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Evaluation of Tenascin C expression as a prognostic marker in Hepatocellular Carcinoma

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INDEX

SUMMARY

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of cancer mortality worldwide; in developed countries, the incidence of HCC has increased since the 1980s. The most common risk factors are chronic viral hepatitis, alcoholic cirrhosis, aflatoxins exposure and non-alcoholic fatty liver disease.

Tenascin C (TNC) is a large hexameric extracellular matrix glycoprotein highly expressed in fetal tissues but lowly represented in adult tissues; it has been demonstrated that TNC has multiple functions in cell adhesion and tissue remodeling, and that it plays an important role in tumor progression in various solid tumors such as colorectal cancer, breast cancer, ductal pancreatic adenocarcinoma, glioblastoma and glioma.

Aim of the Thesis

We evaluated the role of Tenascin C as a prognostic marker for hepatocellular cancer in patients treated with radical surgical resection.

Expression of TNC has been related to patient's age, sex, tumor size, grading, underlying liver disease and overall survival.

Study presentation

We performed a retrospective analysis on clinical outcome related to TNC immunohistochemical expression in a series of 44 patients that underwent surgical radical resection for hepatocellular cancer.

Inclusion criteria for the present study were: age > 18 years, definitive histological diagnosis of primitive hepatocellular carcinoma, radical liver surgery with negative resection margins, adequate material for histological and immunohistochemical evaluation and regular follow – up.

TNC immunoreactivity was expressed by a score composed of the extension of positivity area and staining intensity. We also evaluated the TNC expression pattern on the basis of the localization of TNC reactivity (nucleus, cytoplasm, interstitial space).

Statistical analysis was performed with MedCalc (version 7) and GraphPad PRISM 5 software.

We tested the correlations between levels of TNC immunohistochemical expression and patients' clinical – pathological characteristics with Spearman's non parametric correlation test; survival curves were obtained with Kaplan – Meier method, and differences in terms of overall survival (OS) were evaluated with the log – rank test. Statistical significance has been set at p – value <0.01.

Results

44 patients (35 males, 79.55%; 9 females, 20.45%) were included in the present study.

The median age was 71 years (range 38 – 87 years); 27 patients (61.36%) were affected by chronic hepatitis C virus infection, 7 (15.91%) by alcoholic liver disease, 5 (11.36%) by non - alcoholic fatty liver disease, and 5 (11.36%) by chronic hepatitis B virus infection.

The median tumor size was 39mm (range 13 – 70mm).

22 patients (50%) underwent minor hepatectomy (including wedge resection, segmentectomy, bisegmentectomy; major hepatectomy (trisegmentectomy, left or right hepatectomy) was performed in the remaining cases.

Perioperative morbidity was 9.09% (1 case of liver failure, 2 cases of bile leakage needing re – operation and 1 case of peritoneal collection treated with percutaneous drainage).

At the end of the observation period (31/10/2019), 25 patients (56.82%) were deceased.

Histological section staining demonstrated a great variability in TNC expression in terms of extension and intensity.

25 cases (56.82%) showed TNC expression in less than 10% of the tissue area, 10 cases (22.73%) between 11% and 25%, 7 cases (15.91%) between 26% and 50%, 1 case (2.27%) between 51% and 75%, and 1 case (2.27%) more than 76%.

TNC expression was absent in 9 cases (20.45%); in the remaining 35 specimens, staining intensity was low in 11 cases (25%), moderate in 13 (29.55%) and high in 11 (25%).

Patients were also divided in groups based on TNC expression localization: in 13 cases (29.55%) TNC was mainly expressed in the interstitial and in peri - sinusoidal space; in the other cases in which TNC was expressed, staining was more intense in the nucleus or cytoplasm.

Peri - sinusoidal tenascin expression was absent in 17 cases (38.64%), low in 16 (36.36%), moderate in 3 (6.82%), and high in 8 (18.18%).

At univariate analysis, no correlations between overall survival and age, sex, tumor size, grading, concomitant liver disease, TNC global intensity staining or area expression have been demonstrated.

Moderate and intense expression of TNC in the peri - sinusoidal space was found to be significantly related to a worse prognosis (shorter survival after curative treatment, high rate of tumor local recurrence and metastatization) if compared with patients with absent or low peri - sinusoidal space staining ($p<0.01$).

Discussion

Hepatocellular carcinoma is still one of the most common causes of cancer – related deaths, with a low global 5 - year survival rate (4.4 – 6%) and high 5 - year recurrence rate (43.5 – 61.5%) even after curative treatment; the high metastatic potential, particularly involving lymph nodes, is the main reason of therapeutic failure.

The validation of prognostic biomarkers that would provide a tailored treatment could be a new challenging frontier in liver cancer oncological treatment.

The critical role of the cancerous microenvironment (cellular and non - cellular) is increasingly recognized as an important factor, markedly influencing hepatocarcinogenesis, epithelial – mesenchymal transition, tumor invasion and metastasis.

Tenascin C is mainly expressed during embryonic development. In adults, TNC has a limited pattern of expression, but protein levels rise dramatically under various physiological and pathological conditions, such as tissue remodeling, neovascularization, and inflammation. TNC is thought to influence cancer growth by affecting cell adhesion and motility, thus promoting invasion and metastasis.

Conclusions and future perspectives

Our experience demonstrated that moderate and intense TNC expression in tumor extracellular space is significantly related to high rates of disease recurrence and early mortality in patients affected with early-stage hepatocellular carcinoma treated with a radical surgical approach.

Further studies on larger patient series are needed in order to validate the analysis of the TNC expression pattern as a prognostic marker in potentially curable cases of HCC.

RIASSUNTO

Introduzione

Il Carcinoma Epatocellulare (HCC) è la sesta forma più comune di neoplasia e la seconda causa di morte per tumore nel mondo; nei Paesi Industrializzati, l'incidenza di HCC è nettamente incrementata dagli anni 80. I più comuni fattori di rischio sono l'epatite virale cronica, la cirrosi alcolica, l'esposizione ad aflatossine, e la steato – epatite non alcoolica.
La Tenascina C (TNC) è una grossa glicoproteina esamerica della matrice extracellulare altamente espressa nei tessuti fetali ma scarsa nel tessuto adulto sano; è stato dimostrato che la TNC riveste molteplici funzioni nell'adesione cellulare e nel rimodellamento cellulare, ed ha un ruolo decisivo nella progressione tumorale in svariate neoplasie solide quali il carcinoma colo – rettale, il carcinoma mammario, l'adenocarcinoma duttale del pancreas, il glioblastoma ed il glioma.

Scopo della Tesi

Abbiamo analizzato il ruolo della Tenascina C quale marcatore prognostico nei pazienti affetti da epatocarcinoma, trattati con resezione chirurgica radicale.

Presentazione dello studio

È stata condotta una analisi retrospettiva sul decorso clinico correlato alla espressione di TNC in una serie di 44 pazienti sottoposti a resezione chirurgica radicale per epatocarcinoma.
I criteri di inclusione per il presente studio sono stati: età superiore ai 18 anni, diagnosi istologica definitive di HCC, resezione chirurgica radicale con margini adeguati, materiale adeguato ad analisi anatomopatologica ed immunoistochimica, follow – up regolare.
L'immunoreattività della TNC è stata analizzata tramite uno score costituito dall'estensione dell'area di espressione e dall'intensità della colorazione. È stata inoltre analizzata la distribuzione della tenascina C a livello del tessuto tumorale (nucleo, citoplasma o interstizio).
L'analisi statistica è stata Condotta mediante MedCalc (versione 7) ed I software GraphPad PRISM 5.
Abbiamo testato la correlazione tra le caratteristiche dell'espressione immunoistochimica di TNC ed il decorso clinic dei pazienti con il metodo parametrico di correlazione di Spearman. Le curve di sopravvivenza sono state ottenute con il metodo di Kaplan – Meier e le differenze di overall survival (OS) sono state valutate con il log – rank test.
La significatività statistica è stata stabilita ad un valore di $p < 0.01$.

Risultati

44 pazienti (35 maschi, 79.55%; 9 femmine, 20.45%) sono stati inclusi nel presente studio.

L'età media era di 71 anni (range 38 – 87 anni); 27 pazienti (61.36%) erano affetti da epatite C cronica, 7 (15.91%) da cirrosi alcolica, 5 (11.36%) da steatoepatite non alcolica, 5 (11.36%) da epatite B cronica.

Le dimensioni medie del tumore erano 39 mm (range 13 – 70mm).

22 pazienti (50%) sono stati sottoposti ad epatectomia maggiore (inclusi wedge resection, segmentectomia, bisegementectomia); le epatectomie maggiori (trisegmentectomia, epatectomia destra o sinistra) sono state eseguite negli altri 22 casi.

La morbidità perioperatoria è stata pari a 9.09% (1 caso di insufficienza epatica, 2 casi di leakage biliare che hanno reso necessario un re intervento ed 1 caso di ascesso peritoneale trattato con drenaggio percutaneo).

Alla fine del periodo di osservazione (31/10/2019), 25 pazienti erano deceduti per progressione di malattia (56.82%).

L'immunoistochimica ha evidenziato una grande variabilità nella espressione di TNC in termini di estensione, distribuzione ed intensità.

24 casi (54.55%) mostravano espressione di TNC in meno del 10% dell'area tessutale, 10 casi (22.73%) tra 11% and 25%, 7 casi (15.91%) tra 26% and 50%, 1 caso (2.27%) tra 51% and 75%, 1 caso (2.27%) più di 76%.

In 9 casi TNC non era espressa (20.45%); nei restanti 35 campioni, l'intensità di colorazione risultava bassa in 11 casi (25%), moderata in 13 (29.55%) and elevata in 11 (25%).

I pazienti sono stati inoltre suddivisi in gruppi sulla base della localizzazione di TNC: in 13 casi (29.55%) TNC era prevalentemente espresso nell'interstizio e nello spazio perisinusoidale; negli altri casi in cui la TNC era espressa, la colorazione era più intensa nel nucleo e nel citoplasma.

L'espressione di TNC nello spazio perisinusoidale era assente in 17 casi (38.64%), bassa in 16 (36.36%), moderata in 3 (6.82%), elevata in 8 (18.18%).

Ad una analisi univariata, non sono state trovate correlazioni tra overall survival ed età, sesso, dimensioni del tumore, grading, epatopatia sottostante, intensità di colorazione ed estensione dell'area di espressione della TNC.

L'espressione moderata ed intense di TNC nello spazio perisinusoidale si è dimostrata correlata significativamente con una prognosi peggiore (sopravvivenza minore dopo l'intervento radicale, alta percentuale di recidiva locale e metastatizzazione) se paragonati ai pazienti in cui l'espressione di TNC nello spazio perisinusoidale era assente ($p<0.01$).

Discussione

L'epatocarcinoma risulta ancora una delle principali cause di morte correlate a tumore, con una bassa sopravvivenza globale a 5 ani (4.4 – 6%) ed un alto tasso di recidiva entro i 5 anni (43.5 – 61.5%) anche dopo trattamenti curativi; la principale causa di fallimento terapeutico sembra essere l'elevato potenziale di metastatizzazione, in particolare ai linfonodi.

La validazione di marcatori prognostici che porterebbero ad un trattamento personalizzato potrebbe essere una nuova frontiera nel trattamento oncologico delle neoplasie epatiche primitive.

Il ruolo critico del microambiente tumorale (cellulare e non cellulare) è sempre maggiormente riconosciuto come un fattore importante che influenza marcatamente l'epatocarcinogenesi, la transizione epiteliale - mesenchimale, l'invasione tumorale e le metastasi.

TNC è espressa principalmente nei tessuti embrionali; nell'adulto, TNC ha un pattern limitato di espressione, ma i livelli di espressione della proteina aumentano sensibilmente in svariate condizioni fisiologiche e patologiche, quali il rimodellamento tissutale, la flogosi e la neoangiogenesi; TNC promuove la crescita del tumore influenzando l'adesione del tumore e la motilità tessutale facilitando l'invasione vascolare e la metastatizzazione.

Conclusioni e prospettive future

La nostra esperienza ha dimostrato che una espressione moderata ed intense di TNC nell'interstizio del tessuto tumorale è correlata ad un alto tasso di ripresa di malattia e mortalità precoce nei pazienti affetti da HCC trattati con approccio chirurgico radicale.

Ulteriori studi su più ampie casistiche di pazienti sarebbero necessari per validare l'analisi di espressione di TNC come marker prognostico in casi di HCC potenzialmente curabili.

INTRODUCTION

Hepatocellular Carcinoma: epidemiology and worldwide distribution.

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of cancer mortality worldwide; incidence rates of liver cancer vary widely between geographic regions and are highest in Eastern Asia and sub-Saharan Africa; in developed countries, the incidence of HCC has increased since the 1980s [1].

In 2016, about one million incident cases and 829,000 related deaths were recorded worldwide [2]; it also represents the leading cause of death in subjects affected by liver cirrhosis [3,4].

There are several epidemiological differences according to the geographical area considered; in detail, hepatitis B virus (HBV) etiology is prevalent in China, South East Asia, and sub – Saharian Africa [5,6], while chronic hepatitis C virus (HCV) is a leading risk factor in western countries and Japan [7,8,9].

Chronic alcoholic liver disease is reported worldwide, with the highest prevalence in Eastern and Central Europe (53% and 46% respectively), sub – Saharian Africa (40%) and North America (37%) [10].

The role of aflatoxin is considerable in Africa and Eastern Asia [4].

Although non-alcoholic steatohepatitis (NASH), autoimmune and cholestatic liver diseases may predispose to HCC, their role seems to be less important [11].

The HCC epidemiological scenario has considerably changed in recent years: literature demonstrates a lower severity of the disease at diagnosis [8], a reduction in incidence in areas with a traditionally great prevalence, but an increase in low prevalence areas [12,13,14]. In China and eastern sub – Saharian Africa, a reduction of new cases was observed in the 1990 – 2015time interval, probably thanks to vaccination programs and to lower exposition to aflatoxins [15].

Studies conducted in developed countries but also in some areas in South America have shown increases in cases of HCV and alcohol related HCC (42% and 56% respectively); in western countries there is also an increasing incidence of post – NASH HCC [16].

HCC prognosis, however, remains poor [10]; in Italy, the 5 – year survival rate is about 20%, the data being worse in southern regions, despite the continuous surveillance program [17].

HCC risk factors

Although several risk factors for primary liver carcinogenesis have been recognized, most of them causing chronic parenchymal flogosis, the most common pathological conditions associated with the development of HCC

at present are chronic viral hepatitis, alcoholic cirrhosis, aflatoxin exposure and non - alcoholic fatty liver disease [18].

HBV remains the leading cause of HCC, despite the great success achieved by the universal vaccination in infants; In 2017, the World Health Organization estimated that about 3.5% of the world population is affected by chronic hepatitis B, with the highest hepatitis B surface antigen (HBsAg) prevalence (of 6.2%) in the Western Pacific Region [19]. The role of HBV in carcinogenesis appears to be complex and may involve both direct and indirect mechanisms. Integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion, and it has been shown to induce direct insertional mutagenesis of diverse cancer-related genes in several cases. Chronic liver inflammation and hepatic regeneration induced by cellular immune responses may favor the accumulation of genetic alterations in infected hepatocytes. Prolonged expression of the viral regulatory protein HBx and the large envelope protein LHBs may contribute to deregulating the cellular transcription program and proliferation control, and to sensitizing liver cells to carcinogenic factors. Moreover, the rate of chromosomal alterations is significantly increased in HBV-related HCC compared to tumors associated with other risk factors. HBV might play a role in enhancing genomic instability [20].

Oncogenetic pathways related to HBV is summarized in figure 1.

