy-D-glucose, a glucose analogue that impairs glycolysis, enhanced the clinical efficacy of radio- and chemotherapy in glioblastomas[48] and in anaplastic thyroid cancer[49]. Consistent with the role of glucose availability in cancer progression, it was found that a higher dietary glucose intake significantly associated with an increased risk of recurrence and mortality in stage III colon cancer patients[50].

Here we have shown that the concurrent deletion of PTEN and mutation of p53 exacerbates glucose uptake and consumption in FTC133 cancer cells, which undergo cell cycle arrest and apoptosis, cell migration arrest and manifest defective autophagy when subjected to glucose-restriction. Therefore, targeting together the PI3k-(PTEN)-AKT-mTOR and the p53 pathways, that control both glucose uptake and autophagy, could be an efficacious strategy to cure aggressive cancers.

MATERIALS AND METHODS

Cells and treatments

The WRO and FTC133 follicular thyroid cancer cell lines were kindly provided by Dr. Francesco Frasca, University of Catania, Italy. WRO cells were cultured in RPMI 1640 (with L-glutamine) completed by foetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%); the FTC133 cells were cultured in Dulbecco's Modified Eagle Medium, Nutrient mixture F-12 (1:1, by volume) completed by FBS (10%), penicillin/streptomycin (1%) and L-glutamine (1%). All culture reagents were purchased from Sigma-Aldrich (Germany). For studies on glucose deprivation, the cells were incubated in complete culture medium or medium without glucose (R1383 and D5030, Sigma-Aldrich) for up to 48h. For the experiments, growing cells were plated on sterile plastic dishes or on sterile glass coverslips and allowed to adhere for at least 24 h before the use. The PI3K inhibitor 3-methyladenine (3MA; Sigma-Aldrich) was used at 10 mM. Ammonium Chloride (NH_4Cl) was used at 10 mM. At the end of the incubations, the cells or coverslips were collected and processed as detailed below.

Cell proliferation, cell cycle and cell death assay

Cell growth was assessed by manual and hemocytometer cell counting of adherent viable (trypan blue-excluding) cells. Doubling Time (Dt) was calculated using the free software Doubling Time Online Calculator (http://www.doubling-time.com/compute.php). Cell death was assessed by counting the trypan blue-stained cells (necrotic cells) and by cytofluorometric analysis of Annexin V-Propidium Iodide double stained cells, as previously reported[51,52]. For this purpose, floating (dead) cells and attached cells were combined. Cell cycle analysis was performed by cytofluorometry of Propidium Iodide labelled cells using the software WinMDI 2.9.

Wound-healing migration assay

The cells were plated on Petri dishes and cultured for at least 48 h till confluence. A wound was made by dragging a sterile blue pipette tip along the centre of the plate[53]. Detached cells were washed out twice, and cultures were then incubated for up to 48 h with no changes of medium in Glucose-containing or Glucose-free medium. Images of cell monolayers were taken at the time indicated under the phase-contrast microscope with a digital camera. The wound wideness was calculated by measuring the mean distance between the margins of the wound in randomly selected fields, directly on photographs. Migration was quantified by calculating the area of wound at time points t_0(time of wound), t_{24} (24 h after wound) and t_{48} (48 h after wound). Normalization was obtained by the formula [area(t_0) − area($t_{24\ or\ 48}$)]/ area(t_0). Overall, four independent experiments were performed.

In other experiments, the cell were cultured up to confluence on sterile glass coverslips and wounded as described above, except that a yellow pipette tip was used. At the end of the incubations, the cells on coverslips were fixed, permeabilized and processed for immunofluorescence staining. Alternatively, the living cells were assayed for glucose uptake.

Glucose uptake assay and measurement

The fluorescent glucose analogue 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose (2-NBDG; Life Technologies Co, Carlsbad, CA, USA) was used to detect glucose uptake in living cells[20]. Cells grown on coverslips were incubated with 50 μM of 2-NBDG for 1 h before the end of the treatments, washed twice with PBS 1x and rapidly imaged under the fluorescence microscope. Image processing and data quantification of the area and of the intensity of fluorescence images were performed with the software ImageJ 1.48v (http://imagej.nih.gov/ij/). At least five randomly chosen fields for a total of minimum 50 cells were analysed. Fluorescence Intensity (FI) is given in arbitrary units as an average value per cell in the selected representative fields.

Immunofluorescence staining

The cells adherent on sterile glass coverslips were fixed in cold methanol for 20 min and permeabilized with 0.2% Triton X-100 in PBS1X for 10 min. Overnight incubation in cold room was performed with the following primary antibodies: rabbit polyclonal anti-GLUT1 (Millipore, Darmstadt, Germany; dilution 1:50); rabbit polyclonal anti-PTEN (Millipore; dilution 1: 200); mouse monoclonal anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:100) and mouse monoclonal anti-LC3 (nanoTools, Teningen, Germany; dilution 1:100). As secondary antibody (dilution 1:600) the IRIS-2 (green fluorescence)-conjugated goat-anti-rabbit IgG secondary or IRIS-3 (red fluorescence)-conjugated goat-anti-mouse IgG (Cyanine Technologies SpA, Turin, Italy) was used as appropriate for 1 h at room temperature in a humid chamber. Nuclear chromatin was stained with the fluorescent dye 4,6-diamidino- 2-phenylindole-dihydrochloride (DAPI). As negative control, the primary antibody was omitted or substituted with pre-immune antiserum. Antibodies were diluted in PBS containing 0.1% Triton X-100 and 10% FBS. Stained cells were mounted with Slow-FADE (Light AntiFADE Kit, Molecular Probes Invitrogen, Carlsbad, CA, USA) and observed under a fluorescence microscope (Leica DMI6000B, Leica Microsystem AG, Wetzlad, Germany).

Western blotting assay

Immunoblotting was performed following standard procedures as previously reported.[54] Cell homogenates were prepared by freeze–thawing and ultrasonication in a buffer containing detergents and protease inhibitors. 30 μg of cell proteins were denatured with Laemmli sample buffer, separated by electrophoresis on a 12.5% SDS-containing polyacrylamide gel and then electroblotted onto PVDF membrane (Carlo Erba reagents, Milan, Italy). The filter was first probed with the antibody specific for the protein of interest. The following primary antibodies were used: Rabbit polyclonal anti-PTEN (EX-BIO, Vestec, Czech Republic); rabbit polyclonal anti-LC3 (Sigma-Aldrich); rabbit polyclonal anti-phospho p53 (Ser15) (Cell Signaling technology, Danvers, Massachusetts, USA) and mouse monoclonal anti-p53 (Santa Cruz Biotechnology). The filter was subsequently stripped and re-probed with an antibody specific for β-tubulin (Sigma-Aldrich), as an index of homogenate protein loading in the lanes. Immunocomplexes were revealed by using a peroxidase-conjugated secondary antibody (Bio-Rad, California, USA), as appropriate, and subsequent peroxidase-induced chemiluminescence reaction (PerkinElmer, Massachusetts, USA). Densitometry of Western Blot bands was performed with the Quantity One-4.5.0 software (Bio-Rad) and with the free software Image J (1.48v; http://imagej.nih.gov/ij/).

Small interference RNA and plasmid transfections

The reagents and the methods have been previously described[55]. Post-transcriptional gene silencing was obtained using a specific small interference RNA (siRNA) directed against the mRNA of PTEN (the sense strand was 5′–AGACUUGAAGGCGUAUACA-3′) or of p53 (the sense strand was 5’-AAGAAACCACUGGAUGGAGAAUAUUUC-3’). A control duplex was used for sham-transfection. Duplexes of nucleotide siRNA were purchase from MWG Biotech AG (Ebersberg, Germany). In brief, the cells were plated and let adhere on coverslips for at least 24 h, then incubated for 6 h with 30 pmol RNA-duplexes in the presence of 7.5 μl Lipofectamine 3000 (Invitrogen Co) diluited in 250 μl of Optimem (Life Technologies Co). The cells were then washed and further incubated 36 h post-transfection to allow maximal effect on protein down-regulation before use.

Transgenic expression of *wild type* PTEN was obtained by Lipofectamine 3000 (Invitrogen Co) transfection with a pcDNA3.1Zeo+ plasmid containing the specific full-length cDNA. An empty pcDNA3.1Zeo+ plasmid was used for sham transfection. In brief, the cells adherent on coverslips were incubated for 6 h with 5 μg/μl of DNA in the presence of 7.5 μl Lipofectamine 3000 (Invitrogen Co) diluited in 250 μl of Optimem (Life Technologies Co). Then washed and incubated 36 h post-transfection.

Statistics

Unless otherwise specified, all experiments were replicated independently three times and in double or triplicate. Data are given as average ± S.D. Statistical

significance was taken for p values <0.05.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

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7. PTEN Regulates Plasma Membrane Expression Of Glucose Uptake in Thyroid Cancer

Federica Morani, Suratchanee Phadngam, Carlo Follo, Rossella Titone, Gianluca Aimaretti, Alessandra Galetto, Oscar Alabiso and Ciro Isidoro

Glucose represents an important source of energy for mammalian cells. In highly proliferating cancer cells glucose is preferentially converted into lactate despite the presence of oxygen and functional mitochondria. This aberrant metabolism of glucose, known as the Warburg effect or aerobic glycolysis, is much less convenient in terms of energy gain, and imposes the consumption of an elevated quantity of glucose in proliferating cells. This effect, has been proven to be useful for imaging metabolically active tumors in cancer patients by 18F-fluorodeoxyglucose positron emission tomography (FDG–PET). Glucose is internalized in the cells by glucose transporters (GLUTs) belonging to the GLUT family. GLUT1 (SLC2A1) is the most prevalent isoform in more aggressive and less differentiated thyroid cancer histotypes. In a previous work, we found that loss of expression of PTEN was associated with increased expression of GLUT1 on the plasma membrane (PM) and probability of detecting thyroid incidentalomas by FDG–PET. Herein, we investigated the molecular pathways that govern the expression of GLUT1 on the PM and the glucose uptake in WRO (expressing WT PTEN) and FTC133 (PTEN null) follicular thyroid cancer cells cultured under glucose-depleted conditions. The membrane expression of GLUT1 was enhanced in glucose-deprived cells. Through genetic manipulations of PTEN expression, we could demonstrate that the lack of this oncosuppressor has a dominant effect on the membrane expression of GLUT1 and glucose uptake. We conclude that loss of function of PTEN increases the probability of cancer detection by FDG–PET or other glucose-based imaging diagnosis.

Personal Contribution

In this paper, I contributed to the assessment of GLUT1 expression and localization in PTEN-transfected cells (and additional experiments not shown in the paper).

PTEN regulates plasma membrane expression of glucose transporter 1 and glucose uptake in thyroid cancer cells

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Abstract

Glucose represents an important source of energy for the cells. Proliferating cancer cells consume elevated quantity of glucose, which is converted into lactate regardless of the presence of oxygen. This phenomenon, known as the Warburg effect, has been proven to be useful for imaging metabolically active tumours in cancer patients by ^{18}F-fluorodeoxy-glucose positron emission tomography (FDG–PET). Glucose is internalised in the cells by glucose transporters (GLUTs) belonging to the GLUT family. GLUT1 (SLC2A1) is the most prevalent isoform in more aggressive and less differentiated thyroid cancer histotypes. In a previous work, we found that loss of expression of PTEN was associated with increased expression of GLUT1 on the plasma membrane (PM) and probability of detecting thyroid incidentalomas by FDG–PET. Herein, we investigated the molecular pathways that govern the expression of GLUT1 on the PM and the glucose uptake in WRO (expressing WT *PTEN*) and FTC133 (*PTEN* null) follicular thyroid cancer cells cultured under glucose-depleted conditions. The membrane expression of GLUT1 was enhanced in glucose-deprived cells. Through genetic manipulations of PTEN expression, we could demonstrate that the lack of this oncosuppressor has a dominant effect on the membrane expression of GLUT1 and glucose uptake. We conclude that loss of function of PTEN increases the probability of cancer detection by FDG–PET or other glucose-based imaging diagnosis.

Key Words

- PTEN
- glucose
- Warburg effect
- FDG–PET imaging, cancer
- GLUT

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Introduction

Glucose is a fundamental source of energy for mammalian cells. Normally, in the presence of oxygen, glucose is oxidised through the mitochondrial respiration pathway with the highest rate of production of ATP. However, in intensely proliferating cancer cells, glucose is preferentially converted into lactate despite the presence of oxygen and functional mitochondria. This aberrant metabolism of glucose, known as the Warburg effect or aerobic glycolysis, is much less convenient in terms of energy gain and imposes a large consumption of glucose in proliferating cells (Newsholme *et al*. 1985, Koppenol *et al*. 2011). This fact is being exploited for diagnostic imaging of tumours in patients injected with the probe ^{18}F-fluoro-deoxyglucose (FDG), a radioactive analogue of glucose,

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which can be detected by positron emission tomography (PET) (Busk *et al*. 2008). As the uptake of FDG competes with that of circulating glucose, the patient undergoing FDG–PET must avoid the intake of any source of glucose during the 6 h preceding the start of the PET study (Boellaard *et al*. 2010). This technique has the potential to reveal unsuspected tumours, commonly indicated as 'incidentaloma' because of their accidental discovery.

Glucose, similar to its radioactive analogue ^{18}FDG and its fluorescent equivalent 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG), is internalised in the cells by glucose transporters (GLUTs) belonging to the GLUT family (Szablewski 2013). GLUT1 (SLC2A1) is the most prevalent isoform in highly aggressive and less differentiated thyroid cancer histotypes (Hosaka *et al*. 1992, Samih *et al*. 2000, Matsuzu *et al*. 2005).

Only a few studies have addressed the signalling pathways involved in the glucose uptake by GLUT1 in thyroid cancer cells. There are indications that the surface expression of GLUT proteins is under the control of the phosphoinositide 3-kinase (PI3K)/AKT pathway (Samih *et al*. 2000). Recently, AMPK has been shown to play a pivotal role in the membrane expression of GLUT1 and glucose uptake in rat follicular thyroid cells (Andrade *et al*. 2012). In a small cohort of patients, we found that loss of expression of the oncosuppressor PTEN, a protein–lipid phosphatase that switches off the AKT pathway, was associated with an increased probability of detecting thyroid incidentalomas by FDG–PET (Morani *et al*. 2012). In this work, we analysed the signalling pathways that govern the expression of GLUT1 on the plasma membrane (PM; and the uptake of glucose) in two follicular thyroid cancer cell lines: WRO, expressing WT *PTEN*, and FTC133 *PTEN* null cells. Genetic manipulations indicated that the lack of PTEN expression has a dominant effect on the membrane expression of GLUT1 and glucose uptake. This finding substantiates our hypothesis that loss of function of PTEN increases the probability of cancer detection by FDG–PET or other glucose-based imaging diagnosis (Morani *et al*. 2012).

Materials and methods

Thyroid cancer cell lines, cell culture conditions and pharmacological treatments

The WRO and FTC133 follicular thyroid cancer cell lines were kindly provided by Dr Francesco Frasca, University of Catania, Italy.

The WRO cell line was cultured in RPMI-1640 media (with L-glutamine) supplemented with foetal bovine serum (FBS, 10%) and penicillin/streptomycin (1%); the FTC133 cell lines were cultured in DMEM, nutrient mixture F-12 (1:1, by volume), supplemented with FBS (10%), penicillin/streptomycin (1%) and L-glutamine (1%). All culture reagents were purchased from Sigma–Aldrich. For studies on glucose deprivation, the cells were incubated in a glucose-free medium (R1383 and D5030, Sigma–Aldrich). For the experiments, growing cells were plated on sterile plastic dishes and allowed to adhere for at least 24 h before use. The PI3K inhibitor 3-methyladenine (3MA; Sigma–Aldrich) was used at a concentration of 10 mM. At the end of the incubations, media and cells or coverslips were collected and processed for protein analysis as detailed below.

siRNA silencing of *PTEN*

Post-transcriptional silencing was achieved by the siRNA technology. Duplexes of nucleotide siRNA were synthesised by MWG Biotech AG (Ebersberg, Germany). The sequence and use of the siRNA for Sham transfection have been described previously (Trincheri *et al*. 2007). Two siRNA oligos targeting two different exons were used for the silencing of *PTEN*, and the sense strands were 5′-AGACUUGAAGGCGUAUACA-3′ and 5′-AGAAUCAUCUGGAUUAUAG-3′. Adherent cells (plated at a density of 30 000/cm^2 in a Petri dish) were incubated for 6 h with 100 pmol RNA duplexes in the presence of 6 μl Lipofectamine 2000 (Invitrogen Co.) in 500 μl Optimem (Life Technologies Co.). The cells were then washed and treated 36 h after transfection to allow for a maximal effect on protein down-regulation.

