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GENETIC PROFILES OF FIELD CANCERIZATION AND INTRATUMORAL HETEROGENEITY IN ORAL SQUAMOUS CELL CARCINOMA

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1.HEAD AND NECK SQUAMOUS CELL CARCINOMA AND ORAL SQUAMOUS CELL CARCINOMA

1.1. Definition

The term head and neck cancer (HNC) defines a group of malignant cancers that arise in different anatomical districts in the region of the head and neck including oral cavity, pharynx and larynx.

However different definitions have been proposed.

For example the National Institute of Health, and more precisely, the National Cancer Institute reports that HNC correspond to “Cancer that arises in the head or neck region (in the nasal cavity, sinuses, lips, mouth, salivary glands, throat, or larynx).(1) Differently, UK’s National Health service describes HNC as a miscellaneous group of cancers from 30 different organs or tissues in the head and neck region including “eye cancer, nasal and paranasal sinus cancer (cancers in the nasal cavity and in the sinuses around the nose),nasopharyngeal cancer (the area that connects the back of the nose to the back of the mouth), mouth and oropharyngeal cancer (cancers of the tongue, the gums, cheeks, lip and floor and roof of the mouth), larynx or laryngeal cancer (cancer of the voice box) and oesophageal cancer (cancer of the food pipe or gullet).”(2)

Almost 90% of HNC are squamous cell carcinomas deriving from epithelial cells of mucosal upper digestive tract.

Head and Neck Squamous Cell Carcinoma (HNSCCs) are strongly associated with tobacco exposure, alcohol, areca nut, low vitamins intake.

Recently, also oncoviruses infection has been recognized as a risk factor for HNSCC development and in particular HPV 16,18, EBV, HHV-8.(3)

HNSCC usually adopts an aggressive clinical behaviour due to its ability to invade rapidly adjacent tissue and cervical lymph nodes.(4)

Moreover, HNSCC local destructive action is linked to high risk of subsequent local relapses or distant lymph node metastasis which comprises several demolitive surgeries.

The tendency of multiple aggressive local or distant relapses is biologically explained by the theory of “field cancerization “. This theory was first proposed by Slaughter in 1953 and defines a genetically altered mucosal filed extended far beyond the border of the primary tumour, in which several squamous cell carcinomas may arise regardless surgical excision.(5)

Treatment modalities include surgical excision of the neoplasia, associated to radiotherapy or adjuvant chemotherapy in advanced stages.

Early diagnosis proved to impact positively overall survival and treatment efficacy. Nevertheless, despite clinical efforts in improving early diagnosis the survival rate hasn’t significantly improve in the last decade.(6)

Therapy for HNSCC includes also the use of Cetuximab, a monoclonal antibody against Epidermal Growth Factor Receptor (EGFR) approved by the FDA (Food and Drug Administration).(7)

1.2. Epidemiology:

Incidence of HNSCC worldwide varies significantly depending on the anatomical site included in the statistics. On average, HNSCC classifies as the 6th more common cancer accounting for 4,3 % of the tumours, 599.637 new cases and 224.834 new deaths each year. (Figure)

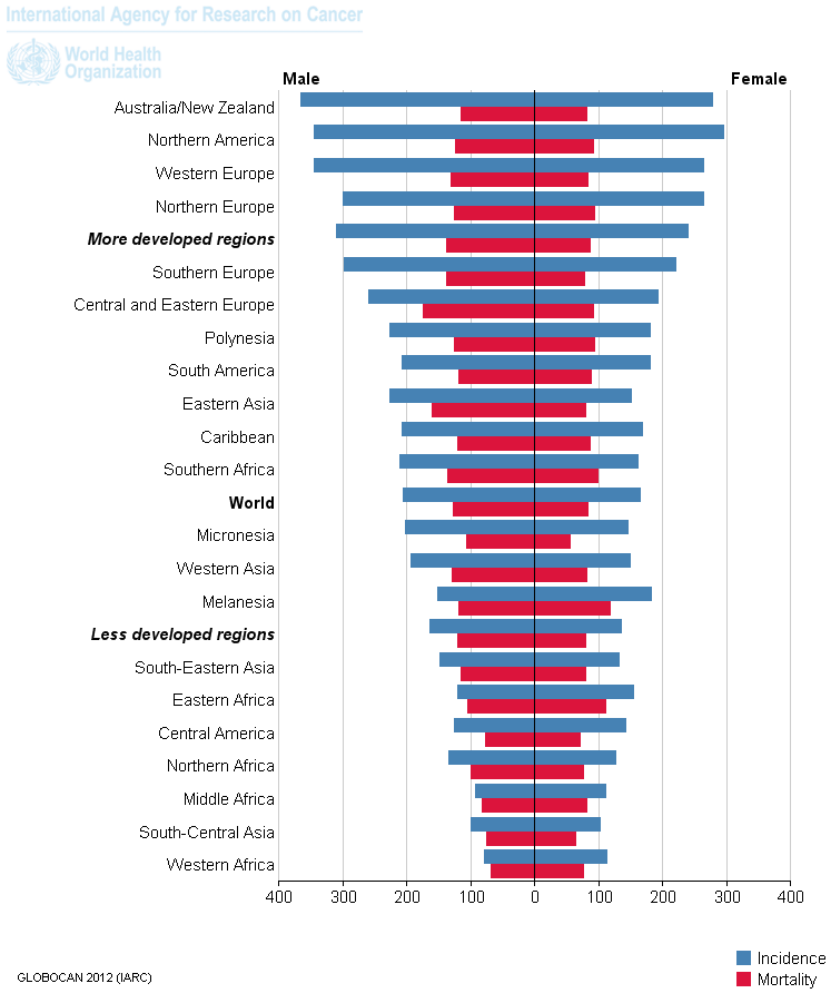


FIG.1 The Graph shows the value of incidence (blue) and mortality(red) of Head and Neck Cancer worldwide. Left horizontal bars shows values for male population while right horizontal bars the values for women. Source: https://gco.iarc.fr/

However, if squamous cell carcinoma of the upper 2/3 of the oesophagus was also included in the HNSCC group, incidence would rise sharply, classifying as the third more frequent carcinoma in men worldwide.

As far as oral squamous cell carcinoma (OSCC) is concerned data estimates that 300000 cases new cases per year and the mortality rate reaches levels around 145 000 cases per year in Europe.(8)

However, higher incidence levels occur in Asia and in particular in Melanesia and India. Widespread use of areca nut and betel leaves in these regions explains the results.

Unfortunately, high incidence levels of OSCC are followed by similar data on mortality. In Taiwan, for example, OSCC embodies the first cause of death among men aged 25-44 years old. Low access to health services is a significant co-factor of high mortality in the region.(9)

HNSCC incidence is greater in men. Lifestyle plays an important role in these findings. In fact, despite men are traditionally more exposed to well known risk factors for oral squamous cell carcinoma such as tobacco smoke or alcohol consumption, incidence of OSCC in women is increasing as effect of similar exposure to risk factors in both sexes. Additionally, deficit in estrogenic hormones has also been advocated to play a role in OSCC tumorigenesis. (10)

OSCC tends to affect men in their sixth decade of life. Epidemiologic studies confirm worse trends in South Asia (fifth decade of life) with respect to North America where mean age of developing an OSCC stands above the threshold of seventh and eighth decade of life.(11)

However, recent data shows that the number of young patients affected by OSCC is increasing. So far, No scientific evidence seems to support that HPV is responsible for the increase of OSCC in young patients.

Yet, HPV related OSCC show better responsiveness to therapies and better survival rates. (12)

1.3. Risk Factors

Tobacco smoke and alcohol abuse are major risk factors in OSCC development. Nearly 80% of OSCCs seem to be related by the action of these two factors. Accordingly, suspension of tobacco or alcohol exposure would reduce approximately the incidence of OSCC by 80%.(13)

Additional risk factors include viral agents (i.e. HPV) chronic oral trauma, UV exposure and immunodepression.

Tobacco:

The risk of developing OSCC is 5-9 times greater in smokers with respect to non-smokers. OSCC risk is dose-dependent. (14)The risk of developing OSCC doubles if more of 20 cigarettes/day are smoked. Additionally, in patients who do not quit smoking after diagnosis, the risk of developing a second neoplasia after resective surgery is up to six times higher the risk of patients who quit.(15)

Cancerogenetic action of tobacco smoke is mainly due to the presence of mutagenic compounds that can be found in products of combustion. More than 70 carcinogenetic compounds have been identified.

These include : polycyclic aromatic hydrocarbons (benzopyrene, anthracene) nitrosamines of tobacco (N-nitrosonornicotine, N-nitrosodimethylamine) aromatic amines (2-toluidine), aldehydes (formaldeyde) metals and organic compounds.(16)

Direct termical irritation of tobacco smoke on mucosae seems to play an important role as co-factor.(17) In fact, reverse smoking, or the habit of smoking a cigarette with the lit end inside the mouth which is widespread in Andhra Pradesh and in Phylipinnes,correlates with higher risk of malignant transformation.(17)

Tobacco exposure other than smoking is also related to OSCC development.

For example, in India and South East Asia, the diffusion of chewing betel leaves in association with tobacco, areca nut and other irritating compounds not only induces the onset of submucous fibrosis, a pre-malignant lesion at high risk of cancer, but also reflects greater rates of OSCC incidence.(18)

Alcohol

Recent data indicate that daily alcohol consumption exceeding 10g g/day cause negative effects on general health. Epidemiological data partially correlates an increase in HNSCC in young adults in UK with heavy alcohol drinkers.(19)

Carcinogenetic effect of alcohol seems to synergic to tobacco smoking. In fact, the risk of OSCC in both heavy smokers and heavy drinkers appears to be 13 fold greater the risk of tobacco or alcohol considered independently.(20)

Similar to tobacco smoking carcinogenetic action, also alcohol action seems to be dose dependant in increasing OSCC development . Alcohol abuse in the post-operative interval resulted in a significant reduction in survival rates, not necessarily related to disease relapses.(21)

Experimental studies proved that ethanol acts as a mutagen as well as can act as solvent for other mutagens. Direct oncogenetic action seems to be related to acetaldehyde, one of the primary metabolites of alcohol and a powerful mutagen. (22,23)

Fungal and viral infection

Yeats and viruses have been investigated for years as potential triggers or co-factors of malignant transformation in OSCC.(24)

In particular Candida spp. is able to produce powerful mutagens such as N-Nitroso bezilmetilamine which seems to play a pivotal role in cancer development. Candida spp. is also associated with premalignant lesions at moderate- high risk of evolving into OSCC such a hyperplastic chronic candidiasis.(25)

However, neither Candida associated premalignant lesions nor the presence of Candida spp in samples from OSCC are able to differentiate between Candida direct carcinogenesis

and Candida super-infection of premalignant/malignant lesions. (26)Consequently, role of Candida spp. in oral carcinogenesis are yet to be fully understood.

HPV infection has been recently related to HNSCC development, and in particular genotypes 16 and HPV 18. HPV has preferential tropism in the pharynx where lymphoid tissue is abundant, and invasion of the mucosal barrier is easier. (26)

In the oral cavity HPV associated OSCC tend to arise posteriorly, at the base of the tongue and in proximity to palatine tonsils while are far less frequent in the anterior part of the oral cavity.(27)

Oncogenetic effect of HPV is mediated by HPV associated oncoproteins such as E6 and E7 which can interfere with in many important pathways such as TP53 inducing tumoral degeneration.(28)

UV radiations and immunodepression

Long-time exposure to UV is a well-known risk factor for the development of OSCC of the lower lip.

In the last years awareness of risk factors among general population has improved incidence rates for lip OSCC, whose prognosis is less aggressive than other OSCCs.(29)

Immunodepression has a deep negative impact on tumorigenesis. As far as OSCC is concerned, bone marrow transplant and related immunosuppressant regimes expose the patient to a 6-10 folds higher risk of developing OSCC.(30)

Similarly, HIV+ patients suffering from AIDS show and augmented risk of developing OSCC with respect to general population.