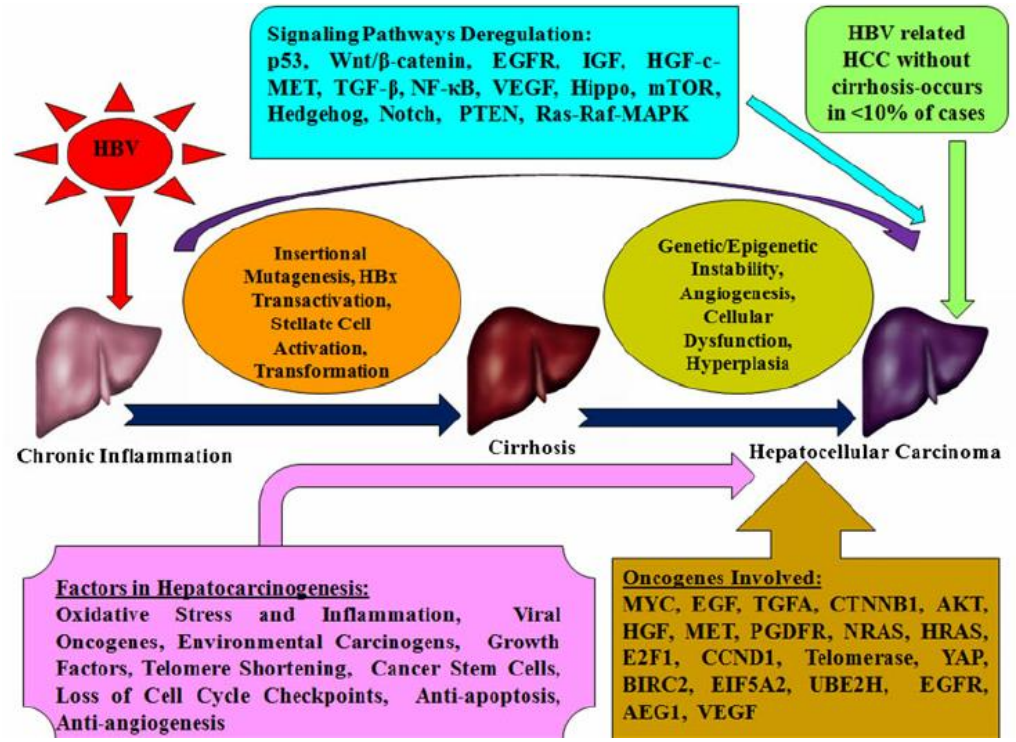

Figure 1: Oncogenetic pathways involving HBV and chronic infection.

HCV represents the other important infective risk factor for HCC; globally, about 71 million people worldwide are affected by HCV chronic infection, with an estimated number of HCV – related deaths of 399,000 / year, mainly due to decompensated liver cirrhosis and HCC development [21].

Traditionally, the HCV direct carcinogenetic potential has been underestimated when compared to HBV; recently, some studies have demonstrated that HCV can directly cause neoplastic transformation: recent researches revealed that the viral protein NS4B activates the expression of several members of the PKC superfamily, stimulates the

ERK/JNK signaling cascades, and represses SOCS3 expression, resulting in the activation of STAT3 by enhancing its phosphorylation. Activated STAT3 then stimulates MMP-2 and Bcl-2 expression, thereby resulting in deregulation of cell transformation and apoptosis [22].

Figure 2 shows carcinogenic mechanisms related to HCV chronic infection.

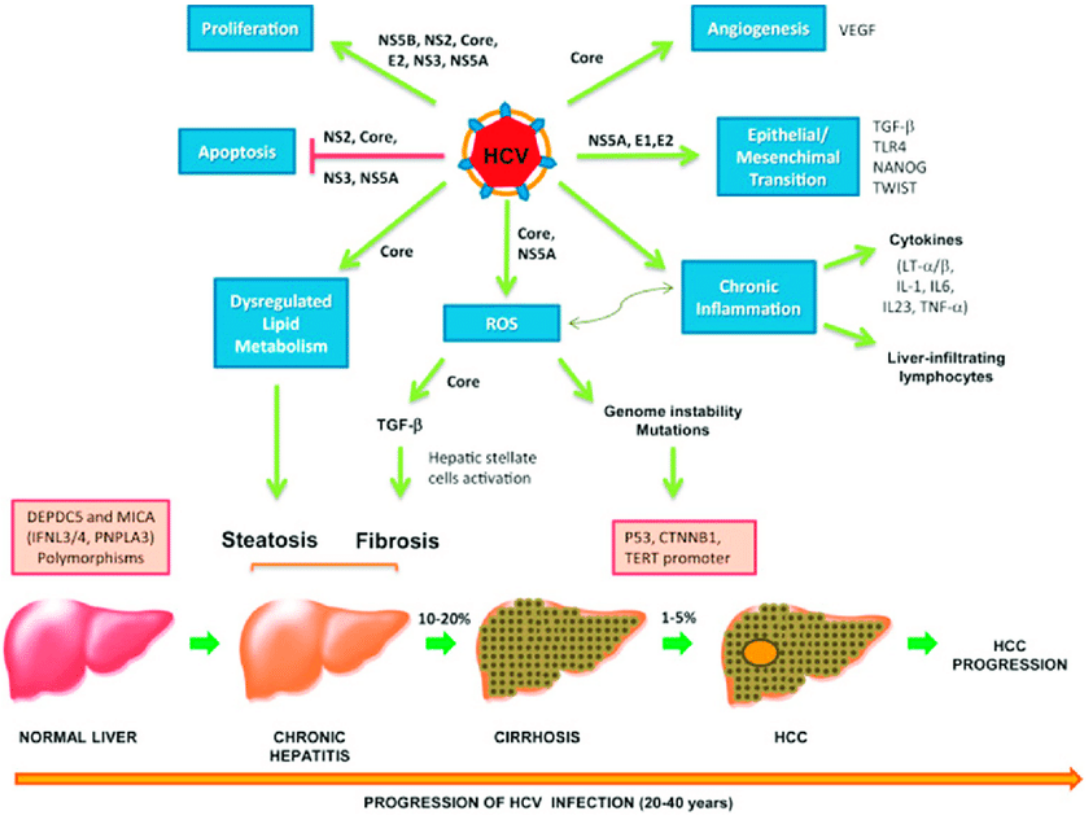

Figure 2: Biomolecular pathways involved in HCV chronic infection.

In the near future, the HCC burden related to HCV infection may strongly be reduced by the diffusion of new antiviral drugs [23].

Alcohol also may affect carcinogenesis by various mechanisms (figure 3); ethanol is oxidized to acetaldehyde by ADH, which acts as a carcinogen and binds to DNA. This metabolism is modified by polymorphisms or mutations in the gene encoding metabolizing enzymes. Acetaldehyde can form hybrid‐adducts with reactive residues (e.g., malondialdehyde adduct) acting on proteins, mediating lipid peroxidation and nucleic acid oxidation. Excessive alcohol consumption leads to the induction of CYP2E1 pathway and may indirectly contribute to acetaldehyde development and ROS production. Excessive alcohol enhances catabolism of retinoic acid by alcohol‐induced CYP2E1 [24].

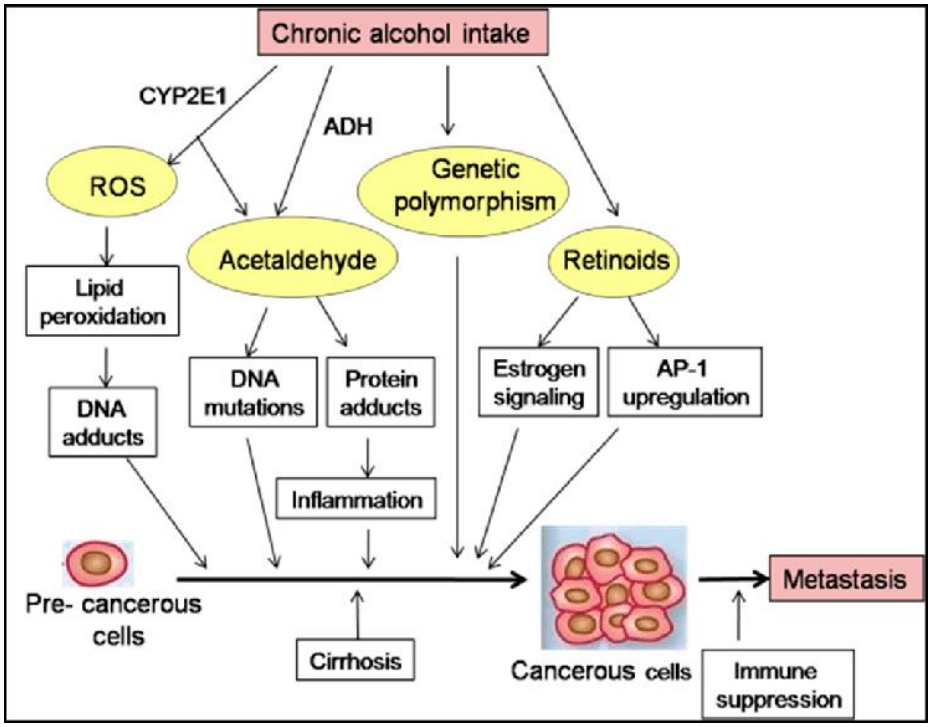

Figure 3: Mechanisms involved in carcinogenesis related to chronic alcohol abuse.

A recent study on animal models has demonstrated that HCC development in alcohol abuse is associated with marked increase of the hepatic expression of pro-inflammatory IL-17A and its receptor IL-17RA, IL-17A being a tumor promoting cytokine which critically regulates alcohol-induced hepatic steatosis, chronic inflammation, fibrosis, and cirrhosis [25].

Non - alcoholic fatty liver disease (NAFLD) is an emerging condition of chronic liver disease; the prevalence of this disease is rapidly increasing nowadays due to the spreading of obesity, type 2 diabetes and metabolic syndrome. NAFLD covers a large spectrum of clinical conditions, ranging from steatosis to aggressive Non - Alcoholic Steato – Hepatitis (NASH), that may lead to liver fibrosis and cirrhosis; NAFLD promotes carcinogenesis mainly through chronic liver parenchymal inflammation (Figure 49 [26].

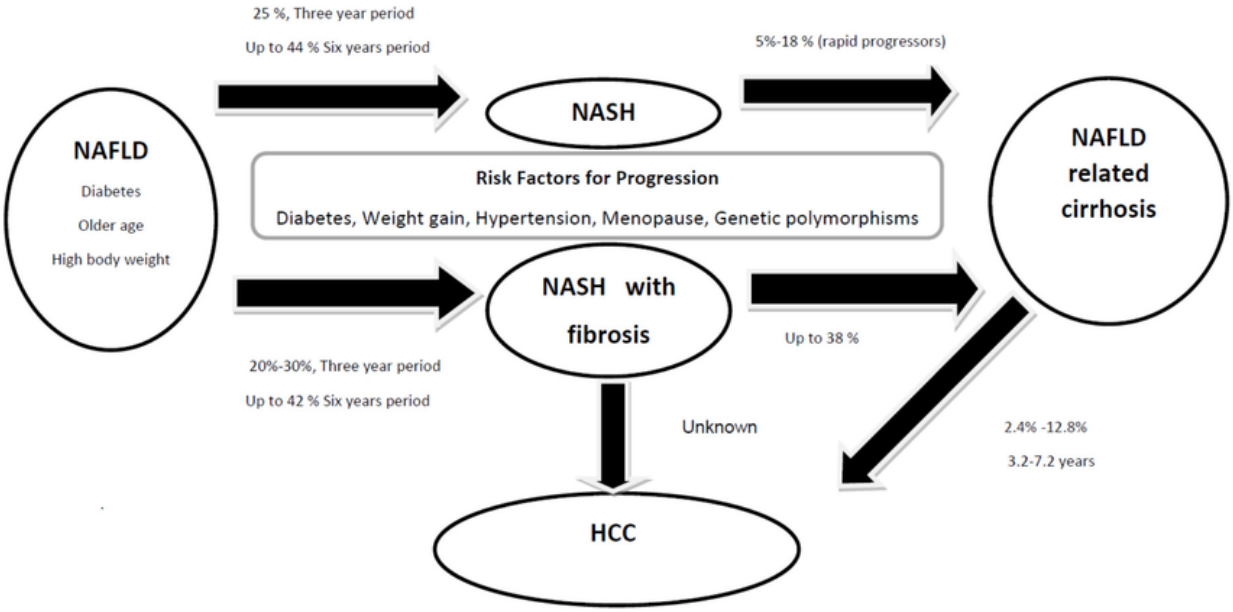

Figure 4: Progression from NAFLD to liver fibrosis, cirrhosis, and HCC.

Aflatoxin dietary exposure is a leading cause of HCC especially in low-income countries; Aflatoxins are mycotoxins produced by Aspergillus flavus and Aspergillus parasiticus.

Aflatoxin B1, a genotoxic hepatocarcinogen, may cause cancer by inducing DNA adducts, leading to genetic changes in target liver cells.

Aflatoxin B1 is metabolized by cytochrome - P450 enzymes to the reactive intermediate AFB1-8, 9 epoxide (AFBO) which binds to liver cell DNA, resulting in DNA adducts. DNA adducts interact with the guanine bases of liver cell DNA and cause a mutational effect in the P53 tumor suppressor gene at the codon 249 hotspot in exon 7, which may lead to HCC (Figure 5) [27].

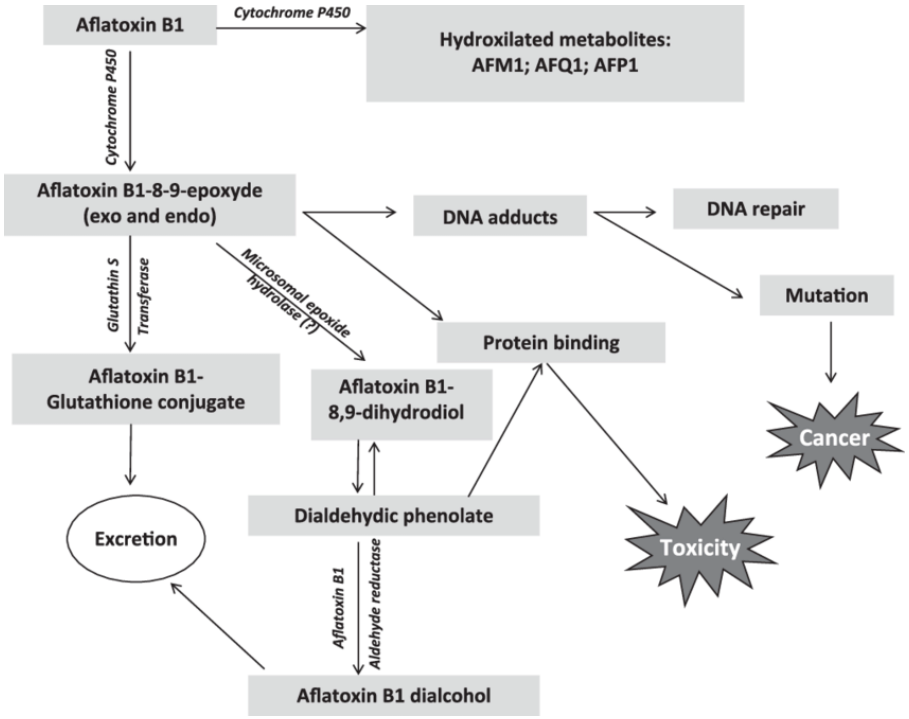

Figure 5: Aflatoxin B1 biotransformation pathway.

Histopathological aspects of Hepatocellular Carcinoma

Hepatocellular Carcinoma may appear as a single (figure 6) or multifocal encapsulated nodular lesion, usually in the context of a liver parenchyma showing micro or macronodular cirrhosis.