Plasmid transfections

pcDNA3.1Zeo+ plasmid containing the full-length WT *PTEN* cDNA were transfected by Lipofectamine 2000 (Invitrogen Co.) following the manufacturer's protocol. The full-length *PTEN* cDNA was cloned from OAW42 ovarian cancer cells mRNA and fully sequenced to confirm the WT sequence. OAW42 total RNA was extracted according to the TRIzol LS reagent protocol (Life Technologies Co.). Total RNA (3 μg) was retrotranscribed using the RevertAiD H Minus FirstStrand cDNA Synthesis Kit (Fermentas, Burlington, CA, USA). RT-PCR was performed according to the manufacturer's instructions with DyNzyme EXT DNA Polymerase (Finnzymes OY, Espoo, Finland) starting from 2 μl cDNA and using a final

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concentration of 10 μM *PTEN* primers (forward primer, CATTTCCATCCTGCAGAAGAAG and reverse primer, CCCAATACAGATTCACTTCCTTTAG). *PTEN* cDNA was subcloned into the plasmid pcDNA 3.1 Zeo (Life Technologies Co.) and subjected to automated sequencing (ABI PRISM 3100, Applied Biosystems). Primers were obtained from MWG-BIOTECH AG (Ebersberg, Germany). An empty pcDNA3.1Zeo+ plasmid was used as control transfection (Sham).

Glucose uptake assay

The fluorescent glucose analogue 2-NBDG (Life Technologies Co.) was used to detect glucose uptake in living cells (O'Neil *et al.* 2005). Cells grown on coverslips were incubated with 50 μM of 2-NBDG for 1 h before the end of the treatments, washed twice with PBS 1× and rapidly imaged under a fluorescence microscope (Leica DMI6000B, Leica Microsystem AG, Wetzlad, Germany).

Immunofluorescence staining

At the end of treatments, cells adherent on sterile glass coverslips were fixed in cold methanol for 20 min and permeabilised with 0.2% Triton X-100 in PBS 1× for 10 min. Antigens were revealed with a primary specific antibody followed by IRIS2 (green fluorescence)-conjugated goat anti-rabbit IgG or IRIS3 (red fluorescence)-conjugated goat anti-mouse IgG (Cyanine Technologies SpA, Turin, Italy) secondary antibody, as appropriate, for 1 h at room temperature in a humid chamber. The nucleus was evidenced by staining the chromatin with the fluorescent dye 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). As negative control, the primary antibody was omitted or substituted with pre-immune antiserum. Antibodies were diluted in PBS containing 0.1% Triton X-100 and 10% FBS. The following primary antibodies were used: rabbit polyclonal anti-GLUT1 (Millipore, Darmstadt, Germany), rabbit polyclonal anti-PTEN (Millipore) and mouse monoclonal anti-Rab5 (BD Transduction Laboratories, San Jose, CA, USA). Stained cells were mounted with Slow-FADE (Light AntiFADE Kit, Molecular Probes Invitrogen) and observed under a fluorescence microscope (Leica DMI6000B, Leica Microsystem AG). Observations were performed by two independent investigators. Representative images of at least three independent experiments are shown. Image processing was carried out using the ImageJ Software (freely available at http://imagej.nih.gov/ij/, see below).

Western blotting assay

Immunoblotting was carried out following standard procedures as reported previously (Castino *et al.* 2007). Cell homogenates were prepared by freeze–thawing and

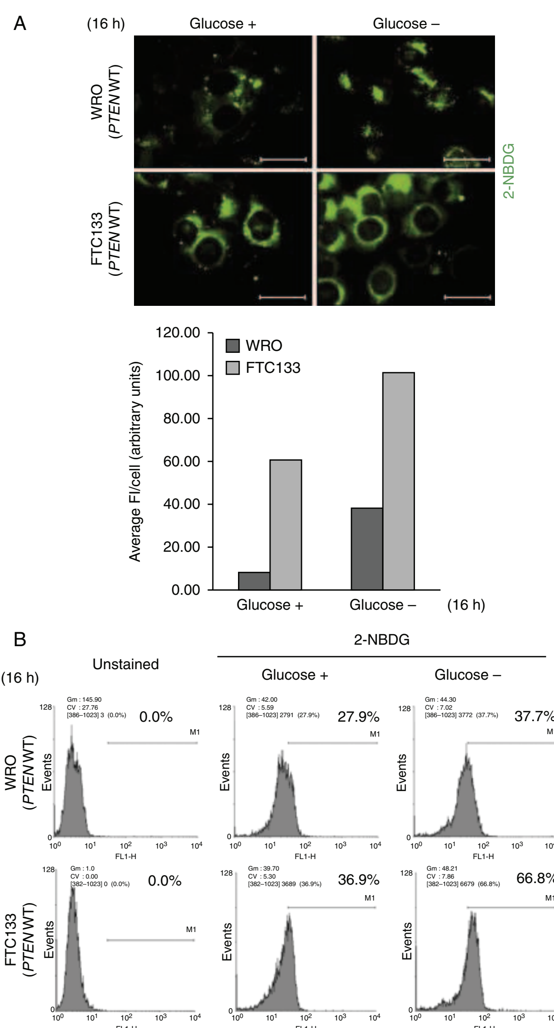

Figure 1
Differential uptake of glucose in WRO and FTC133 cells. (A) The uptake of the glucose fluorescent analogue 2-NBDG (in green, 50 μM) was monitored for 16 h in both cell lines cultured in a glucose-containing or glucose-free medium. The probe was added to the culture medium 1 h before the end of the incubation. Representative microscopic fields are shown in the upper panel. Quantification of 2-NBDG uptake was done using the ImageJ Software directly on the microscopic images of labelled cells (lower panel). (B) A parallel culture of cells plated on Petri dishes and labelled as above was used for cytofluorometric quantification of 2-NBDG-positive cells. The level of 2-NBDG uptake was higher in FTC133 cells than in WRO cells and greatly increased when the cells were pre-incubated in a glucose-free medium. Representative data of four experiments are shown. Scale bar=20 μm. Magnification=63×. Data shown in this figure have been reproduced six times independently. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0118.

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ultrasonication in a buffer containing detergents and protease inhibitors. Approximately 30 μg of cell proteins were denatured with Laemmli sample buffer, separated by electrophoresis on a 12.5% SDS-containing polyacrylamide gel and then electroblotted onto a PVDF membrane (Carlo Erba Reagents, Milan, Italy).

Protein of interest was detected with the following primary antibodies: rabbit polyclonal anti-PTEN (EX-BIO, Vestec, Czech Republic); rabbit polyclonal anti-phospho AKT (Ser473) (Cell Signaling Technology, Danvers, MA, USA); rabbit polyclonal anti-AKT (Cell Signaling Technology); rabbit polyclonal anti-GLUT1 (Millipore) and rabbit polyclonal anti-HIF1α (GeneTex, Irvine, CA, USA). As an index of homogenate protein loading in the lanes, β-tubulin was used (Sigma–Aldrich). Immunocomplexes were revealed using a peroxidase-conjugated secondary antibody (Bio-Rad), as appropriate, and subsequent peroxidase-induced chemiluminescence reaction (Perkin-Elmer, Waltham, MA, USA). Western blotting data were reproduced at least three times independently.

Quantification analysis: methods and statistics

Unless otherwise specified, all experiments were replicated three times independently and in duplicate or triplicate. For measurement of glucose uptake, two methods were employed. In adherent 2-NBDG-loaded cells, data quantification of the area and of the intensity of fluorescence images was performed using the ImageJ 1.48v Software (freely available at http://imagej.nih.gov/ij/). At least five randomly chosen fields for a total of minimum 50 cells were analysed. Fluorescence intensity (FI) is given in arbitrary units as an average value per cell in the selected representative fields.

Alternatively, the 2-NBDG-loaded cells were subjected to cytofluorometry analysis. Adherent cells were incubated with 50 μM of 2-NBDG for 1 h before the end of the treatments, washed twice with PBS 1×, collected by trypsinisation, centrifuged at 100 ***g*** at 4 °C for 10 min and re-suspended in OPTIMEM (Life Technologies Co.). Cells were analysed using a Becton Dickinson FACS SCAN cytofluorometer (BD Bioscience, Franklin Lakes, NJ, USA).

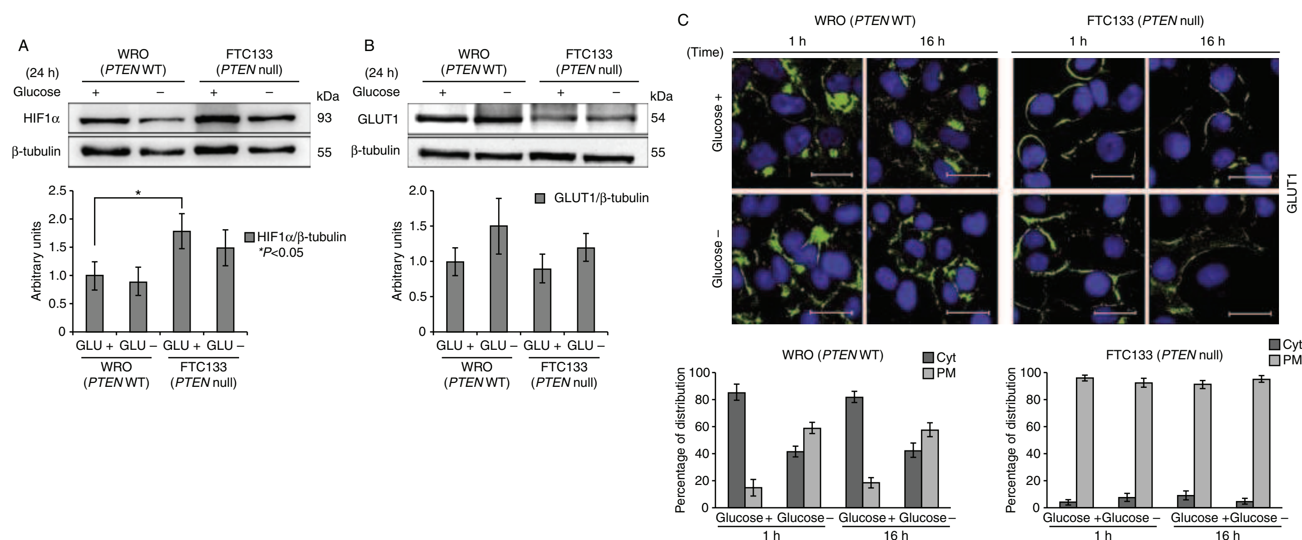

Figure 2

Differential subcellular localisation of GLUT1 in WRO and FTC133 cell lines. (A) Western blotting of HIF1α in homogenates from WRO (*PTEN* WT) and FTC133 (*PTEN* null) thyroid cancer cell lines incubated for 24 h in a glucose-containing or glucose-free medium. Densitometry of three experiments is included. The basal level of HIF1α expression was higher in FTC133 cells than in WRO cells. (B) Western blotting of GLUT1 in homogenates prepared as for A. Densitometry of three experiments is included. Differences in the expression of GLUT1 in WRO and FTC133 cells were not statistically significant. Glucose starvation apparently did not alter the levels of HIF1α and GLUT1 expression in both cell lines. (C) Immunofluorescence assay of GLUT1 expression in WRO and FTC133 cells cultured for 1 or 16 h in a glucose-containing or glucose-free medium. Representative fields of immunofluorescence staining are shown in the upper panels, and the corresponding fluorescence quantification of GLUT1 in the cytoplasmic (Cyt) and plasma membrane (PM) compartments is shown in the lower panels. In WRO cells cultured in a glucose-containing medium, GLUT1 was localised to the cytoplasm (~85%) and, under glucose-depleted conditions, it translocated onto the PM (from ~15 to ~60%). In FTC133 cells, GLUT1 permanently localised to the PM (~95%), regardless of the presence or absence of glucose in the culture medium. Nuclei are stained with DAPI. The images shown in C have been reproduced four times independently. Fluorescence quantification is based on the analysis of a minimum of 50 cells for each of the three independent experiments. Scale bar = 20 μm. Magnification = 63×. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0118.

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Data were acquired using the CellQuest Software (BD Bioscience) (10 000 gated events/sample, on FL-1) and analysed using the WinMDI 2.9 Software (http://facs.scripps.edu/software.htm). Unstained parallel cultures were used for setting the instrument. The proportion of cells positive for 2-NBDG uptake is given as the percentage of the whole population. One (out of three or four) representative cytofluorogram is shown per condition. Alternatively, data are expressed as average ± S.D. of $n=4$.

Quantification of protein expression in fluorescence images of adherent cells was performed using the ImageJ Software. Data were collected from at least three independent experiments. Five randomly chosen fields for a total of minimum 50 cells were analysed in each coverslip. Data on subcellular distribution of the protein of interest (GLUT1) are expressed as percentage (± S.D.) of FI in the PM and cytoplasmic (Cyt) compartments of the cells in the selected representative fields.

Densitometry quantification of western blotting bands was performed using both the ImageJ (1.48v; http://imagej.nih.gov/ij/) and Quantity One 4.5.0 Softwares (Bio-Rad), with consistent results. Data are expressed in arbitrary units as average ± S.D. of at least three independent experiments. The P values <0.05 were considered statistically significant.

Results

FTC133 cells take up glucose more avidly than WRO cells

To monitor the ability of cells to take up glucose, we employed 2-NBDG, a glucose analogue fluorescently labelled at position 2, that is a substrate for GLUTs (Fig. 1). Quantification of 2-NBDG uptake was performed measuring the fluorescence in the cell monolayer (Fig. 1A, lower panel) as well as by cytofluorometry after

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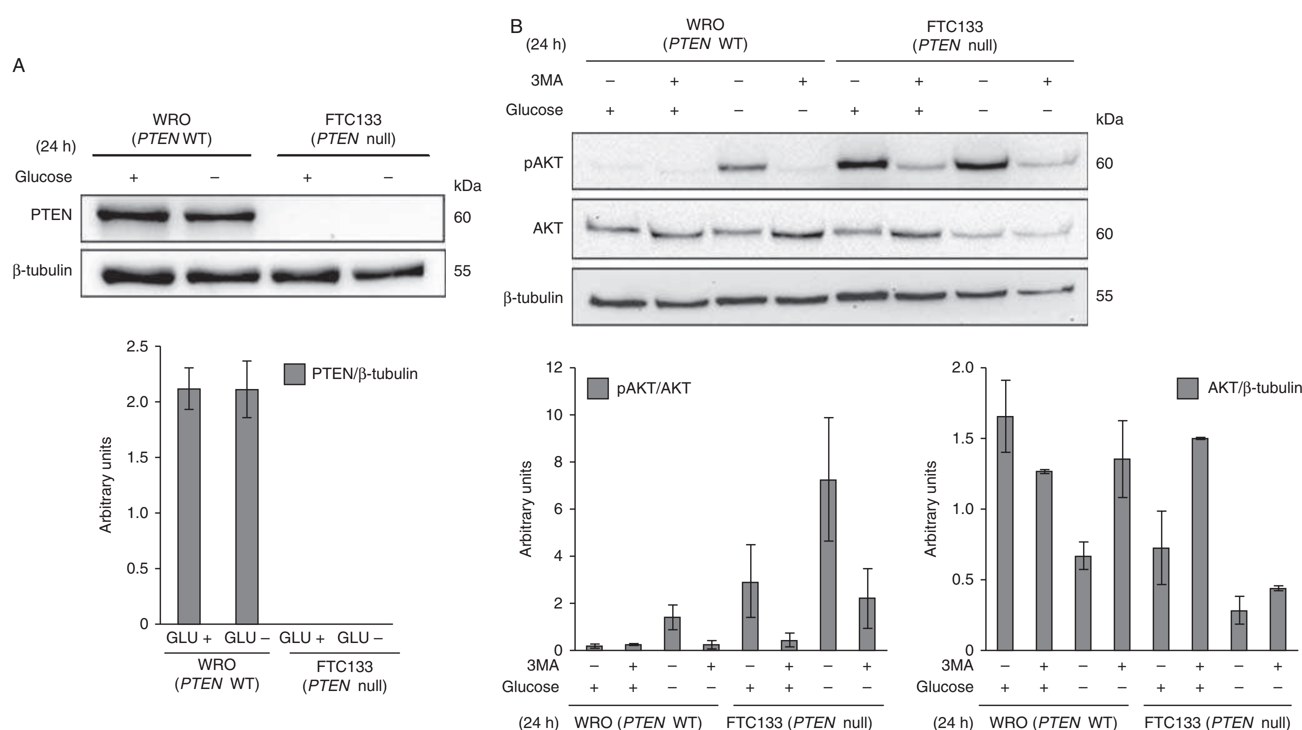

Figure 3

Involvement of the PI3K signalling pathway in the glucose-deprivation response in WRO and FTC133 cells. (A) PTEN status was analysed by western blotting in WRO and FTC133 cell lines after 24 h of incubation in a glucose-containing and glucose-free media. FTC133 cells do not express detectable levels of PTEN. In WRO cells, the expression of PTEN is not subjected to glucose-dependent modulation. (B) AKT status, as a readout of PI3K activity, was analysed by western blotting in WRO and FTC133 cells cultured for 24 h in the presence or absence of glucose and of the PI3K inhibitor 3MA (10 mM). AKT phosphorylation on Ser473 was basally active in FTC133 cells, and it was activated under glucose-depleted conditions in WRO cells. 3MA effectively decreased AKT phosphorylation. Densitometry of the bands corresponding to PTEN (A) and of pAKT and AKT (B), normalised vs β-tubulin, is included. Data shown in this figure have been reproduced three times independently.