In these patients immune system is compromised and tumours can escape immune surveillance more easily with more descriptive clinical effects. (31)

2. TNM CLASSIFICATION*:*

TNM classification systems is based on the clinical extension of the disease and includes three parameters: dimension of primary tumour (T), nodal involvement (N), presence of distant metastasis (M).

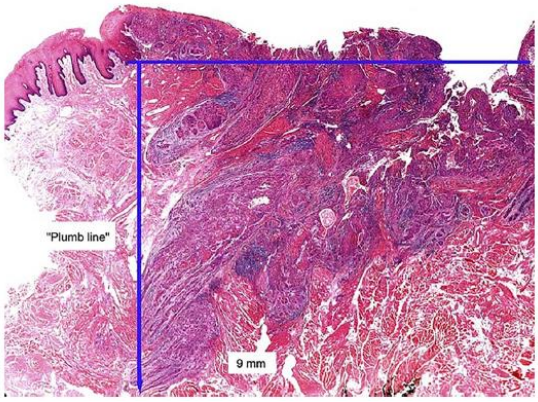
Clinical classification (cTNM) is based on data obtained before surgical treatment. After tumour resection, cTNM is normally compared to histological classification (pTNM).

In HNSCC T parameter differs depending on the site of primary tumour. N classification of cervical nodes, on the contrary, is almost universal and only nodal involvement of nasopharynx has an independent classification.(32)

TNM CLASSIFICATION TO DEFINE LIP AND ORAL CAVITY SQUAMOUS CELL CARCINOMA

TAB1. The Table shows the criteria of classification for T category according to TNM classification. Source: AJCC 8th Edition

T CATEGORY	T CRITERIA
TX	Primary tumor cannot be assessed
Tis	Carcinoma in situ
T1	Tumor ≤2 cm, ≤5 mm depth of invasion (DOI) (DOI is depth of invasion and not tumor thickness)
T2	Tumor ≤2 cm, DOI >5 mm and ≤10 mm *or* tumor >2 cm but ≤4 cm, and ≤10 mm DOI
T3	Tumor >4 cm *or* any tumor >10 mm DOI
T4	Moderately advanced or very advanced local disease
T4a	Moderately advanced local disease: (lip) tumor invades through cortical bone or involves the inferior alveolar nerve, floor of mouth, or skin of face (ie, chin or nose); (oral cavity) tumor invades adjacent structures only (eg, through cortical bone of the mandible or maxilla, or involves the maxillary sinus or skin of the face); note that superficial erosion of bone/tooth socket (alone) by a gingival primary is not sufficient to classify a tumor as T4
T4b	Very advanced local disease; tumor invades masticator space, pterygoid plates, or skull base and/or encases the internal carotid artery



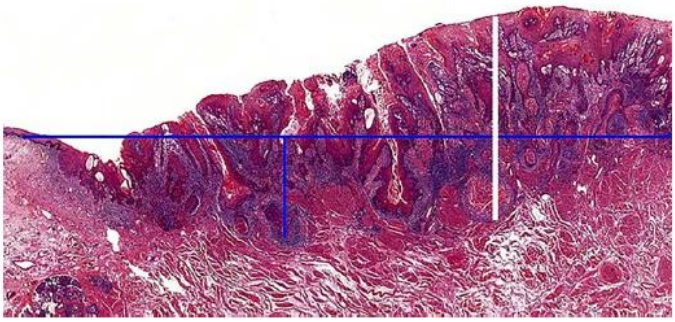


FIG.2 (left) The mesasure of depth of invasion is assessed by dropping a "plumb line" from the horizon (level of basement membrane relative to the closest intact squamous mucosa)
FIG.3 (right) Difference between "depth of invasion" (blue bar) and tumour thickness (white bar)

TAB2. The Table shows the criteria of classification for N category according to TNM classification.
Source: AJCC 8th Edition

N CATEGORY	N CRITERIA[b]
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension and ENE-negative
N2	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension and ENE-positive; *or* more than 3 cm but not more than 6 cm in greatest dimension and ENE-negative; or metastases in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension and ENE-negative; *or* metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension, ENE-negative
N2a	Metastasis in a single ipsilateral or contralateral lymph node 3 cm or less in greatest dimension and ENE-positive; *or* metastasis in a single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension and ENE-negative
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension and ENE-negative
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension and ENE-negative
N3	Metastasis in a lymph node more than 6 cm in greatest dimension and ENE-negative; or metastasis in a single ipsilateral lymph node more than 3 cm in greatest dimension and ENE-positive; *or* metastasis in multiple ipsilateral, contralateral, or bilateral lymph nodes, with any ENE-positive
N3a	Metastasis in a lymph node more than 6 cm in greatest dimension and ENE-negative
N3b	Metastasis in a single ipsilateral node more than 3 cm in greatest dimension and ENE-positive; *or* metastasis in multiple ipsilateral, contralateral, or bilateral lymph nodes, with any ENE-positive

3. OSCC AND THE THEORY OF FIELD CANCERIZATION:

3.1 Introduction:

The term " field cancerization" was introduced by Slaughter and colleagues in 1953 (5) to explain the appearance of multiple oral squamous cell carcinomas (OSCC)in the same patient. This model for head and neck cancer is still actual nowadays.

3.2 Slaughter's principles and first evidences.

Analysing a population of 783 patients with OSCC Slaughter et al.(5) recorded in a publication titled "field cancerization in oral stratified squamous epithelium. Clinical implications of multicentric origin" that 1) oral cancers usually had the tendency of spreading more easily in laterality than in depth 2) that the mucosa surrounding the neoplasia frequently harboured clinical or morphological atypia. 3) that 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.

A similar apparently independent multifocality, they acknowledged, was "well above the statistical possibility of chance occurrence, therefore they concluded that multiple OSCC should be the effect of a "field cancerization," in which an "area of epithelium has been preconditioned by an as-yet-unknown carcinogenic agent. Such a carcinogenic influence if operative long enough in time and intense enough in exposure produces an irreversible change in cells and cell groups in the given area, so that change of the process toward cancer becomes inevitable. "

Nowadays we know that not only was Slaughter's intuition correct but also that it deeply helped our understanding of the natural history of OSCC. At that time, however, molecular techniques could hardly be performed hence it should not be surprising that a work citing the theory of field cancerization appeared no sooner than 16 years after Slaughter's publication.

In 1969 Roth et al (33) studying the healing performances of UV-induced damaged cells acknowledged that epithelium from patients affected by oral and upper aero digestive tract squamous cells carcinoma showed a reduced repairing ability. They reported that a similar deficit could be the result of a damage at DNA level predisposing for further tumour development.

Albeit Slaughter's theory had gained some clinical evidence it still lacked proper scientific basis and it could not gain widespread popularity.

In 1982, Incze et al (34) observed, at electron microscope level, biopsies from normal appearing epithelium in patients with squamous cell carcinoma of the upper aero digestive tract and they reported that morphologic abnormalities were consistent with the concept that carcinogenesis is a multistep process of sequential neoplastic development extending over a long period of time.(Fig.1)

Only two years later, in 1984, Strong et al (35) observed that Field cancerization could be demonstrated by supravital staining with toluidine blue or by electron microscopic study of random biopsies taken from apparently normal mucosa.

Both authors not only confirmed Slaughter's statements with clinical and morphological means but also identified tobacco and alcohol exposure as the "yet-unknown carcinogenic agent" able to condition mucosal behaviour towards cancer development.

3.3 Biomolecular evidences of Slaughter's field cancerization

In 1996 Califano et al(36), in response to the lack of knowledge surrounding genetic progression of head and neck squamous cell carcinoma and genetic basis for field cancerization, published a PCR based analysis of loss of heterozygosis (LOH) at selective genes putatively involved in head and neck carcinogenesis. They studied a population of eighty-seven lesions of the head and neck, including pre-invasive lesions and benign lesions associated with carcinogen exposure. Observing the level of accumulation of gene losses at different degrees of pre-neoplastic lesions they found that it was possible to

identify a model that could explain the progression of a mutated epithelium towards squamous cell carcinoma. However, despite some mutations were more likely to occur at specific stages or pre-malignancy, they suggested that accumulation rather than the order of genetic events led mutated cells in the progression towards cancer.

Genes studied by Califano and colleagues(36) included the 9p21 locus corresponding to an area of genetic loss common to many solid tumours containing p16, a cyclin-dependent kinase inhibitor involved in cell cycle regulation. Region 11q13 that includes the bcl-1/int-2 locus, an amplicon carrying the proto-oncogene cyclin D1, the p53 gene locus located at 17p13, the 3p21 locus and 13q21 locus that contains an area with frequent LOH near the retinoblastoma locus(36).

In particular, 3p and 17p losses were more frequent in mucosa undergoing dysplastic modification while 11q and 13 q losses could be found in epithelium preceding malignant transformation.

Califano's results were confirmed by Patridge et al. in 1997 (37) and Lydiatt(38,39) in 1998 where LOH was found in dysplastic tissue surrounding tumours and histologically normal mucosa respectively.

Genetic approach paved the way to a large number of studies with different molecular techniques that tried to disclose hidden mechanisms of oral carcinogenesis with the aim at discovering clinically relevant biomarkers to be used in clinical practice.

As a consequence, the extension of the field and the related premalignant lesion whence the field could have been arisen, became a major concern.

Noteworthy, Ai H. (39) in 1999 detected by FISH chromosome aneuploidy in mucosa distant from the carcinoma. Interestingly, 9/10 tested patients were smokers with respect to only one non-smoker patient with aneuploidy. Molecular tests can be of difficult application in daily practice, therefore several authors tested immunohistochemical markers useful to evidence areas od filed cancerization.

Van Oijen e Slootweg in 2000 (40)demonstrated that immunostaining of P53 and EGFR was abnormal in apparently normal mucosa of smokers when compared to non-smokers subjects. (Fig.2)

Tabor et al. (41)demonstrated that the presence of Ki67 positive mature keratinocytes , corresponded to areas of LOH. This observation has been validated by Montebugnoli et al(42–44)who demonstrated that the immunohistochemical analysis of ki67 in oral mucosa located in the cheek opposite to the OSCC can act as prognostic biomarker as, when overexpressed, had an impact on the aggressiveness of the primary tumour.

On the other hand, studies speculated on how far the field could extend. Interestingly Griffioen GH, et al.(45) In 2015 reported that patients successfully treated for head and neck cancer died of primary lung cancers suggesting that the field could extend not only through the entire upper aero digestive tract, but also in the deep respiratory system.(Fig.3)

3.4 Multiple Oral Lesions: Different models for field cancerization and the problem of clonality

The theory of field cancerization derives from the effort of explaining the increased occurrence of local secondary tumours in oral cavity and upper aero digestive tract. However, the exact mechanisms through which this phenomenon occurs are still matters of debate and different theories has been formulated so far.

In 1999 Garcia et al (46)described the presence of cluster of cells usually positive for TP53 in the normal mucosa of patients with head and neck squamous cell carcinoma. He called these clusters “patches”.

Assuming a monoclonal origin for oral squamous carcinoma, he hypothesized that these clusters of genetically altered cells could clonally expand as a consequence of proliferative advantages over non mutated cells generating greater area of mutated mucosa, better defined as “field”. Hence, according to Califano’s model of carcinogenesis(47), the

accumulation in the field of further mutations could lead to the development of independent neoplastic events.

On the other hand, an alternative theory reviewed by van Oijen et al. in 2000(40), reported that the occurrence of multiple pre(neoplastic) events could also result from a migration of mutated cells that, acting as micro metastasis, could generate different tumours at different local sites.

Apart from field cancerization theoretical models, the distinction between a local recurrence (LR) a metastasis or a second primary tumour (SPT) always represented a critical clinical issue able to deeply influence therapy and prognosis.