From a clinical point of view, HCC can be classified as peripheral (or subcapsular) or central. Peripheral nodes are usually easier to treat by surgical resection or ablation, but they may infiltrate the diaphragm of the abdominal wall; subcapsular tumors are exposed to the risk of spontaneous rupture, which is responsible for massive peritoneal bleeding or trans celomatic dissemination. Central nodes can infiltrate hilar structures such as the common bile duct, the hepatic artery, or the portal vein, thus being technically unresectable.

Histopathologically, HCC can be described as a well vascularized tumor with wide trabeculae, acinar pattern, small cell changes, cytological atypia, mitotic activity, vascular invasion, absence of Kupffer cells and the loss of the reticulin network. The most common histologic growth patterns are: trabecular-resembling normal liver tissue, pseudo - glandular or acinar with possible bile or fibrin content and a compact or solid pattern. Bile production is common. The histopathological characteristics of HCC are shown in figure 7.

Histological differentiation is usually expressed by 4 grades (G1 – well differentiated, G2 – moderately differentiated, G3 – poorly differentiated, G4 – completely undifferentiated. G1 – HCC could be difficult to diagnose due to the similarity with regeneration nodes.

Trabecular HCC is the most common primitive liver neoplastic lesion; however, there are some rare histopathological variants, such as fibrolamellar, sarcomatous, and scirrous, “clear cells” with lymphoid stroma [28,29].

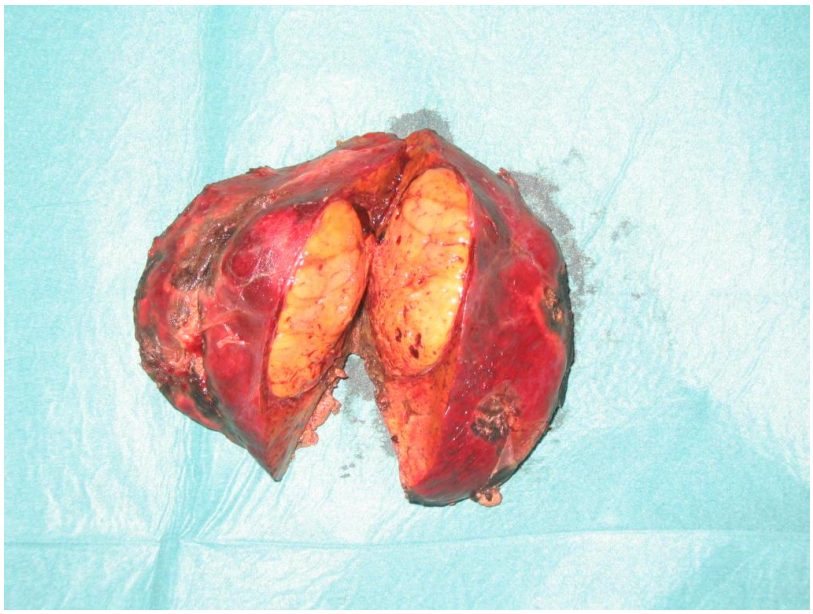

Figure 6: Macroscopic aspect of a hepatocellular carcinoma located on segment 6

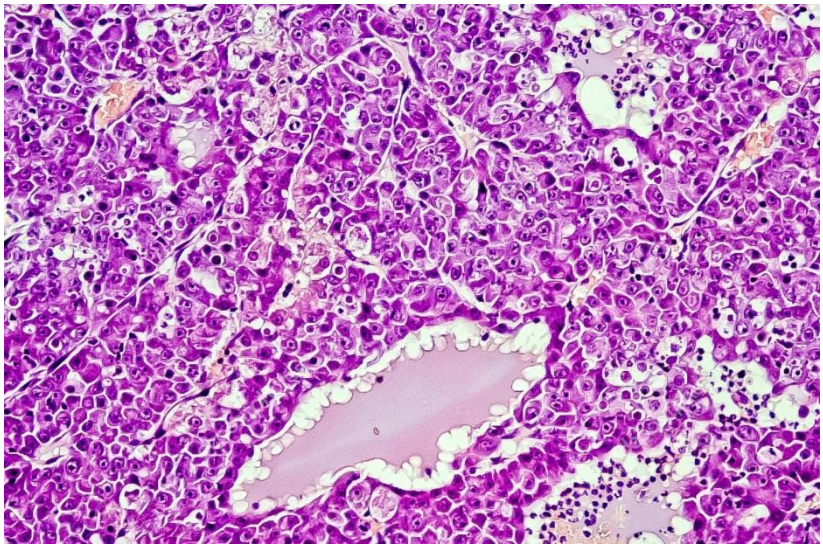

Figure 7: Microscopic aspects of trabecular hepatocellular carcinoma

Physiopathology and evolution of HCC

Hepatocarcinogenesis appears to be a complex and multistep process that usually occurs in a context of chronic liver inflammation. The natural history of HCC follows a sequence of events starting with the

development of regenerative nodules with low grade dysplasia, followed by high grade dysplasia, early - stage HCC and advanced stage HCC [30].

HCC is the result of the accumulation of somatic genomic alterations in passenger and driver cancer genes [31]. In each tumor nodule a mean number of 40 functional somatic alterations are accumulated in coding regions, so each tumor is the result of a unique combination of genetic alterations combined with epigenetic modifications [32]. In any case, genomic alterations are not accumulated randomly, thus suggesting the fact that several specific pathways, likely to be related to risk factors, can cooperate in hepatocarcinogenesis [33].

The process of hepatocyte malignant transformation includes Wnt/β-catenin pathway activation, re – expression of fetal genes, deregulation of protein folding machinery and response to the oxidative stress. Telomere maintenance and telomerase complex controlling the repeated sequence TTAGGG, play a key role in hepatocarcinogenesis in cirrhotic livers [34].

It is well known that constitutive inactivation of the TERT gene (which encodes the Telomerase Reverse Transcriptase), is associated with a high risk of developing cirrhosis [35].

Progression to HCC requires a second step, with telomerase reactivation in order to promote liver carcinogenesis and tumor proliferation (the so – called telomerase switch [36]).

In normal liver tissue, TERT is not expressed; TERT results as encoded in about 6% of low - grade dysplastic nodes, 20% of high - grade dysplastic nodes and 60% of HCC [37]. TERT mutations appears to be pro – oncogenetic, and in most HCC cases (more than 90%), telomerase activation is selected during malignant transformation and tumor progression [38].

TERT promoter activation is required at an early step of carcinogenesis in order to bypass the replicative senescence of cirrhotic hepatocytes. On the contrary, acquisition of genomic diversity appears to be a late event in tumor development [39].

Chronic viral infections seem to provide early mutations involved in malignant transformation. In the case of HBV, DNA insertion in hepatocytes occur within the TERT promoter and activate telomerase and other oncogenes including lysine (k) – specific methyltransferase 2B (KMT2B), cyclin E1 and SUMO1/sentrin specific peptidase 5 (SENP5) [40].

Several pathways have been recognized regarding tumor progression.

- *Telomere maintenance:* Recognized in about 90% of HCC, it contributes to the evasion of cellular senescence [37].
- *Wnt/β- catenin pathway:* It is frequently activated in HCC through CTNNB1 activation; it contributes to carcinogenesis in 11 – 37% of cases and is often related to HBV infection [41].

- *Inactivation of p53 and alteration of cell cycle:* It is more frequent in HCC related to Aflatoxin exposure and contributes to a worse prognosis [42,43].
- *Alteration of chromatin remodeling complexes:* These alterations include mutations in the BRG1 or HRBM – associated factors (BAF) and other pathways influencing DNA methylation [44].
- *Activation of the Ras/Raf/MAP and PI3K/AKT-mTOR pathways:* These abnormalities are caused by the amplification of a region that includes fibroblast growth factor 3, 4 and 19 (about 5% of HCC) [36].
- *Oxidative stress pathway*: It is constitutively activated by the activation of the nuclear factor erythroid 2 – related factor 2 (NFE2L2) or by the inactivation of kelch - like ECH – associated protein 1 (KEAOP1), in about 5 – 15% of HCC cases [45].

DNA amplifications are also recognized in HCC development, with the most common high - level amplifications sited on chromosomal regions 11q13 and 6p21; in particular, amplification of 6p21 leads to an over expression of Vascular Endothelial Growth Factor A [46].

Some genomic studies have revealed the presence of HCC molecular subclasses; two main groups, each comprehending about 50% of cases, have been identified: proliferative and non - proliferative HCC; the proliferative subclass usually presents the activation of Ras, mTOR, insulin – like growth factor (IGF) signaling and FGF19 amplification, and

it is usually associated with HBV etiology and poor prognosis [47]. The non-proliferative subclass is more heterogeneous, but often characterized by CTNNB1 mutations; it is usually related to alcohol abuse and HCV infection [48].

Diagnosis

Hepatocellular carcinoma, especially in its typical trabecular form, is usually incidentally diagnosed at routine follow up ultrasonography in patients affected by chronic hepatitis or cirrhosis. Elevated levels of Alpha-fetoprotein (AFP) could be considered as an effective biomarker for diagnosis of HCC [49]; however, more than 30% of patients affected by hepatocellular carcinoma, even in an advanced stage, present low AFP serum levels [50]; An AFP > 400ng/mL is currently considered diagnostic for HCC, but less than 50% of cases reach this concentration [51]. For the above cited reasons, AFP specificity results close to 100%, but sensitivity falls below 45%, and the positive predictive value ranges from 9% to 32% [52]. The AFP serum dosage alone is not taken into consideration in common clinical practice. Accuracy improves in the diagnosis of tumor recurrence after treatment, but only in patients affected by AFP producing tumors [53].

Ultrasonography can be taken into consideration as first step diagnostic tool for HCC; typically, HCC appears as a hypoechoic, well defined or

infiltrative nodular lesion; ultrasonography accuracy does not reach high levels because of the difficulties in distinguishing small HCC from regenerative nodes in cirrhotic liver.

A CT scan with multiphasic contrast imaging of the liver is to be considered the most important second level imaging in the case of suspected - HCC; HCC radiological aspects at triphasic hepato – specific CT usually detects a well-defined node with rapid arterial contrast enhancement and rapid dismission at the venous phase [54].

An MRI uses the same concepts as the CT scan for diagnosis of HCC; its accuracy is similar to a CT, but is less accurate for HCC nodes with a diameter of less than two centimeters.

Liver biopsy was always used in the past to confirm the malignancy; nowadays histological confirmation by a percutaneous biopsy is not necessary in the presence of a liver node with typical radiological behavior in a patient affected by liver cirrhosis or chronic liver disease. Liver biopsy remains mandatory in cases of uncertain radiological diagnosis and in young patients not affected by liver cirrhosis [54].

Therapeutic strategies

HCC prognosis and indications for treatment are based upon conventional staging systems such as Barcelona Clinic Liver Cancer (BCLC), Hong Kong Liver Cancer and liver function (figure 8). Current radical

therapeutic procedures include transplantation, surgical resection and ablation with radiofrequency or microwave energy. Current palliative therapies for advanced hepatocellular carcinoma are chemoembolization, radioembolization and MAP kinase inhibitors (Sorafenib) [55].

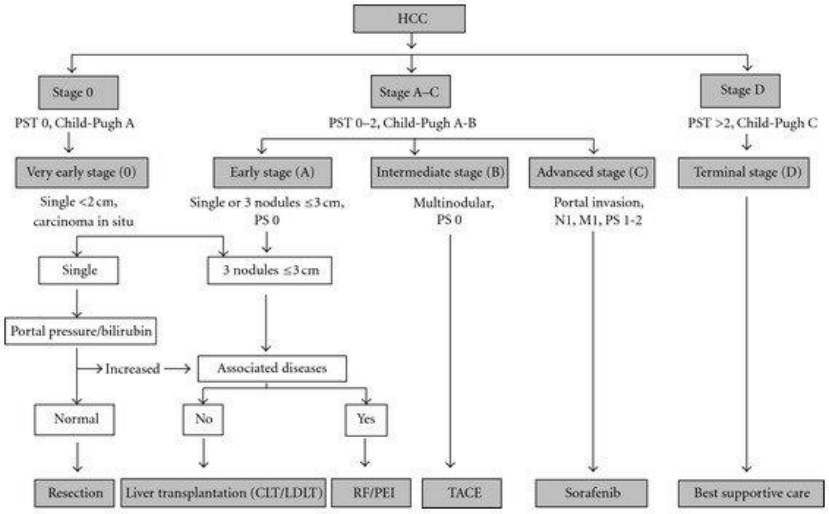

Figure 8: BCLC flow chart for HCC treatment

Liver transplantation for HCC

Orthotopic liver transplantation (OLT) is the only treatment option available for radically removing both the tumor and the main risk factor for recurrence, i.e., the cirrhotic liver. In any case, the admittance of patients with HCC to the waiting list for OLT is restricted, due to the limited resources and the risk of tumoral recurrence in the transplanted liver; the risk increases with the size and number of neoplastic lesions in the explanted liver. Various criteria have been established in order to reduce recurrence and provide a survival rate comparable to that of

patients transplanted for cirrhosis without HCC [56]. The most widely adopted are the Milan Criteria, consisting in the presence of a single tumor < 5cm in diameter, or up to three tumors with none exceeding 3cm in diameter [57, 58].

The most critical problem for patients awaiting OLT for HCC is tumor progression, leading to the overcome of criteria; studies evaluating the doubling time of HCC and its natural story without treatments suggest that about 25 - 45% of the patients will develop tumor related contraindications to OLT during the first 12 months of waiting; the presence of multiple nodes or of a single node > 3cm in diameter, and a previous liver resection for HCC are predictors of drop - out [59].

Patients adequately selected for transplantation show excellent outcomes (5 years survival up to 75%), hence the need of a short waiting list in order to treat patients before exclusion due to tumor progression [60]. Since the waiting time is increasing everywhere and has become longer than 10 - 12 months in most European and American centers, it is mandatory to seek an effective strategy to prioritize cirrhotic patients with HCC or to maintain these patients within the criteria for transplantation while waiting for OLT. To this end, several form of adjuvant strategies have been tested as a bridge to transplantation (liver resection, trans - arterial chemoembolization, percutaneous ablation); each of them show

interesting results, but also risks of liver decompensation and or tumor seeding [61, 62].

Liver resection

The best treatment strategy for small HCC in patients with preserved liver function is controversial. Studies have shown superior survival results after transplantation compared to resection, especially in terms of disease - free survival [63]. However, other experiences have demonstrated similar overall survival rates after resection and transplantation for small HCC, and hence have concluded that resection rather than transplantation should be the first - line treatment for small HCC in patients with preserved liver function [64]; moreover, the donor organ shortage has been a major deterrent to the use of liver transplantation for resectable HCC [65].

Cirrhosis is a known risk factor for a higher incidence of postoperative complications, including bile leak, liver failure and death. When considering hepatectomy for treatment of HCC, consideration of baseline liver function is crucial. Resection should not only follow general oncologic principles (R0), but it must also be performed in a way to maximize recovery, minimize morbidity and mortality, and preserve adequate liver function. Limiting the extent of resection based on tumor location and with segment - oriented procedures can allow for a safe

hepatectomy in patients who otherwise would not tolerate extended hepatectomies [66].

In selected patients with recurrence after liver resection, OLT can be performed as a rescue treatment with satisfactory outcomes [67].

Thermal ablation

HCC ablation is currently accepted as a curative option in many HCC treatment guidelines due to its excellent outcomes performed in less invasive manner [68].

Recent EASL - EROTC guidelines recently recommend ablation as a first line operation for very early stage (single < 2cm) HCC rather than surgical resection [69].