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re-suspension of the labelled cells (Fig. 1B). In WRO cells cultured in a glucose-containing standard medium, the uptake of 2-NBDG was negligible, while it increased upon incubation in a glucose-free medium (Fig. 1). The simplest explanation of this phenomenon is that 2-NBDG clearly competes with the glucose present in the culture medium. However, we noted that, in FTC133 cells, the basal uptake of 2-NBDG was much higher than in WRO cells, despite the presence of glucose in the culture medium, and it increased further when incubated in a glucose-free medium (Fig. 1).

Glucose depletion differentially affects the membrane localisation of GLUT1 in WRO and FTC133 thyroid cancer cells

Hypoxia triggers a metabolic switch towards aerobic glycolysis through HIF1α-mediated expression of GLUT proteins and glycolytic enzymes (Semenza 2012, Szablewski 2013). The metabolic stress induced by glucose depletion shares a common signalling pathway with hypoxia. The level of HIF1α (HIF1A) expression was

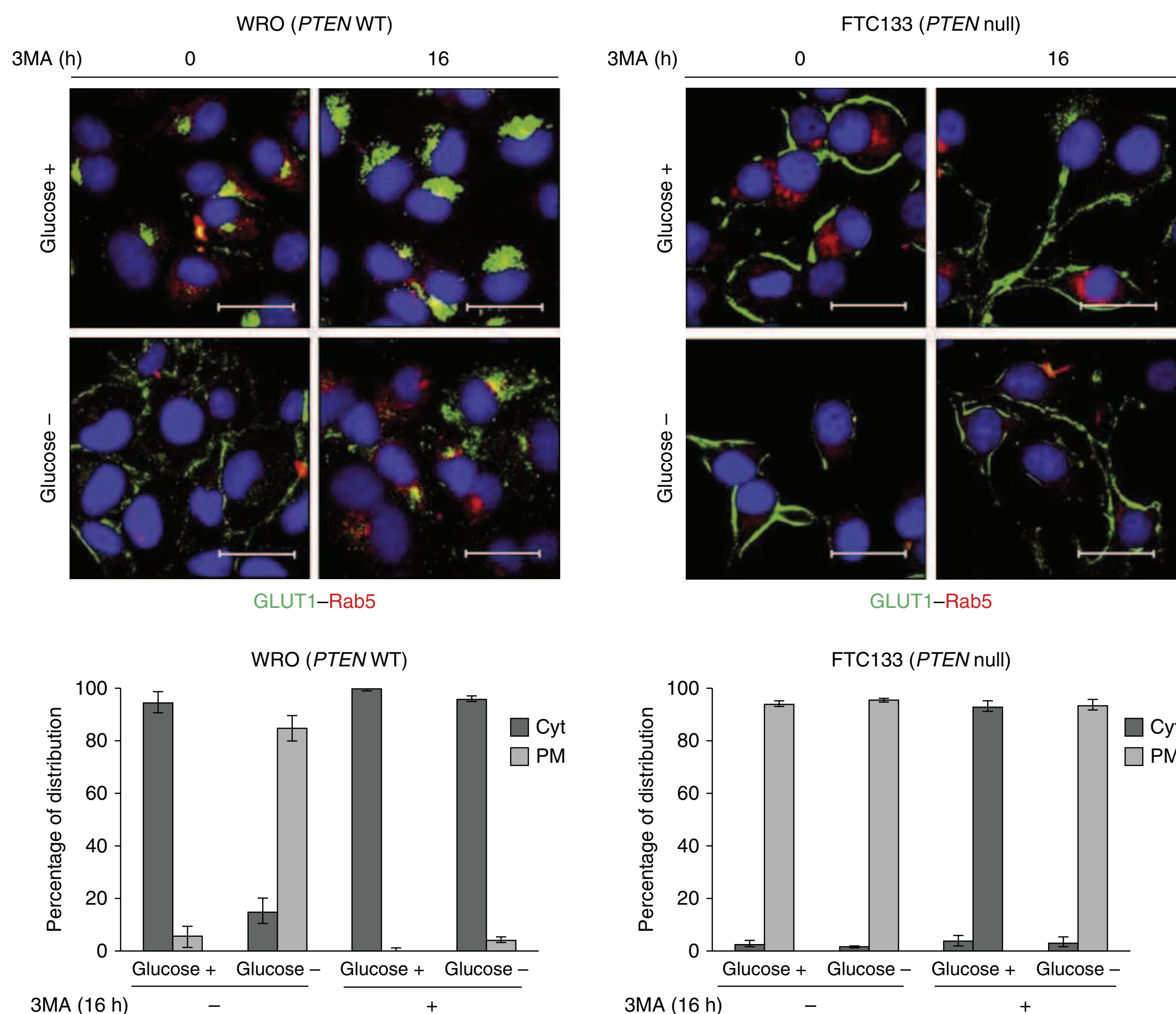

Figure 4

3-Methyladenine abrogates glucose starvation-induced plasma membrane (PM) translocation of GLUT1 in WRO cells. The cells adherent on coverslips were incubated in the presence or absence of 3MA for 16 in a glucose-containing or glucose-free medium. At the end, the coverslips were processed for immunofluorescence staining of GLUT1 (in green) and Rab5 (in red; a marker of endocytic vesicles). Nuclei were stained with DAPI. Glucose starvation induced the PM relocation of GLUT1 in WRO cells. This effect was abrogated by 3MA. In FTC133 cells, GLUT1 was permanently localised to the PM, regardless of the presence or absence of glucose. Quantification of the relative distribution in cytoplasm (Cyt) vs PM of GLUT1 is included. Representative images and distribution of fluorescence (in percentage) of three independent experiments are shown. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0118.

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higher in FTC133 cells than in WRO cells (Fig. 2A). Then, we looked at the expression of GLUT1, a GLUT that is abnormally over-expressed in highly aggressive and less differentiated thyroid cancers (Hosaka *et al.* 1992, Samih *et al.* 2000, Matsuzu *et al.* 2005). GLUT1 was expressed at a comparable level in WRO and FTC133 cells, and its level slightly increased upon incubation in a glucose-free medium (Fig. 2B). The above data suggested that membrane localisation rather than protein expression of GLUT1 could be the mechanistic cause for the differential uptake of glucose in the two cell lines. In the large majority (>80%) of WRO cells cultured in a glucose-containing medium, GLUT1 was essentially localised to the cytoplasm, and it promptly translocated onto the PM on switching the culture to a glucose-free medium (Fig. 2C). Under glucose-depleted conditions, GLUT1 persisted on the PM of WRO cells for up to 48 h (not shown). On switching to a glucose-depleted medium, the membrane-associated GLUT1 fluorescence in WRO cells increased by approximately fourfold. By contrast, in FTC133 cells, GLUT1 was found permanently residing on the PM (>95% of GLUT1 fluorescence), regardless of whether the cells were cultured in the presence or the absence of glucose (Fig. 2C).

In the following experiments, we investigated the signalling pathway(s) involved in the glucose uptake and the membrane translocation of GLUT1 in response to glucose deprivation in the two thyroid cancer cell lines.

PI3K drives the membrane translocation of GLUT1 in glucose-deprived WRO cells

In FRTL-5 rat thyroid cells, the PM translocation of GLUT1 was shown to depend on the PI3K/AKT pathway (Samih *et al.* 2000). It has been reported that the activation of this pathway leads to increased HIF1α levels (Zundel *et al.* 2000). Indeed, it is well documented that the PI3K–AKT pathway is abnormally activated in thyroid cancers (Saji & Ringel 2010). Given that this pathway is controlled by the lipid kinase activity of PTEN, the above data could be explained with the altered expression of this oncosuppressor in the two cell lines. In fact, WRO cells express WT *PTEN*, whereas FTC133 cells are subjected to monoallelic deletion and bear an R130STOP mutant allele for PTEN (Weng *et al.* 2001, Saiselet *et al.* 2012). The latter leads to a truncated PTEN isoform that is not detectable in western blotting (Fig. 3A). The expression level of PTEN in WRO cells did not change after 24 h of incubation in a glucose-free medium (Fig. 3A). To determine as to what extent the AKT pathway was driving the membrane expression of GLUT1 in WRO and FTC133 thyroid cancer cells, we pharmacologically inhibited this pathway with 3MA, an inhibitor of PI3K. The PI3K–AKT pathway, as mirrored by Ser473 phosphorylation of AKT, was basally active (regardless of the presence or absence of glucose in the medium) in FTC133 cells, consistent with the lack of PTEN, and it was activated in WRO cells upon cultivation under glucose-free condition (Fig. 3B). In WRO cells, Ser473 phosphorylation of AKT was completely abrogated in the presence of 10 mM 3MA (Fig. 3B). By contrast,

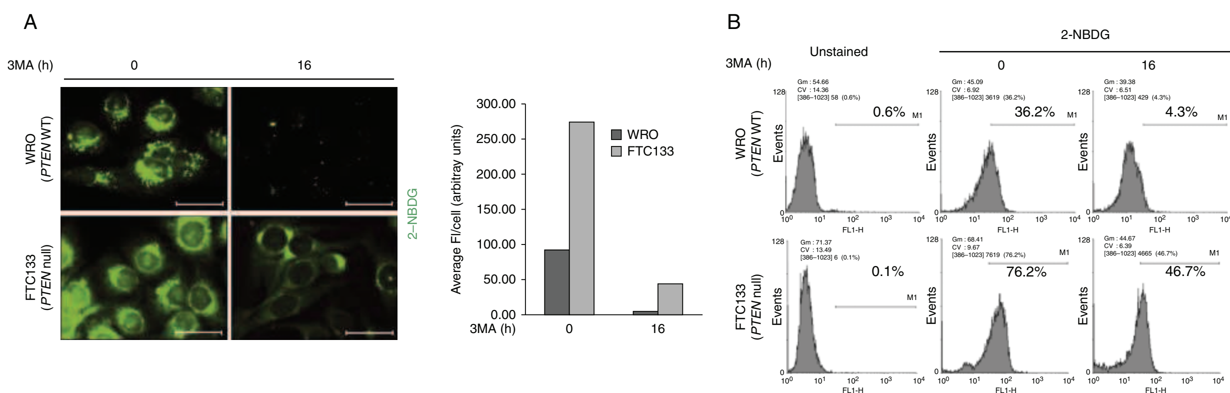

Figure 5

3MA differentially affects the uptake of glucose in WRO and FTC133 cells. (A) 2-NBDG uptake was monitored in both cell lines cultured for 16 h in a glucose-free medium in the absence or presence of 3MA. 3MA completely arrested the uptake of 2-NBDG in WRO cells under glucose-depleted conditions, while it had a milder effect in FTC133 cells. ImageJ quantification of 2-NBDG in the cells of representative fields is shown in the right panel. Scale bar = 20 μm. Magnification = 63×. (B) Cytofluorometry assessment of 2-NBDG uptake in the cells cultured as above. Representative cytofluorograms are shown. This experiment further confirmed the inhibitory effect of 3MA on the uptake of glucose. Data shown in this figure have been reproduced four times independently. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0118.

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residual phospho-Ser473–AKT was still detectable in FTC133 cells even in the presence of 3MA, especially in glucose-depleted conditions (Fig. 3B). To be noted, the cellular level of total AKT was reduced in glucose-deprived cultures, especially in FTC133 cells.

We then looked at the phenotypic consequences of the activation and inhibition of the AKT pathway. 3MA had no effect on the membrane expression of GLUT1 in FTC133 cells, whereas, in WRO cells incubated in a glucose-free medium, it strongly limited the membrane translocation of GLUT1, which accumulated in a para-Golgian area (Fig. 4). This observation was confirmed by image quantification of the expression of GLUT1 in the Cyt and PM compartments (Fig. 4, lower panels).

Inhibition of the PI3K–AKT pathway differentially affects the glucose uptake in WRO and FTC133 cells

Then, we assessed the effects of 3MA on the uptake efficiency of glucose in WRO and FTC133 cells. The cells were plated on coverslips and incubated with the 2-NBDG probe in the absence or presence of 3MA in a glucose-free medium. At the end, the cells were imaged under the microscope and cell-associated fluorescence was quantified using the ImageJ Software. 3MA completely abrogated the uptake of 2-NBDG in WRO cells, while it had a milder effect in FTC133 cells (Fig. 5A). Parallel cultures in Petri dishes were used for cytofluorometric quantification of fluorescence-positive cells. Cytofluorometry data indicated that a large

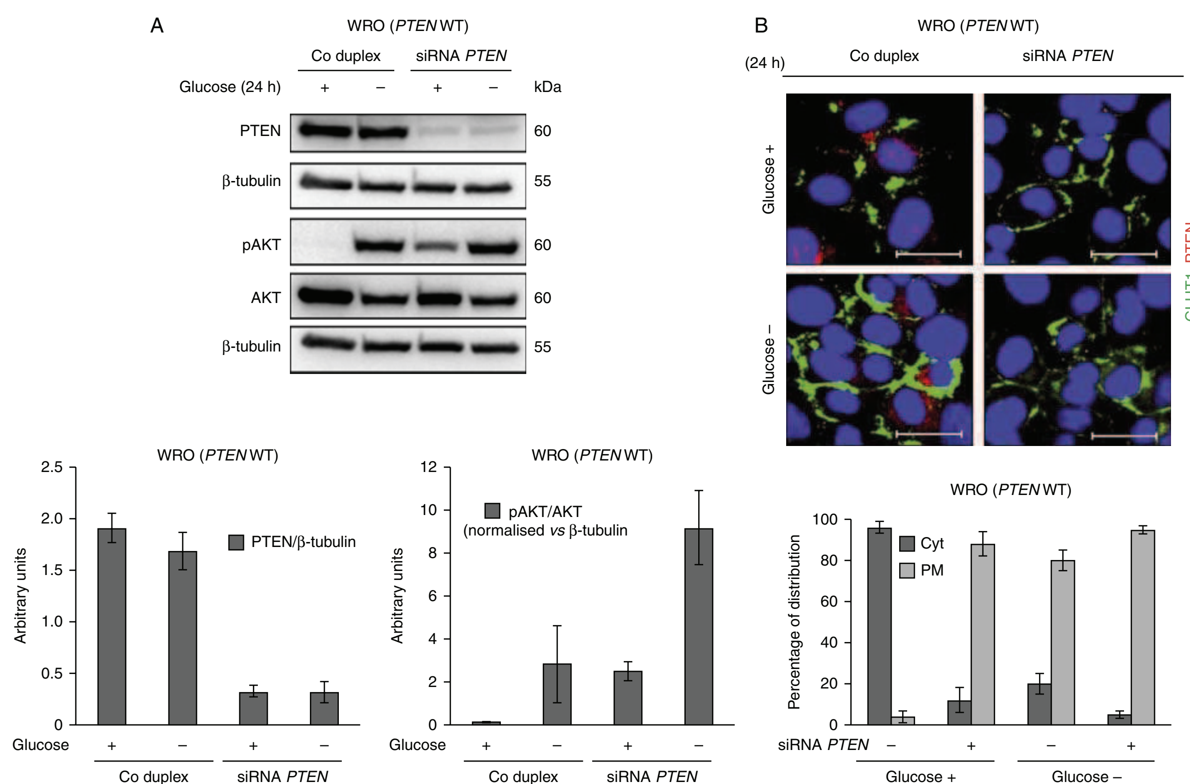

Figure 6

Silencing of *PTEN* in WRO cells determines the membrane translocation of GLUT1. (A) Knockdown of *PTEN* mRNA efficiently reduced (>80%) the expression of the PTEN protein in WRO cells (data obtained with one of the two siRNA oligonucleotides targeting two different exons of *PTEN* are shown). The activation status of the AKT pathway was assessed by western blotting after siRNA silencing of *PTEN* and 24 h of incubation in a glucose-containing or glucose-free medium. Basal phosphorylation of AKT increased upon silencing *PTEN* and further increased under glucose starvation. Densitometry quantification of the AKT activation status ($n=3$) is reported in the lower panels (pAKT/AKT was normalised vs β-tubulin). (B) Subcellular expression of GLUT1 (in green) and PTEN (in red) after siRNA silencing of *PTEN* and 24 h of incubation in a glucose-containing or glucose-free medium. Nuclei are stained with DAPI. Down-regulation of PTEN promoted the cytoplasmic (Cyt) to plasma membrane (PM) relocation of GLUT1. ImageJ quantification of this phenomenon is included. Note that >80% of the *PTEN* siRNA-transfected cells were negative for PTEN staining. Scale bar = 20 μm. Magnification = 63×. Representative images and distribution of fluorescence (in percentage) of three independent experiments are shown. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0118.

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proportion of FTC133 cells could take up glucose despite the presence of 3MA (Fig. 5B).

