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ORIGINAL ARTICLE

"Curette technique" and FISH analysis for the assessment of oral field cancerization

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ABSTRACT

BACKGROUND: As tumorigenesis is a multi-step process driven by an accumulation of genetic changes, oral field cancerization still represents a valid theory for the development of recurrences and second primary tumors in oral squamous cell carcinomas (OSCCs). The aim of this preliminary study was to assess and validate the use of Scraping technique in association with FISH analysis as an early detection method for a specific chromosome 3q26.3 amplicon in mucosal sites that were considered clinically normal adjacent to OSCCs, keeping in mind the concept of oral field cancerization.

METHODS: Eleven patients with OSCCs were included in the study. During surgical intervention 4 microbiopsies in clinically normal tissue adjacent to the tumoral lesion were taken by curette technique. The collected samples were sent for FISH analysis with a 3q26.3 region probe.

RESULTS: In three patients genetic abnormalities in 3q26.3 were identified. Two of the three patients that presented positivity to FISH analysis had both risk factors (smoke and alcohol), were male and were the youngest in the study population.

CONCLUSIONS: The use of the curette technique in combination with FISH to detect copy number amplification of 3q26.3 could represent a valuable tool for the assessment of oral field cancerization.

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Although advances in treatment have reduced patient morbidity, the survival rates of oral squamous cell carcinoma (OSCC) in advanced stages have remained low.[1-11]

OSCC is often associated with widespread epithelial histopathological alterations, and even when successful treatment of a primary tumor is achieved, there is a high likelihood of occurrence of a second primary tumor or a recurrence.[1-6]

The high frequency of multiple primary tumors within the same tissue region has led to the hypothesis of 'oral field cancerization'.[1-6]

The concept of field cancerization was first coined by Slaughter *et al.*[7] in a paper from 1953 in which it is proposed that normal tissue adjacent to the primary tumor harbor preneoplastic alterations that can lead to the development of local recurrence and second primary tumors. In the initial phase, a cell acquires genetic alterations, divides and forms a patch, a clonal unit of daughter cells. Then, additional acquired alterations transform the patch into a proliferating field that gradually displaces the normal mucosa, and from this field tumors develop.[1-3] In particular, Slaughter *et al.*[7] observed that: 1) oral cancers usually have the tendency of spreading more easily in laterality than in depth; 2) the mucosa surrounding the neoplasia frequently harbors clinical or morphological atypia; 3) OSCC may consist of multiple independent foci that eventually may converge; 4) OSCC may develop multifocally in distant

areas presenting preneoplatic features; 5) the persistence of altered epithelium after surgical resection may induce the formation of new carcinomas.[1-3, 7] This model for head and neck cancer is still actual nowadays.

As tumorigenesis is a multistep process driven by an accumulation of genetic changes resulting in deregulation of the mechanisms controlling cell proliferation and differentiation, potential biomarkers for tumorigenesis include indicators of the degree of generalized and specific genetic changes in the tissue at risk for tumor development.[1-5]

Chromosome 3q26-ter or 3q26-27 amplicons, spanning around 20 cM, are targets that are altered in malignant disease. In fact, clusters of putative oncogenes, such as *TERC* (3q26.2), *PI3KCA* (3q26.32), *ZASC1* (3q26.33), *SCCRO* (3q26.3), *TP63* (3q27.2), have been recognized to be mapped to this amplicon. Furthermore, recent articles have indicated that 3q26 amplification is associated with clinical outcome.[1-7]

Recently, a number of fluorescence *in situ* hybridization (FISH) technique variants were used to detect chromosome or genomic imbalances in interphase cells. Essentially, FISH allows for a comprehensive characterization of the chromosomal alterations and assessment of topographical distribution of the most prominent changes in a tumor on a single-cell basis, yielding information on tumor heterogeneity and progression. Moreover, FISH is fast to perform and only requires a small amount of cells, which make it suitable for routine screening of tumorigenesis.[1-3, 6]

In order to obtain a sufficient amount of cells for FISH analysis by a not invasive technique, we hypothesized that 'scraping' using a dermatological curette could be an option to investigate OSCC by cytology and microhistology of the obtained "micro-biopsies".[1-6]

Therefore, the aim of this preliminary study was to assess and validate the use of Scraping technique in association with FISH analysis as an early detection method for a specific chromosome 3q26.3 amplicon in mucosal sites that were considered clinically normal adiacent to OSCCs, keeping in mind the concept of oral field cancerization.