From a technical point of view, complete and accurate ablation is essential to achieve the best outcomes. Different energy sources are currently used. Microwave ablation has recently gained popularity around the world because of its intrinsic advantages of faster ablation and lower susceptibility to the heat sink effect (heat dispersion when energy is applied near a blood vessel), compared to radiofrequency. These days, alcohol ablation has been largely abandoned, due to the unsatisfactory results and the risk of toxicity, except for when treating extremely small single nodes (< 1cm). Ablation can be achieved by percutaneous radiological guided probe placement. Laparoscopy can be very useful in

cases of HCC found in difficult anatomical sites (subphrenic, central) [70].

Trans - Arterial Chemoembolization (TACE) and Trans Arterial Radioembolization (TARE)

Since the early years of 2000, TACE has become the standard of care therapy for patients with intermediate stage HCC, with a proven survival benefit [71].

The rationale for TACE is that HCC is primarily nourished by branches of the hepatic artery, so complete tumor ischemia can be potentially achieved, with higher drug concentration within the target area [72, 73].

Patients affected by intermediate stage HCC (BCLC B), not candidates for curative strategies, with well-preserved liver function, are currently the best targets for TACE; median survival rate is about 47.7 months [74].

The TACE approach can also be taken into consideration in cases of patients with early - stage HCC with nodes which are centrally located or larger than 3cm and who are not candidates for ablation due to the high risk of bile duct or vessels injury. The 5 - year survival rate is 81%, not significantly different from patients treated with surgical resection or ablation [75]. TACE can also be combined with Sorafenib administration in patients with advanced HCC, with advantages in term of survival [76].

TARE employs the same rationale of TACE, with intra-arterial injection of Y90 glass microspheres. The anti - tumoral effect of Y90 is thought to be secondary to irreversible damage to tumor epithelial and to stromal and endothelial cells [77]. Current clinical indications for Y90 TARE include patients with advanced HCC (BCLC C) due to partial or branch portal vein thrombosis, patients with intermediate HCC (BCLC B) who are poor candidates for TACE due to large tumor size, multifocal disease and advanced age, patients who did not respond to prior TACE and patients who are not eligible for potentially curative treatments but could become eligible as a result of a reduction in tumor burden or downstaging of the disease [78].

Systemic treatment of advanced stage HCC

Sorafenib, a Ras Kinase inhibitor, is the most employed targeted drug for patients with advanced stage HCC [79]. Sorafenib suppresses tumor cell proliferation by inhibiting Raf-1, B-Raf and kinase activity in the Ras/Raf/MEK/ERK signaling pathways. In addition, Sorafenib is capable of targeting platelet - derived growth factor receptor, vascular endothelial growth factor 2, hepatocyte factor receptor (c-KIT), and other proteins to inhibit tumor angiogenesis. Several studies confirm the efficacy of Sorafenib as a first line treatment for advanced HCC stages. Unfortunately, only approximately 30% of patients can benefit from

Sorafenib, and this population usually acquires drug resistance within 6 months. Adverse events identified in patients administered Sorafenib mainly include gastrointestinal or skin disease (e.g., hand and foot skin reaction, weight loss, diarrhea); in serious cases, Sorafenib can cause abdominal pain and hypertension [80]. The tumor microenvironment plays a crucial role in Sorafenib resistance, mainly by regulating cell stemness, mesenchymal state and epigenetic regulations [79]. Hence the employment of second line target drugs (Regorafenib), or Immune checkpoint inhibitors (Pembrolizumab) [81].

Tumour - matrix interactions in the development of HCC

Most of the patients affected by HCC suffer from an underlying chronic liver disease and chronic liver damage caused by persistent inflammation associated with deregulated growth of hepatocytes, often resulting in the formation of regenerative nodules, dysplastic nodes and eventually HCC [82,83]. The available data demonstrate that hepatocytes deriving from a cirrhotic liver present a cellular phenotype that indicates a switch from MAPK-independent to MAPK-dependent cell survival signalling. These cells also show an increased expression of markers of mesenchymal cells such as Vimentin and Type 1 Collagen, indicating the so - called “epithelial-mesenchymal transition”, a process in which epithelium loses its phenotypic characteristics and acquires features typical of

mesenchymal cells [84,85]. Epithelial-mesenchymal transition is a feature of embryogenesis and adult tissue repair after damage, and it is thought to be a critical connection between inflammation and progression toward liver cirrhosis and HCC [86]. In this setting, the tumour micro-environment plays a very important role in the development and progression of HCC by modulating not only epithelial-mesenchymal transition, but also tumour invasion and metastasis. The HCC microenvironment consists of a variety of cells, including liver stellate cells (HSC), fibroblasts, Kupffer cells, vascular endothelial cells, and immune cells, among which there are lymphocytes, natural killer (NK) and NKT cells [87].

In normal livers, Hepatic Stellate Cells (HSCs), also known as Ito cells or perisinusoidal cells, are responsible for the turn-over of the extracellular matrix. In response to hepatic damage and inflammation, HSCs undergo activation, acquiring myofibroblast phenotype and producing large amounts of extracellular matrix [88]. HSCs are activated by several stimuli, including cytokines such as Platelets Derived Growth Factor (PDGF), Tumour Growth Factor β1 (TGF-β1), metalloproteases, Insulin-Like growth factor binding protein 5, cathepsins B and D, HBV X protein, and HCV non-structural proteins [88]. Activated HSCs are the main cause of the development of liver fibrosis during chronic hepatic injury [88]. Furthermore, activated HSCs also infiltrate the stroma of liver

tumours and localize around tumour sinusoids, fibrous septa, and capsule, thus playing an important role in HCC development [83,89]. In fact, activated HSCs can release a variety of cytokines, chemokines, and growth factors, including hepatocyte growth factor HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) and interleukin-1 (IL-1), which are capable of mediating cancer cell survival, proliferation, and migration [90]. Activated HCS are often indistinguishable from cancer associated fibroblasts (CAF), which are the most important cells of neoplastic stroma in many tumours. In the case of CAF, HCC cells stimulate the proliferation of activated HSCs, thus suggesting a complex tumour - stromal interaction [91]. Among the factors responsible for sustaining activated HSC and CAF functions in HCC, TGF- plays a key role. TGF-β1 is released in the extracellular matrix in a latent form that is activated by Matrix Metallo - Proteases 2 or 9 (MMP-2, MMP-9), which are richly expressed in the tumour microenvironment [92]. By binding to TGF receptor II, TGF-β1 activates downstream signalling involving Smad-2 and Smad-3 transcription factors one, which induce HSC proliferation and sustain collagen production [93]. TGF-β1 normally acts as a tumour suppressor in pre-malignant conditions, through the inhibition of hepatocyte proliferation and by favouring apoptosis. This anti-oncogenic effect may change into pro-oncogenic potential by several mechanisms. For instance, during chronic viral infection, HBx and HCV

can shift liver TGF-β1 signalling from the tumour-suppressive pSmad3C pathway to the pro-oncogenic pSmadL3 pathway. Thus, in the context of a primitive neoplastic liver node, TGF-β1 can increase migration, vascular invasion, angiogenesis, tumour - stromal cross talk and the risk of metastasis [94].

HCC usually develops in livers affected by chronic flogosis. Inflammation drives a continuous reparative reaction and stimulates liver cell death and regeneration, eventually associated with the development of dysplastic nodules and cancer. HCC associated with persistent production of Th2 - like cytokines (IL-4, IL-8, IL-10, and IL-5) usually presents higher aggressiveness and metastatic phenotype compared to tumours secreting higher levels of Th1-like cytokines (IL-1α, IL-1β, IL-2, TNFα) [95]. A further important aspect of the interaction between matrix and tumour involves the production of IL-6 by Kupffer Cells, which represents an important stimulus for hepatocyte survival and proliferation and favours the development of HCC from regenerative and dysplastic nodes [53]. Moreover, IL-6 elevation in the serum is related to a poor prognosis [96] while suppression of HGF and IL-6 by oestrogens represses HCC metastasis [97]. Besides, IL-6, IL-10 and IL-22 are over expressed in HCC tumour stroma, and the upregulation of these cytokines leads to cancer growth, inhibition of apoptosis and promotion of metastasis [98].

High levels of IL-10 are also associated with a high risk of progression after HCC radical surgical treatment [99].

Along with inflammation, immune responses in the tumour microenvironment are important in HCC development and progression [100]. T - lymphocytes are well evident within HCC matrix and unlike adjacent peritumoral tissue $CD4^+/CD25^+$ regulatory T cells (Tregs), they predominate over cytotoxic $CD8^+$ T Cells in the tumour environment. The excess of Tregs impairs $CD8^+$ T cell proliferation as well as their cytotoxic action. In line with this, low $CD8^+$ T cells and high representation of regulatory T cells are related to worse prognosis [100]. Besides the immunosuppressive action of Tregs, the HCC tumour microenvironment can contribute to the evasion of the immune response through TGF-β mediated impairment of $CD8^+$ T and an enhanced expression of programmed death ligand-1 (PDL-1) by Kupffer cells [100]. In fact, the interaction of PD-L1 with PD1 in $CD8^+$ T cells impairs cytotoxic $CD8^+$ T cell function in human HCC. Conversely, blocking the interaction between PD-L1 on Kupffer cells and PD1 on $CD8^+$ T cells restores cytotoxic $CD8^+$ T cell function [101].

A key aspect in tumour-stroma interactions is represented by angiogenesis, which plays a very important role in hepatocarcinogenesis from the early stages. Chronic liver diseases stimulate neo-angiogenesis within the liver parenchyma, and the progression to fibrosis and cirrhosis

increases the secretion of MMP, PDGF, TGFβ-1, FGF and Vascular Endothelial Growth Factor (VEGF), which are all pro-angiogenetic factors, while the deposition of extracellular matrix and the anatomical alterations occurring during the fibrogenic process cause resistance to blood flow and affect oxygen exchanges, thus favouring hypoxia [102]. The Vascular Endothelial Growth Factor (VEGF) is to be considered the most critical pro-angiogenetic factor, already expressed in dysplastic nodules, and further increasing in HCC carcinogenesis. VEGF promotes endothelial cell replication and migration [103], and its expression correlates with HCC aggressiveness [104]. VEGF also promotes the proliferation of cancer cells by expressing the VEGF-A receptor through downstream Akt/mTOR signalling [105]. Fibroblast Growth Factor (FGF), a member of heparin-binding growth factors, acts synergistically with VEGF to induce angiogenesis, while PDGF is involved in cell migration and new vessel maturation. HCC cells secrete PDGF by a paracrine mechanism involving endothelial cells and fibroblasts, and PDGF secretion correlates with cancer progression [106]. Interestingly, vascular endothelial cells associated with HCC differentiate from the sinusoidal endothelial present in healthy liver, in that they show rapid cell turn over, enhanced motility, migration, and high expression of CD105 and TGF-β1. Notably, TGF-β1 is a specific chemo-attractant for CD105 expressing endothelial cells and thus promotes tumour angiogenesis [107].

Even though HCC is highly vascularized, the tumour environment is often hypoxic. The reduced availability of oxygen induces the expression of hypoxia inducible factor-1 (HIF-1), a transcriptional factor responsible for regulating the expression of genes critical in angiogenesis, cell survival, invasion and metastatization. High levels of HIF-1 in HCC are also related to a poor prognosis [108].

Tenascin C in tumour matrix interactions

As outlined above, the tumour microenvironment plays a crucial role in cancer development, local progression and metastatization [109,110]. Regarding this, several studies have investigated the role of specific extracellular matrix proteins in neoplastic diseases [111,112]. Among the extracellular matrix proteins that have received attention in recent years, Tenascin (TNC) deserves special consideration. TNC is an extracellular matrix glycoprotein with a hexameric structure that belongs to the Tenascin family (figure 9). Each of the monomers are made of four domains: a) a cysteine rich N-terminus TNC assembly domain; b) epithelial growth factor EGF-like repeat domain; (is the term EGF - like repeat domain or repetition domain?) c) Fibronectin-type III domain capable of alternative splicing; d) a calcium rich COOH fibrinogen globe. These domains have distinct functions and binding affinity for several cell surface receptors, extracellular matrix proteins and glycolipids.

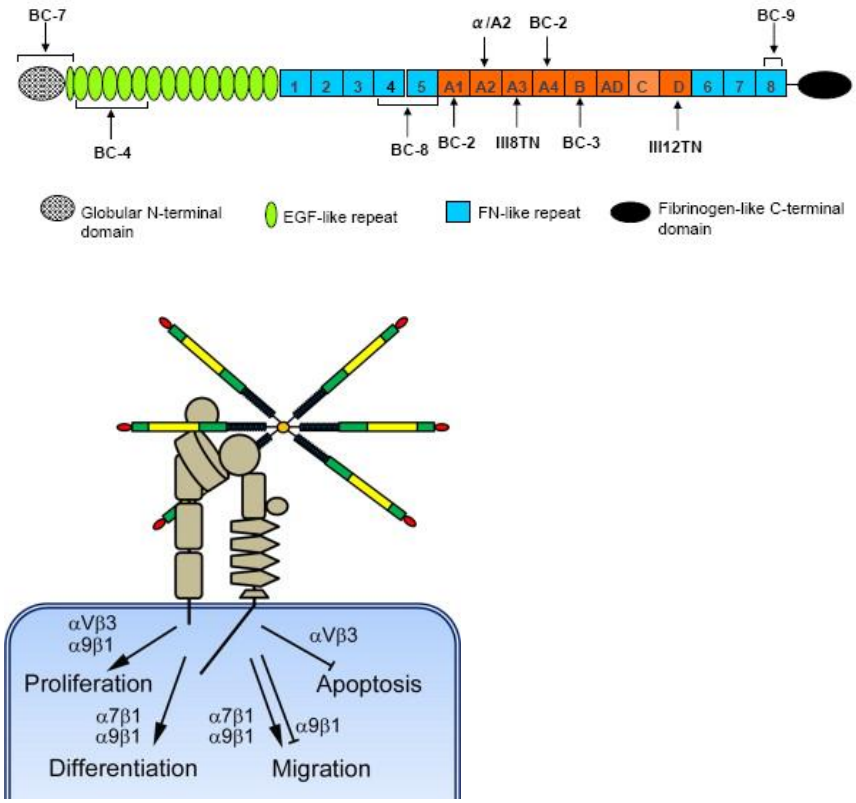

Figure 9: TNC structure and functions

TNC could be considered as the founding member of the Tenascin gene family. All Tenascins show a similar ultrastructural architecture, consisting of heptad repetitions next to each chain N-terminus, which supports trimerization. Two TNC trimers can then join to generate a hexabrachion [113]. From the functional point of view, TNC facilitates cell interactions with the extracellular matrix and many growth factors, thus regulating cell adhesion, differentiation, migration, and survival [114]. In line with these findings, TNC knockout mice show abnormalities in brain cytoarchitecture, in organ morphogenesis and in trauma response. In vitro cell behaviour is also influenced by TNC, which

influences the cell spreading and signalling mediated by fibronectin and integrins [115].

TNC can be found in several isoforms generated by modifications at both transcriptional and post-translational levels. In human TNC, eight of the Fibronectin-type III (FNIII) repeats are always present, but the ninth FNIII repeat located between FNIII 5 and FNIII 6 are subject to alternative splicing, thus potentially originating 511 different isoforms [116]. Furthermore, TNC also contains several glycosylation sites and glycosylated, which are important for functional activities [117]. TNC can also be modified post-translationally through the conversion of arginine residues to citrulline. So far, five different sites of citrullination have been identified [118]. It is noteworthy that citrullination increases the immunogenicity of the C-terminal residues of TNC, leading to the generation of autoantibodies. These autoantibodies are detectable in patients affected by rheumatoid arthritis, and their presence is an independent predictor of clinical outcome [119].