Genetic manipulations of the PTEN expression and trafficking of GLUT1

The above findings are consistent with the involvement of the PI3K/AKT pathway in the trafficking of GLUT1-positive vesicles in both WRO and FTC133 cancer cells.

To determine whether PTEN is involved in the trafficking of GLUT1 in thyroid cancer cells, we genetically manipulated the expression of PTEN in WRO and FTC133 cells. First, we post-transcriptionally silenced *PTEN* in WRO cells. The transfection with a PTEN-specific siRNA elicited (on average) a >80% down-regulation of the expression of PTEN protein (Fig. 6A). The genetic silencing of *PTEN* expression was reflected on the activation of the AKT pathway. To be noted, under glucose-free condition, AKT was phosphorylated regardless of the expression level of PTEN (Fig. 6A, densitometry in lower panels). Parallel cultures were set on coverslips for immunofluorescence staining of GLUT1 and PTEN. The latter was included to monitor the cells that had been effectively silenced for PTEN. As a result of *PTEN* silencing, in a large proportion of the cells, GLUT1 was found on the PM (Fig. 6B). As predictable, this effect was more evident in the culture incubated in a glucose-free medium (Fig. 6B). Quantification by ImageJ analysis confirmed the PM translocation of GLUT1 upon *PTEN* knockdown.

Then, we transgenically over-expressed *PTEN* in *PTEN*-deficient FTC133 cells (Fig. 7A). The ectopic

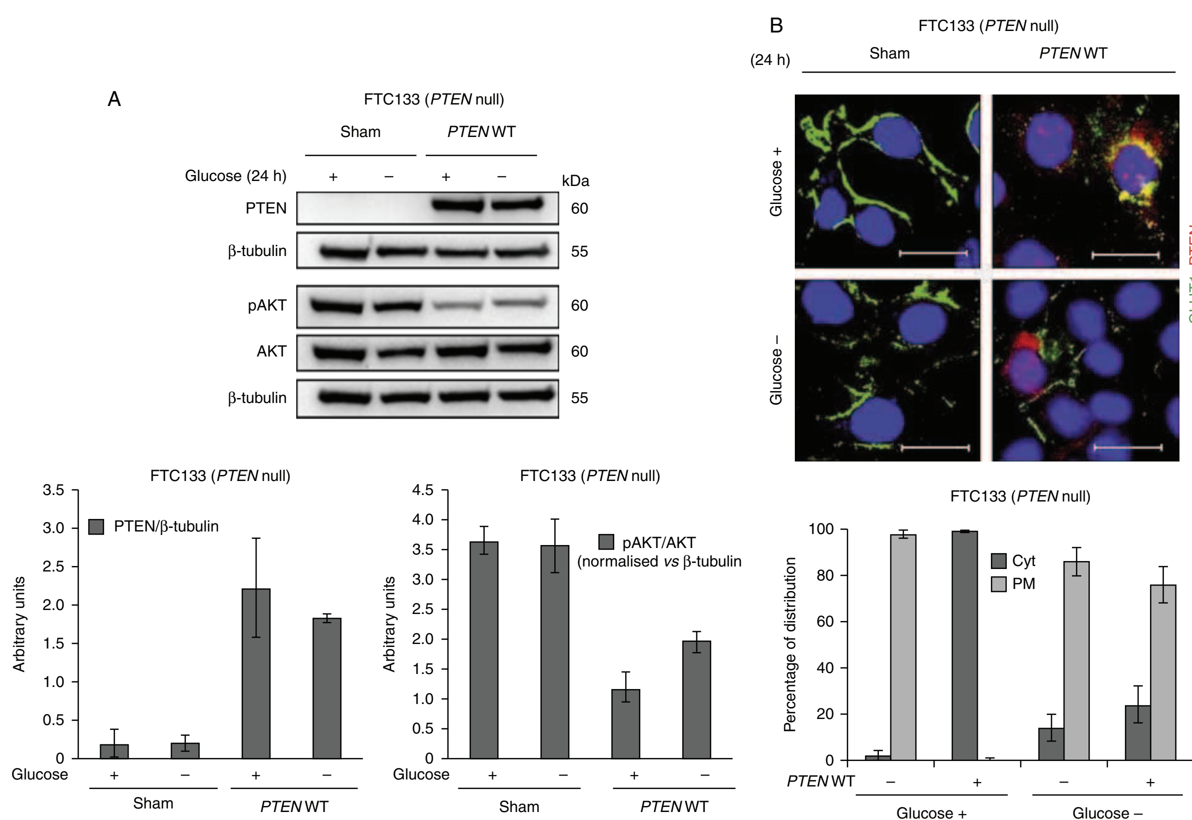

Figure 7

Transgenic expression of *PTEN* in FTC133 cells precludes the membrane translocation of GLUT1. (A) Transgenic expression of WT *PTEN* in FTC133 cells was controlled by western blotting assay. The blot was stripped and re-probed to assay the phosphorylation status of AKT. Data show that ectopic expression of PTEN greatly inhibited the phosphorylation of AKT independent of the presence or absence of glucose in the culture medium. Densitometric analysis ($n=3$) of the AKT activation status is included (pAKT/AKT was normalised vs β-tubulin). (B) Subcellular expression of GLUT1 (in green) and PTEN (in red) in transfected FTC133 cells after 24 h of incubation in a glucose-containing or glucose-free medium. Nuclei are stained with DAPI. Under glucose-containing culture condition, GLUT1 appears clustered in a perinuclear–para-Golgian area and, in glucose-free culture condition, only a small amount of GLUT1 appears to reach the plasma membrane (PM). In *PTEN*-transfected cultures, >60% of the cells were positive for PTEN immunofluorescence. Ectopic expression of PTEN largely precluded the PM relocation of GLUT1. ImageJ quantification of this phenomenon is included. Representative images and distribution of fluorescence (in percentage) of three independent experiments are shown. Cyt, cytoplasm. Scale bar = 20 μm. Magnification = 63×. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-14-0118.

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expression of PTEN was paralleled by the inactivation (~75%) of the AKT pathway (see densitometry in lower panels), probably reflecting the de-phosphorylation of PIP3 in PIP2 operated by PTEN. To be noted, in *PTEN*-transfected cells, AKT remained largely inactive even when cultured in a glucose-free medium (Fig. 7A). In a parallel experiment, the cells adherent on coverslip were stained for GLUT1 and PTEN. In the transfected cells (positive for PTEN), GLUT1 was mainly clustered in a perinuclear–para-Golgian region, though this effect was somehow mitigated when the cells were cultured in a glucose-free medium (Fig. 7B). Based on ImageJ quantification, ectopic expression of PTEN almost completely abrogated the PM-associated GLUT1 fluorescence translocation of GLUT1 in transfected FTC133 cells cultured in the presence of glucose (Fig. 7B).

To determine the functional consequences of the PTEN-dependent relocation of GLUT1 on glucose uptake, we measured the uptake of 2-NBDG in the transfected cell populations. The proportion of cells positive for 2-NBDG almost doubled in *PTEN*-silenced WRO cells compared with control duplex-transfected cells and almost halved in transfected cells expressing transgenic *PTEN* compared with the control Sham-transfected counterparts (Fig. 8).

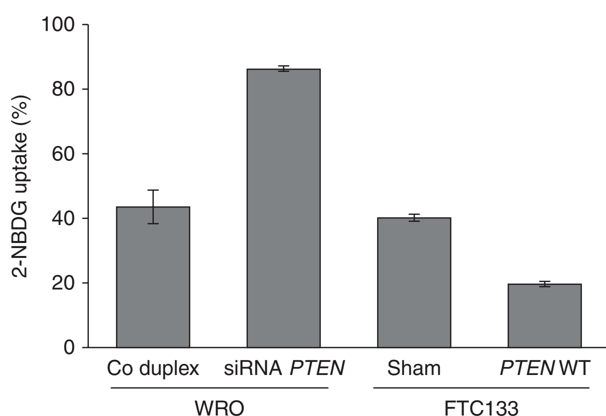

Figure 8
PTEN drives the glucose uptake in thyroid cancer cells. WRO and FTC133 cells adherent on Petri dishes were transfected with a PTEN-specific siRNA or with a plasmid harbouring the *PTEN* cDNA respectively. Sham-transfection was included as a control. The cells were incubated for the optimal time to attain the highest efficiency of endogenous *PTEN* knockdown or of ectopic PTEN expression in WRO and in FTC133 cells respectively. The cells were then loaded with the fluorescent 2-NBDG probe and analysed by cytofluorometry. The histogram shows the quantification of uptake (average ± S.D.) of four experiments. Note that control duplex-transfected WRO cells show an abnormally high uptake of 2-NBDG, probably due to the stress associated with Lipofectamine transfection. The data clearly indicate that PTEN is a master regulator of glucose uptake in WRO and in FTC133 cells.

Discussion

To sustain their high proliferative rate, cancer cells adapt their metabolism towards the aerobic glycolysis (Tong *et al*. 2009). Thyroid cancer cells also show a high rate of glycolysis (Andrade *et al*. 2012). Immunohistochemical studies have shown that enhanced glucose uptake in cancer cells correlates with over-expression of GLUTs, in most cases GLUT1 (Haber *et al*. 1997, Ciampi *et al*. 2008). Herein, we show that the oncosuppressor PTEN plays a dominant role in the membrane expression of GLUT1 and glucose uptake in thyroid cancer cells. It has been shown that the ectopic hyper-expression of PTEN contrasts the uptake and the large glycolytic consumption of glucose observed in proliferating cancer cells (Garcia-Cao *et al*. 2012). Consistently, we found that the transgenic reintroduction of *PTEN* in FTC133 abrogates the membrane expression of GLUT1. This effect was evident in glucose-fed cells, while, in the glucose-deprived cells, the absence of glucose triggered anyhow the membrane translocation of GLUT1 (Fig. 7B). Thus, the absence of glucose is 'dominant' in driving GLUT1 membrane translocation, regardless of the presence of PTEN as in fact occurs in WRO cells as well (Figs 2C, 4 and 6B). Glucose depletion is known to activate the AMPK pathway, which is also involved in the membrane expression of GLUT1 (Andrade *et al*. 2012).

Conversely, we show that the lack of PTEN expression has a dominant effect on the membrane expression of GLUT1 independent of the level of glucose available. Notably, in PTEN-expressing cancer cells, the scarce availability of glucose favoured the expression of GLUT1 on the PM. The PTEN-dependent relocation of GLUT1 had functional consequences in terms of glucose uptake, as the silencing of *PTEN* in WRO cells greatly increased, and conversely the ectopic expression of PTEN in FTC133 cells greatly reduced, the uptake rate of 2-NBDG. These facts have relevance in terms of imaging diagnosis. Imaging by FDG–PET has the potential to reveal unsuspected tumours, including thyroid tumours, commonly indicated as 'incidentaloma' because of their accidental discovery (Bogsrud *et al*. 2010, Hsiao *et al*. 2011, Pagano *et al*. 2011, Bertagna *et al*. 2012). Novel non-radioactive methods for imaging the glucose internalisation by cancer cells are being developed (Walker-Samuel *et al*. 2013), underscoring the importance of glucose uptake as a diagnostic readout of cancer proliferation.

The uptake of glucose in thyroid cancer cells has been shown to be inversely correlated with the uptake of iodide (the so-called 'flip–flop' phenomenon), a feature

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of highly aggressive thyroid tumours that are associated with increased lethality (Filetti *et al*. 1986, Frilling *et al*. 2001, Bläser *et al*. 2006, Grabellus *et al*. 2012). In addition, a link between the expressions of GLUT molecules and the pGp responsible for chemoresistance has been shown (Seo *et al*. 2009). Therefore, understanding the signalling pathways that govern the membrane expression of GLUTs and the glucose uptake in thyroid cancer cells also has prognostic and therapeutic implications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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8. Turmeric Toxicity in A431 Epidermoid Cancer Cell Associates with Autophagy Degradation of Anti-apoptotic and Anti-autophagic p53 Mutant

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Turmeric is a commonly used dietary spice in the Asiatic cuisine. It is derived from the rhizome of the plant Curcuma longa, and it has been used since centuries in the Asiatic traditional medicine as a natural remedy for skin diseases, among others.
The cutaneous Squamous Cell Carcinoma (SCC) is the second most common form of non-melanoma skin cancer, and its incidence is increasing in the last decades. Topical application or oral administration of curcumin has been shown to prevent chemically induced skin carcinogenesis. The power of curcumin anticancer activity relies on the fact that it targets several cancer-related pathways. In this work we show that similarly to its bioactive component Curcumin, Turmeric could induce both apoptosis and autophagy in A431 cells, and these effects were concomitant with degradation of p53. In this work, we studied the cytotoxic properties of turmeric in keratinocyte-derived SCC A431 cells. These cells express the R273H mutant p53 that has been shown to confer chemoresistance to conventional chemotherapeutic pro-apoptotic drugs to confer invasiveness and metastatic properties and to exert inhibitory effect on BECLIN 1-dependent autophagy. Turmeric and curcumin also stimulated the activity of mTOR, which notoriously promotes cell growth and acts negatively on basal autophagy. Rapamycin-mediated inhibition of mTOR synergized with turmeric and curcumin in causing p53 degradation, increased the production of autophagosomes and exacerbated cell toxicity leading to cell necrosis. Small-interference mediated silencing of the autophagy proteins BECLIN 1 or ATG7 abrogated the induction of autophagy and largely rescued p53 stability in Turmeric-treated or Curcumin-treated cells, indicating that macroautophagy was mainly responsible for mutant p53 degradation. These data uncover a novel mechanism of turmeric and curcumin toxicity in chemoresistant cancer cells bearing mutant p53.

Personal Contribution

In this paper, I contributed to the siRNA BECLIN 1 transfection experiments, data analysis and interpretation.

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Turmeric Toxicity in A431 Epidermoid Cancer Cells Associates with Autophagy Degradation of Anti-apoptotic and Anti-autophagic p53 Mutant

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The keratinocyte-derived A431 Squamous Cell Carcinoma cells express the p53R273H mutant, which has been reported to inhibit apoptosis and autophagy. Here, we show that the crude extract of turmeric (*Curcuma longa*), similarly to its bioactive component Curcumin, could induce both apoptosis and autophagy in A431 cells, and these effects were concomitant with degradation of p53. Turmeric and curcumin also stimulated the activity of mTOR, which notoriously promotes cell growth and acts negatively on basal autophagy. Rapamycin-mediated inhibition of mTOR synergized with turmeric and curcumin in causing p53 degradation, increased the production of autophagosomes and exacerbated cell toxicity leading to cell necrosis. Small-interference mediated silencing of the autophagy proteins BECLIN 1 or ATG7 abrogated the induction of autophagy and largely rescued p53 stability in Turmeric-treated or Curcumin-treated cells, indicating that macroautophagy was mainly responsible for mutant p53 degradation. These data uncover a novel mechanism of turmeric and curcumin toxicity in chemoresistant cancer cells bearing mutant p53. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: skin cancer; phytochemicals; autophagy; apoptosis; rapamycin; p53R273H.

INTRODUCTION

The cutaneous Squamous Cell Carcinoma (SCC) is the second most common form of non-melanoma skin cancer, and its incidence is increasing in the last decades (Lauth and Unden, 2004; Hussain *et al*., 2010; Ratushny *et al*., 2012). The most relevant cause of cutaneous SCC is the exposure to UVB (Lauth and Unden, 2004). Polyphenol-containing herb extracts can provide protection against skin cancers (Chun *et al*., 2002; Surh and Chun, 2007). For instance, the natural dietary spice curcumin has been shown to prevent skin diseases including cancer (Chun *et al*., 2003; Dujic *et al*., 2007; Thangapazham *et al*., 2007). Topical application or oral administration of curcumin has been shown to prevent chemically induced skin carcinogenesis (Conney *et al*., 1991; Azuine and Bhide, 1992; Huang *et al*., 1992; Limtrakul *et al*., 1997). The power of curcumin anticancer activity relies on the fact that it targets several cancer-related pathways (Goel *et al*., 2008; Teiten *et al*., 2013), which are cell type context specific.

Turmeric is a commonly used dietary spice in the Asiatic cuisine. It is derived from the rhizome of the plant *Curcuma longa*, and it has been used since centuries in the Asiatic traditional medicine as a natural remedy for skin diseases, among others (Balunas and Kinghorn, 2005; Gurib-Fakim, 2006). We have recently shown that turmeric (dichloromethane) extract protects human keratinocytes against UVB-induced toxicity and DNA mutagenicity (Thongrakard *et al*., 2014). The yellow pigment curcumin is one of Turmeric's main bioactive component (Gupta *et al*., 2013). Curcumin-free turmeric aqueous extract was also shown to prevent chemically induced carcinogenesis (Deshpande *et al*., 1997; Deshpande *et al*., 1998), suggesting that components other than curcumin present in turmeric may synergistically exert anticancer activity. Notably, besides curcumin, other compounds found in turmeric (e.g. Furanodiene and Xanthorrhizol) have been shown to possess anticancer activity (Guo *et al*., 2013; Kim *et al*., 2013; Xu *et al*., 2014).