Before genetic approach, Second Primary Tumours (SPT) were usually defined by many researchers using Warren and Gates(48) criteria developed in 1932. This method was based on clinical and morphological parameters. In particular, SPTs were defined as two neoplastic lesions showing definitive and distinct pictures of malignancies after excluding that one could be the metastasis of the other. However, in case of two lesions both in the same anatomical area, the minimal distance to exclude a local recurrence was controversial as some researchers accepted 2 cm while others 1.5 cm.

Furthermore, second events may chronologically develop synchronously or methachronously depending on whether the interval between the two carcinomas is lower or higher than six months respectively. Similarly, to spatial criteria, also time of occurrence could easily be confounding in the diagnosis of a second event.

As aforementioned criteria were easily matter of debate and scarcely took into account the theory of field cancerization, in 2002 Braakhuis (49), proposed a modification based on genetic profiles.

Evidence deriving from many works on LOH and *TP53* mutations led Braakhuis to identify four types of second events: Local recurrence, metastasis, second primary tumours and second field tumours.

In particular, common molecular profiles between the primary tumours and the second lesions should be interpreted as consistent with local recurrence or metastasis depending if the second event developed in adjacent or distant site respectively. On the other hand, partially different genetic profile between the two lesions, should be suggestive of a secondary field tumour that, arising from a preconditioned field but followed different carcinogenetic pathways. Second Primary Tumours, being genetically and clinically independent should instead show different genetic profiles.

Further studies(50–53) confirmed the utility of Braakhius modified criteria and disclosed new genetic methodologies to perform clonal analysis of multiple squamous cell carcinomas. Remarkably, the high frequency rate of mtDNA mutations in tumors, especially those found in the D-loop region, a non-coding region, along with numerous mitochondrial genomes present in a single cell, has made mtDNA a reliable marker for clonality assays from microdissected paraffin-embedded tissue samples. (54,55)

As a result, mtDNA analysis not only demonstrated higher diagnostic sensibility when compared to clinical and temporal criteria, but also resulted more informative with respect to TP53 analysis, reflecting the ability of some squamous cell carcinoma to follow carcinogenic pathways different from *TP53* mutations.

In addition, mtDNA analysis resulted highly useful in disclosing possible mechanisms behind the cancerization of skin graft after surgery such as the spread of the clonal cell population to the cutaneous flap stimulated by cytokines produced by the grafted skin.

In fact, as reported by Foschini et al,(50) in all three studied cases, the neoplastic lesions arising in the skin graft showed a clonal relationship with the previous OSCC and, on the basis of the results obtained by mtDNA analysis, could be considered as a recurrence of the primary OSCC.

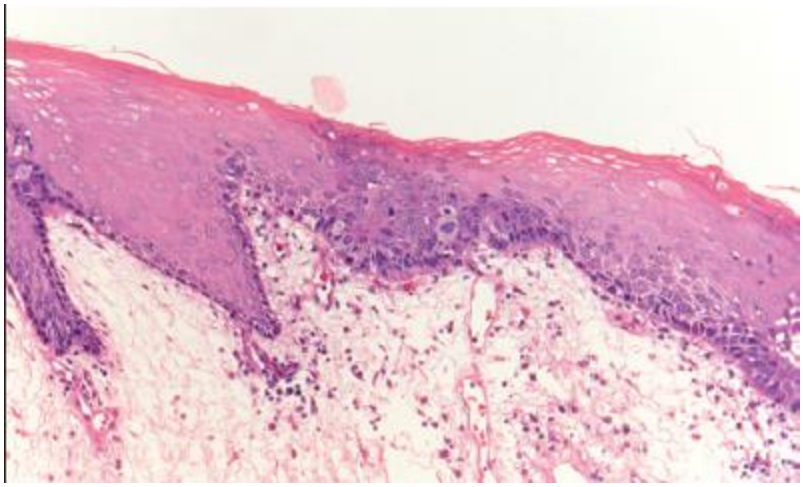


FIG.3: Apparently normal oral mucosa can present small areas of altered epithelial keratinocytes

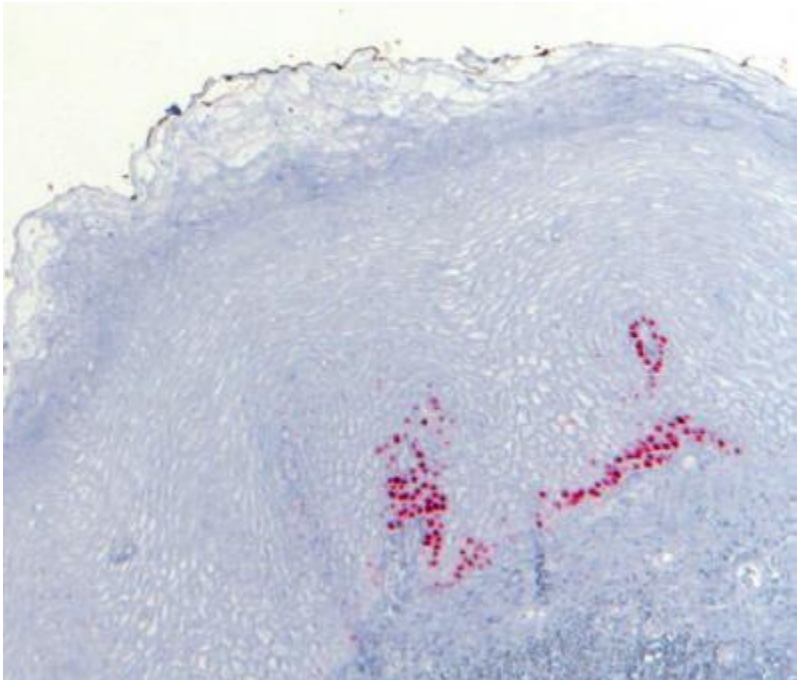


FIG.4: Immunohistochemical staining for TP53 in a restricted “patch” of oral epithelium supports the theory according to which the progression of a field of genetically altered kerationcytes precedes and promotes oral carcinogenesis

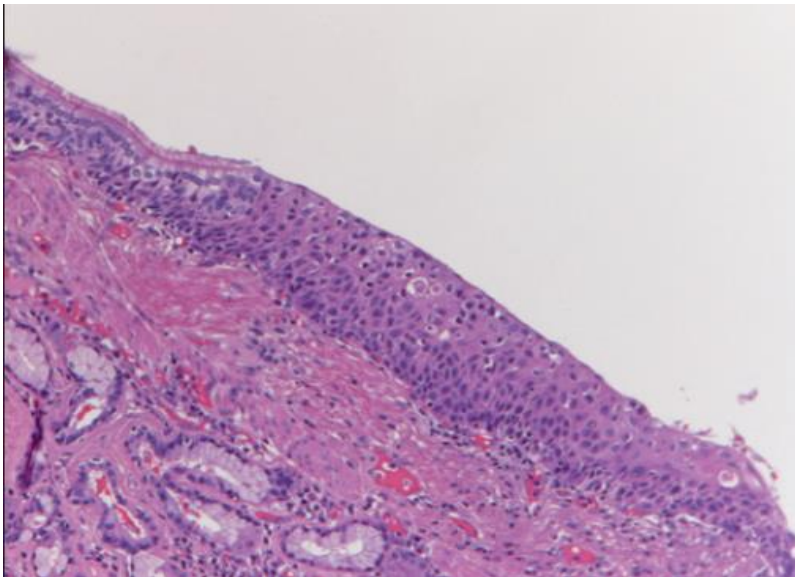


FIG 5: Smokers can present small areas of dysplasia in bronchial epithelium

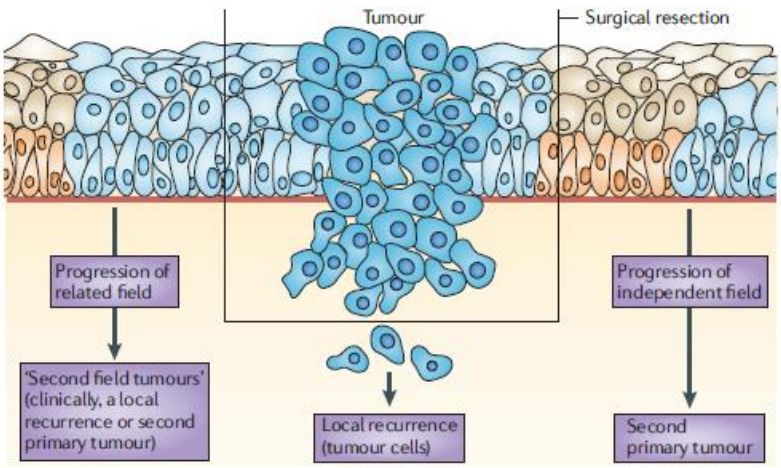


FIG 6: According to field cancerization theory three different types of second events can develop after primary tumour resection: Local Recurrence, Second Primary tumour and second field tumour. Source: https://www.tandfonline.com/doi/pdf/10.1586/14737159.2016.1126512

4. THE CONCEPT OF INTRATUMORAL HETEROGENEITY:

Introduction

The idea of heterogeneity among tumours and within tumours is not new. The observation that microscopic morphology may not be uniform throughout the entire extension of a primary tumor suggested that differences may exist among tumours cells.

Slaughter et al, studying morphology of OSCC recorded that OSCC may consist of multiple independent foci that eventually may converge. (5)

In addition, the hypothesis of tumoral heterogeneity was supported by clinical data even before bimolecular investigations, as tumours with similar morphology but deeply different behaviour and prognosis.

However, only in recent years, thank to improved sequencing technologies it was possible to study tumor heterogeneity at a deeper level.

Tumours should thus be regarded as complex biological entities. Heterogeneity seems to involve clinical, phenotypic, genetic and epigenetic variables as disclosed by recent studies of molecular biology applied to tumours.

Two theories have been proposed to explain intratumour heterogeneity: clonal evolution models and cancer stem cells models (CSC).(56,57)

Both theories assume that tumours are initiated by single cells that acquire molecular aberrations that confer on them proliferative advantages and escape from programmed death and believe that microenvironment plays an important role in tumor progression. Nevertheless, the two theories also display important differences.

4.1 Clonal evolution theory:

In 1976 Nowell proposed a theory of clonal evolution assuming that a single cell could give rise to a tumor through a continued accumulation of genetic mutations.(58)

Genetic instability could then result in progressive formation of clonal populations contributing to intra-tumor heterogeneity.

Two types of clonal evolution have been described : linear and branched evolution.

In linear evolution mutations are sequential and fitter clones tend to replace their predecessors. The grade of heterogeneity in linear evolution is low and can be observed only when new clones has only partially replaced the old ones.

Linear evolution has been observed in acute myeloid leukaemia and multiple myeloma.

In the branched evolution theory, different subclones coexist and evolve as the branches of a tree.

Early mutations, being shared by all subclones, can be seen as the trunk of the tree and reflect the genetic profile of the founder cell. During the evolution, genetic instability induces the formation of new subclones who acquire new genetic features.

Clonal evolution, due to its branching evolution, reflects higher grade of heterogeneity.

However, it is important to mention that proliferation and expansion of a given subclone is the result of selective pressures that follow Darwinian rules.

4.2 Cancer stem cell theory:

This theory claims that only a small amount of cells with great self renewal ability features has the potential to promote tumor progression. These cells are cancer-stem-cells (CSCs) When CSCs lose their “stemness” and differentiate into a non CSC phenotype they give rise to subclones with individual genetic profile contributing to intratumor heterogeneity.

The CSC theory was firs demonstrated in hematopoietic tumours and later in solid tumours. A set of membrane surface markers permits to identify CSCs (i.e $CD44^{+}$ /CD 24 ^{low}).(59) Experimental studies demonstrate that cell with CSCs profiles can be isolated and if injected in xenografts are able to induce tumor formation and progression.