TNC is widely present in bone, cartilage, and tendons and in the central nervous system of embryos, however, its expression in adult tissues is limited to stem cell niches and tendons [113]. In adult tissue, TNC is transiently increased following tissue injury and mediates the inflammatory and the fibrotic responses necessary for tissue repair. For instance, TNC is over - expressed after liver resection, and it has a

predictive role in liver failure after resection in experimental models and in patients treated with hepatectomy [120]. TNC increase is also evident in other pathological conditions causing local and generalized inflammation, such as systemic sepsis, asthma, autoimmune diseases, and inflammatory bowel disease. A recent study has shown that high serum TNC levels are significantly associated with the severity of clinical conditions and predict low survival in patients affected by systemic sepsis [121]. A further aspect of TNC involvement in human disease is that it is also abundant in the stroma of solid tumours [113].

Role of TNC in cancer development, progression and metastatization

TNC is highly expressed in many epithelial tumours which are mainly produced by stromal cells, including myofibroblasts and vascular endothelial cells. TNC expressed in cancer tissues often involves large splice variants that appears to be partially related to the tumour origins. Nonetheless, TNC can also be produced by parenchymal cancer cells both in spontaneous tumours and in cell line form, from melanomas, colon and breast cancer. In the latter, TNC expression correlates with the capacity of producing lung metastasis by surrounding epithelial tumour cell aggregates during blood vessel invasion and by promoting extravasation of tumour cells from pulmonary blood vessels into the lung parenchyma [122]. Consistently, TNC knockdown in melanoma cells inhibits their

ability to colonize the lungs while it does not significantly affect the growth of subcutaneous tumours [123]. Functional studies using mouse models have confirmed that TNC contributes to cancer progression by supporting cell proliferation, epithelial-mesenchymal transition, and cell migration, underscoring its contribution to the development of metastasis [124]. In fact, TNC has a pleiotropic role in the metastatic process by promoting migratory and invasive cell behaviour, angiogenesis, and cancer cell viability under stress. Furthermore, TNC is an essential component of the metastatic niche and modulates stem cell-mediated signals within the niche [124]. This may be crucial for the survival of disseminated cancer cells confronted with a foreign environment in secondary organs, which can exert a strong selective pressure on invading cells. Figure 10 shows the metastatic pathway of HCC. In line with these findings, clinical evidence indicates that TNC expression is associated with poor clinical outcome for cancer patients. Histology of tissue sections from breast cancers indicates that TNC expression is often prevalent at the tumour edge, which predicts exceedingly poor overall survival. Similarly, autocrine TNC expression in cancer cells predicts poor overall survival in colon cancer patients [124].

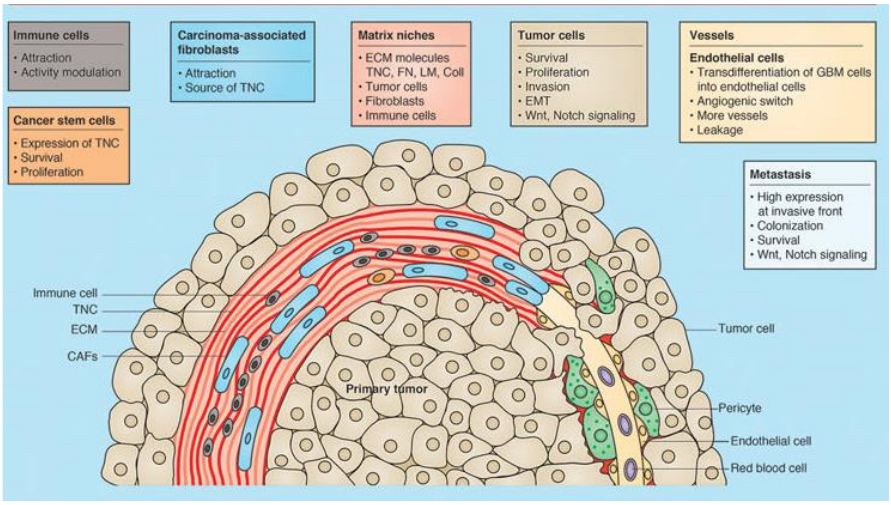

Figure 10: metastatic pathway of HCC.

So far, few studies have addressed the involvement of TNC in HCC development. Available data indicate that TNC is up-regulated in chronic hepatitis and cirrhotic livers, where it is detectable at the interface between parenchyma and mesenchyme, especially in the regions of piecemeal necrosis [125,126], with HSCs being the primary cellular source of TNC [127]. Conversely, TNC deficiency attenuates development of fibrosis [127]. Along with that, TNC concentration is also increased in the plasma of patients affected by cirrhosis and further increased in cases of HCC [128]. Consistently, immunohistochemistry shows that TNC reactivity is generally stronger in HCCs than in cirrhosis [129,130]. Furthermore, TNC expression is higher in metastatic than in the non-metastatic HCC tissues and high TNC staining has been associated with lower survival rate in patients with advanced HCC [131]. Along this line, Benbow and co-workers [128] have reported that an

enhanced TNC production by hepatic stellate cells accompanies hepatic inflammation in an animal model of HCC associated with diet-induced obesity, and that TNC stimulates hepatic inflammation by interacting with Toll-like receptors 4 in myeloid cells. On the other hand, pro - inflammatory cytokines such as TNF-α can stimulate in vitro TNC production by hepatoma cells, thus suggesting a complex interplay between parenchyma and matrix in regulating TNC production in HCCs. Altogether, these data indicate the TNC can be an important player in the tumour-matrix interactions occurring during hepatic carcinogenesis, and they suggest the possibility that TNC expression in HCC might represent a possible marker for detecting patients at risk of rapid tumour evolution [131].

STUDY PRESENTATION

Aim of the work

Although several experimental studies have demonstrated the involvement of TNC in the processes associated with cancerogenesis and metastasis dissemination, the clinical prognostic value of TNC in different types of tumours continues to remain controversial.

This is also the case of HCC. In fact, the over-expression of TNC in liver tumours is as well established as it is in chronic liver disease and fibrosis. Nonetheless, so far only one study has addressed the prognostic values of TNC expression in advanced HCC and related to an increased risk of metastasis [131]. Thus, although HCC is one of the tumours in which the association between chronic inflammation and cancer development and progression is well established [130], relatively little is known about the involvement of TNC in non-metastatic early stage HCCs.

From this background, the present retrospective study aim is to evaluate the expression pattern of TNC in specimens coming from surgical radical resection of very early or early stage HCCs, in order to highlight potential correlations with different clinical outcomes.

Patients and methods

By scanning an electronic database including all adult subjects who were consecutively diagnosed with primitive liver cancer from January 01, 2003 to October 31, 2019 at an Academic Center in Northern Italy (Novara), we retrieved data from 286 patients' records.

78 patients underwent surgical radical treatment and entered a regular follow – up.

Inclusion criteria for the present study have been the followings:

- age > 18 years;
- histological definitive diagnosis of primitive hepatocellular carcinoma;
- radical liver surgery with negative resection margins;
- adequate material for histological and immunohistochemical evaluation;
- regular follow – up.

34 patients have been excluded, 21 because of inadequate histological material, 13 because of histological postoperative diagnosis of cholangiocarcinoma or metastatic tumor.

44 patients (35 males, 79.55%; 9 females, 20.45%) have been included in the present study.

The median age was 71 years (range 38 – 87 years); 27 patients (61.36%) were affected by chronic hepatitis C virus infection, 7 (15.91%) by alcoholic liver disease, 5 (11.36%) by non-alcoholic fatty liver disease, 5 (11.36%) by chronic hepatitis B virus infection.

The median tumor size was 39mm (range 13 – 70mm).

22 patients (50%) underwent minor hepatectomy (including wedge resection, segmentectomy, bisegementectomy); major hepatectomy (trisegmentectomy, left or right hepatectomy) was performed in the remaining 22 cases.

Preoperative management

All patients have been referred to our Surgical Department after the detection of an HCC node during the regular follow up for chronic liver disease or cirrhosis. No cases of fibro – lamellar hepatocellular carcinoma in healthy liver have been included in the study.

All patients with suspected HCC at ultrasonography underwent triphasic CT abdominal CT scan; tumor staging was completed with a thorax CT scan.

Tru – Cut needle percutaneous liver biopsy was necessary only in 9 cases (20.45%) because of an atypical radiological behavior of the liver mass at CT scan.

Preoperative liver function was assessed by calculating Child – Pugh and MELD scores; all patients underwent noninvasive research of signs of portal hypertension (upper gastrointestinal tract endoscopy, platelet counts, spleen diameter measurement).

Patients' general conditions were evaluated with the American Society of Anesthesiologists (ASA) score.

Surgical procedure

Patients were admitted to the hospital the day before the surgical procedure; no intestinal preparation had been administered.

Intravenous Cefazolin (2g) was administered as short antibiotic prophylaxis 1 hour before surgery.

Surgical interventions were conducted either laparoscopically (5 cases of minor hepatectomy) or with traditional open surgery (39 cases, including minor and major hepatectomies).

Postoperative management

Patients were mobilized within 24 hours from surgery. The gastric tube was routinely removed the first day after the procedure and oral fluid intake restarted immediately after the tube removal.

The drainage was usually removed 3 – 4 days after surgery.

Histological and immunohistochemical evaluation

All surgical specimens had been reduced and fixed in formalin for 48 - 72 hours, processed and included in paraffin.

The first sections had been stained with hematoxylin – eosin and analyzed for definitive histological diagnosis.

Specimens from the patient included in the study underwent 3 *u*m microtome dissection for immunohistochemical staining; sections were placed in water at 40°C, collected on polarized glasses, heated to 60°C in order to eliminate the paraffin, immersed in Xylene and subsequently in ethanol for tissue rehydration.

Glasses were immersed in EDTA at pH 8 and heated with microwaves for 15 minutes, in order to restore the antigen pattern of tissues that could have been altered by fixation.

Glasses were analyzed by 2 pathologists; TNC immunoreactivity was expressed by a score composed of the extension of positivity area and staining intensity (see table 1).

Area	**Intensity**
1 = 0-10%	0 = negative
2 = 11-25%;	1 = low
3 = 26-50%	2 = moderate
4 = 51-75%	3 = high
5 = 76-100%	

Table 1: Immunoreactivity score for TNC expression

Objective of the study

We evaluated the role of Tenascin C as a prognostic marker for hepatocellular cancer in patients treated with radical surgical resection.

Expression of TNC was related to patient's age, sex, tumor size, grading, underlying liver disease, overall survival.

Statistical analysis

Statistical analysis was performed with MedCalc (version 7) and GraphPad PRISM 5 software.

We tested the correlations between levels of TNC immunohistochemical expression and patients' clinical – pathological characteristics with Spearman's non - parametric correlation method; survival curves were obtained with the Kaplan – Meier method, and differences in terms of overall survival (OS) were evaluated with the log – rank test.

Statistical significance was set at p – value <0.01.

Results

No perioperative mortality occurred. Perioperative morbidity was 9.09% (1 case of liver failure, 2 cases of bile leakage needing re – operation and 1 case of peritoneal collection treated with percutaneous drainage).

The mean hospital stay was 5 days (range 3 – 21 days).

At the end of the observation period (31/10/2019), 25 patients (56.82%) had died; all deaths were cancer – related.

The median survival was 1248 days (approximately 42 months); 5 - years survival rate was 25%; tumor recurrence rate was 70.37%.

Figure 11 shows the global survival Kaplan – Meier curve.

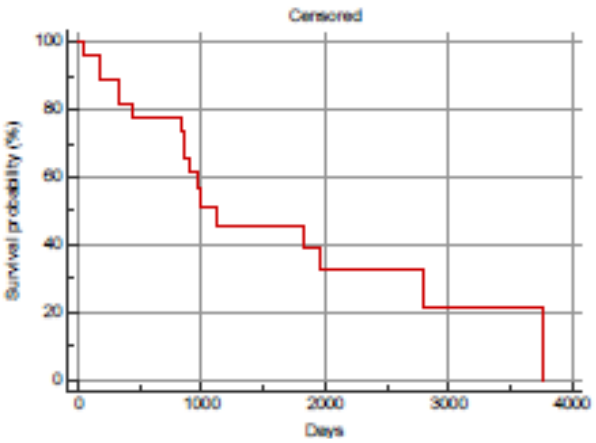

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Figure 11: global survival Kaplan – Meier curve

Histological sections staining demonstrated a great variability in TNC expression in terms of extension and intensity.

25 cases (56.82%) showed TNC expression in less than 10% of the tissue area, 10 cases (22.73%) between 11% and 25%, 7 cases (15.91%)

between 26% and 50%, 1 case (2.27%) between 51% and 75%, and 1 case (2.27%) more than 76%.

TNC expression was absent in 9 cases (20.45%); in the remaining 35 specimens, staining intensity was low in 11 cases (25%), moderate in 13 (29.55%) and high in 11 (25%).

Patients were also divided in groups based on TNC expression localization: in 13 cases (29.55%) TNC was mainly expressed in the interstitial and in peri - sinusoidal space; in the other cases in which TNC was expressed, staining was more intense in the nucleus or cytoplasm.

Peri - sinusoidal tenascin expression was absent in 17 cases (38.64%), low in 16 (36.36%), moderate in 3 (6.82%), and high in 8 (18.18%).

Figure 12 shows immunohistochemical samples from our patients' series with different patterns of TNC expression.

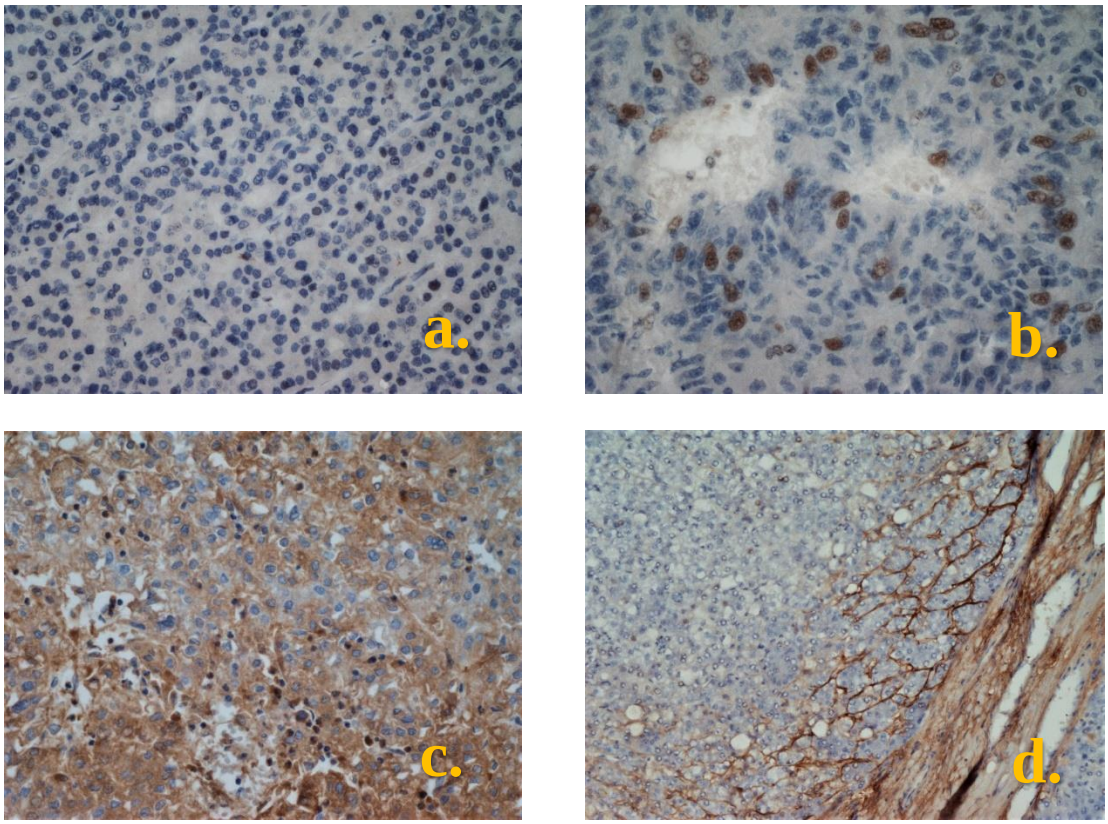

Figure 12: TNC histological specimens (HCC), with TNC immunohistochemical staining.
a - no TNC expression
b - nuclear TNC expression
c - cytoplasmatic TNC expression
d - intense TNC interstitial expression

At univariate analysis, no correlations between overall survival and age, sex, tumor size, grading, concomitant liver disease, TNC global intensity and extension of area expression have been demonstrated (Figure 13, 14, 15).