In this work, we studied the cytotoxic properties of turmeric in keratinocyte-derived SCC A431 cells. These cells express the R273H mutant p53 (Kwok *et al*., 1994) that has been shown to confer chemoresistance to conventional chemotherapeutic pro-apoptotic drugs (Wong *et al*., 2007; Schilling *et al*., 2010), to confer invasiveness and metastatic properties (Muller *et al*., 2009) and to exert inhibitory effect on BECLIN 1-dependent autophagy (Morselli *et al*., 2008a). Autophagy, a lysosomal driven degradation pathway, plays two opposite roles in carcinogenesis eliciting either pro-survival or pro-death effects depending on the cellular context and the

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extracellular stimuli (Lorin *et al.*, 2013). Turmeric and pure curcumin induced both apoptosis and autophagy in A431 cells, and the concomitant inhibition of mTOR further increased autophagy and led to cell necrosis. These cytotoxic effects were associated with extensive degradation of the mutant p53, which was mediated by macroautophagy as it could largely be prevented by knocking down the expression of the autophagy genes *BECLIN 1* or *ATG7*.

In conclusion, the data here reported: (i) Demonstrate that turmeric extract may exert the same cytotoxic effects as the pure substance curcumin; (ii) Uncover a novel mechanism through which turmeric and curcumin can circumvent the resistance of cancer cells expressing the anti-apoptotic p53R273H mutant; and (iii) Support the utilization of turmeric and of mTOR inhibitors as ingredients of topical or oral medicaments to prevent or cure skin cancers.

MATERIALS AND METHODS

Chemicals and reagents. Analytical grade reagents were used. Dulbecco's Modified Eagle's Medium, fetal bovine serum, L-glutamine solution, penicillin-streptomycin solution, propidium iodide, rapamycin and chloroquine were all purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Immune-blot polyvinylidene fluoride membrane and nitrocellulose membrane were purchased from Biorad (Hercules, CA, USA). Trypan blue solution and lipofectamine® 2000 transfection reagent were purchased from Invitrogen (Carlsbad, USA). Z-VAD-fmk was purchased from Alexis Biochemicals (San Diego, CA, USA). The western lightning™ Chemiluminescence Reagent Plus was purchased from PerkinElmer (Waltham, MA USA). The dichloromethane extract of turmeric was prepared and characterized as previously reported (Thongrakard *et al.*, 2014). Pure (>95%) curcumin was prepared as previously reported (Changtam *et al.*, 2010).

Antibodies. The mouse monoclonal anti-LC3B (L7543), anti-β-tubulin (T5293) and anti-actin (A5316) antibodies were from Sigma-Aldrich Co. The monoclonal rabbit anti-S6 ribosomal protein (2217), monoclonal rabbit anti-phospho-S6 ribosomal protein (Ser235/236) (4856), the polyclonal rabbit anti-BAX (2772) and polyclonal rabbit anti-Ser15phospho-p53 (9284) antibodies were from Cell Signalling Technology (MA, USA). The rabbit polyclonal anti-BECLIN 1 (sc-11427) and the monoclonal mouse anti-p53 (sc-126) antibodies were from Santa Cruz Biotechnology (Texas, USA). The rabbit monoclonal anti-ATG7 (04-1055) antibody was from Millipore Corporation, USA. The mouse monoclonal anti-LAMP1 (555801) antibody was from BD Pharmingen™ (California, USA). IRIS-2 (green fluorescence)–conjugated goat anti-rabbit IgG (2WS-08) and IRIS-3 (red fluorescence)–conjugated goat anti-mouse IgG (3WS-07) antibodies were from Cyanine Technology SpA (Turin, Italy). Goat Anti-Rabbit IgG-HRP Conjugate (170-6515) and Goat Anti-Mouse IgG-HRP Conjugate (170-6516) were from Biorad, Hercules (CA, USA).

Cell culture and drug treatments. A431 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere at 5% CO_2. The cells were seeded and adhered on Petri dishes for 24 h before starting the treatment. A431 cells were treated with turmeric (10 and 20 μg/mL) or curcumin (20 and 30 μM) for 24 h. Where indicated, the cells were pre-treated for 1 h with Z-VAD-fmk (30 μM), with Chloroquine (ClQ, 50 μM) or with rapamycin (Rap, 10 μM).

Viable cell counting. The cells were counted with a haemocytometer. To discriminate viable cells, the Trypan blue dye exclusion test was used.

Flow cytometry analysis. A431 cells were trypsinized and treated for Propidium Iodide staining as previously described (Trincheri *et al.*, 2008). The samples were analysed by flow cytometry (Becton Dickinson, USA). The data were interpreted using the winMDI software. (http://facs.scripps.edu/software.htm)

Immunofluorescence. The cells were plated on sterile cover slip and were adhered and grown at least 24 h prior to start the incubation with the substances. After the indicated time of incubation, the cells were processed for immunofluorescence staining with primary antibodies against LC3, as a marker of autophagosome, and against LAMP1, as a marker of lysosomes, or against BAX, as a marker of apoptosis, following standard procedures (Trincheri *et al.*, 2008; Castino *et al.*, 2010). Images were captured using a Leica DMI600 fluorescence microscope (Leica Microsystems AG, Wetzlad, Germany) equipped with an imaging acquisition system.

Western blotting analysis of protein expression. Cell homogenization and western blotting were performed following our published procedures (Cagnin *et al.*, 2012). The filters were subsequently probed and (after stripping) re-probed with antibodies as indicated. Finally, the filters were probed with anti-β tubulin or anti-actin antibodies to assess homogenate protein loading. The signal was detected by peroxidase-conjugated secondary antibody and subsequent peroxidase-induced chemiluminescence reaction. Intensity of the bands was estimated by densitometry (Quantity one software, Biorad; ImageJ software).

Small interfering RNA transfection. The sequence and validation of the oligonucleotides used for the silencing of BECLIN 1 mRNA [Small interfering RNA (siRNA)-BECLIN] and for sham-transfection as control have been published previously (Trincheri *et al.*, 2008). In brief, adherent A431 cells at about 40–50% confluence were transfected with 100 μM siRNA specific for BECLIN1 (5′GGA ACU CAC AGC UCC AUU ACU UAC CAC 3′) or for ATG7 (5′GGG UUA UUA CUA CAA UGG UGT T 3′) or with control duplex (5′AGG UAG UGU AAU CGC CUU GTT 3′) using Lipofectamine 2000 transfection reagent for 6 h in antibiotic-free and serum-free Opti-MEM® reduced-serum medium. After transfection, the cells were cultured in complete medium for 48 h (changing the medium every 24 h) before use.

Propidium iodide staining of unfixed cells. The cells, grown on cover slip and treated as indicated, were stained unfixed with 0.3 μg/mL Propidium Iodide for

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5 min in the dark at 37 °C (Castino *et al.*, 2010). Fluorescence was imaged with a Leica DMI600 fluorescence microscope (Leica Microsystems AG, Wetzlad, Germany).

Statistical analysis. All experiments were performed independently at least three times in triplicate. The siRNA experiment was performed two times in duplicate with reproducible results. Data on cell growth are expressed as the mean ± standard error of the mean. Densitometric data are expressed as the average ± SD of three independent experiments, unless otherwise stated. Statistical significance was determined using the Student's *t*-test (statistical level of significance was set at $p < 0.05$).

RESULTS

Turmeric induces apoptosis and autophagy in A431 cells

Apoptosis in A431 cells was evaluated by cytofluorometric analysis of the SubG1 peak (which measures the proportion of apoptotic bodies containing fragmented chromatin) in the cultures exposed to turmeric or curcumin in the absence or presence of the pan-caspase inhibitor z-VAD-fmk. After a 24-h exposure to turmeric or curcumin, approximately 30% of the cell population contained a sub-diploid amount of DNA, and the co-treatment with zVAD-fmk, which inhibits the caspase-dependent apoptosis pathway, nearly completely abrogated the appearance of this sub-population of cells (Fig. 1a). By cell counting, zVAD-fmk rescued, albeit not completely, cell loss from the monolayers of the cultures incubated with turmeric or curcumin (Fig. 1b). A431 cells harbour the p53R273H mutant (Kwok *et al.*, 1994), which has been associated with resistance to caspase-dependent drug toxicity (Wong *et al.*, 2007). We therefore investigated mechanisms other than apoptosis possibly involved in the cytotoxic effect of turmeric.

The autophagy-lysosomal degradation pathway plays a fundamental role in cell survival and cell death (Mariño *et al.*, 2014). To determine the possible induction of autophagy in the cells exposed to turmeric or curcumin, we assayed by western blotting the presence of the lipidated isoform II of LC3, a protein derived from the microtubule-associated protein MAP-LC3, that associates with autophagosomal membranes (Klionsky *et al.*, 2012). A parallel set of cultures were co-treated with Chloroquine, a lysosomotropic weak base that impairs the lysosomal degradation of autophagosomes. The comparison of the cellular level of LC3-II in the absence and in the presence of Chloroquine allows to measure the autophagy flux, that is, the ratio of autophagosome formation and consumption under a given treatment (Klionsky *et al.*, 2012). Turmeric and curcumin *per se* increased the actual level of LC3-II in the cells (Fig. 1c). As expected, in the presence of Chloroquine LC3-II accumulated in the cells, and it further increased both in turmeric-treated and curcumin-treated cultures, the latter being the strongest inducer. By densitometry (Fig. 1c, lower panel), it was found that turmeric and curcumin increased by twofold and threefold, respectively, the actual level of LC3 II, and in the presence of Chloroquine the increase was of about 10% and 20%, respectively, compared with control culture. These data indicate that turmeric and curcumin induce the formation of autophagosome and also stimulate the autophagy flux (i.e. the consumption of autophagosomes). The cellular level of LC3-II isoform *bona fide* reflects the presence of autophagosomes and autophagolysosomes. Ongoing autophagy was further confirmed by immunofluorescence detection of vacuoles positive for LC3. In the presence of Chloroquine, the autophagosome produced cannot be degraded because of the inefficient fusion with lysosomes and the pH-dependent inhibition of the lysosomal hydrolytic enzymes (Klionsky *et al.*, 2012). Consistent with data in Fig. 1c, in the presence of Chloroquine, the proportion of cells positive for LC3 and the intensity (number and dimension of LC3-positive vacuoles) of the staining greatly increased in the culture exposed to turmeric and curcumin, reflecting the accumulation of the autophagic vacuoles produced during the 24 h of incubation (Fig. 1d). A parallel, though of much less extent, increase in LC3 staining was also observed in control cultures, reflecting the level of ongoing basal autophagy (Fig. 1d).

Turmeric and curcumin induce mTOR-independent autophagy

The mTORC1 complex exerts a tonic inhibition of basal autophagy (Chang *et al.*, 2009), while it positively regulates protein synthesis (Hara *et al.*, 1998). Upon treatment with the mTOR inhibitor rapamycin (10 μM for 16 h), the downstream ribosomal protein S6 was inactivated, consistent with a general block of the protein synthesis pathway (Fig. 2a). Yet, under this condition, the co-treatment with either turmeric or curcumin maintained the mTOR-p70S6k pathway active to some extent (Fig. 2a). By densitometry, the actual level of total p70S6 was increased by approximately twofolds in turmeric-treated or curcumin-treated cells compared with controls. When normalized to β-Tubulin, the total amount of phospho-S6 in the cells treated with turmeric or curcumin remained relatively high (approximately 25%) despite the concomitant exposure to rapamycin. Noteworthy, turmeric and curcumin could induce autophagy despite the mTOR-p70S6k pathway was still active, as indicated by the increased production of LC3-II in concomitance with S6 phosphorylation (Fig. 2a and b). Rapamycin raised up the level of basal autophagy, as expected, and further increased the production of LC3-II in turmeric-treated and curcumin-treated cells (Fig. 2b). The rapamycin induction of autophagy was definitively confirmed by the increased immunofluorescence staining for LC3 (as a marker of autophagosome) and for LAMP1 (as a marker of lysosome), as shown in Fig. 2c. Taken together, these data indicate that turmeric and curcumin could induce autophagy through an mTOR-independent pathway, likely involving Adenosine MonoPhosphate Kinase (AMPK).

Inhibition of mTOR exacerbates turmeric and curcumin cytotoxicity

The aforementioned data demonstrated that turmeric and curcumin tend to stimulate the S6-mediated anabolic pathway and in parallel induce mTOR-independent

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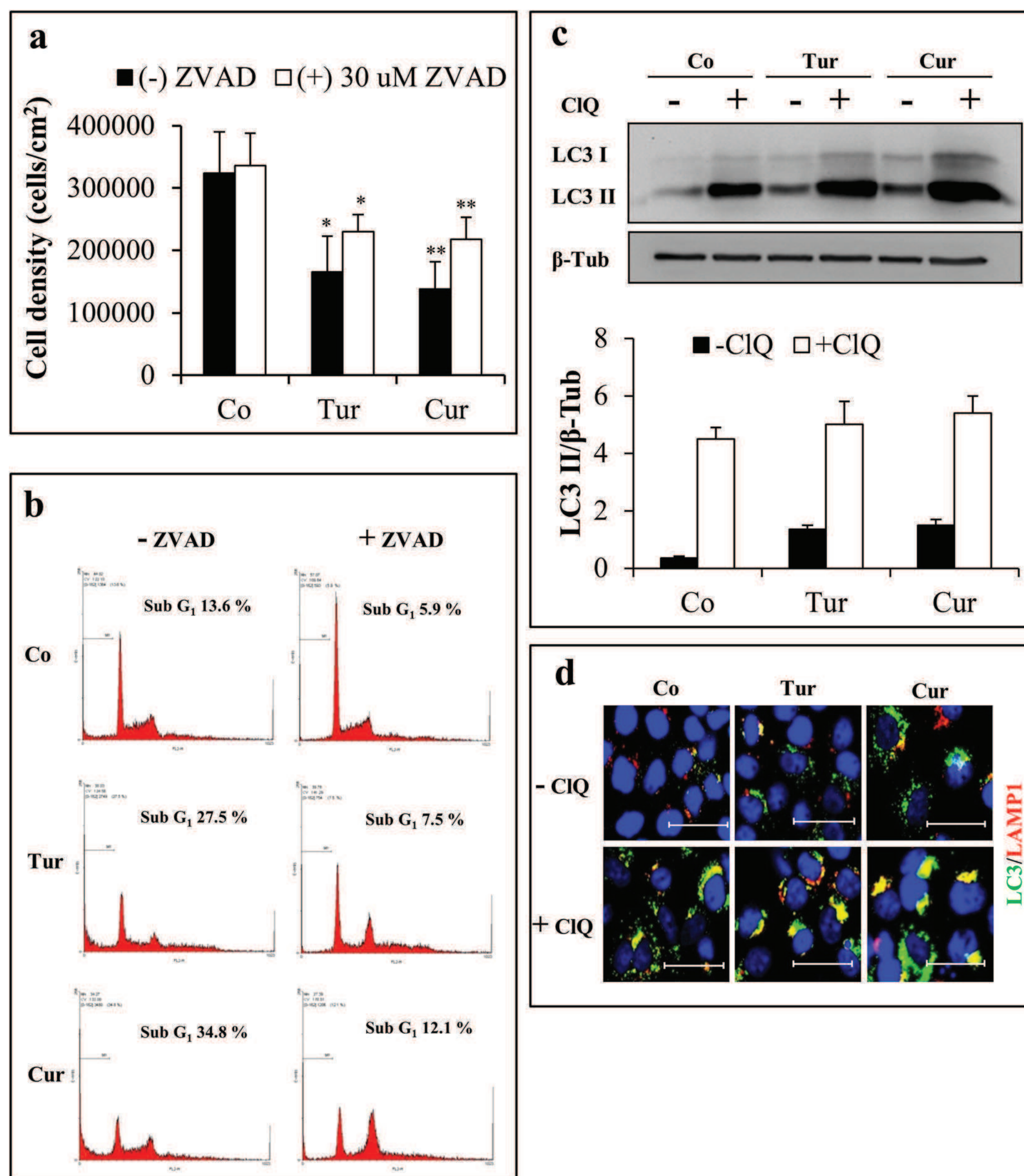

Figure 1. Turmeric induces apoptotic cell death and autophagy in A431 cells. A431 cells were treated with turmeric (20 μg/mL) or curcumin (30 μM) for 24 h. (a) The cells were pre-incubated 1 h with Z-VAD-fmk prior to the treatment with turmeric or curcumin, and thereafter, viable adherent cells were counted. Statistical significance: $*p<0.05$; $**p<0.01$; $***p<0.001$, significant difference between comparison groups (b) flow cytometry analysis showing the subG1 peak as an indirect measure of apoptosis. (c) A431 cells were treated with turmeric or curcumin and co-treated or not with chloroquine (ClQ, 50 μM). Upper panel: western blotting analysis shows LC3-I and LC3-II (the latter isoform is associated with autophagosomal membranes); lower panel: densitometric analysis of specific bands (average ±SD of three independent experiments). (d) Immunofluorescence showing the presence of autophagy as indicated by the staining of LC3, a marker of autophagosomes, and LAMP1, a marker of lysosomes. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

autophagy, which further increases in the presence of the mTOR inhibitor rapamycin. While performing these experiments, we noticed a time-dependent exacerbation of cell toxicity in the latter condition. Indeed, while the inhibition of mTOR was *per se* not toxic, it revealed to be extremely toxic when the cells were concomitantly exposed to turmeric or curcumin (Figs. 3a). Curcumin and (to a lesser extent) turmeric induced necrosis, which markedly increased in the presence of rapamycin, as shown by the trypan blue staining data (Fig. 3b), and the Propidium Iodide staining of unfixed cells (Fig. 3c). Bax-mediated apoptosis also increased in Turmeric-treated and Curcumin-treated cells following the concomitant inhibition of mTOR (Fig. 3d). Co-staining of LC3 confirmed the hyper-induction of autophagy in this condition (Fig. 3d). These synergistic toxic effects were somehow unexpected, given that A431 cells express a mutant p53 known to inhibit both caspase-dependent apoptosis (Wong *et al.*, 2007; Schilling *et al.*, 2010) and autophagy (Morselli *et al.*, 2008a). Phosphorylation at the Ser15 position is essential for the activation and stabilization of wild-type p53. We therefore looked at the status of the mutant p53 in the aforementioned conditions. Of note, while the levels of p53 expression and Ser15-phosphorylation were not affected by rapamycin, they were greatly reduced in the cells exposed to turmeric or curcumin and even more in those co-treated with rapamycin (Fig. 3e). By densitometry (Fig. 3e, lower panel), it was found that in turmeric-treated or curcumin-treated cultures the actual cellular amount of p53 dropped to approximately 50% and 30%, respectively, of the value in control untreated cells, and a large proportion of it was still phosphorylated.