CSCs divide asymmetrically, resulting in a self-renewal CSC and a non.CSC. Non CSCs represent the majority of tumor mass but contribute less to tumor proliferation.(60)

Interestingly, according to CSC model, tumor cells show cellular plasticity. In fact, not only CSC may differentiate into non.CSC but also a non-CSC may reversibly switch to a CSC particular conditions.(61)

Tumor biology is thus regulated not only through a hierarchic organization of cells (with and without self-renewal ability) but also by a homeostatic equilibrium between CSCs and non CSCs.(62)

Cancer stem cell model has gained increasing attention in recent years and studies have tried to study CSC markers in both OSCC and adjacent mucosa. The goal was to integrate our understanding of OSCC biology with evidence that markers associated with a stem-cell like behaviour.

Noteworthy, Simple et et al have proposed an intriguing theoretical model for field cancerization driven by genetically mutated stem-cells. In particular, according to that model, field cancerization is initiated by the carcinogen assault leading to genetic mutations (p53/p16) in a stem cell residing in normal epithelium. This cancer stem cell will proliferate and initially form a patch), which ultimately spreads to form a field. Histologically, at this

stage the cells remain in dysplastic or a premalignant stage. After getting a subsequent hit (RB), one of the cells in the field will form the primary tumor. The tumor will also host the increase in expression of different CSC-specific genes and other downstream markers of HNSCC tumorigenesis.

The progression of the field occurs either by the monoclonal or the polyclonal mode of cancerization. The CSCs of the field can also migrate (CD44h/ALDH1A1h) laterally to spreadthe field or get implanted at a new site and form a genetically similar tumor at a later stage signifying the monoclonal mode of field cancerization. On the other hand, multiple hits to the normal stem cells in the epithelium will lead to the development of independent clones (polyclonal mode). (63)

Nevetheless, as pointed out by Gonzales-Moles et al, results are divergent depending on which marker of CSC is used. Hence, the search for specific markers to identify these cells in routine laboratory workup are strongly encouraged.(64)

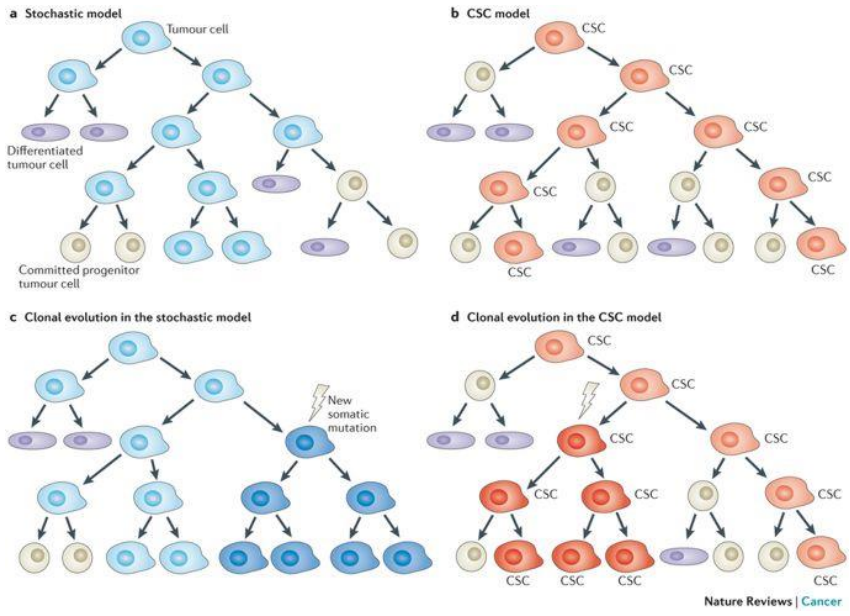


FIG 7 Stochastic model and CSC model describe different patterns of tumour evolution but both include the formation of genetic heterogeneity among tumour cells.Source: www.nature.com/articles/nrc3597

EXPERIMENTAL SECTION:

1. AIMS OF THE PHD PROJECT (ABSTRACT):

Worldwide high mortality rate of Oral squamous cell carcinoma (OSCC) gives rise to a considerable global public health burden (65). Despite the currently available therapeutic strategies, comprising the surgical excision of malignant tissue and a combination of radiotherapy and chemotherapy, the 5-year survival rate is still poor (66). The high mortality rate is usually attributed to late diagnosis, but some cases of OSCC surgically treated at an early stage still present with aggressive behaviour and disease progression (67,68) The aggressive behaviour of OSCC has been related to the “field cancerization concept” as the mucosa surrounding the primary mass is characterized by genetically altered epithelial cells that can escape clinical and histological examination and that may be responsible for cancer progression(69).

Recently, also the existence of a heterogeneous population of cells within the tumour mass (intratumor heterogeneity) has been linked to tumour aggressiveness and resistance to therapy.

Therefore, current research efforts focus on the discovery of new therapeutic strategies to determine the risks of OSCC occurrence, progression, and metastatic spread, and thereby to reduce mortality rates. Aim of the present PhD project was to investigate field cancerization and intratumour heterogeneity in OSCC adopting a biomolecular approach.

PROJECT 1: INVESTIGATION OVER IMMUNOHISTOCHEMICAL EXPRESSION OF KI67 IN DISTANT MUCOSAE AS A PROGNOSTIC FACTOR IN ORAL SQUAMOUS CELL CARCINOMA (PUBBLISHED AS: Ki67 Overexpression in mucosa distant from oral carcinoma: A poor prognostic factor in patients with long-term follow-up, Journal of Cranio-Maxillofacial Surgery, Volume 44, Issue 9, September 2016, Pages 1430-1435)

AIMS :

Genetically altered cells may escape macroscopic or histopathological examination and may require sophisticated biomolecular approaches to investigate genetic abnormalities of the epithelial field surrounding the tumor.(41,70–72). Immunohistochemistry is a simple, low-cost procedure that is frequently used to improve histological diagnosis. The Ki67 index is a good predictor of the presence of genetically altered cells in oral mucosa (41,73–76) and a good surrogate of sophisticated analyses, i.e., loss of heterozygosity (LOH)(41) when applied to the “non-neoplastic” mucosa surrounding a primary OSCC.

Aim of this project was to investigate whether Ki67 in distant mucosa from a long-term follow-up data from a cohort of patients treated for OSCC is associated with a poor prognosis in terms of locoregional control (LRC) of disease (appearance of local recurrence, second primary tumor, and lymph node metastasis) and disease-specific survival (DSS).

METHODS

Patients and procedures

The studied population consisted of 55 patients with a histological diagnosis of OSCC referred to our Department with a minimum of 12 months follow-up. The diagnosis and surgical treatment of OSCC were performed at the Department of Biomedical and Neuromotor Sciences University of Bologna, Sections of Oral Sciences, Anatomic Pathology at Bellaria Hospital and the Maxillofacial Surgery Unit, Sant'Orsola Hospital.

Patient assessment at presentation, before surgery, included examination and diagnostic imaging (head and neck computed tomography [CT] scan and/or magnetic resonance imaging [MRI]). Patient treatment consisted of a composite resection, including excision of the primary tumor with ipsilateral or bilateral neck dissection. Selective supraomohyoid neck dissection with lymph node evaluation on frozen sections was performed simultaneously with tumor resection in all cases diagnosed as cN0. If a lymph node metastasis was disclosed on frozen section, the neck dissection was extended to levels IV and V. Radical or modified radical neck dissection was performed for stage cNþ patients. During surgery, a specimen of macroscopically non-neoplastic mucosa was removed from the cheek opposite the primary OSCC for immunohistochemical evaluation of Ki67.

The work was approved by the ethical committee of the University of Bologna and all patients gave their written informed consent (CH-MAX-HNC Markers code 037/2008/O/Tess). The cohort of the present study included some of the patients analysed in previous studies(42,77)

Adjuvant therapy was administered according to the National Comprehensive Cancer Network guidelines(78) in locally advanced T3 and T4 lesions, lesions with high-grade histology or positive or close to the margins of surgical resection, and in cases with an N-stage higher than N1.

Follow-up was performed every 2 weeks for the first 2 months after surgery and then monthly during the first year after surgery, every 3 months during the second year after surgery, and finally every 6 months. A CT scan or MRI was requested every 6 months during the first 3 years after surgery and then once a year. Clinicopathological information obtained from each patient included: age; sex; tumor location; tumor stage, according to the TNM classification of the International Union Against Cancer(79); CT and clinical examination before surgical management to identify all patients with clinically positive cervical lymph node metastasis (LNM) using criteria defined in(80); histological grade,

defined according to(65); status of surgical margins assessed at the closest point to the surgical resection margin and classified in four categories according to the guidelines of the Royal College of Pathologists in the United Kingdom(81) as follows:

▪ cleared no evidence of microscopic carcinoma or presence of epithelial precursor lesions (EPLs) within 5 mm of the margin

▪ closed histological evidence of carcinoma between 5 and 1 mm of the margin but not at the margin

▪ involved when neoplastic cells appeared on the inked margin (cases with involved margin were excluded from the present study)

▪ EPL of moderate-to-severe dysplasia (high grade squamous intra-epithelial lesions according to the Ljubljana classification) or in situ carcinoma (82) but not invasive carcinoma within 5 mm of the margin.

Ki-67 expression was evaluated in each patient from both the biopsy samples obtained within the tumor mass and the clinically non-neoplastic mucosa located in the cheek opposite the primary OSCC. No distant site showed any sign of premalignant transformation.

All areas in the opposite cheek chosen for the biopsy sample were free of any trauma or clinically visible abnormal condition. All tissues were fixed in 10% formalin and embedded in paraffin according to routine practice. Serial sections were cut from each block and stained with hematoxylin and eosin for histologic evaluation and immunohistochemical analysis using the anti-Ki-67 monoclonal antibody (clone MIB 1, diluted 1:200 Dako, Glostrup, Denmark). The processing was performed in an automatic stainer (Autostainer, Ventana Medical Systems, Tucson, AZ, USA). Counting the percentage of positive nuclei in 400 consecutive epithelial cells from selected areas yielded a semiquantitative evaluation of the immunohistochemical results. In addition, the presence of mature

keratinocytes positive for Ki67 was recorded. Cut-off values of Ki67-positive cells of 42% in OSCC and 20% in non-neoplastic mucosa served to separate cases with “high” and “low” proliferative indexes(42,77) . These cut-off values in normal tissue were taken as reference according to previous works and were based on literature validated data. All slides were evaluated by a pathologist who was unaware of the clinical and follow-up information. The mucosal biopsy obtained from the cheek opposite to the OSCC was evaluated for the presence of oral potentially malignant lesions, which were reviewed and graded according to the recently proposed Ljubljana system(82) The disease-free survival endpoints were defined as the duration between treatment completion and the diagnosis of recurrence, lymph node or distant metastasis, death, or the last follow-up visit.

Statistical analysis

The outcomes of interest of the present study were disease specific survival (DSS) and locoregional control (LRC). DSS was defined as the time from diagnosis of the primary tumor to death from OSCC; LRC was defined as time from OSCC diagnosis to appearance of local recurrence (LR), second primary tumor (SPT), or LNM. The patient's age, sex, primary localization, T score, clinical staging, lymph node at appearance, tumor stage, histological grade, status of surgical margins, perineural and vascular invasion, Ki67 within tumor mass, and Ki67 from distant mucosa were analysed for their relationship to DSS and LRC. The survival rate was estimated using the Kaplan Meier method. Statistical significance was evaluated using the log-rank test. For those variables found to be statistically significant by univariate analysis, the Cox proportional hazards method with forward selection was used for further evaluation by multivariate survival analysis. Time was defined as the period between treatment and the target event (DSS or LRC) or last follow-up. P values <0.05 were considered to be statistically significant in all analyses.