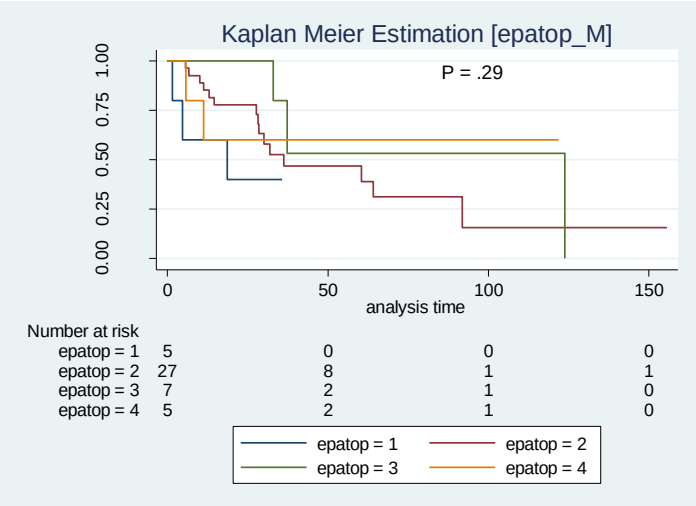

Figure 13: Kaplan Meier Estimation curves based on underlying chronic disease Epatop 1= HBV; Epatop 2= HCV; Epatop 3= Alcohol; Epatop 4=NASH

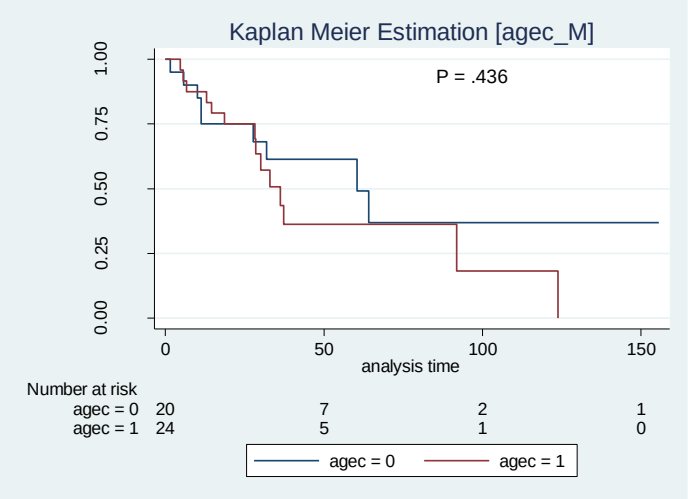

Figure 14: Kaplan Meier estimation curves based on patient age (agec=0 → age > 75 years; agec=1: age < 75 years)

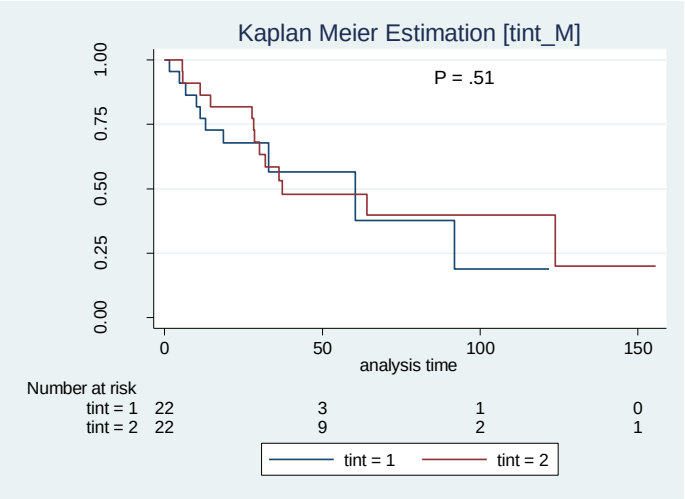

Figure 15: Kaplan Meier Estimation Curves based on surgical procedure
tint 1= major hepatectomy tint 2=minor hepatectomy

Moderate and intense expression of TNC in the peri - sinusoidal space was found to be significantly related to a worse prognosis (shorter survival after curative treatment, high rate of tumor local recurrence and metastatization) than in patients with absent or low peri - sinusoidal space staining ($p<0.01$) (Figure 15).

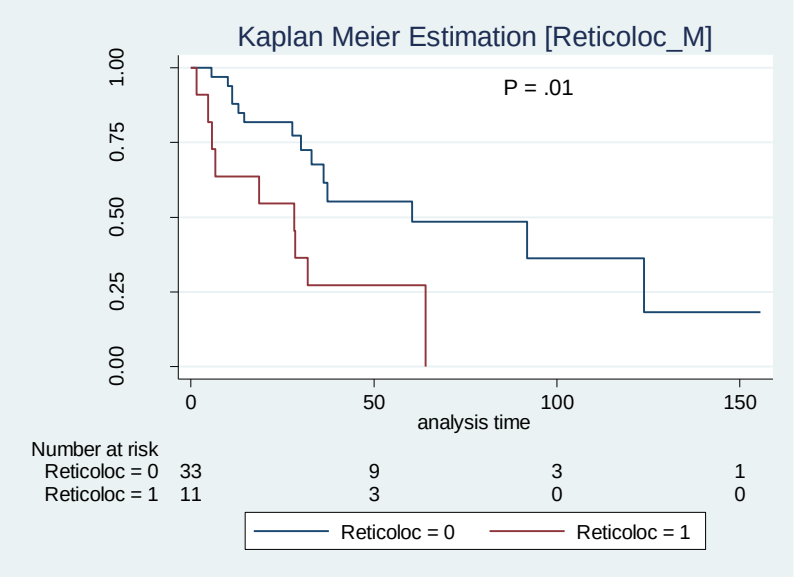

Figure 15: Kaplan – Meier survival curves showing a significant lower survival rate in patients with moderate and high Tenascin C expression in peri - sinusoidal space.
reticoloc 0=TNC expression in peri - sinusoidal space absent or low; reticoloc 1=TNC expression in peri - sinusoidal space moderate or high.

Discussion

Hepatocellular carcinoma is still one of the most common causes of cancer – related deaths, with a low global 5 - years survival rate (4.4 – 6%) and high 5 - year recurrence rate (43.5 – 61.5%) even after curative treatment [123]. The high metastatic potential, particularly involving lymph nodes, is the main reason of therapeutic failure [132].

Clinical experience provides many curative options for patients affected with very early and early - stage HCC.

Liver transplantation is an established treatment for HCC. and is currently the best option for cirrhotic patients affected with a primary liver tumor.

Compared to any other available treatments for liver cancer, liver transplantation presents the highest potential for cure, because of its ability to remove at the same time both the seeded – HCC and the damaged hepatic tissue where carcinogenesis and chronic liver disease have progressed together [132].

Unfortunately, not all cirrhotic patients, even those affected by early - stage HCC are eligible for liver transplantation. In 1996 the so – called "Milan Criteria" gave the first guidelines for selecting potentially curable patients with liver transplantation for hepatocellular cancer; a patient would be suitable for transplantation if he presents with a single node inferior or equal to 5 centimeters in diameter, or up to 3 nodes, each inferior or equal to 3 centimeters.

In recent years, criteria for liver transplantation in patients affected with hepatocellular carcinoma evolved, and at present a vast heterogeneity can be found in Literature. Another controversy is the management of HCC recurrence after liver transplantation, because there is little evidence available regarding improvement of survival with any treatment after liver transplantation [133].

Many experiences in Literature show that ablative techniques and surgical resection can be used in curative treatment of HCC with good results; the main advantage of percutaneous radiofrequency or microwaves application, particularly in patients with compromised liver functions and high stage cirrhosis is that these techniques are less invasive than surgical approaches.

Surgical nodulectomy and ablation are both valid methods that can be used for patients affected by HCC who are not eligible for liver transplantation but can undergo a curative treatment.

Indications are related to two factors: tumor size and localization; the two techniques seem to have similar outcomes when applied to patients with cancers up to 4 cm of diameter.

The advantages of both treatments are different: on the one hand surgical resection allows for the complete removal of the lesion and histological confirmation and for intraoperative exploration of liver parenchyma “in toto”, with the help of contrast – enhancement ultrasonography. It also

allows for the treatment of other tumor nodes incidentally found at operation. On the other hand, ablative techniques are less invasive.

Patients with HCC and underlying cirrhosis often present altered liver functions that lead to coagulative disorders; for this reason, surgical resection and percutaneous ablation can be followed by adverse events. In any case, the incidence of life – threatening complications is relatively low, RF ablation having slightly lower complication rates than surgery, particularly for single HCC smaller than 3cm [134].

Patients affected with more advanced stage HCC are unfortunately not eligible for radical curative procedures.

Trans – arterial chemoembolization (TACE) is universally recognized as a suitable therapy, improving the survival of patients with hepatocellular carcinoma who cluster into the “intermediate” BCLC. Some radiological markers are useful for predicting prognosis in patients undergoing TACE; in our recently published study we demonstrated that tumor size larger than 7cm, intratumor necrosis and arterial ectatic neovascularization are significantly related to worse prognosis [135].

The validation of prognostic biomarkers that would provide a tailored treatment could be a new challenging frontier in very early or early - stage liver cancer surgical and oncological approach.

The critical role of the cancerous microenvironment (cellular and non-cellular) is increasingly recognized as an important factor markedly

influencing hepatocarcinogenesis, epithelial – mesenchymal transition, tumor invasion and metastasis [136]; a recent study demonstrates the potential role of 78-kDa glucose – regulated protein and Galectin-3 as biomarkers for lymph node hepatocellular carcinoma metastasis in a murine model [137].

Tenascin C is mainly expressed during embryonic development. In adults, TNC has a limited pattern of expression, but protein levels rise dramatically under various physiological and pathological conditions, such as tissue remodeling, neovascularization, and inflammation. TNC is thought to influence cancer growth by affecting cell adhesion and motility, thus promoting invasion and metastasis [138, 139].

Berndt et Al recently assessed the crucial role of TNC re expression and alternative splicing in promoting migration and epithelial to mesenchymal transition in urothelial carcinoma of the urinary bladder and oral squamous cell carcinoma [140].

Simultaneous expression of TNC and other stromal proteins (Twist 1) in fibroblasts are positively associated with tumor location, pT stage, lymph node metastasis, clinical stage, and poor prognosis in gastric cancer [141].

TNC also seems to promote breast cancer progression by immobilizing infiltrating T lymphocytes, thus influencing immune host reaction to tumor development [142].

TNC is expressed in the bone endosteum and is associated with the formation of prostate bone metastases. Metastatic cells cultured on osteo – mimetic surfaces coated with TNC exhibit enhanced adhesion and colony formation. Reactive stroma response in the bone endosteum accompanies prostate cancer metastasis to the trabecular bone, with potential implications to therapeutically target this process in patients [143].

Hagiwara et Al, studying human pancreatic cancer specimens, demonstrated that the co - expression of large splicing TNC variant and Annexin A2 (a cell surface receptor for TNC) is an independent indicator of poor prognosis [144].

In glioma, TNC is expressed by tumor and stromal cells; high expression of TNC is correlated with tumor progression and poor prognosis; besides promoting invasion and angiogenesis, TNC has been found to affect the morphology and function of tumor – associated microglia / macrophages. Clinically, TNC can serve as a biomarker for tumor progression, and TNC antibodies have been utilized as an adjuvant agent to deliver anti - tumoral drugs to target glioma [145].

TNC has been demonstrated to play a role in vasculogenic mimicry in gastric cancer (which is the leading cause for the failure of anti-angiogenesis therapy in advanced stage patients). Furthermore, knockdown of TNC significantly inhibited angiogenesis and proliferation of cancer cells in vitro and in vivo, with a reduction in cell migration and

invasion, by the phosphorylation of EMT. Combining inhibition of TNC and ERK may be a potential therapeutic approach to inhibit gastric cancer growth and metastasis and decrease antiangiogenetic therapeutic resistance [146].

TNC may also promote epithelial to mesenchymal transition – like change and proliferation, leading to poor prognosis in patients affected with colorectal cancer [147].

Parek et Al, in a clinical study conducted on patients surgically treated for non-small cell lung cancer, demonstrated that the increased expression of TNC at the site of the tumor correlates with recurrence, probably because of TNC's capability of inhibiting tumor infiltrating lymphocyte proliferation and cytokine expression [148].

In primary liver cancer, due to the strong correlation between TNC and inflammation, it would be plausible to hypothesize that TNC strongly contributes to tumor progression.

Literature reports a great number of experimental studies regarding TNC and tumor genesis, development and metastatization but few clinical experiences have been published to date. Nong et Al presented a retrospective analysis conducted on a large series of patients, assessing that high expression of TNC is related to a worse prognosis but 50% of patients included in the study already presented with portal infiltration [149].

Our study, even if conducted retrospectively on a series of 44 cases, demonstrated that a specific pattern of TNC expression, i.e., an intense extracellular expression in the peri - sinusoidal space, is related to tumor recurrence after curative surgical resection and shorter survival, probably due to the ability of the protein to promote lymph node metastasis, which is the leading cause of distant spreading and mortality.

Our experience, conducted on a series of selected patients suitable for radical liver resection, seems to demonstrate that TNC expression in tumor stroma is strongly related to a worse prognosis (i.e., high rates of recurrence and early mortality rate). A limit of the present study is of course the limited number of patients series; this could be explained by considering the fact that only very few cases of HCC are actually suitable only for surgical resection without other radical or palliative treatments (liver transplantation, ablation, chemoembolization, drug treatment, best supportive care). Further investigations are needed in order to validate the evaluation of the expression of TNC as a prognostic marker of HCC (i.e., enlarging patients' series, prospective studies). Literature and medical practice guidelines currently suggest that radiological typical behavior of a liver mass in a context of cirrhosis is sufficient to diagnose HCC, thus avoiding biopsy; the validation of TNC expression as a prognostic marker would lead to a renewed discussion of the utility of the biopsy, at least in

patients suitable for radical treatment, in order to tailor the follow up and surveillance.

Conclusions and future perspectives

Our experience demonstrated that moderate and intense TNC expression in tumor extracellular space is significantly related to high rates of disease recurrence and early mortality in patients affected with early stage hepatocellular carcinoma treated with a radical surgical approach.

Further studies on larger patients' series are needed to validate the analysis of TNC expression pattern as a prognostic predictor in potentially curable cases of HCC, in order to justify the reintroduction of the preoperative liver biopsy for a better characterization of cases with a high risk of recurrence.

REFERENCES

1 - Tang A, Hallouch O, Chernyak V, Kamaya A, Sirlin CB. Epidemiology of hepatocellular carcinoma: target population for surveillance and diagnosis Abdom Radiol (NY) 2017 Jun 24.