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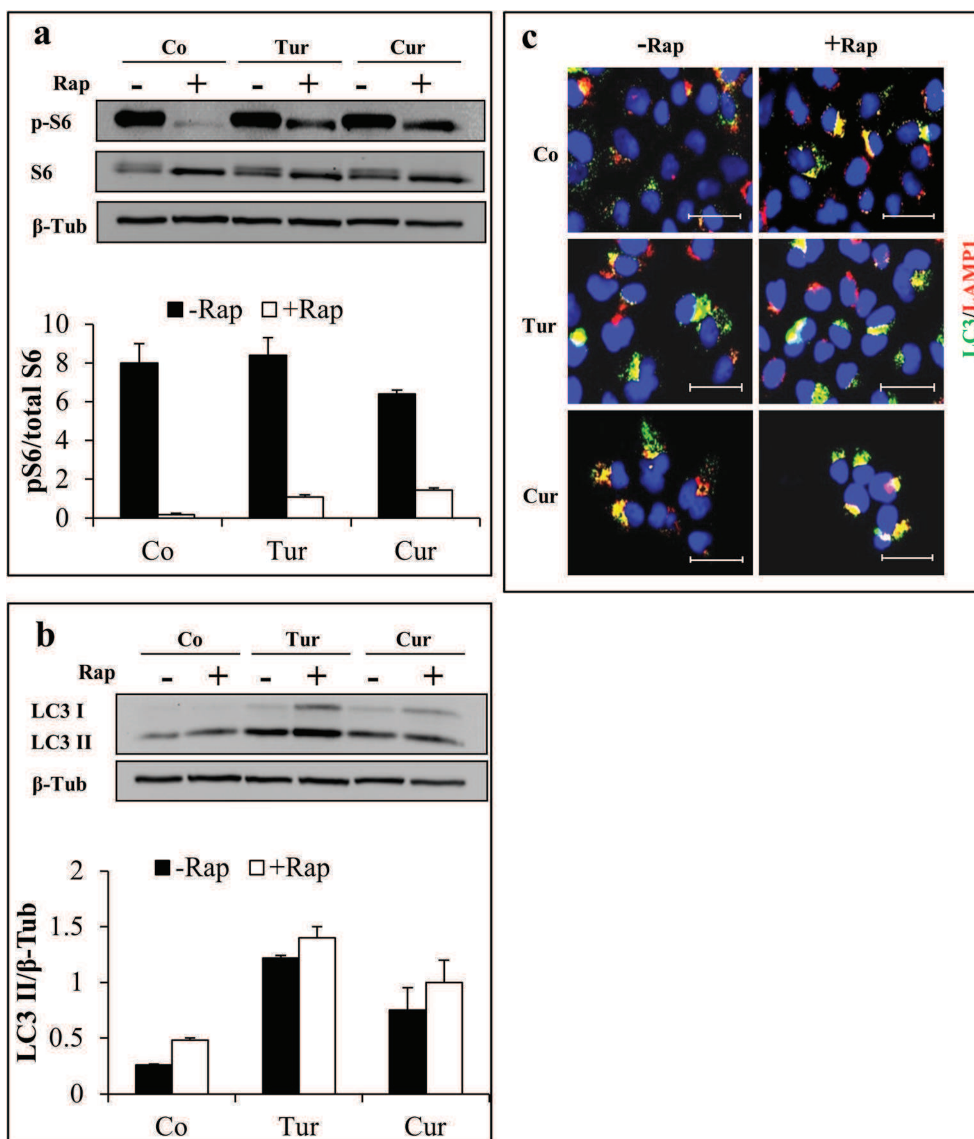

Figure 2. Turmeric and curcumin induce mTOR-independent autophagy. A431 cells were pre-treated for 1 h with 10 μM rapamycin (Rap) to inhibit mTOR and then exposed for 16 h to turmeric (20 μg/mL) or curcumin (30 μM). (a) Upper panel: western blotting showing the inhibitory effect of rapamycin and the stimulating effect of turmeric and curcumin on mTOR activity. Lower panel: densitometric analysis of specific bands (average ±SD of three independent experiments). The phosphorylation status of the ribosomal S6 protein was assumed as a read out of the activity of mTOR. (b) Upper panel: western blotting analysis of the expression of LC3-II as a read out of ongoing autophagy in the cells treated as indicated; lower panel: densitometric analysis of specific bands (average ±SD of three independent experiments). (c) Immunofluorescence staining of autophagosomes and autophagolysosomes as detected by anti-LC3 and anti-LAMP1 antibodies. It is evident the hyper-induction of autophagy in the cells co-treated with Rapmycin. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Knockdown of either the autophagy-related proteins BECLIN 1 or ATG7 prevents mutant p53 degradation induced by turmeric and curcumin

The aforementioned data suggested a possible mechanistic link between the degradation of mutant p53 and the sensitization to the toxic effects of turmeric and curcumin. It is known that degradation of wild-type p53 occurs through the MDM2-proteasome pathway (Kubbutat *et al.*, 1997) and is triggered during induction of autophagy (Tasdemir *et al.*, 2008). We suspected that autophagy could be functional to apoptosis sensitization by degrading the mutant p53. To test this hypothesis, two autophagy-related genes, namely *BECLIN 1* and *ATG7*, were transiently silenced with specific siRNAs. The endogenous level of BECLIN 1 and of ATG7 proteins in specific siRNA-transfected cells dropped down to approximately 20% of that in control sham-transfected cells (Fig. 4a). Under this condition, the induction of autophagy by turmeric or curcumin was nearly completed abrogated, as the level of LC3 II isoform, assumed as a marker of autophagosome production and accumulation in the cell, was comparable in treated and non-treated samples (Fig. 4a). In the sham-transfected counterpart, LC3 II isoforms was generated upon treatment with turmeric or curcumin as expected (Fig. 4a). Interestingly, the incubation with turmeric or curcumin led to increased expression of the autophagy protein ATG 7 (Fig. 4a). Consistent with previous results (Fig. 3e), in sham-transfected cells, the level of p53 and of Ser15-p-p53 was reduced upon exposure of the cells to either turmeric or curcumin. By contrast, in BECLIN 1 or ATG7 knocked-down cells, the stability of p53 and of Ser-phosphorylated p53 was maintained, although not completely, also in the cells exposed to turmeric or curcumin (Fig. 4b). Remarkably, p53 stabilization was more evident in ATG7-silenced

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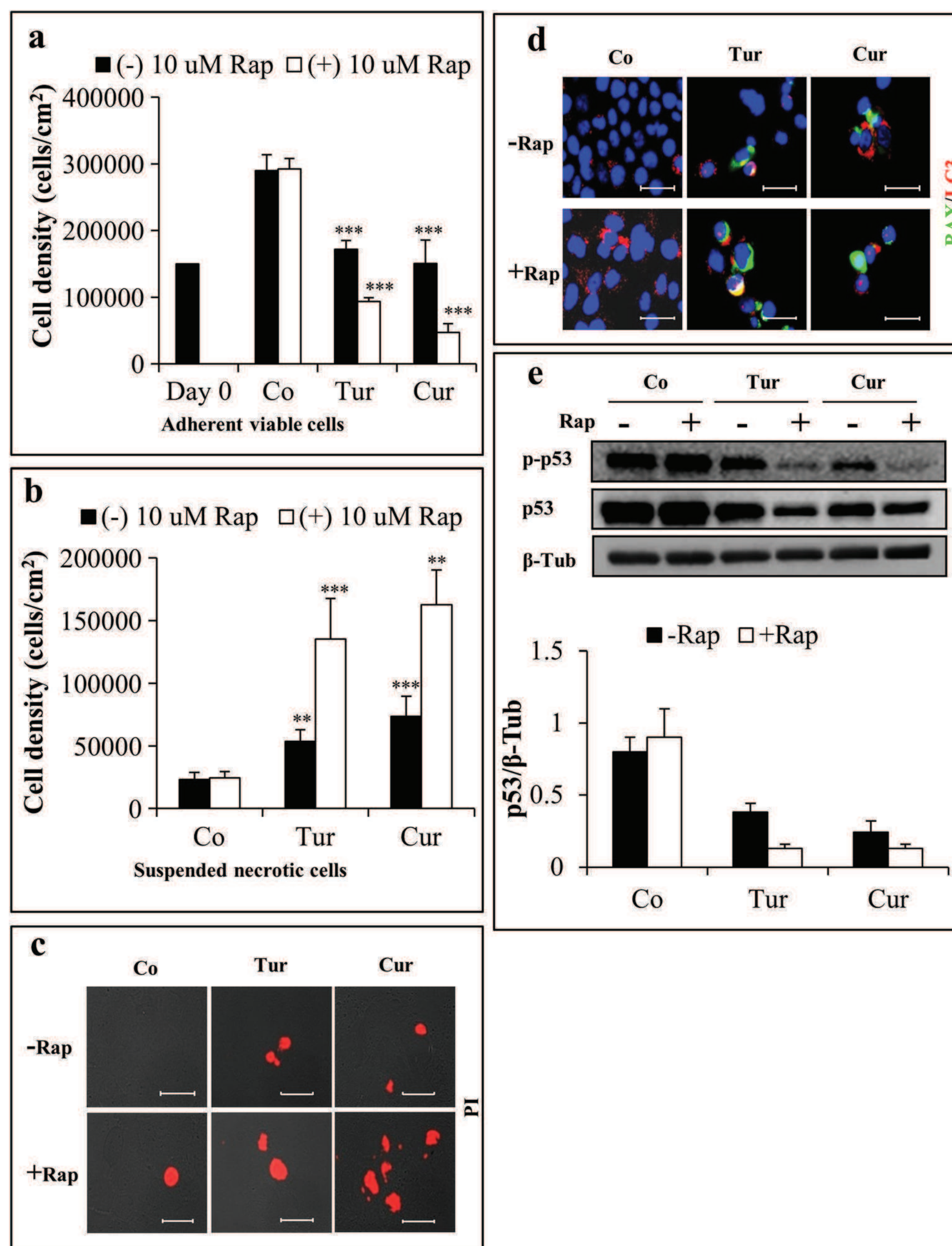

Figure 3. Inhibition of mTOR exacerbates turmeric and curcumin toxicity and p53 degradation. Biological consequences of the combinatorial treatment of rapamycin (10 µM) with turmeric (20 µg/mL) or curcumin (30 µM). (a) Cell count of adherent viable cells at 16 h post-treatment; (b) cell count of suspended trypan blue positive cells; (c) the cells plated on cover slips and treated as indicated were unfixed and stained with PI, which in this condition enters and labels the DNA of only the necrotic cells. The proportion of necrotic cells increased in the cultures treated with turmeric or curcumin along with rapamycin; (d) immunofluorescence co-staining of LC3, as a marker of autophagy, and of BAX, as a marker of apoptosis, in A431 cells plated on cover slips and treated as indicated; (e) expression of the Ser15-phosphorylated and of total p53 proteins in the cultures treated as indicated. Upper panel: western blotting; lower panel: densitometric analysis of specific bands (average ±SD of three independent experiments). Rapamycin synergized with turmeric and curcumin in inducing p53 degradation. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

cells, in which the abrogation of autophagy (as mirrored by LC3 II level) was more efficient (Fig. 4b). These data demonstrate that macroautophagy is responsible, to some extent, for the degradation of mutant p53 induced by turmeric and curcumin.

DISCUSSION

In the Asiatic traditional medicine, turmeric is by large the true spice employed for the preparation of topical medicaments, including cosmetics (Lee *et al.*, 1999), as compared with its component curcumin. However, while the potency of curcumin to prevent chemically induced skin carcinogenesis has been well documented (Conney *et al.*, 1991; Azuine and Bhide, 1992; Huang *et al.*, 1992; Limtrakul *et al.*, 1997), little is known about the effects and the pathways triggered by turmeric in skin cancer cells. In this work, we employed the epidermoid cancer A431 cell line, which harbours the R273H mutant p53. The oncosuppressor p53 is a master regulator of the cell fate in cells subjected to genotoxic stress and prevents carcinogenesis. Accordingly, the combined topical

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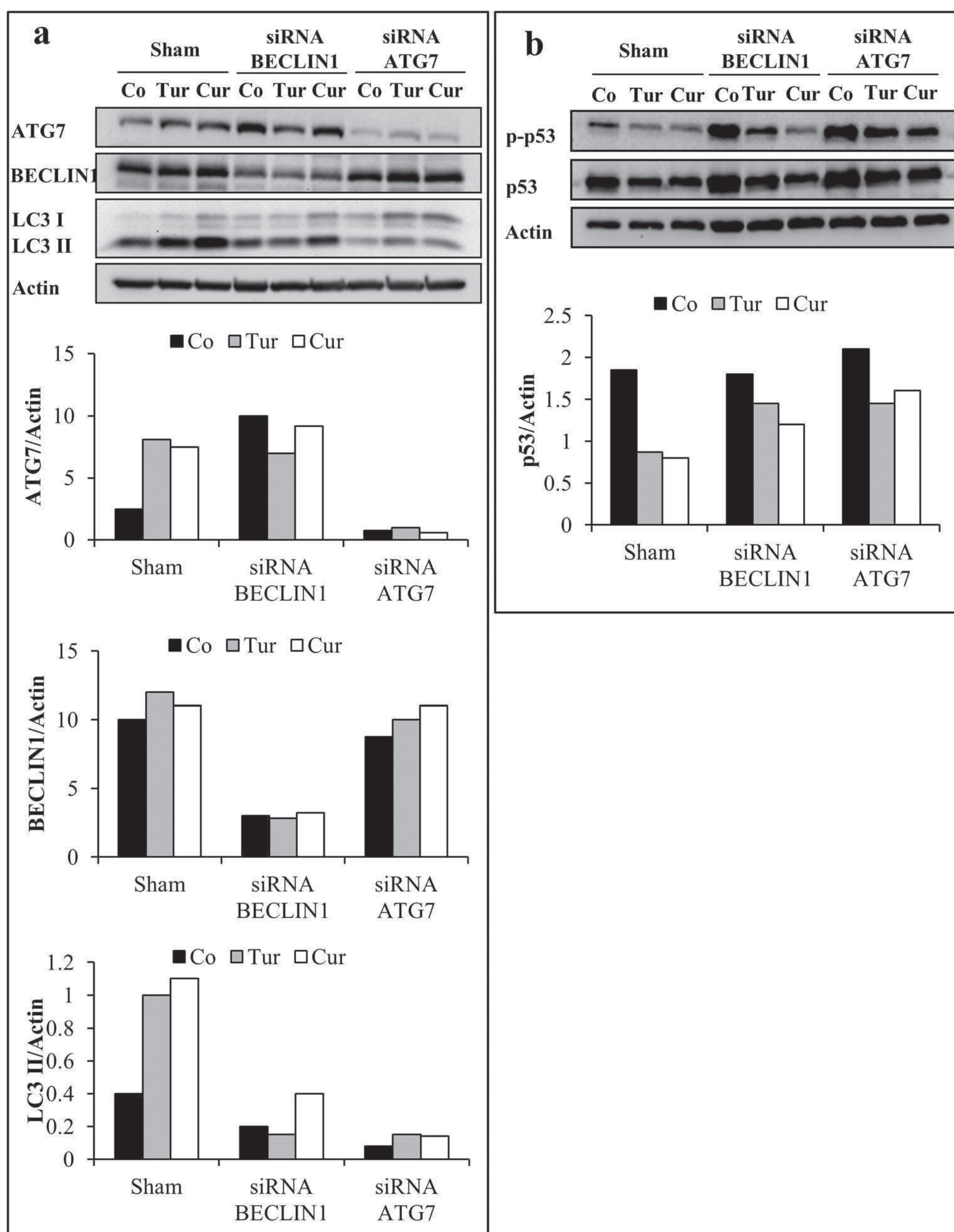