Materials and methods

Patients with OSCC referring to Maxillofacial Surgery and Otolaryngology Divisions of Novara Hospital, Novara, Italy were considered for this study. Inclusion criteria were: unilateral carcinoma, first diagnosis of OSCC, absence of distance metastasis (M1), good performance status of the patient, need of surgery as a treatment for OSCC. Patients with multiple cancer localizations and patients affected by cancer with other histological diagnosis were excluded.

Demographic data (age, gender), smoke and/or alcohol use, tumor data (location, TNM stage), and tumor grade were collected for all cases.

Tumor stage was determined by the criteria established by the fifth edition of the American Joint Committee on Cancer staging system. The staging information was confirmed by review of tumor maps, operative records, and physicians' notes.

The included patients, after the first clinical, imaging and pathological examinations, underwent multidisciplinary head and cancer group assessment for the establishment of the most appropriate treatment. As aforementioned, only patients with indications to surgery were included in this study. During surgical intervention, immediately after surgical excision of the primary tumor, 4 microbiopsies were taken by curette technique ("Acu-Dispo Curette"), in addition to a control check at tumoral site, with repeated scraping movements to reach the superficial layers of connective tissue: the first from the macroscopic healthy margin in correspondence of the greater diameter of the lesion, the second one at 5 mm from resection margin, the third one at 10 mm from resection margin, and the last one contralaterally.

The control check (Figure 1) and collected samples for each patient were immediately sent for FISH analysis with

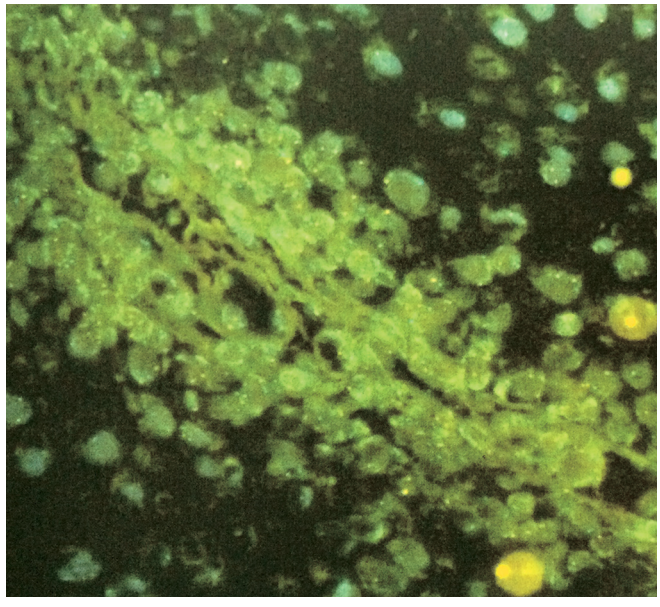

Figure 1.—FISH analysis of oral squamous cell carcinoma specimen: amplification of 3q26.3 locus in most cancer cells.

TABLE I.—*Patients and personal data.*

Patient	Age	Gender	Smoke	Alcohol	Tumor location	TNM	Grading
1	80	M	Yes	Yes	Tongue	T2 N0	G1
2	83	M	Yes	No	Retromolar trigon	T3b N2b	G3
3	68	F	No	No	Cheek	T1 N0	G1
4	80	M	Yes	No	Retromolar trigon	T4 N2b	G3
5	62	M	Yes	Yes	Tongue	T1 N0	G2
6	63	M	Yes	Yes	Tongue	T1 N0	G2
7	65	M	Yes	No	Tongue	T1 N2b	G2
8	78	M	Yes	No	Tongue	T2 N0	G2
9	82	M	Yes	No	Tongue	T3 N1	G2
10	35	M	Yes	Yes	Tongue	T1 N2	G1
11	70	F	Yes	No	Tongue	T1 N0	G3

a probe characterized by 3q26.3 region, following the protocol described by Singh *et al*.[2] The main specimen was of course sent for histopathology examination to confirm the preoperative diagnosis and staging.