2 - Fitzmaurice C, Fitzmaurici C, Akinyemiju et al. Global, regional and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2016: a systematic analysis for the global burden of disease study. JAMA Oncology. 2018;4(11):1553-1568.

3 - Fact Sheets by Population-Globocan-IARC n. D. December 18, 2017 http://globocan.iarc.fr/Pages/fact_sheets_population.aspx,(accessed).

4 - Njei B, Rotman Y, Ditah I,Lim K. Emerging trends in hepatocellular carcinoma incidence and mortality. Hepatology 2015;61(1):191-199.

5 - Bossetti C, Turati F, La Vecchia C. Hepatocellular carcinoma epidemiology. Best Practice and Research Clinical Gastroenterology. 2014;28(5):753-770.

6 - Goh GBB, Li JW, Chang PE, Chow KY, Tan CK. Deciphering the epidemiology of hepatocellular carcinoma through the passage of time: a study of 1,401 patients across 3 decades. Hepatology communications. 2017;1(6):564-571.

7 - Wallace MC, Preen D, Jeffrey GP, Adams LA. The evolving epidemiology of hepatocellular carcinoma: a global perspective. Exp Rev Gastroenterol Hepatol.2015;9(6):765-779.

8 - Soresi M, La Spada E, Giannitrapani L et al. Hepatocellular carcinoma: comparison of two different periods at the same center. Eur J Int Med.2010;21(2):127-130.

9 - Cazzagon N, Trevisani F, Maddalo G et al. Rise and fall of HCV-related hepatocellular carcinoma in Italy: a long-term survey from the ITA.LI. CA centers. Liver International.2013;33(9):1420-1427.

10 - Galle PR, Forner A, Llovet JM. European association for the study f the liver: EASL clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol.2018;69():182-236.

11 - El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology2007;132(7):2557-2576.

12 - Bucci L, Garuti F, Camelli V et al. Comparison between alcohol and hepatitis C virus-related hepatocellular carcinoma: clinical presentation, treatment and outcome. Alimentary Pharmacology and Therapeutics. 2016;43(3):385-399.

13 - Masotto A, Nardone G, Colecchia A et al. The evolutionary scenario of hepatocellular carcinoma in Italy: an update. Liver International.2017;37(2):259-270.

14 - Liu Z, Jiang Y, Yuan H et al. The trends in incidence of primary liver cancer caused by specific etiologies: results from the global burden of disease study2016 and implications for liver cancer prevention. J Hepatol.2019;70(4):674-683.

15 - Akinyemiju T, Abera S, Ahmed M et al. The burden of primary liver cancer and underlying etiologies from 1990 to 2015 at the global, regional and national level. JAMA Oncology. 2017;3(12):1683-1691.

16 - Giannitrapani L, Zerbo M, Amodeo S, Pipitone E, Galia M, Li Cavoli TV, Minissale MG, Licata A, Schiavone C, Brancatelli G, Montalto G, Soresi M. The Changing Epidemiology of Hepatocellular Carcinoma: Experience of a Single Center. Biomed Res Int.2020 Feb 27; 2020:5309307.

17- https://www.regstritumori.it/cms/sites/default/files/pubblicazioni/2018_NumeriCancro-operatori.pdf

18 - Debes JD, Chan AJ, Balderramo B, Kikuchi L et Al. Hepatocellular Carcinoma in South America: Evaluation of Risk Factors, Demographics and Therapy Liver Int 2017 Jun 22.

19 - Shen S, You H, Niu J et Al.
Baseline Characteristics and Treatment Patterns of the Patients Recruited to the China Registry of Hepatitis B. J Clin Transl Hepatol. 2019 Dec 28; 7(4):322-328.

20 - Cougot D, Neuveut C, Buendia MA. HBV induced carcinogenesis. J Clin Virol 2005 Dec;34.

21 - WHO 2017 – Hepatitis C infection

22 - Rusyn I, Lemon SM. Mechanisms of HCV – induced liver cancer: What did we learn from in vitro and animal studies? Cancer Lett. 2014 Apr 10; 345(2): 210 – 215.

23 - Burra P, De Martin E, Zanetto A, Senzolo M, Russo FP, Zanus G, Fagiuli S. Hepatits C virus and liver transplantation; where do we stand? Transpl Int. 2016 Feb; 29(2): 135-52

24 - Ratna A, Mandrekar A, Pranoti G. Alcohol and Cancer: Mechanisms and Therapies. Biomolecules. 2017; 7:61.

25 - Ma HY, Yamamoto G, Xu J, Liu X et Al. IL – 17 signaling in steatotic hepatocytes and macrophages promotes hepatocellular carcinoma in alcohol related liver disease. J Hepatol. 2019 Dec 31.

26 - Calzadilla Bertot L, Adams LA. The Natural Course of Non – Alcoholic Fatty Liver Disease Int J. Mol. Sci. 2016. 17,774.

27 - Hamid AS, Tesfamariam IG, Zhang Y, Zhang ZG. Aflatoxin B1 – induced hepatocellular carcinoma in developing countries: Geographical distribution, mechanism of action and prevention (Review) Oncology Letters. 2013; 5:1087-1092.

28 - Schlageter M, Terracciano LM, D'Angelo S, Sorrentino P. Histopathology of hepatocellular carcinoma. World J Gastroenterol 2014 Nov 21;20(43):15955-15964.

29 - Vyas M, Zhang X. Hepatocellular Carcinoma: Role of Pathology in the Era of Precision Medicine. Clin Liver Dis. 2020 Nov;24(4):591-610.

30 - Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Shwarts M,Sherman M, Gores G. Hepatocellular Carcinoma Nat Rev Dis Primers. 2016 Apr 14; 2:16018.

31 - Alizadeh AA et al Toward understanding and exploiting tumor heterogeneity. Nat Med. 21, 846 – 853 (2015).

32 - Guichard C et al. Integrated analysis of somatic mutations and focal copy number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet. 44, 694 – 8 (2012)

33 - Schulze K et al. Exome sequencing of hepatocellular carcinoma identifies new mutational signatures and potential therapeutic targets. Nat Genet. 47, (2015).

34 - Gunes C et al. The role of telomeres in stem cells and cancer Cell 152, 390 – 3 (2013).

35 - Hartmann D et al. Telomerase gene mutations are associated with cirrhosis formation. Hepatology 53, 1608 – 17 (2011).

36 - Lechel A et al. Telomerase deletion limits progression of p-53 mutant hepatocellular carcinoma with short telomeres in chronic liver disease Gastroenterology 132, 1465 – 75 (2007).

37 - Totoky Y et al. Trans – ancestry mutational landscape of hepatocellular carcinoma genomes. Nat Genet. 46, 1267 – 73 (2014)

38 - Pilati C et al. Genomic profiling of hepatocellular adenomas reveals recurrent FRK – activating mutations and the mechanisms of malignant Transformation Cancer Cell 25; 428 – 41 (2014).

39 - Zucman – Rossi J, Villanueva A, Nault JC, Llovet JM. The genetic landscape and biomarkers of hepatocellular carcinoma. Gastroenterology 5, 1226 – 1239 (2015).

40 - Sung WK et al. Genome – wide survey of recurrent HBV integration in hepatocellular carcinoma Nat Genet 44, 765 – 9 (2012).

41 - Audard V et al. Cholestasis is a marker for hepatocellular carcinoma displaying beta– catenin mutations. J Pathol. 212, 345 – 52 (2007)

42 - Bressac B, Kew M, Wands J, Ozturk M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from Southern Africa Nature 350, 429 – 31 (1991)

43 - Amaddeo G et al. Integration of tumor and viral genomic characterization in HBV – related hepatocellular carcinomas Gut 64, 820 – 9 (2015).

44 - Villanueva A et al. DNA – methylation-based prognosis and epidrivers in hepatocellular carcinoma Hepatology 61, 1945 – 56 (2015)

45 - Sporn MB, Liby KT. NRF2 and cancer: the good, the bad and the importance of context. Nat Rev Cancer. 12, 564 – 71 (2012).

46 - Sawey ET et al. Identification of a therapeutic strategy targeting amplified FGE19 in liver cancer by Oncogenomic screening. Cancer Cell 19, 347 – 58 (2011).

47 - Chiang DY et al. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma Cancer Res. 68, 6779 – 6788.

48 - Lachenmayer A et al. Wnt – pathway activation in two molecular classes of hepatocellular carcinoma and experimental modulation by sorafenib. Clin Cancer Res. 18, 4997 – 5007 (2012).

49 - Qi F, Zhou A, Yan L, Yuan X, Wang D, Chang R, Zhang Y, Shi F, Han X, Hou J, Wei L, Zhang. The diagnostic value of PIVKA-II, AFP, AFP-L3, CEA, and their combinations in primary and metastatic hepatocellular carcinoma. J Clin Lab Anal. 2019 Dec 10: e2315

50 - Colombo M. Screening for cancer in viral hepatitis. Clin Liver Dis 2001; 5:109–22.

51 - Gupta S, Bent S, Kohlwes J. Test characteristics of alphafetoprotein for detecting hepatocellular carcinoma in patients with hepatitis C.A systematic review and critical analysis. Ann Intern Med 2003; 139:46–50.

52 - Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. Gastroenterology 2002; 122:1609–19.

53 - Johnson PJ. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. Clin Liver Dis 2001; 5:145–59

54 - Bialeki ES, Di Bisceglie AM. Diagnosis of hepatocellular carcinoma. HPB, 2005;7:26-34.

55 - Zhong JH, Peng NF, You XM, Ma L, Xiang X, Wang YY, Gong WF, FX, Xiang BD, Li LQ. Tumor stage and primary treatment of hepatocellular carcinoma at a large tertiary hospital in China: A real-world study. Oncotarget 2017 Mar 14;8:(11):18296-18302.

56 - Bolondi L, Piscaglia F, Camaggi V, Grazi GL, Cavallari A. Review article: liver transplantation for HCC. Treatment options on the waiting list. Aliment Pharmacol Ther 2003; 17(Suppl. 2): 145 - 150.

57 - Mazzaferro V, Regalia E, Doci R et al. Liver transplantation for the treatment of small hepatocellular carcinoma in patients with cirrhosis. N Engl J Med 1996; 334: 693 - 9.

58 - Barbara L, Benzi G, Gaiani S et al. Natural history of small untreated hepatocellular carcinoma in cirrhosis: a multivariate analysis of prognostic factors of tumor growth rate and patients' survival. Hepatology 1992: 16: 132 - 7.

59 - Yao FY, Bass NM, Nikolai B et al. Liver transplantation for hepatocellular carcinoma: Analysis of survival according to the intention - to - treat principle and dropout from the waiting list. Liver Transplant 2002; 8: 873 - 83.

60 - Sarasin FP, Majno PE, Llovet JM, Bruix J, Mentha G, Hadengue A. Living donor liver transplantation for early hepatocellular carcinoma: a life - expectancy and cost - effectiveness perspective. Hepatology 2001; 33: 1073 - 9.

61 - Sarasin FP, Giostra E, Mentha G, Hadengue A. Partial hepatectomy or orthotopic liver transplantation for the treatment of resectable hepatocellular carcinoma? A cost - effectiveness perspective. Hepatology 1998: 28: 436 - 42.

62 - Metha N, Bhangui P, Yao FY et al. Liver Transplantation for Hepatocellular Carcinoma. Working Group Report from the ILTS Transplant Oncology Consensus Conference. Transplantation 2020; 104: 1136 - 1142.

63 - Figueras J, Jaurrieta E, Valls C et al. Resection or transplantation for hepatocellular carcinoma in cirrhotic patients: outcomes based on indicated treatment strategy. J Am Coll Surg 2000; 190: 580 - 57.

64 - Yamamoto J, Iwatsuki S, Kosuge T, et al. Should hepatomas be treated with hepatic resection or transplantation? Cancer 1999; 86: 1151 - 1158.

65 - Strong RW. Transplantation for liver and biliary cancer. Semin Surg Oncol 2000; 19: 189 - 199.

66 - Orcutt ST, Anaya DA. Liver Resection and Surgical Strategies for Management of Primary Liver Cancer. Cancer Control 2018; 25(1): 1 - 15.

67 - Poon RT, Fan ST, Lo CM, Liu CL, Wong J. Long - Term Survival and Pattern of Recurrence After Resection of Small Hepatocellular Carcinoma in Patients with Preserved Liver Function. Ann Surg 2002; 235(3): 373 - 382.

68 - Heimbach JK, Kulik LM, Finn RS, et al. Guidelines for the treatment of hepatocellular carcinoma. Hepatology 2018; 67: 358 - 80.

69 - European Association for the Study of the Liver. EASL clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2018; 69: 182 - 236.

70 - Zhu F, Rhim H. Thermal ablation for hepatocellular carcinoma: what's new in 2019. Chin Clin Oncol 2019; 8(6): 58.

71 - Llovet JM, Real MI, Montana X et al. Arterial embolization of chemoembolization versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomized controlled trial. Lancet 2002; 359(9319): 1734 - 1739.

72 - Wallace S, Carrasco CH, Charnsagavej C et al. Hepatic artery infusion and chemoembolization in the management of liver metastases. Cardiovasc Intervent Radiol. 1990; 13(3): 153 - 160.

73 - Anderson JH, Gianturco C, Wallace S. Experimental transcatheter interarterial infusion - occlusion chemotherapy. Invest Radiol. 1981; 16(6): 496 - 500.

74 - Lencioni R, de Baere T, Soulen MC, Rilling WS, et al. Lipiodol transarterial chemoembolization for hepatocellular carcinoma: a systematic review of efficacy and safety data. Hepatology 2016; 64(1): 106 - 116.

75 - Kis B, El - Haddad G, Sheth RA, Parikh NS, Ganguli S, Shyn PB, Choi JC, Brown KT. Liver - Directed Therapies for Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma. Cancer Control 2017; 24(3): 1 - 19.

76 - Geshwind JF, Kudo M, Marrero JA et al. TACE treatment in patients with sorafenib - treated unresectable hepatocellular carcinoma in clinical practice: final analysis of GIDEON. Radiology 2016; 279(2): 630 - 640.

77 - Folkman J, Camphausen K. Cancer. What does radiotherapy do to endothelial cells? Science. 2001; 293(5528): 227 - 228.

78 - Mazzaferro V, Sposito C, Bhoori S et al. Yttrium - 90 radioembolization for intermediate - advanced hepatocellular carcinoma: a phase 2 study. Hepatology. 2013; 57(5): 1826 - 1837.

79 - Xia S, Pan Y, Liang Y, Xu J, Cai X. The microenvironmental and metabolic aspects of sorafenib resistance in hepatocellular carcinoma. EBioMedicine 51 (2020) 102610.

80 - Tang W, Chen Z, Zhang W, Cheng Y, Zhang B, Wu F, Wang Q, Wang S, Rong D, Reiter FP, De Toni EN, Wang X. The mechanisms of sorafenib resistance in hepatocellular carcinoma: theoretical basis and therapeutic aspects. Signal Transduct Target Ther. 2020 Jun 10;5(1): 87.

81 - Chiang CL, Chjan SK, Lee SF, Wong OL, Choi CW. Cost - effectiveness of Pembrolizumab as a Second - Line Therapy for Hepatocellular Carcinoma. JAMA Network Open. 2021(4); 4(1): e2033761.

82 - Bruix J, Boix L, Sala M, Llovet JM. Focus on hepatocellular carcinoma. Cancer Cell 2004; 5:215-19.

83 - Amann T, Bataille F, Spruss T, Muhlbauer M, Gabele E, Scholmerich J. et al. Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma. Cancer Sci 2009; 100:646-53.

84 - Nitta T, Kim JS, Mohuczy D, Behrns KE: Murine cirrhosis induces hepatocyte epithelial – mesenchymal transition and alterations in survival signaling pathways. Hepathology 2008; 48:909-19.