Figure 4. Post-transcriptional silencing of either the autophagy genes *BECLIN* 1 or *ATG7* prevents induction of autophagy and of p53 degradation by turmeric and by curcumin. A431 cells were transfected with a control duplex oligonucleotide (sham) or with siRNAs for the specific silencing of BECLIN 1 or ATG7 mRNAs. After 48 h, the medium was re-freshed and the cells were exposed or not for 24 h to turmeric or curcumin. (a) Western blotting showing the expression of the autophagy proteins BECLIN 1 and ATG7 in sham-transfected and in siRNA-transfected A431 cells. Based on densitometry, the specific siRNA reduced the expression of BECLIN 1 and of ATG7 of approximately 80% the value in sham-transfected cells; (b) Western blotting expression of p53 (total and its Ser15-phosphorylated form) in the same conditions as in the succeeding text. Representative western blotting and average densitometric data of two independent experiments are shown.

application of Resveratrol and Black Tea Polyphenols was shown to suppress mouse skin carcinogenesis through the activation of p53 (George *et al*., 2011). In addition, polyphenol-induced Ser15-phosphorylation and increase in the expression level of p53 correlated with apoptosis and inhibition of chemically induced skin carcinogenesis (Kalra *et al*., 2008; Roy *et al*., 2009; George *et al*., 2011). The anti-proliferative activities of wild-type p53 extend from its nuclear transcription to its cytoplasmic pro-apoptotic functions. While nuclear p53 transcribes, among others, the pro-apoptotic BAX protein and the cell cycle inhibitor p21WAF1/Cip1 (Miyashita *et al*., 1994; Waldman *et al*., 1995), the cytosolic p53 associates with the mitochondria, sequesters the anti-apoptotic BCL-2 proteins and promotes BAX oligomerization (Morselli *et al*., 2008b), thus allowing cell death through the intrinsic pathway. However, the p53R273H mutant is likely unable to direct the transcription of anti-proliferative proteins (such as BAX or p21), and accordingly, it has been shown to confer resistance to drug-induced apoptosis (Wong *et al*., 2007; Schilling *et al*., 2010). Here, we show that turmeric and curcumin can be toxic to A431 cells bearing the p53R273H mutant. When in the cytosol, wild-type p53 has been shown to also inhibit basal autophagy by interacting with FIP200/ULKC (Morselli *et al*., 2011), and this correlates with increased mTOR activity and concomitant inactivation of the AMPK pathway (Tasdemir *et al*., 2008). Consistently, the p53R273H mutant, which shows a preferential cytoplasmic

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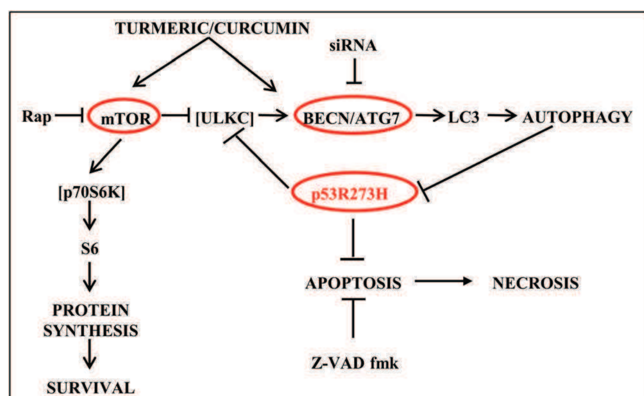

Figure 5. Interpretative scheme of the mechanisms of turmeric and curcumin toxicity in A431 cells. Turmeric and curcumin induce autophagy and cell death in A431 cells. Autophagy occurs through activation of both mTOR-dependent and mTOR-independent pathways. Autophagy leads to degradation of the p53R273H mutant, which was reported to oppose both apoptosis and autophagy. Knockdown of either the autophagy protein BECLIN 1 or ATG7 leads to the stabilization of Ser15-phospho-p53. Concomitant inhibition of mTOR by rapamycin increases autophagy, degradation of the mutant p53 and exacerbates cell death with onset of apoptosis and necrosis. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

localization, has been proven to inhibit BECLIN 1-dependent autophagy (Morselli *et al.*, 2008a). Surprisingly, we found that turmeric and curcumin are capable of inducing a rise of basal autophagy in A431 cells. Autophagy occurred (at least partly) independently of mTOR, very likely through activation of the AMPK pathway (as suggested by the Ser15-p53 phosphorylation). Consistent with the activation of a pathway alternative to the AKT-mTOR, rapamycin further increased the level of autophagy in the cells exposed to turmeric and curcumin. We searched for a mechanistic link between the induction of autophagy and onset of apoptosis in turmeric-treated or curcumin-treated A431 cells. It is known that both mTOR-dependent and mTOR-independent triggers of autophagy lead to the MDM2-proteasome mediated degradation of p53 (Tasdemir *et al.*, 2008). Recently, it has also been reported the degradation of p53 mutants through the chaperon-mediate autophagy pathway (Vakifahmetoglu-Norberg *et al.*, 2013). Here, we show that the activation of mTOR-independent (macro) autophagy-lysosomal pathway by turmeric or curcumin may play a pivotal role in the degradation of mutant p53. In fact, in BECLIN1 and in ATG7 knocked-down cells, the actual level of p53 and of Ser15-phosphorylated p53 was increased.

Thus, we describe a new pathway through which turmeric and curcumin can circumvent the chemoresistance of cancer cells bearing mutant p53. It has also to be stressed that turmeric extract was shown to have similar anticancer activity as its purified bioactive component curcumin. This finding is of translational relevance, given that the absorption and bio-availability of turmeric are higher than that of pure curcumin.

Noteworthy, while in itself not toxic at the concentration used, rapamycin synergized with turmeric and curcumin in causing the degradation of the p53R273H mutant and greatly exacerbated cell death triggering both apoptosis and necrosis, likely as a result of an over-stimulation of autophagy. A two-hit stress that hyper-induces autophagy may in fact lead to cell death (Castino *et al.*, 2005; Castino *et al.*, 2010). Further, *in vivo* investigations are warranted to demonstrate whether the combination of turmeric and rapamycin (rapalogs) at low concentrations could efficaciously prevent or cure skin cancer, as well as other carcinomas bearing the p53R273H mutation. It is worth mentioning, in this respect, that turmeric and curcumin could also inhibit A431 cell migration (our unpublished data). An interpretative scheme of the present findings is reported in Fig. 5.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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Unpublished Data

Below I report the experimental data obtained in the last two years which focused on the BECLIN 1 interactome in ovarian cancer cells.

The study revealed the presence of splicing isoforms of BECLIN 1 which differentially regulate the autophagy response to starvation depending on the interaction with BCL2 or VPS34.

The research is still ongoing and the preparation of the manuscript is in progress.

Below, I attach the 'Progress Report' on these novel data.

CROSS-TALK BETWEEN AUTOPHAGY AND APOPTOSIS: INTERACTING DOMAINS, COMPARTMENTALIZATION AND ROLE OF BECLIN 1 AND ITS PARTNERS IN THE AUTOPHAGY INTERACTOME

Project aim/objectives

Autophagy is a lysosomal degradative pathway for redundant or damaged cellular self-constituents. Autophagy acts as a survival mechanism under conditions of stress, maintaining cellular integrity by regenerating metabolic precursors and clearing subcellular debris. *(Levine and Kroemer, 2008; Ravikumar et al. 2010)*. This process contributes to basal cellular and tissue homeostasis. Moreover, autophagy can provide protection against aging and a number of pathologies such as cancer, neurodegeneration, cardiac disease and infection *(Lavallard et al., 2012)*.

Autophagy participates in the turnover of mitochondria (through the selective process of mitophagy) and other organelles (e.g., endoplasmic reticulum and peroxisomes) *(Johansen and Lamark 2011; Wang and Klionsky 2011)*. Furthermore, autophagy is involved in the clearance of polyubiquitinated protein aggregates (i.e., aggrephagy), which accumulate during stress, aging, and disease owing to perturbations in protein structure or folding *(Lamark and Johansen 2012)*. Autophagy has also been implicated as a regulator of lipid metabolism (i.e., lipophagy) *(Singh et al. 2009)*. Autophagy primarily acts as a protective mechanism that may prevent cell death, though it can also precipitate into cell death if hyper-activated beyond the point-of-no-return. Interaction between regulatory elements of both autophagy and apoptosis (e.g., the inhibitory interaction between BCL-2 and BECLIN 1 and the interaction between LC3B and Fas) (*Pattingre et al. 2005; Chen et al. 2010*) suggests, in fact, that a complex cross-talk exists between these two processes.

Our aim is to define the role of BECLIN 1-dependent autophagy and the molecular factors that may regulates its activation in cell subjected to metabolic and genotoxic stresses.

Experimental plan and methods

For our study, the ovarian cancer cell lines OVCAR3 and SKOV3 and the breast cancer cell line MCF7 were employed. The cells were grown in normal standard culture medium (D-MEM) or in EBSS (Earle`s Balanced Salt Solution), a culture medium depleted of aminoacids, serum growth factors and containing a reduced amount of glucose (1 mg/L). Culturing the cells in EBSS corresponds to a condition of 'starvation', which represents a classically used strong stimulus of autophagy.

Then we isolated BECN1 mRNA from an ovarian cancer cell line, NIH-OVCAR3, and amplified these mRNAs by PCR and Nested-PCR. We found four different isoforms of BECN1, that have been sub-cloned in pcDNA zeo (-) and sequenced.

In addition, for our study, we prepared deletion mutants for the interacting domains with bcl-2 and UVRAG. In a preliminary work, we have also prepared NES-mutants of BECLIN-1 tagged with GFP to study the role of nuclear vs cytoplasmic compartmentalization of BECLIN-1.

The impact of BECLIN 1 mutants on the autophagic flux induced by starvation was assessed by Western Blotting and Immunofluorescence of LC3 (a canonical autophagosomal marker) and BECLIN 1, in cells transfected with an empty plasmid (sham) or with plasmids harbouring either of the 4 different isoforms of Becn1 N2, N3, N6, N7 (subcloned into pcDNAzeo (-)).

Results

1) First, we analysed the autophagic flux in three different cancer cell line, MCF7 (breast cancer), OVCAR3 and SKOV3 (both ovarian cancer) cultured in standard and in starvation conditions by LC3 II Western Blotting. During nutrient deprivation, authophagy provides the constituents required to maintain the metabolism essential for survival. Therefore to induce autophagy, we cultured the cell in starvation condition for 0.5 , 1, 4 and 24 hour. These cultures conditions allow a direct comparison of the basal and induced autophagy in the cell (Fig.1). We added to the treatment also the ammonium chloride, that blocks the autophagic flux by lowering the intralysosomal pH and inhibiting the autophagosome/lysosome fusion and the subsequent degradation of the autophalysosome. In the presence of ammonium chloride all autophagosomes produced during the incubation accumulate. From the comparison of the levels of LC3II accumulated in the absence and presence of ammonium

chloride it is possible to evaluate the rates of production and of consumption of the autophagosomes.

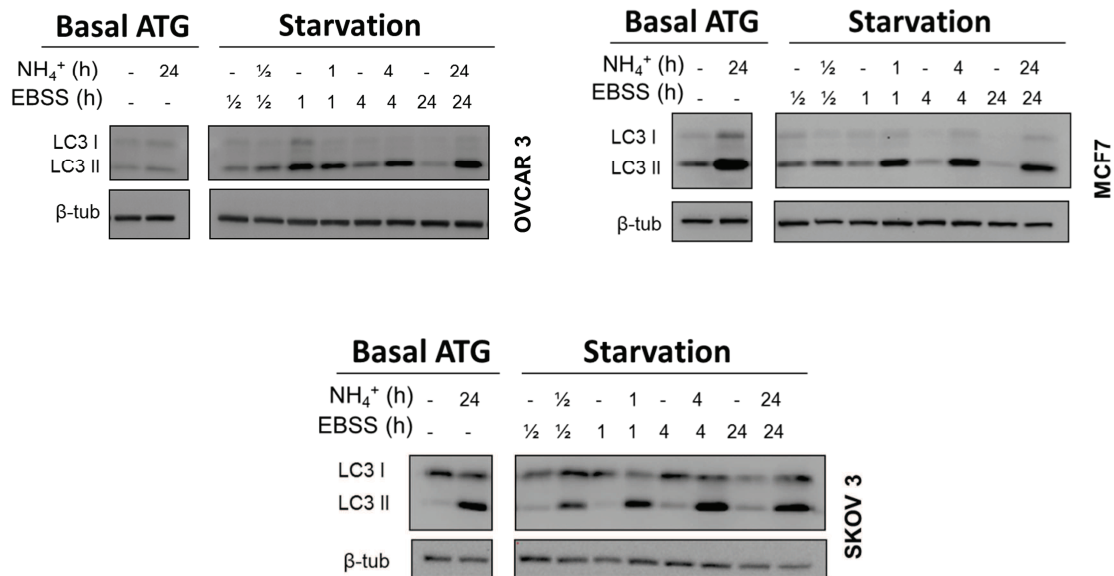


Fig. 1. *Western Blotting LC3 I/II OVCAR3, MCF7, SKOV3 cell lines, time course starvation conditions (EBSS)*

Our data shows that the basal autophagy (ATG) is different in the three cell lines, being higher in MCF 7 and SKOV 3 than in OVCAR3 cells. This suggest the possibility that production of autophagosomes under basal conditions is defective or very limited in OVCAR3 cell line. Induced autophagy under starvation condition occurs in OVCAR3 cells. However, the kinetics of autophagosomes formation and consumptions differ between the three cell types, though in all cases the accumulation of autophagosomes peaks at 4 h, then declines by 24 h.

2) To investigate the defect in the authofagosome formation of NIH-OVCAR 3 cell line, under normal conditions, we examined the expression of BECLIN1, a protein that plays a central role in the early stage of starvation-induced autophagy. Our previous data showed the presence in NIH-OVCAR3 of four isoforms of BECLIN1, that we named respectively BECLIN1 N2, N3, N6, N7. While N2 corresponds to the wild-type (WT) and is expressed at a high level, N2, N3 and N4 are expressed at very low levels and present with domains deletion

in the protein as schematically represented in Fig.2. Our goal is to determine whether the defective autophagosome formation in NIH-OVCAR3 cells could be attributed to the presence of these isoforms of BECLIN 1.

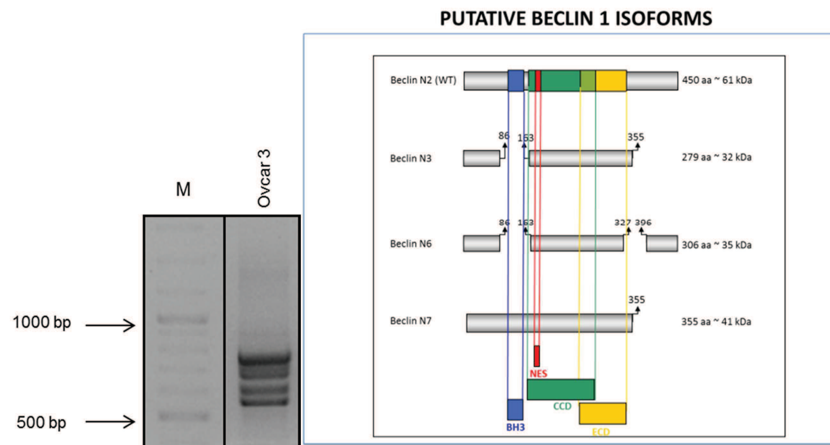


Fig.2 Nested-PCR Becn1 in OVCAR3 cell line and putative isoforms of BECLIN 1

To analyze the different isoform of BECLIN 1 in NIH-OVCAR-3, we have sub-cloned the isoforms in pcDNA zeo (-). We sequenced the isolated BECLIN-1 isoforms (Fig.3A) and designed the putative BECLIN-1 (Fig. 3B) peptide by CLUSTAL W (European Bioinformatics Institute, EBI), an alignment software.