RESULTS

Descriptive analysis

A total of 55 cases met the inclusion criteria. Of the 55 patients, 32 were male and 23 were female, with a median age of 61.9 ± 16.1

years. Index tumor locations were the following: in 24 of 55 patients, the tongue; in 9, the floor of mouth; in 9, the cheek; in 1, the soft palate; in 11, the gingival and hard palate; and in 1, the lower lip. In all, 46 patients were treated with surgery alone, and 9 patients were treated with adjuvant radiotherapy. All disease-free patients had a minimum of 12 months' follow-up (mean 53.7 ± 32.4 months; range 12e110 months). During the follow-up, 23 of 55 patients (41.8%) experienced a second locoregional neoplastic manifestation: 6 of 55 (10.9%) developed an LR, 12 (21.8%) developed an SPT, and 11 (20%) an LNM. Six of 55 patients presented with multiple second locoregional neoplastic manifestations. One of 55 patients (1.8%) developed a distant metastasis. Eleven of 55 patients (20%) died of OSCC; all presented with a locoregional neoplastic manifestation before death. The features of the study population in relation to DSS and LRC are summarized in Table 3.

Ki-67 values in the OSCC

The Ki-67 values in the OSCC tumor mass ranged from 8% to 90% with a mean Ki67 value of 46.6 ± 22.3; 17 of 50 patients showed “low” Ki67 values and 33 showed “high” Ki67 values in the tumor mass (>42%).

Mucosal biopsies performed on the cheek opposite the OSCC:

None of the biopsies presented features of squamous intraepithelial lesions (82). The Ki-67 values in the clinically and histologically “non-neoplastic” mucosa distant from the primary OSCC ranged from 2% to 41% with a mean Ki67 value of 18.69 ± 9.1. Low Ki67 values (<20%) were observed in the 37 of 55 patients with “low” Ki67 values. Eleven patients with “low Ki67 values” (29.7%) presented with a second locoregional neoplastic event, of whom

4 died of disease after a time interval ranging from 11 to 52 months (mean 25.5 ± 18.2 months). High Ki67 values (>20%) and the presence of Ki67-positive mature keratinocytes were observed in 18 of 55 patients. Twelve patients (66.7%) with “high” Ki67 values (>20%) presented with a second locoregional neoplastic manifestation, of whom 7 died of disease after a time interval ranging from 1 to 66 months (mean 14.6 ± 23.1 months). Results of Kaplan Meier analysis showed that “high” Ki67values resulted in a variable significant association with worse LRC (χ^2=10.6; $p < .05$). “High” Ki67 values also resulted in a variable significant association with worse DSS (χ^2=6.5; $p < .05$) (Figs. 8 and 9). Histological grade also resulted in a variable significant relation with worse LRC (χ^2= 6.02; $p < .05$) and DSS (χ^2=16.8; $p < .01$); 3 of 5 patients (60%) with a poorly differentiated OSCC showed a second locoregional neoplastic manifestation, compared with 15 of 31 patients (48.4%) with a moderately differentiated OSCC and 5 of 19 (26.3%) with a diagnosis of well-differentiated OSCC.

Three of 5 patients (60%) with a poorly differentiated OSCC died during follow-up (mean 5.6 ± 3.05 months; range 3e9 months), compared with 8 of 31 patients (48.4%) with a moderately differentiated OSCC (mean 23.37 ± 23.26 months; range 1e56 months).

All patients with a diagnosis of well-differentiated OSCC survived during the follow-up period of the present study.

Finally, Kaplan Meier analysis showed that LNM at presentation was related to lower survival in terms of LRC (χ^2=3.8;p ¼ 0.04): 8 of 14 patients (57.1%) with LNM presented with a locoregional manifestation during follow-up, in comparison to 15 of 41 patients (36.6%) with N0 at presentation. N status was also related to worse DSS (χ^2= 4.3; p ¼ 0.03): 5 of 14 patients (35.7%) with a positive N value died during follow-up, in comparison to 6 of 41 patients (14.6%) with a negative N value at presentation.

Predictivity of LRC by multivariate analysis using the Cox proportional hazards model showed that “high” Ki67 values in clinically and histologically normal distant mucosa were

the most powerful prognostic factor (Table 4). Predictivity of DSS by multivariate analysis using the Cox proportional hazards model showed that histological grade was the most powerful prognostic factor (Table 5).

Considering the group of 31 T1-2N0 OSCCs, univariate analysis demonstrated that the only variable statistically related to a worse LRC (χ^2 =9.5; p < .01) and DSS (χ^2= 5.51; p < .05) was the presence of “high” Ki67 values in non-neoplastic distant mucosa : 7 of 11 patients (63.6%) with “high” Ki67 values (>20%) showed the appearance of a second locoregional event, in comparison to 4 of 20 (20%) patients with “low” Ki67 values (<20%). All 3 patients in the T1-2N0 group who died during follow-up showed “high” Ki67 values (>20%) (Figs. 10 and 11).

TAB.3 Univariate analysis for potential prognostic variables related to locoregional control and disease-specific survival. Entries in boldface and asterisk indicate statistically significant p values.

Prognostic variable	Patients	Local recurrence/second primary tumor/lymph node metastasis			Death from OSCC		
		n Patients	% Patients	p Value	n Patients	% Patients	p Value
Sex							
Male	32 (58.2%)	13	40.6%	0.941	7	21.9%	0.5727
Female	23 (41.8%)	10	43.4%		4	17.4%	
Age, y							
<65	29 (52.7%)	10	34.5%	0.31	5	17.3%	0.571
>65	26 (47.3%)	13	50.0%		6	23.1%	
Site							
Tongue	24 (43.6%)	9	37.5%	0.9	4	16.7%	0.1
Floor of mouth	9 (16.4%)	2	22.2%		0	0%	
Cheek	9 (16.4%)	5	55.5%		3	33.4%	
Soft palate	1 (1.8%)	1	100%		1	100%	
Gingiva	11 (20.0%)	6	54.5%		3	27.2%	
Inferior lip	1 (1.8%)	0	0%		0	0%	
Grading							
G1	19 (34.5%)	5	26.3%	**0.04***	0	0%	**0.0004***
G2	31 (56.4%)	15	48.4%		8	25.8%	
G3	5 (9.1%)	3	60.0%		3	60%	
T							
T1	18 (32.7%)	8	44.4%	0.976	3	16.7%	0.32
T2	19 (34.5%)	7	36.8%		2	10.5%	
T3	2 (3.6%)	1	50.0%		1	50%	
T4	16 (29.1%)	7	43.8%		5	31.3%	
N							
N0	41 (74.5%)	15	36.6%	**0.04***	6	14.6%	**0.04***
N1	7 (12.7%)	3	42.9%		3	42.9%	
N2	7 (12.7%)	5	71.4%		2	28.6%	
% Ki67 in tumor mass							
<40	17 (34.0%)	6	35.3%	0.345	3	17.6%	0.67
>40	33 (66.0%)	14	42.4%		7	21.2%	
% Ki67 in cheek opposite OSCC							
<20	37 (67.2%)	11	29.7%	**0.001***	4	10.8%	**0.01***
>20	18 (32.7%)	12	66.7%		7	38.8%	
Perineural infiltration							
Yes	7 (13.0%)	2	28.6%	0.627	2	28.6%	0.39
No	47 (87.0%)	20	42.6%		8	17.02%	
Vascular infiltration							
Yes	3 (5.6%)	1	33.3%	0.961	0	0%	0.44
No	51 (94.4%)	21	41.2%		10	19.6%	
Margin of resection tumor							
Clear	48 (90.6%)	20	41.7%	0.741	7	14.6%	0.34
Close	2 (3.8%)	1	50%		1	50%	
Epithelial precursor lesion involved	3 (5.7%)	1	33.3%		1	33.3%	

FIG.8 Kaplan-Meier for disease free survival rate by Ki67 expression in the "normal" mucosa from the cheek opposite the OSCC. Significantly locoregional control(LCR p<0.01) was found for patients with high (>20%) Ki67 scores.

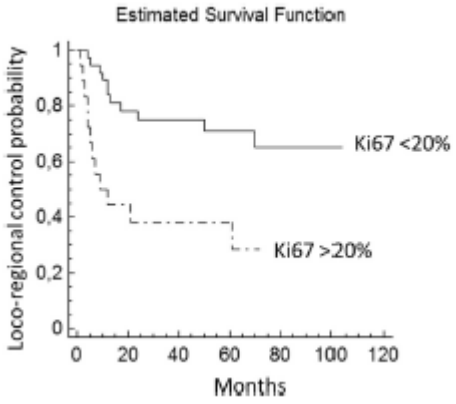
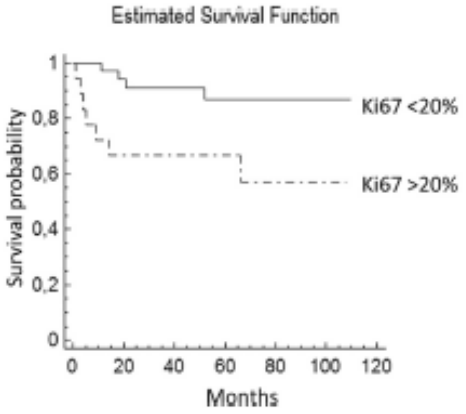


FIG.9 Kaplan-Meier for disease free survival rate by Ki67 expression in the "normal" mucosa from the cheek opposite the OSCC. Significantly worse Disease Specific Survival (DSS p<0.01) was found for patients with high (>20%) Ki67 scores.



TAB4.Statistically significant variables by multivariate analysis for predicting locoregional control

Variable	χ^2	df	p Value
Ki67 in distant mucosa	12.7045	1	0.0004
Histological grade	5.57444	2	0.0616
Lymph node metastasis at presentation	1.82594	1	0.1766

FIG.10 Kaplan-Meier for disease free survival rate by Ki67 expression in the "normal" mucosa from the cheek opposite the OSCC. Significantly locoregional control(LCR p<0.01) was found for T1-2N0 patients with high (>20%) Ki67 scores.

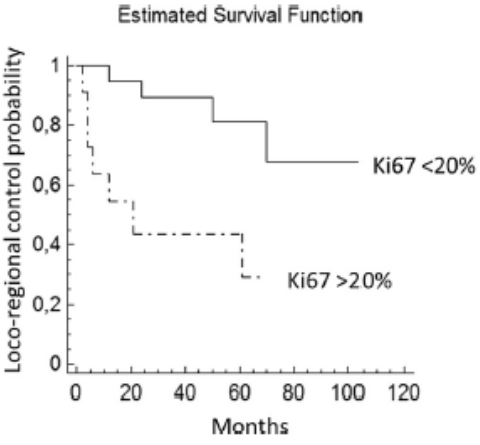
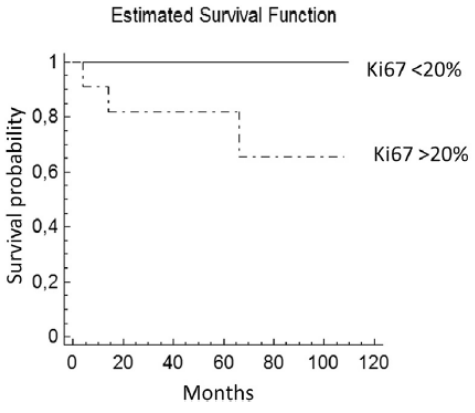


FIG.11 Kaplan-Meier for disease free survival rate by Ki67 expression in the "normal" mucosa from the cheek opposite the OSCC. Significantly worse Disease Specific Survival (DSS p<0.01) was found for T1-2N0 patients with high (>20%) Ki67 scores.