85 - Thiery JP. Epithelial mesenchymal transitions in tumor progression. Nat Rev Cancer 2002; 2:442-54.

86 - Kalluri R, Weinberg RA. The basics of epithelial – mesenchymal transition. J Clin Invest 2009; 119:1420 – 8.

87 - Yang JD, Nakamura I, Roberts LR. The Tumor Microenvironment in Hepatocellular Carcinoma: Current Status and Therapeutic Targets. Semin Cancer Biol. 2011 Feb;21(1):35-43.

88 - Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol 2008; 214:199-210.

89 - Dubuisson L, Lepreux S, Bioulac – Sage P, Balabaud C, Costa AM, Rosenbaum J, et al. Expression and cellular localization of fibrillin – 1 in normal and pathological human liver. J Hepatol. 2001; 34:514-22.

90 - Schulze – Krebs A, Preimel D, Popov Y, Bartenschlager R, Lohmann V, Pinzani M et al. Hepatitis c virus – replicating hepatocytes induce fibrogenic activation of hepatic stellate cells. Gastroenterology 2005; 129:246-58.

91 - Fransvea E, Mazzocca A, Antonaci S, Giannelli G. Targeting transforming growth factor (TGF)-betaR1 inhibits activation of beta 1 integrin and blocks vascular invasion in hepatocellular carcinoma. Hepatology 2009; 49:839-50.

92 - Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev. 2000; 14:163–76. Ramaiah SK, Rittling S.

93 - Eghbali Fatourechi G, Sieck GC, Prakash YS, Maerklein P, Gores GJ, Fitzpatrick LA. Type 1 procollagen production and cell proliferation is mediated by transforming growth factor beta in a model of hepatic fibrosis. Endocrinology 1996; 137:1894-903.

94 - Fransvea E, Mazzocca A, Antonaci S, Giannelli G. Targeting transforming growth factor (TGF) betaR1 inhibits activation of beta integrin and blocks vascular invasion in hepatocellular carcinoma. Hepatology 2009; 49:839-50.

95 - Tilg H, Wilmer A, Vogel W et al. Serum levels of cytokines in chronic liver diseases. Gastroenterology 1992; 103: 264 – 74.

96 - Wang YC, Xu GL, Jia WD et al. Estrogen suppresses metastasis in rat hepatocellular carcinoma through decreasing interleukin – 6 and hepatocyte growth factor expression. Inflammation 2012; 35:143 – 9

97 - Jiang R, Tan Z, Deng L et al. Interleukin – 22 promotes human hepatocellular carcinoma by activation of STAT3. Hepatology. 2011; 54:900 – 9

98 - Beckebaum S, Zhang X, Chen Z et al. Increased levels of interleukin – 10 in serum from patients with hepatocellular carcinoma correlate with profound numerical deficiencies and immature phenotype of circulating dendritic cell subsets. Clic Cancer Res. 2004; 10:7260 – 9.

99 - Li W, Gomez E, Zhang Z. Immunohistochemical expression of stromal cell derived factor – 1 (SDF-1) and CXCR4 ligand receptor system in hepatocellular carcinoma. J Exp Clin Cancer Res. 2007; 26:527 – 33.

100 – Ringelhan M, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. Nat Immunol. 2018; 19:222-232.

101 - Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, et al. Inflammatory tumor microenvironment is associated with superior survival in hepatocellular carcinoma patients. Gastroenterology 2007; 132:2329-39.

102 - Zhu AX, Duda DG, Sahani DV et al. HCC and angiogenesis: possible targets and future directions. Nat Rev Clin Oncol. 2011; 8:292 – 301.

103 - Coulon S, Heindryckx F, Geetrs A et al. Angiogenesis in chronic liver disease and its complications. Liver Int. 2011; 31:146 – 62.

104 - Bronzert DA, Pantazis P, Antoniades HN et al. Synthesis and secretion of platelet – derived growth factor by human breast cancer cell lines. Proc Natl Acad Sci USA. 1987; 84:5763 – 7.

105 - Yang JC, Teng CF, Wu HC, Tsai HW, Chuang HC, Tsai TF, et al. Enhanced expression of vascular endothelial growth factor – A in ground glass hepatocytes and its implications in hepatitis B virus hepatocarcinogenesis. Hepatology 2009; 49:1962-71.

106 - Budhu A, Forgues M, Ye QH et al. Prediction of venous metastases, recurrence and prognosis in hepatocellular carcinoma based on a unique response signature of the liver microenvironment. Cancer Cell. 2006; 10:99 – 111.

107 - Benetti A, Berenzi A, Gambarotti M, Garrafa E, Gelati M, Dessy E, et al. Transforming growth factor – beta 1 and CD 105 promote the migration of hepatocellular carcinoma – derived endothelium Cancer Res. 2008; 68:8626-34.

108 - Guichard C, Amaddeo G, Imbeaud S et al. Integrated analysis of somatic mutations and focal copy – number changes identify key genes and pathways in hepatocellular carcinoma. Nat Genet. 2012; 44:694 – 8.

109 - Jones FS, Jones PL.The tenascin family of ECM glycoproteins: structure, function and regulation during embryonic development and tissue remodeling. Dev Dyn 2000; 218: 235-259.

110 - Oskarsson T, Acharrya S, Zhang XH, Vanharanta S, Tavazoie SF, Morris PG, Downey RJ, Manova-Todorova K, Brogi E, Massegue J. Breast cancer cell produce tenascin C as a metastatic niche component to colonize the lungs. Med 2011; 17: 867-874.

111 - Esposito I, Penzel R, Chaib-Harrieche M, Barcena U, Bergman F, Riedl S, Kayed H, Giese N, Kleef J, Friess H, Schimaker P. Tenascin C and annexin II expression in the process of pancreatic carcinogenesis. J Pathol 2006; 208: 673-685.

112 - Tang ZY, Ye SL, Liu YK, Sun HC, YE QH, Wang L, Zhou J, Qiu SJ, Li Y, Ji XN, Liu H, Xia JL, Wu ZQ, Fan J, Ma ZC, Zhou XD, Lin ZY, LIU KD. A decade's studies on metastasis of hepatocellular carcinoma J Cancer Res Clin Oncolo 2004; 130: 187-196.

113 - Midwood KS, Chiquet M, Tucker RP, Orend G. Tenascin – C at a glance. Journal of Cell Science (2016) 129,4321-4327.

114 - Mishra P, Kiebish MA, Cullen J, Srinivansan A, Patterson A, Sarangarajan R, Narain NR, Dobi A. Genomic alteration of Tenascin C in highly aggressive prostate cancer: a meta – analysis. Genes & Cancer 2019; 10:5-6.

115 - Yoshida T, Akatsuka T, Imanaka – Yoshida K. Tenascin C and integrins in cancer. Cell Adh. Migr. (2015); 9:96 – 104.

116 - Giblin SP., Midwood KS. Tenascin – C form versus function Cell Adh. Migr. (2015); 9:48-82.

117 - Yagi H, Yanagisawa M, Suzuki Y, Nakatani Y, Ariga T, Kato K, Yu RK. HNK-1 epitope carrying tenascin – C spliced variant regulates the proliferation of mouse embryonic neural stem cells. J. Biol. Chem. (2010); 285:37293-37301.

118 - Schwerzer A, Jiang X, Mikuls TR, Payne JB, Sayles HR, Quirke AM, Kessler BM, Fischer R, Venables PJ, Lundberg K et al. Identification of an immunodominant peptide from citrullinated tenascin – C as a major target for autoantibodies in rheumatoid arthritis. Ann. Rheum. Dis. (2015); 75:1876-1883.

119 - Li H, Ge X, Pan K, Sui M, Cai H, Cui C, Li C, Lu S. The Predictive Role of Tenascin – C and cellular communication Network Factor 3 (CCN3) in Post Hepatectomy Liver Failure in a Rat Model and 50 Patients following Partial Hepatectomy. Med Sci Monit, 2019;25:6755-6766.

120 - Yuan W, Zhang W, Yang X, Zhou L, Hanghua Z, Xu K. Clinical significance, and prognosis of serum tenascin – C in patients with sepsis BMC Anesthesiology. 2018; 18:170.

121 - Gkountela S, Castro - Giner F, Sczerba BM, Vetter M, Landin J, Scherrer R et Al. Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. Cell. 176(2019) 98 – 112.

122 - Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, Drexler S, Sofat N, Kashiwagi M, Orend G, Brennan F, Foxwell B. Tenascin – C is an endogenous activator of Toll – like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nat Med 2009, 15:774-780.

123 - Mancuso A, Perricone G. Hepatocellular Carcinoma and Liver Transplantation: State of the Art. J Clin Transl Hepatol. 2014 Sep;2(3):176-81

124 - Lowy CM, Oskarsson T. Tenascin C in metastasis: A view from the invasive front. Cell Adh Migr. 2015; 9:112-124.

125 - Yamada S, Ichida T, Matsuda Y, Miyazaki Y, Hatano T, Hata K, Asakura H, Hirota N, Geerts A and Wisse E. Tenascin expression in human chronic liver disease and in hepatocellular carcinoma. Liver 1992; 12: 10-16.

126 - Van Eyken P, Sciot R and Desmet VJ. Expression of the novel extracellular matrix component tenascin in normal and diseased human liver. An immunohistochemical study. J Hepatol 1990; 11: 43-52.

127 - El-Karef A, Yoshida T, Gabazza EC, Nishioka T, Inada H, Sakakura T, Imanaka-Yoshida K. Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice. J Pathol. 2007, 211: 86-94.

128 - Benbow JH, Thompson KJ, Cope HL, Brandon-Warner E, Culberson CR, Bossi KL, Li T, Russo MW, Gersin KS, McKillop IH, deLemos AS, Schrum LW. Diet-Induced Obesity Enhances Progression of Hepatocellular Carcinoma through Tenascin-C/Toll-Like Receptor 4 Signaling. Am J Pathol. 2016; 186:145-158.

129 - Zhao M, Laissue JA, Zimmermann A. Tenascin and type IV collagen expression in liver cell dysplasia and in hepatocellular carcinoma. Histol Histopathol. 1996 Apr; 11:323-333.

130 - Okamura N, Yoshida M, Shibuya A, Sugiura H, Okayasu I and Ohbu M. Cellular and stromal characteristics in the scirrhous hepatocellular carcinoma: comparison with hepatocellular carcinomas and intrahepatic cholangiocarcinomas. Pathol Int 2005; 55: 724-731.

131 - Nong Y, Wu D, Lin Y, Zhang Y, Bai L, Tang H. Tenascin-C expression is associated with poor prognosis in hepatocellular carcinoma (HCC) patients and the inflammatory cytokine TNF-α-induced TNC expression promotes migration in HCC cells. Am J Cancer Res 2015; 5:782-791

132 - Garavoglia M1, Oldani A, Gentilli S, Portigliotti L, D'Agostino G. Percutaneous radiofrequency ablation versus surgical radiofrequency-assisted nodulectomy in treatment of small single nodes of hepatocellular carcinoma: our experience. Minerva Chir. 2013 Aug;68(4):367-75.

133 - Pinato DJ, Pai M, Reccia I, Patel M, Giakoustidis A, Karamanakos G, Rushd A, Jamshaid S, Oldani A, Grossi G, Pirisi M, Tait P, Sharma R. Preliminary qualification of a novel, hypoxic-based radiologic signature for trans-arterial chemoembolization in hepatocellular carcinoma. BMC Cancer. 2018 Feb 20;18(1):21

134 - Melnikova VO, Bar-Eli M. Inflammation and melanoma metastasis. Pigm Cell Melanoma Res 2009; 22: 257 – 267

135 - Luo S, Wang S, Zhou H, Cao J, Hu Y, Zhang J. Caveolin – 1 upregulates CD147 glycosilation and the invasive capability of murine hepatocarcinoma cell lines. Int J Biochem Cell Biol 2006; 38: 1584-1593.

136 - Zhu W, Owusu L, Zang S, Zhang S, Xin Y, Yan C. GRP78 and GAL3, differentially regulated by lymph node homogenates, as potential biomarkers for lymph node metastasis in mouse hepatocellular carcinoma cells. Oncology Letters 4 2012; 4: 1374-1378.

137 - Paron I, Berchtold S, Voros J, Shamarla M, Erkan M, Hofler H, Esposito I. Tenascin – C Enhances Pancreatic Cancer Cell Growth and Motility and Affects Cell Adhesion through Activation of the Integrin Pathway. PloS ONE 6(6): e 21684, doi 10.1371/journal.pone.0021684.

138 - Ekblom M, Fassler R, Tomasini – Johanson B, Nilsson K, Ekblom P. Downregulation of Tenascin expression by glucocorticoids in bone marrow stromal cells and fibroblasts. J. Cell. Biol. (1993); 123:1037-1045.

139 - Zheng H, Tsuneyama K, Cheng C, Takahashi H, Cui Z, Nomoto K, Murai Y,Takano Y. Expression of KAI1 and tenascin, and microvessel density are closely correlate with liver metastasis of gastrointestinal adenocarcinoma. J Clin Pathol 2007; 60:50-56.

140 - Berndt A, Richter P, Kosmehl H, Franz M. Tenascin - C and carcinoma cell invasion in oral and urinary bladder cancer. Cell Adh Migr. 2015;9(1-2):105-11.

141 - Qi W, Yang Z, Li H, Cui Y, Xuan Y. The role of Tenascin - C And Twist1 in gastric cancer: cancer progression and prognosis. APMIS. 2019 Feb;127(2):64-71.

142 - Murdamothoo D, Sun Z, Ylmaz A, Riegel G, Faycal CA et Al. Tenascin - C immobilizes infiltrating T lymphocytes through CXCL12 promoting breast cancer. EMBO Mol Med. 2021 Jun 7;13(6):e13270.

143 – San Martin R, Pathak R, Jain A, Jung SY, Hilsenbeck SG, Pina-Barba MC, Sikora AG, Rowley DR. Tenascin - C and Integrin a9 Mediate Interaction of Prostate Cancer with the Bone Microenvironment. Cancer Res. 2017 Nov 1;77(21):5977-5988.

144 - Hagiwara K, Harimoto N, Yokobori T et Al. High Co - expression of Large Tenascin C Splice Variants in Stromal Tissue and Annexin A2 in Cancer Cell Membranes is Associated with Poor Prognosis in Pancreatic Cancer. Ann Surg Oncol. 2020 Mar;27(3):924-930.

145 - Yalcin F, Dzaye O, Xia S. Tenascin - C Function in Glioma: Immunomodulation and Beyond. Adv Exp Med Biol. 2020; 1272:149-172.

146 - Kang X et Al. Tenascin - c knockdown suppresses vasculogenic mimicry of gastric cancer by inhibiting ERK - triggered EMT. Cell Death Dis. 2021. PMID: 34903721.

147 - Yang Z, Zhang C, Qi W, Cui C, Cui Y, Xuan Y. Tenascin - C as a prognostic determinant of colorectal cancer through induction of epithelial - to - mesenchymal transition and proliferation. Exp Mol Pathol. 2018 Oct;105(2):216-222.

148 - Parek K, Ramachandran S, Cooper J, Bigner D, Patterson A, Mohanakumar T. Tenascin - C over expressed in lung cancer down regulates effector functions of tumor infiltrating lymphocytes. Lung Cancer. 2005 Jan;47(1):17-29.

149 - Nong Y, Wu D, Lin Y, Zhang Y, Bai L, Tang H. Tenascin – C expression is associated with poor prognosis in hepatocellular carcinoma (HCC) patients and the inflammatory cytokine TNF – α – induced TNC expression promotes migration in HCC cells. Am J Cancer Res 2015;5(2):782 – 791.

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