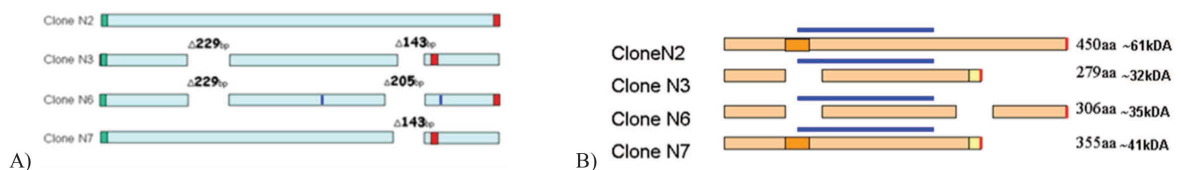


Fig.3 *A) Isolated BECLIN-1 isoforms,* ***in green the START codon, in blue point mutation, in red the STOP codon***

B) Putative BECLIN-1 peptides

We found four isoforms named N2, N3, N6, and N7 in NIH-OVCAR-3 cells.

- The clone N2 shows only one polymorphism in B103A.
- The clone N3 have a deletion of 229 bp, that removes the putative domain of interaction with bcl-2 and part of the potential coiled-coil domain; and a deletion of 143 bp that results in a frame-shift and in a premature STOP codon.

- The clone N6 have a deletion of 229 bp, that removes the putative domain of interaction with bcl-2 and part of the potential coiled-coil domain; and the deletion of 205 bp with preservation of reading frame.
- The clone N7 have a deletion of 143 bp with the frame-shift as in N3.

Therefore, our data provide the tools to modulate the composition of the autophagy interactome, to study the autophagy flux and to analyze the cross-talk between autophagy and apoptosis. Understanding the molecular factors that influence the formation of the BECLIN-1 complexes is of great importance to determine which signaling pathways should be stimulated or inhibited in order to activate a pro-survival autophagy (as desired in the case of neurodegeneration) or a pro-death autophagy (as desired in the case of cancer).

3) The MCF7 breast carcinoma cells present a mono-allelic deletion of BECLIN 1 (as reported in the literature). Accordingly, the analysis of the protein by Western Blotting shows a very low level of BECLIN 1 expression in MCF7, compared to OVCAR3 and SKOV3 cells (Fig.4). The expression does not change during the 4 h incubation in starvation medium

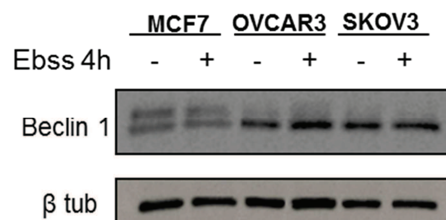


Fig. 4 *Western Blotting BECLIN1 in normal and starvation (EBSS) conditions*

To investigate the possible role of the BECLIN 1 isoforms in the regulation of autophagy, under basal and starvation-induced conditions, we transgenically expressed these isoforms separately in MCF7 cells (Figure 5A), and analysed the level of LC3II, as a readout of autophagosome formation (Figure 5B), at 4 hours of starvation (corresponding to the maximal peak of induction, see above, Fig.1).

In sham-transfected cells, we observe a reduced accumulation of LC3 II after starvation, this could be due to an impairment in the induction of autophagy induction or an accelerated degradation of LC3 due to increased autophagic flux.

COMMENT: to clarify this point we will need to introduce a treatment (e.g. ammonium chloride) that blocks the autophagosome-lysosome fusion step.

Of note, the ectopic expression of Becn1 N2 (WT) leads to an increased accumulation of LC3II upon starvation. This could be interpreted as follow: 1. in sham-transfected cells the level of endogenous BECLIN 1 is too low for allowing an efficient response to starvation in terms of autophagosome production, though it is enough for allowing basal autophagy; 2. in BECLIN 1-N2 transfected cells, the amount of BECLIN 1 WT (see Figure 5A) available for autophagy signalling is increased and allows to produce autophagosome in response to starvation. On the other hand, the transgenic expression of the mutant BECLIN 1 N3 slightly increases the accumulation of autophagosomes under basal conditions, but is not efficient for allowing the production of autophagosomes under starvation-induced conditions (Figure 5B). The mutant N6, and even more the mutant N7, when ectopically expressed in MCF7 cells do not allow an efficient production of autophagosomes under starvation conditions (Figure 5B).

COMMENT: The above data indicate that in MCF7 cells, which express low levels of endogenous BECLIN 1, the ectopic expression of BECLIN 1 mutants strongly affects the signalling at the early stage of autophagy induced by starvation.

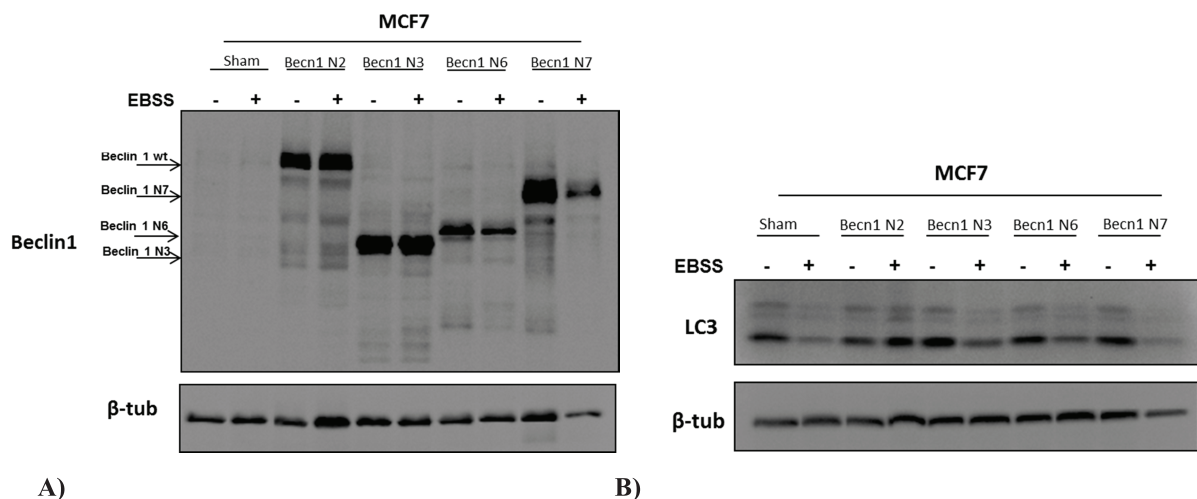


Fig.5 Western Blotting BECLIN 1 (A) and LC3I/II (B) in MCF7 cell line after ectopic expression of Becn1 N2 (WT), N3, N6, N7, in normal and in starvation conditions (EBSS)

4) To see if the effects of BECLIN 1 mutants on the formation of autophagosomes is cell context dependent (given that MCF7 cells are monoallelic deleted for Belcin 1), we expressed

the WT and the mutants (Becn1 N2 (WT), N3, N6, N7) in the ovarian cancer cell line SKOV 3, which express both alleles at physiologic level.

Our data on LC3 Western Blotting (Fig.6) show that in SKOV3 cells BECLIN 1 N3 and N6 mutants, which have a deletion in the BH3 domain, cause an autophagic stimulation in normal (increase in basal autophagy) and in starvation conditions. By contrast, the BECLIN 1 N7 mutant does not affect the basal autophagy, but it impairs the induction of autophagosome formation under starvation conditions.

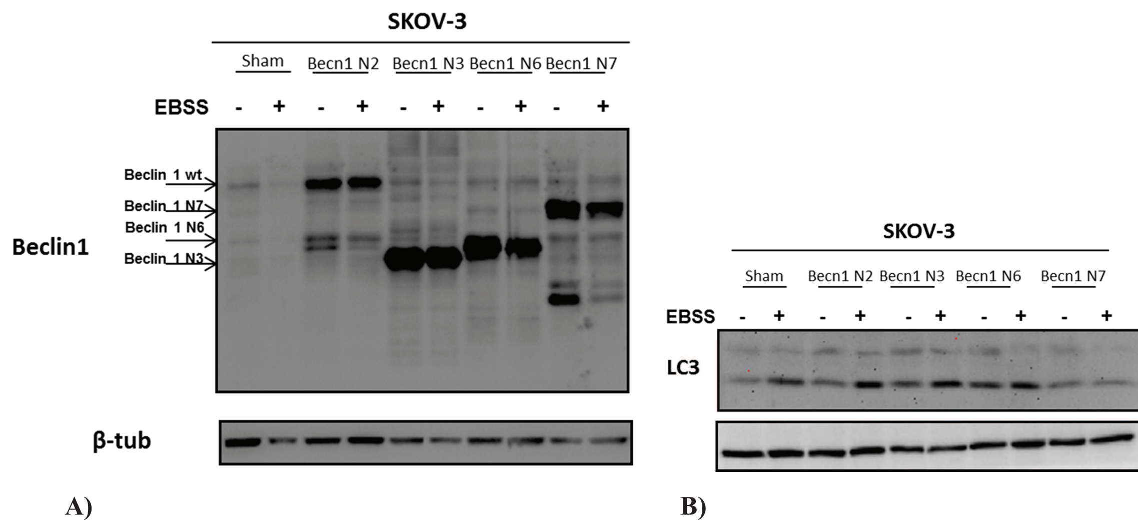


Fig.6 *Western Blotting BECLIN 1 (A) and LC3I/II (B) in SKOV3 cell line after ectopic expression of Becn1 N2 (WT), N3, N6, N7, in normal and in starvation conditions (EBSS)*

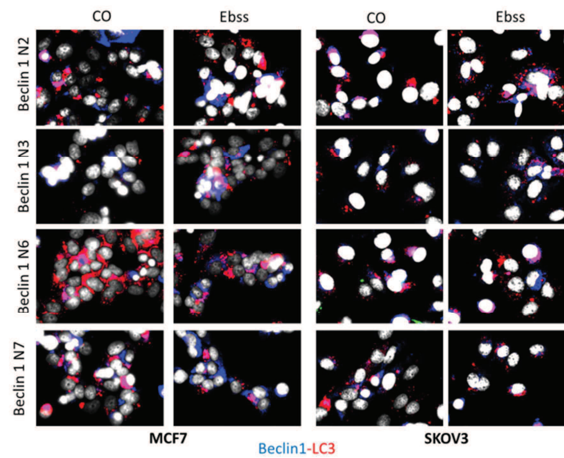
COMMENT: The clone N3 and N6 have a deletion of 229 bp which removes the putative domain of interaction with bcl-2 and part of the potential coiled-coil domain. The deletion of the BH3 domain disrupts the BECLIN 1-Bcl-2 complex, resulting in the stimulation of autophagy *(Ciechomska et al, 2009)*.

5) Finally, we investigate by immunofluorescence the expression and localization of LC3 after transgenic expression of BECLIN1 N2 (WT), N3, N6, N7, in SKOV3 and MCF7.

In these cells we assessed the formation of autophagosomes under basal and starvation-induced autophagy. When LC3 I is converted into LC3 II and moves from the cytoplasm to authophagosomes can be, it can be riconognized by immunofluorescence staining as fluorescence puncta.

The pictures in Figure 7 illustrate an increase of LC3 II in SKOV3 cell line under starvation conditions, and suggest that the transgenic expression of BECLIN1 N3 and N6 mutants

determines an increase in the induction of autophagy under normal and starvation conditions, according to the data obtained with the Western Blotting. In this respect, the MCF7 cell line shows a different behaviour in terms of autophagy induction upon transfection with these



mutants, as already seen in Western Blotting analysis.

Fig. 6 Immunofluorescence BECLIN 1 and LC3 in MCF7 and SKOV3 cell line after ectopic expression of Becn1 N2 (WT), N3, N6, N7, in normal and in starvation conditions (EBSS)

CONCLUSION AND PERSPECTIVES

Modulation of autophagy has a big impact on the tumorigenesis and cancer treatment. Depending on whether it is considered at the precancerous or at the advanced stage, up or downregulation of autophagy may elicit either tumour promoting or tumour-suppressive effects *(Degenhardt et al., 2006; Rosenfeldt and Ryan, 2009)*. Since autophagy protects cells from metabolic stress, it is reasonable that the upregulation of autophagy preserves cellular fitness and genomic integrity and thus prevents tumorigenesis. On the contrary, established tumor cells can utilize autophagy to survive stress like nutrient limitation and hypoxia. Further, tumor cells can activate autophagy as a stress response to survive cancer treatment.

MicroRNAs are recognized as master epigenetic regulators of gene expression that have a great impact on many malignant features of cancer cells, such as the propensity to metastasize and to resist to antineoplastic treatments. In the context of the intricate involvement of autophagy in cancer progression, emerging data point to the role of miRNAs as regulators of autophagy gene expression. In human cancer the role of miRNAs in the control of autophagy could open new avenues for the molecular therapy of cancer. For example chemosensitivity could be rescued by manipulating the level of miRNAs targeting autophagy.
Autophagy can be regulated also by amino acid deprivation, Studies demonstrate that even a single amino acid starvation can active autophagic response in tumor cells *(Kim et al., 2009; Sheen et al., 2011)*. Arginine, besides being required for protein biosynthesis, has other versatile functions in the cell as a precursor of nitric oxide, agmatine, and polyamines and as a regulatory molecule *(Morris, 2006)*. The ovarian cancer cell line SKOV3 exhibits high expression of arginine-succinate synthetase (ASS), we demonstrate that arginine withdrawal rapidly and markedly induces autophagy in this cell line. Our results support a new approach in cancer therapy, in fact the combinational treatment based on arginine deprivation and an autophagy inhibitor (e.g., chloroquine) can potentially be applied as a second line treatment for a subset of ovarian carcinomas deficient in ASS.

Autophagy is deregulated in different kinds of human cancer and is involved in the progression of cancerogenesis. The autophagy gene BECN1 is monoallelically deleted in a high percentage of human breast, ovarian and prostate cancers, and decreased levels of the protein have been found in human breast, ovarian and brain tumors *(Miracco et al., 2007; Mathew et al., 2007; Karantza-Wadsworth and White, 2007)*. We showed that in patients with ovarian and breast cancer high level of BECLIN1 protein and mRNA expressions are correlated with overall survival and good prognosis of the patients. Future clinical trials should evaluate the level of expression of BECLIN1 to predict the response to

chemiotherapeutic or strategy that increase its expression. BECLIN1 is inhibited by interaction with BCL-2 family members and HER2 *(Pattingre et al., 2005; Maiuri et al., 2007; Han et al., 2013)*, the kinase AKT activity and by EGFR postraslational modification *(Wang et al., 2012;Wei et al., 2013)*. Therefore we can increase the BECLIN1 activity and improve clinical outcomes using BECLIN1/BCL-2 binding inhibitors, AKT, EGFR and HER2 inhibitors.

Recent studies highlight the role of autophagy in thyroid cancer chemotherapeutic response. Several oncogene and oncosuppressor gene deregulated in thyroid cancer play also a role in autophagy. In clinic could be possible use autophagy inducer or inhibitor drugs. The inducers may have effect in preventing the development and growth of cancer cells. The inhibitors should improve the efficacy of anticancer therapy in metastatic cancers.
Thyroid cancer cells show a high rate of glycolysis *(Andrade et al., 2012)*. This is a mechanism, that many solid tumors put to use, because of the decrease of glucose and oxygen availability and the cancer cells adapt their metabolism towards the aerobic glycolysis (*Tong et al., 2009*). We show that the oncosuppressor PTEN plays a dominant role on membrane expression of GLUT-1 and glucose uptake in thyroid cancer cells, independent of the level of glucose available. We have demonstrate that deletion of PTEN concurrent with p53 mutation, increases the glucose uptake and consumption in in FTC133 thyroid cancer cella with undergo cell cycle arrest and apoptosis, cell migration arrest and defective autophagy when subjected to glucose restriction. Therefore, targeting together the PI3k-PTEN-AKT-mTOR and p53 pathways, we can control both glucose uptake and autophagy. Understanding the mechanism that link autophagy and thyroid cancer could help to clarify the diagnostic and prognostic potential biomarkers, and to find novel therapeutic approaches based on the modulation of autophagy.

The natural spice curcumin has been shown to have anticancer properties on the fact that it target several cancer related pathways *(Goel et al., 2008; Teiten et al., 2013)*. Our data indicate that curcumin and turmeric (the spice where is extract the curcumin) induce the formation of autophagosome and also stimulate the autophagy flux, probably through an mTOR-indipendent pathway, likely involving AMPK. Rapamycin-mediated inhibition of mTOR synergized with turmeric and curcumin in causing p53 degradation and increasing the production of autophagosomes and exacerbated cell toxicity leading to cell necrosis. Our results offer the opportunity to find new applications in therapy of these two spices. Turmeric and curcumin could circumvent the chemoresistance of cancer cells bearing mutant p53.

The role of autophagy in cancer is complex and different studies exhibit results that are often contradictory. The role of autophagy changes depending on the type of therapy, the tumor microenvironment, and the tumor genotype as well as the tumor stages. Afterwards, the effect of autophagy during cancer progression and therapy is dual. We are working to explain in detail which factors lead to treatment induced autophagic cell death and which factors promote autophagy-driven therapy resistance.

The results reported in this doctoral thesis, in accord with data from other laboratories, support the contention that the proteins involved in the regulation of autophagy can be used as prognostic marker, and could also be exploited as targets for molecular therapies. For sure, the characterization of the autophagy (dys)regulation in cancer may give useful hints for a more appropriate and individualized therapy of the patient.

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