TAB5.Statistically significant variables by multivariate analysis for predicting Disease Specidfic Survival

Variable	χ^2	df	p Value
Ki67 in distant mucosa	5.98224	1	0.0144
Histological grade	11.0891	2	0.0039
Lymph node metastasis at presentation	0.335139	1	0.5626

DISCUSSION

The poor survival of OSCC patients has traditionally been ascribed to the high rate of local recurrence (LR), second primary tumours (SPTs), and deaths due to comorbidity. Despite recent progress, accurate prediction of prognosis and choice of appropriate treatment has been hampered by the fact that even patients with a small tumor may have a fatal outcome (83,84)

In 1953, Slaughter et al. proposed the “field cancerization” concept as a pathogenic pathway for the development of multiple primary OSCCs arising in different areas of the oral cavity, frequently associated with pre-neoplastic lesions. They postulated that OSCCs are often surrounded by genetically altered cells, and that SPTs can develop within this field as a result of independent events affecting multiple cells after continuous exposure to carcinogenic agents(5). Recent studies based on molecular techniques supported this hypothesis(69,74). Two previous studies proposed the analysis of Ki67 expression in areas distant from the original tumor as a prognostic marker, particularly in the clinically and histologically nonneoplastic mucosa located on the cheek opposite the primary OSCC (42,77).

The Ki67 labelling index is a simple, low-cost procedure frequently used to assess cell epithelial turnover in the oral mucosa. Its role as a prognostic marker has been analyzed in several OSCC cohorts showing a good correlation between Ki67 expression in tumor mass and histological grading of OSCC(83,85–87). The authors of two studies proposed overexpression of Ki67 in the tumor mass as an independent prognostic marker(88,89) , although other studies did not find this relationship (90,91).

In an article published in 2010, Gonzales-Moles et al. showed a good relationship between high Ki67 expression in non-tumor epithelium associated with OSCC and the risk of multiple tumours. They identified a significant difference in Ki67 expression in distant (>1 cm) and close (<1 cm) epithelium associated with OSCC in patients who experienced multiple tumours compared to controls and patients with single tumours(73).

The results of both studies conducted in 2009 and 2011 showed that the Ki67 mean value in the oral mucosa distant from the primary mass was significantly higher than that in controls, and 20% of OSCC patients had an abnormally high cell turnover in the clinically and histologically non-neoplastic oral mucosa from the cheek opposite the primary tumor. Moreover, those data disclosed a relationship between abnormally proliferating areas and primary tumor aggressiveness in terms of locoregional recurrence.

However, the limit of both studies was the short follow-up of patients, ranging from 1 to 49 months, with approximately one third of patients followed up for less than 9 months. Instead, the OSCC cohort in the present study has a longer follow-up (mean 53.7 ± 32.4 months; range 12e110 months), and the role of Ki67 expression in clinically and histologically normal distant mucosa was analyzed in relation not only to LRC but also to disease-specific survival (DSS).

The results showed a higher mean Ki67 value in patients who experienced a second neoplastic event and in patients who died of disease complications. High KI67 expression in distant mucosa together with tumor differentiation and LNM at presentation were all

predictive variables for a worse prognosis in terms of LRC and DSS, but Ki67 expression was the most powerful independent prognostic factor related to LRC. Similar results were obtained considering only the group of T1-T2N0 OSCCs, emphasizing that Ki67 expression in distant mucosa is related to LRC and DSS even among early-stage OSCCs.

These results confirm the findings of studies with shorter follow-ups and suggest that Ki67 in clinically and histologically “non-neoplastic” mucosa distant from the primary tumor could be a promising biomarker to better understand the biological nature of OSCC, including its aggressiveness and long-term survival rate.

The long-term prognosis and choice of the most appropriate treatment in OSCC patients are routinely based on clinical and histological staging systems, such as histological grading or LNM(65,92,93)

The present study confirmed the clinical value of these parameters as reliable prognostic markers, but they seem to lose their efficacy in predicting LRC and survival in early stage tumors, whereas Ki67 expression remains a reliable predictive marker also in this group of patients. The use of Ki67 in distant mucosa may be included in the list of clinical pathological biomarkers to be screened preoperatively, in surgical decision making, and as a good prognostic indicator of a more intensive surveillance during follow up.

In conclusion, although our data must be considered with caution due to the relatively small size of the cohort, the results of the present population study with a long-term follow-up period confirm the value of Ki67 expression in distant mucosa as a prognostic marker for OSCC patients.

PROJECT 2 : INVESTIGATION OVER PROGNOSTIC VALUE OF mtDNA PHYLOGENETIC ANALYSIS AS A TOOL FOR CLONAL DIAGNOSIS OF SECONDARY EVENTS IN ORAL SQUAMOUS CELL CARCINOMA(PUBBLISHED AS Clonal analysis as a prognostic factor in multiple oral squamous cell carcinoma.Oral Oncol. 2017 Apr;67:131-137)

AIMS :

A novel classification based on molecular methods to assess clonality defines three types of secondary oral squamous cell carcinoma (OSCC): second primary tumour (SPT) independent from the index tumour, local recurrence (LR), clonally related to the primary tumour, and second field tumour (SFT), derived from the same genetically altered mucosal field as the primary tumour. (49)The present study applied mtDNA analysis in a group of patients experiencing a second loco-regional neoplastic manifestation.

The purpose was to differentiate secondary tumours into LRs, SPTs and SFTs and evaluate the prognostic impact in terms of survival rate.

METHODS :

The study population comprised 23 consecutive patients who experienced a second neoplastic loco-regional manifestation after complete surgical resection of a primary OSCC. The cohort included some patients analysed in previous reports (52,53), but a minimum follow-up of 24 months after the appearance of the second tumour was required for enrolment. The study was approved by the institutional ethical committee (mtDNA01, code 020/2013/U/Tess), and informed consent was obtained from all patients. Twenty (86.9%) second neoplastic manifestations were OSCCs limited to the oral cavity whereas the remaining 3 (13.05%) presented a delayed lymph node metastasis (LNM) as a second event. Patients with LNM were enrolled only when the metastasis appeared 6 months or more after primary OSCC surgery. A surgical margin involved in the primary OSCC was considered an exclusion criterion.

All 23 patients were treated at the Maxillofacial Surgery Unit of Bellaria Hospital, and at the Oral and Maxillofacial Surgery Unit of S. Orsola–Malpighi University Hospital during the period 2002– 2011. They all underwent surgical resection of OSCC in accordance with standard treatment practice(65). Surgery consisted of composite resections, including excision of the primary tumour with ipsilateral or bilateral neck dissection. Microvascular reconstruction was performed for patients with locally advanced stages. Post-operative radiation therapy was performed when indicated, depending on the tumour stage, surgical margins, node involvement, and extra-nodal spread, according to currently accepted criteria(78). Tissue samples of the primary tumour and second neoplastic manifestation were sent for histological analysis to the Sections of Anatomic Pathology of the University of Bologna at Bellaria Hospital and S. Orsola–Malpighi University Hospital. A sample of clinically healthy oral mucosa located on the cheek contralateral to the OSCC was collected from the study population during surgical resection of the index lesion and second tumours. Cells from clinically healthy mucosa were selected and served as control reference DNA for mtDNA analysis and/or to evaluate a potentially altered genetic field distant from the neoplastic lesion. All tissues were formalin-fixed and routinely paraffin-embedded (FFPE). From each block, sections stained with haematoxylin and eosin were obtained for routine diagnosis. Histological diagnoses were made following the criteria proposed in the World Health Organization Blue Book(94). The mucosal biopsy obtained from healthy mucosa was always evaluated histologically to exclude any oral potentially malignant lesions according to the Ljubljana system(82).

Microdissection and DNA extraction

Ten micrometer-thick sections were carefully microdissected for DNA extraction by means of the laser-assisted SLlcut Microtest (MMI GmbH, distributed by Nikon, http://www.mmimicro.com) as previously described(52,53) [14,15] to obtain homogeneous

populations of tumour cells. Cells from normal mucosa were also selected for mtDNA analysis. DNA was purified using the Quick ExtractTM FFPE DNA extraction kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions.

Mitochondrial DNA sequencing and analysis DNA was sequenced for mtDNA D-loop region and for TP53 by 454 platform (GSJunior, Roche, Branford, CT, USA). In brief, mtDNA D-loop sequence analysis was performed by amplifying four segments, covering the whole region from position 15,995 to position 700, according to Anderson et al.(95) as described in the human mitochondrial database (NC_012920 gi:251831106, MITOMAP: a Human Mitochondrial Genome Database. Center for Molecular Medicine, Emory University, Atlanta, GA, USA, http://www.mitomap.org). Primers were designed using primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and their sequences have been reported(52,53). To generate amplicons for the 454 NGS library, fusion primers were designed to contain specific mtDNA primers, the A and B sequencing adapters and the key sequence required for 454 NGS, and one of 14 different10 bp multiplex identifier (MID) barcodes, according to the manufacturer's GS FLX standard sequencing method. PCR reactions were performed in a 20 ll volume containing 5 pmol of each forward and reverse primer using Phusion II HotStart High Fidelity DNA Polymerase, following the instructions of the supplier (ThermoScientific, Pittsburg, PA, USA). PCR products were purified using the AmpPure kit (Agencourt, Beverly, MA, USA). The DNA sequence was analysed bidirectionally by the GSJunior sequencer (Roche, Branford, CT, USA) following the supplier's recommendation with a threshold of at least 5% mutant reads using Amplicon Variant Analyzer software 2.7. Phylogenetic and cluster analyses were conducted using MEGA software version 5.2 (http://www.megasoftware.net) using the NJ method and Kimura-2 parameter with a Gamma model that corrects for multiple hits taking into account transitional and

transversional substitution rates and differences in site substitution. Every NJ tree was tested for standard error by the bootstrap method as previously described(52,53).

Statistical analysis

Disease-specific survival was the outcome of interest in the present study. Patient age, gender, type of second neoplastic event (local event or LNM), localization of secondary OSCC, presence of a phylogenetic relationship between the index OSCC and the secondary tumour, genetic diagnosis of a second neoplastic event following the Braakhuis classification (LR, SPT or SFT) (49)were analysed for their relationship with outcome. Survival rate was estimated using the Kaplan-Meier method. Statistical significance was evaluated using the log rank test. Time was defined as the period between the appearance of the second neoplastic lesion and death of disease, or last follow-up visit. The statistical analysis also evaluated the potential influence of outcome variables related to index OSCC (T score of index OSCC, N score of index OSCC, tumour differentiation of index OSCC, localization of index OSCC). For this second analysis, time was defined as the period between the appearance of the primary neoplastic lesion and death of disease, or last follow-up visit. P values < 0.05 were considered statistically significant in all analyses.

RESULTS:

Study population:

The study population comprised 16 women and 7 men aged 26–89 years with a mean age at second neoplastic event presentation of 63.65 ± 17.69.

Genetic classification of secondary OSCCs in LR, SPT and SFT Secondary tumours were genetically categorized as LR, SPT and SFT following the Braakhuis et al. classification(49) on the basis of the phylogenetic relationship between primary and secondary OSCC, and between the clinically and histologically normal mucosa distant from the index OSCC and

the mucosa distant from the secondary OSCC. Based on mtDNA results, cases were classified as follows:

LR: when second manifestations were phylogenetically related to the index OSCC, and normal mucosa samples clustered together and were not genetically related to the index tumour or the recurrence. In our series seven second neoplastic events (30.4%) were phylogenetically related to the index OSCC. In all 7 cases the normal mucosa samples clustered together and were not genetically related to the index tumour or the recurrence (Fig. 12). On histology the LRs were not associated with epithelial precursor lesions (Fig. 15a).

SPT: when second manifestations were phylogenetically independent to the index OSCC, and normal mucosa samples clustered together and were not genetically related to the index or secondary OSCC. Four cases were classified as SPT as they presented a clonal relationship between the normal mucosa located distant from the tumour, sampled at the time of the primary OSCC and the time of the second manifestation, whereas the two tumours were not clonally related, suggesting the occurrence of two genetically distinct neoplastic events (Fig. 13);

SFT: when second manifestations were phylogenetically independent to the index OSCC and normal mucosa samples did not cluster together but may be genetically related to the index tumour or the recurrence. In such cases the genetic distance in normal mucosa suggested presence an altered genetic field. Twelve patients showed no genetic relationship between the normal distant mucosa at the time of the primary OSCC and the normal distant mucosa at the time of the second manifestation, while both neoplastic events showed a clonal relation with the respective apparently normal mucosa. These features suggested a wide altered mucosal field, leading to a diagnosis of SFT (Fig. 14).