Descriptive statistics were used to summarize study data, as the low numerosity of this preliminary study did not allow further statistics.

IRB approval exempt. We followed Helsinki Declaration guidelines. The study was approved by the committee on research ethics at the institution in which the research was conducted and any informed consent from human subjects was obtained as required.

Results

Eleven patients (9 males, 2 females) were included in the study. Demographic and clinical data are resumed in Table I. Mean age of the study population was 69.6 years (SD, 13.9; median, 70), with 10 patients out of 11 being older than 61 years. Ten patients had history of smoking, whereas just 4 patients referred habitual alcohol use. On the whole, 4 patients had together exposure to both risk factors of smoking and alcohol (patients 1, 5, 6, and 10). Most involved tumor site was the tongue (9 cases), followed by retromolar region (2 cases). Tumor grading was G3 in 3 cases, G2 in 5 cases, and G1 in 3 patients.

Table II presents the results of FISH analysis by 3q26.3 probe following scraping during surgery in the foreseen cases. In three patient, the scraping site n 1 was positive, thus identifying genetic abnormalities in 3q26.3 at the macroscopic healthy margin in correspondence of the greater diameter of the lesion (Figure 2). One of these three patients (patient 5) also presented positivity at scraping site N. 2 at 5 mm from resection margin.

TABLE II.—*Results of FISH analysis by 3q26.3 probe in the different sites of scraping.*

Patient	Scraping site 1	Scraping site 2	Scraping site 3	Scraping site 4
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	+	-	-	-
5	+	+	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	+	-	-	-
11	-	-	-	-

Scraping site 1: macroscopic healthy margin in correspondence of the greater diameter of the lesion; scraping site 2: at 5 mm from resection margin; scraping site 3: at 10 mm from resection margin; scraping site 4: contralaterally.

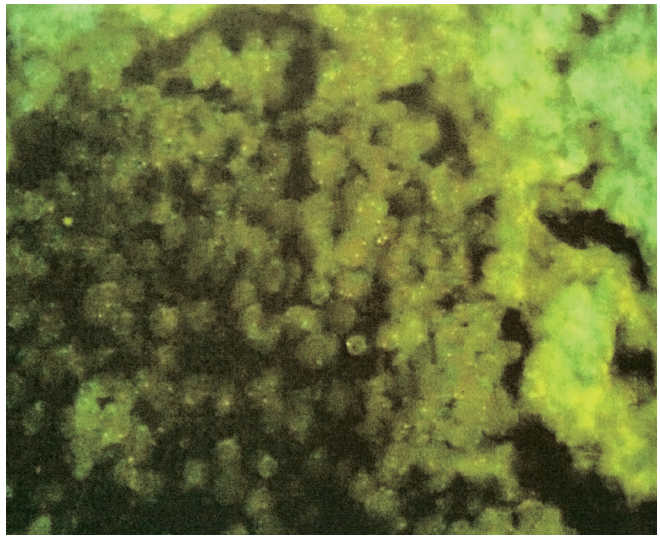

Figure 2.—FISH analysis of not neoplastic mucosa tissue (scraping site 1) in a patient (Patient 5) affected by oral squamous cell carcinoma: amplification of 3q26.3 locus in epithelial healthy cells.

Two of the three patients (patients 5 and 10) that presented positivity to FISH analysis had both risk factors (smoke and alcohol), were male and were the youngest in the study population (62 and 35 years respectively). Instead, tumor anatomical location, TNM staging, and grading did not vary by the 3q copy number status.