On histology SPT (Fig. 15b) and SFT (Fig. 15c) were associated with areas of high grade squamous intraepithelial lesions (HG SIL)(82,94). In SFT cases the areas of HG-SIL were multiple.

The clinical and molecular profile of the second neoplastic event and related index OSCC are summarized in Table 6.

Results from Kaplan-Meier statistics

The log-rank test showed that the only independent prognostic factor related to a better survival rate ($p < 0.05$) was an altered mucosal field in non-clonal patients classifying the second neoplastic

manifestation as SFT; only 2/12 (16.6%) SFT events failed, compared to 5/7 LRs (71.4%) and 3/4 SPTs (75%) (Fig. 16). Results from Kaplan-Meier statistics were summarized in Table 7.

TAB 6. Clinical and molecular profile of the study population

Patient	Age	Sex	Type of second event	TNM index tumour	TNM second tumour	Site of primary tumour	Site of secondary tumour	Phylogenetic relationship between primary oral carcinoma and secondary oral carcinoma	Phylogenetic relationship between distant healthy mucosa of index tumour and distant healthy mucosa of secondary tumour	Classification of second event following Braakhuis classification	Follow up after appearance of secondary tumour
Case 1	77	F	Local event	T1N0M0	T1N0M0	Hard palate	maxilla	Notclonal	Notclonal	SFT	Alive after 57 months
Case 2	77	F	Local event	T4aN1M0	T2N0M0	mandible	Floor of mouth	Clonal	Clonal	LR	DOD after 6 months
Case 3	71	M	Local event	T4aN2bM0	T1N0M0	Floor of mouth	Left check	Notclonal	Notclonal	SFT	Alive after 47 months
Case 4	48	F	Local event	T2N0M0	T2N2bM0	tongue	Floor of mouth	Notclonal	Notclonal	SFT	Alive after 47 months
Case 5	45	F	Local event	T2N1MO	T2N0M0	tongue	tongue	Notclonal	clonal	SPT	DOD after 3 months
Case 6	51	F	Local event	T2N0M0	T2N0M0	tongue	tongue	clonal	Clonal	LR	DOD after 63 months
Case 7	58	F	Local event	T4N0M0	T2N0M0	mandible	mandible	Notclonal	Notclonal	SFT	DOD after 26 months
Case 8	84	F	Local event	T1N0M0	T1N0M0	tongue	tongue	Notclonal	Notclonal	SFT	DDD after 12 months
Case 9	34	F	Local event	T2N1M0	T1N0M0	tongue	Dorsaltongue	Notclonal	Notclonal	SFT	Alive after 17 months
Case 10	82	M	Local event	T2N1M0	T1N0M0	tongue	tongue	clonal	clonal	LR	DOD after 1 months
Case 11	26	F	Local event	T2N0M0	T2N0M0	Tongue	Floor of mouth	clonal	Clonal	LR	Alive after 36 months
Case 12	76	M	Local event	T4N0M0	T1N0M0	palate	gingiva	Notclonal	Notclonal	SFT	Alive after 88 months
Case 13	38	M	Local event	T2N0M0	T2N0M0	tongue	tongue	Notclonal	clonal	SPT	Alive after 48 months
Case 14	52	M	Local event	T4N1M0	T1N0M0	tongue	tongue	Notclonal	Notclonal	SFT	Aliveafter 48 months
Case 15	78	F	Local event	T2N0M0	T1N0M0	maxilla	mandible	Notclonal	Notclonal	SFT	DOD after 3 months
Case 16	70	F	Local event	T2N0M0	T2N0M0	Right mandible	Left mandible	Notclonal	Notclonal	SFT	Alive after 76 months
Case 17	89	F	Local event	T1N0M0	T1N0M0	Cheek	Cheek	Notclonal	Notclonal	SFT	Aliveafter 67 months
Case 18	53	M	LNM	T1N2bM0		Cheek	LNM	Notclonal	Notclonal	SFT	Alive after 72 months
Case 19	58	F	Local event	T4N0M0	T1N0M0	Cheek	Cheek	Notclonal	clonal	SPT	DOD after 35 months
Case 20	71	F	Local event	T1N0M0	T1N0M0	tongue	tongue	Notclonal	clonal	SPT	DOD after 13 months
Case 21	85	F	Local event	T2N0M0	T2N0M0	Superior lip	tongue	clonal	clonal	LR	Alive after 41 months
Case 22	65	M	LNM	T2N2bM0		tongue	LNM	clonal	clonal	LR	DOD after 13 months
Case 23	76	F	LNM	T2N1M0		Cheek	LNM	clonal	clonal	LR	DOD after 6 months

FIG 12 Phylogenetic tree of a case interpreted as local recurrence (LR). The secondary tumour (OSCC2) resulted phylogenetically related to the index tumour (OSCC1) and the respective normal distant mucosa was phylogenetically related.

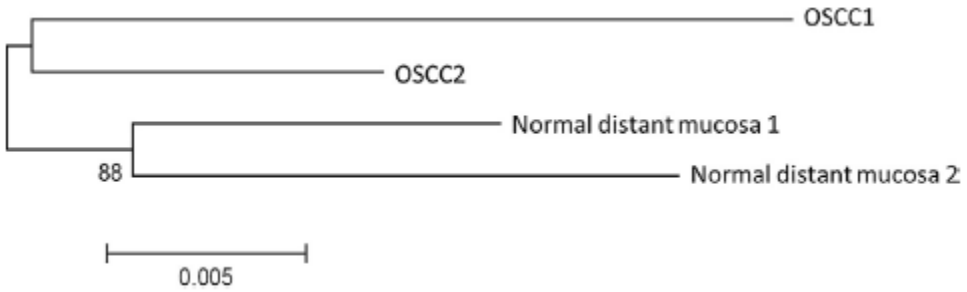


FIG 13 Phylogenetic tree of a case interpreted as second primary tumour (SPT). The secondary tumour (OSCC2) resulted phylogenetically distant from the index tumour(OSCC1) and the respective normal distant mucosa was phylogenetically related.

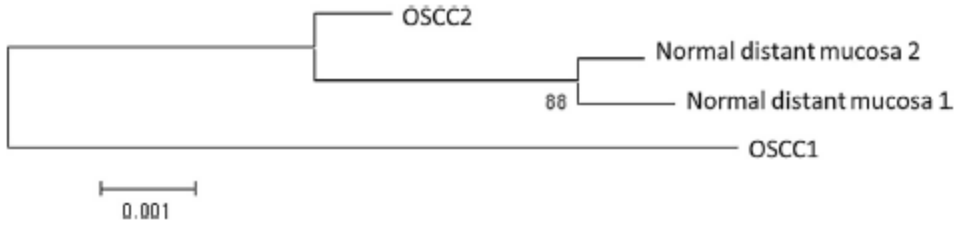


FIG 14 Phylogenetic tree of a case interpreted as second field tumour (SFT). The secondary tumour (OSCC2) resulted phylogenetically distant from the index tumour (OSCC1) and the respective normal distant mucosa did not show a phylogenetic relationship.

Fig.15 Histology: (a) LR is characterized by the presence of neoplastic cells (arrow) in the muscular wall, not related with the epithelium. (b) SPT is associated with HG-SIL.
The interface between non neoplastic oral epithelium and HG-SIL is indicated by the arrow. (c) SFT: the present case is a microinvasive OSCC (star). The surrounding mucosa
presents an area of HG-SIL (empty arrow); normally looking oral epithelium (black arrow) is interposed between microinvasive OSCC and HG-SIL.

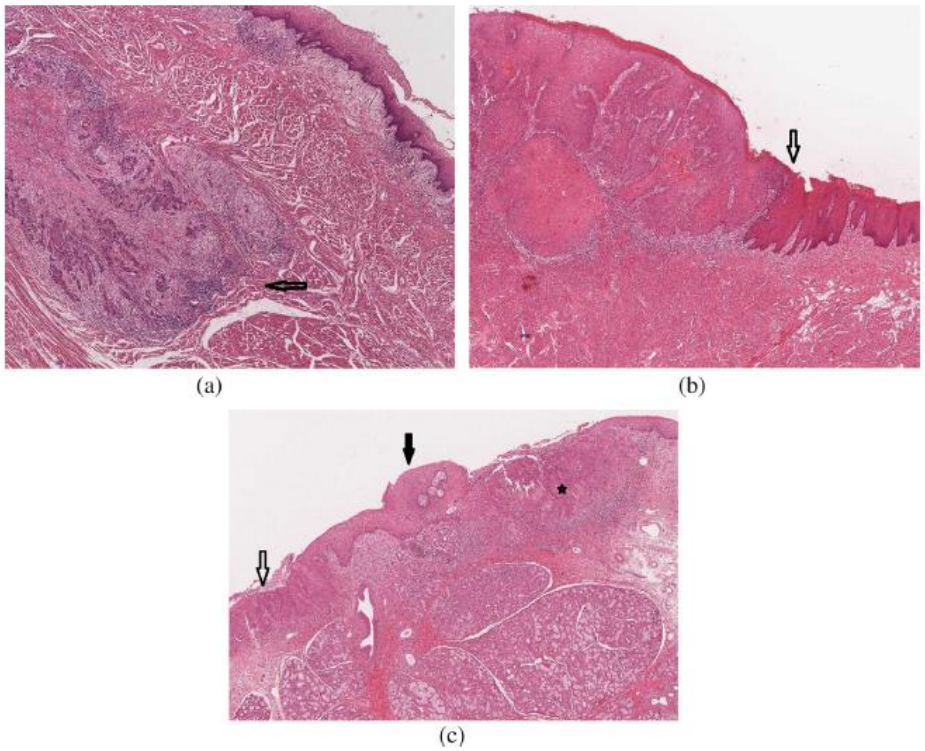
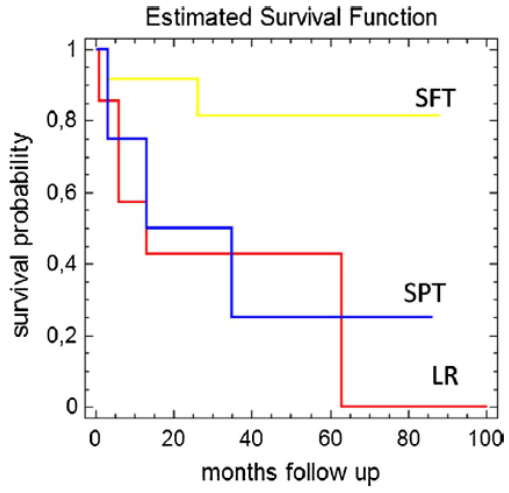


Fig.16 Kaplan–Meier estimate for disease-free survival rate by genetic diagnosis of a second neoplastic event following the Braakhuis classification (LR, SPT or SFT). An altered mucosal field in non-clonal patients was the only prognostic factor related to a significantly better survival rate ($p < 0.05$). Indeed only 2/12 (16.6%) SFTs failed compared to 5/7 LRs (71.4%) and 3/4 SPTs (75%).