Discussion

OSCC represents a continuum of disease that develop along a multistep pathway in tissues that have encountered long periods of carcinogen exposure and thus, have accumulated genetic hits in a number of functional targets that are relevant to tumor evolution. Several genetic abnormalities have been investigated as markers of disease progression and/or outcome in OSCC by a variety of techniques.[1-3, 5]

We applied the curette technique in association with FISH analysis in order to assess and investigate genetic abnormalities in the clinically normal mucosa adjacent to areas invaded by OSCC, based on the oral field cancerization hypothesis.

The theory of field cancerization derives from the effort of explaining the increased occurrence of local secondary tumors in oral cavity and upper aero digestive tract.[1-3, 6]

Three theories about the concept of field cancerization have been proposed: 1) the first theory describes the whole oral cavity as one big field, as the whole oral cavity is exposed to carcinogens

leading to multiple lesions arising independently, either simultaneously or after a period of time. Lesions arising independently would mean that the lesions would be polyclonal; they do not share a common ancestor; the other two theories state that multiple lesions occur either by 2) the migration of mutated cells in the tumor adjacent mucosa, or by 3) the migration of mutated cells through saliva, or other means, as a form of micro metastasis. Migration of mutated cells in both instances would mean that the lesions share a common clonal ancestor; they would be monoclonal.[1-6]

The field cancerization theory also aims to distinguish between four types of second events: local recurrence, metastasis, second primary tumors and second field tumors.

In particular, second lesions with molecular profiles equal to those of primary tumors should be interpreted as local recurrences or metastasis depending if the second event developed in adjacent or distant site respectively. Secondary field tumors present some partial differences as for genetic profile from the primary tumor, suggesting that it has arised from a preconditioned field but followed different carcinogenetic pathways. Second primary tumors, being genetically and clinically independent, should instead show different genetic profiles.[1-6]

As for genetic abnormalities in OSCC, it has been stated that 3q amplification is a transition event in the progression to invasive squamous cell carcinoma.[2]

In particular, genomic alterations of chromosomal region 3q26-3qter are a major feature of neoplastic transformation, determining a higher rate of local recurrence.[2-4] In fact, the presence of 3q amplification was shown to be an independent predictor of clinical tumor behavior and long-term outcome in patients with head and neck cancer, thus suggesting that 3q amplification may represent an important biomarker for the assessment of patients with OSCC.[2]

The identification in this study of copy number amplification of 3q26.3 region supports the concept that oncogenomic 3q26-27 amplification may be a transitional event during oral carcinogenesis.

Limitations of the study

Limitations to the FISH assay include the technical artifacts leading to signal loss or gain. Then, cells in the G2 or late S phase with decondensed DNA may display significantly separated signals for the sister chromatids, leading to an incorrect interpretation as hyperdiploid. The centromeric sequences are highly repetitive sequences in the genome and less specific homology may be recognized by cross-hybridization.[1-5]

Furthermore, this is of course a preliminary study and the low numerosity of the study population do not allow to draw any definitive conclusion, but further studies are needed to validate this technique and to obtain definitive results.

However, we have to consider that the detection of OSCC in the early asymptomatic stages can improve survival rates. Scraping of clinically normal oral mucosa has been valuable for genotyping or cytopathological evaluation. Therefore, our results could be a first step in the assessment and early diagnosis of oral field cancerization in patients affected by OSCC.

Conclusions

In conclusion, we have extended the use of the curette technique in combination with FISH to detect copy number amplification of 3q26.3. Oral field cancerization seems to be confirmed by our results.

Our findings distinguish FISH as an attractive, non-

invasive laboratorial approach for testing field cancerization in individuals affected by OSCC. Finally, this current analysis further indicates that the development of this technique could also represent a tool for the establishment and maintenance of a more appropriate follow up in individuals affected by OSCC; the non-invasive sampling procedure here described seems to be simple, fast to perform, and ensures a regional collection of the cells.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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