TAB 7. Univariate analysis for Potential Prognostic Variables related to disease specific survival. Entry in boldface and with asterisk indicate statistically significant P values

	Variable	N	% OSCC patients died for disease	Significance
	Age (<65 vs. >65)	11/12	41.6 vs. 41.6	.75
	Sex (F vs. M)	16/7	50 vs. 28.6	.389
Index OSCC	Site (tongue and floor of mouth vs. other sites)	11/12	45.5 vs. 41.6	.88
	Grading (well vs. moderate vs. poorly differentiated)	4/15/4	0 vs. 47.8 vs. 75	.13
	T (T1-2 vs. T3-4)	16/7	43.7 vs. 42.9	.68
	N (N0 vs. N+)	14/9	35.7 vs. 55.6	.24
Secondary OSCC	Type of second event (local or lymph node metastasis)	20/3	40 vs. 66.7	.46
	Site of second event (tongue and floor of mouth vs. other sites)	11/12	45.4 vs 41.6	.51
	Phylogenetic relationship between neoplastic manifestations (clonal vs. non clonal)	7/16	71.4 vs. 31.2	.06
	Braakhuis classification (LR vs. SFT vs. SPT)	7/12/4	71.4 vs 16.6 vs 75	**.04***

DISCUSSION:

mtDNA (D-loop) sequence analysis followed by NJ is a useful molecular method to assess tumour clonality [13–16,18–20]. In previous studies it was evaluated the reliability of mtDNA analysis in establishing the clonal relationship between paired neoplastic lesions in OSCC in comparison with the Hong classification based on clinical and histological criteria. Total agreement between mtDNA analysis and the Hong classification was found in 19/25 cases (76%). Specifically, complete agreement was achieved when mtDNA was compared with histopathological criteria, while discrepancies arose only in 6 cases in which the Hong classification was based only on the spatial or temporal distance of the second lesion(52). Subsequently it was evaluated the relationship between primary OSCC and lymph node metastasis in a series of patients with synchronous and metachronous metastases, comparing mtDNA results with those obtained by another clonality test, i.e. TP53 sequence analysis. The results of *TP53* and mtDNA analysis were consistent, showing that all neck metastases clonally related to the index tumour also shared similar mutations in the same *TP53* gene regions(53). Establishing a clonal relationship between a second neoplastic lesion and the index tumour is not simply a problem of classification but yields new insights into the patient's tumour biology and can influence the prognosis and treatment of the second lesion.

Few studies to date have analysed the difference in prognosis of patients with LR or SPT and SFT. Gonzalez Garcia et al. reported a lower survival rate in LR patients compared with patients with SPT(96) whereas Renmeno et al. found no differences in survival(97). However, the two studies used different clinical and histological criteria to differentiate LR from SPT: Gonzalez Garcia et al. used the Hong classification(98) whereas Renmeno et al. used Warren and Gates' criteria(48). To the best of our knowledge, this is the first study to analyse the prognosis of patients with multiple OSCCs following a classification based on molecular clonality assessment. The results revealed that the majority of second OSCC should be considered SFT, i.e. tumours phylogenetically independent from the primary and originating from a genetically altered mucosa. The present series found 12/23 SFT, 7/23 LR and 4/23 SPT. These results confirm the field cancerization theory that the mucosa surrounding the primary OSCC mass is characterized by genetically altered epithelial cells that can escape clinical and histologic examination and might be responsible for cancer progression. This concept is further supported by the presence of multiple HG-SIL areas observed in cases of SFTs. The presence of a genetically altered filed surrounding OSCC, originally proposed by Slaughter et al.(5), has been widely confirmed by biomolecular approaches during the last two decades(99). Recently, Dasgupta et al. identified mitochondrial DNA (mtDNA) mutations in histologically negative resection margins of OSCC(100), confirming that even clinically and histologically radical surgical excision can lead to genetically altered mucosa capable of further neoplastic transformation. Kaplan Meier analysis of our results showed that these second manifestations had a better prognosis (16% failures) than LRs (71% failures) or SPTs (75% failures). The worse prognosis of LRs with respect to SFTs in terms of survival is in accordance with the clinical data of Gonzalez Garcia et al.(96), while the higher failure rate of LRs is probably due to the difficulty of obtaining a radical excision of primary tumours when it is hard to identify microscopic disease remaining after surgery and adjuvant radiotherapy (101). In addition,

the residual disease is often present as multiple neoplastic foci in cases of LR. Conversely, SFT could be considered a new tumour and is usually detected at an earlier stage due to active surveillance applied in patients treated for OSCC, thereby enhancing the prognosis. Unexpectedly, SPT showed an aggressive behaviour similar to LR, as 3/4 patients died of disease 3, 13 and 41 months respectively after the second neoplastic manifestation. In all three cases the second tumour appeared in the same area as the index OSCC and should have been classified as LR, following clinical and histological criteria. This observation suggests an interpretation bias. Primary OSCCs in these patients more likely developed subclones with a higher proliferation rate and aggressive molecular signature, accounting for the wide genetic distance between the primary OSCC and the second event. In these cases, the NJ trees displaying the mutational patterns of mtDNA should be considered only a snapshot of the tumour and do not differentiate them into LRs or SPTs. Additional genomic or epigenomic investigations could be needed to differentiate second events clonally related to primary OSCC (LRs) from clonally unrelated second manifestations in case of primary tumours containing multiple subclones.

PROJECT 3 : STUDY OF INTRATUMOR HETEROGENEITY IN RECURRENT-METASTATIC ORAL SQUAMOUS CELL CARCINOMA BY MEANS OF MULTI REGION NEXT GENERATION SEQUENCING AND mtDNA ANALYSIS(PUBBLISHED AS: Gabusi, A.; Gissi, D.B.; Tarsitano, A.; Asioli, S.; Marchetti, C.; Montebugnoli, L.; Foschini, M.P.; Morandi, L. Intratumoral Heterogeneity in Recurrent Metastatic Squamous Cell Carcinoma of the Oral Cavity: New Perspectives Afforded by Multiregion DNA Sequencing and mtDNA Analysis. J. Oral Maxillofac. Surg. 2019, 77, 440–455.

AIMS:

Improvements in sequencing technologies have revealed that genetic differences among neoplastic cells may reflect clonal expansion.(102) Intratumor heterogeneity (ITH) has been suggested to explain differences in prognosis and treatment response, indicating that personalized medicine is the goal of the future (103). Aim of this project was to study ITH in oral squamous cell carcinoma and to track tumour evolution from adjacent non neoplastic field to local neoplastic events developing after primary tumour.

METHODS:

Sample selection and DNA extraction

ITH evolution was tracked using tissues representing selected stages in oral carcinogenesis. OSCC tends to relapse locally or to spread through lymphatic vessels. We obtained multiple samples from each tumor (tumor cell percentage >70%). The original histological slides were reviewed by two pathologists with experience in oral pathology, and areas ≥0.5 cm apart exhibiting different morphological features were selected for analysis. At least five 10-µm-thick sections of the same areas were macrodissected manually (using a scalpel) to collect about 1 cm^2 tissue. The study was approved by our Institutional Ethics Committee (S. Orsola Hospital, project mtDNA01, approval code 020/2013/U/Tess) and informed consent was obtained from all patients. Additional samples were collected from diagnostic biopsies and mucosae surrounding tumors; again, all samples were ≥0.5 cm apart. Using the same protocol, multiple samples from surgical specimens of nodal metastases/secondary manifestations were collected and analyzed. DNA purification from each sample was performed as described previously(104). In brief, macrodissected tissue was digested at 56°C for 3 h using the solution of the Quick Extract™ FFPE DNA

extraction kit (Epicentre, Madison, WI, USA). After denaturation at 95°C for 5 min, each suspension was centrifuged at 10,000 x *g* at 4°C for 5 min to pellet undigested tissue and solidify floating paraffin. DNA for PCR was collected from the interphase and stored at 4°C.

Next Generation Sequencing:

We subjected the following genes (selected by DriverDBv2(105)) involved in head and neck squamous cell carcinogenesis(106) to deep sequencing: *KRAS* (exons 2–4), *NRAS* (exons 2–4), *HRAS* (exons 2, 3), *BRAF* (exon 15), *PIK3CA* (exons 10, 21), *TP53* (exons 4–9), *NOTCH1* (exons 4, 6, 11, 26, 27), *PTEN* (exons 5–8), *CDKN2A* (exons 1, 2), *EGFR* (exons 18–21), *AKT1* (exon 2), and *CTNNB1* (exon 3). Locus-specific amplicon libraries with tagged primers were generated using overhang adapters based on the 5′ Nextera sequences; these were recognized during the second round of short PCR, which added P5/P7 sequencing adapters (Illumina, San Diego, CA, USA) and sample-specific indices. In the first round of enrichment PCR, seven multiplex PCR tubes were used for parallel amplification of all the above DNA regions using Phusion U (Thermo Fisher, Waltham, MA, USA) as the proofreading enzyme. The amplification products were mixed and purified using MagSi-NGSPREP-Plus beads (Magnamedics, Geleen, the Netherlands), quantified with the aid of a Quantus fluorometer (Promega, Madison, WI, USA), and employed as templates (100 ng) in the second (barcoding) PCR step (eight cycles). The amplicons were purified using MagSi-NGSPREP beads, quantified employing the fluorometer, pooled, and loaded onto a MiSEQ platform (Illumina). FASTQ files trimmed in terms of the multiplex identifiers were used for quality control. Only reads of PHRED quality score > Q30 and length > 100 bp were retained for mapping and variant analysis in a Galaxy Project environment(107). Raw reads were mapped to the hg19 human reference genome sequence, and read alignment files in BAM format were generated with Bowtie2 mapping, GATK local realignment, HaplotypeCaller and Picard MarkDuplicates. The BAM files were analyzed using an Integrative Genomic Viewer (IGV)(108) to manually identify all mutations of a clinical sensitivity threshold > 2%; only bidirectional variant calls with more than 10 reads were reported(109). The depth of coverage was assessed manually using the IGV, and regions with less than 100 reads were discarded. To test the reliability of variant allele frequencies (VAFs), three cell lines with known mutations (SW620: *KRAS pG12V*; CAL62: *KRAS p.G12R*; OCUT: *BRAF p.V600E*) were spiked into a background of wild-type DNA (DNA female pool, code G1521; Promega) at different concentrations, as described

previously(110). ASLNAqPCR (an orthogonal method) was used to derive sensitivities and specificities for detection of *KRAS* codons 12–13 and *BRAF* codon 600(110)(111).

Phylogenetic trees

Phylogenetic trees were constructed as described previously(112)(113)(114), with some modifications. D-loop sequencing featured amplification of four segments covering the region from positions 15,995 to 700 of human mitochondrial DNA (NC_012920 gi:251831106; MITOMAP: a Human Mitochondrial Genome Database, http://www.mitomap.org). Locus-specific amplicon libraries with tagged primers were generated using overhang adapters based on the 5′ Nextera sequences, as described above for OSCC driver genes. FASTQ files were filtered in terms of PHRED score > Q30 and read length > 100 bp, as above, and converted to FASTA format. Four FASTA files representative of each of the four D-loop fragments were created by Perl and processed by Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand) to allow for multiple sequence alignment to identify heteroplasmies. The four consensus sequences were then joined and used to construct phylogenetic trees employing MAFFT (https://mafft.cbrc.jp/alignment/server/), with UPGMA/Jukes-Cantor serving as the substitute model(115).

Evaluation of tumor heterogeneity

The ITH of OSCC somatic mutations was derived by calculating the heterogeneity rates of all affected genes and dividing that sum by the number of affected genes not shared by all tumor regions, as described previously(116). These values were used to compare the ITH of nodal metastases and secondary events (assessed independently). ITH was also calculated for non-neoplastic mucosae surrounding primary tumors, to explore field cancerization.

RESULTS

Patient characteristics

Three of the five patients were male; the median age of all patients was 59.6 (SD, 5.5) years; all were non-smokers. All primary tumors arose in the oral cavity; four were in the tongue (patients 1, 3, 4, and 5) and one arose on the floor of the mouth (patient 2). In terms

of the pTNM classification(79), patient 1 was pT3N2cM0; the others exhibited no nodal involvement at diagnosis and were classified as pT2N0M0, pT4N0M0, pT1N0M0, and pT2N0M0, respectively (Table 1). All patients underwent radical surgery with clear margins. No intraepithelial precursor lesion was detected histologically on the resection margins. During post-surgical follow-up, patients 1, 4, and 5 did not relapse. Patients 2 and 3 developed several neoplastic lesions at different oral cavity sites, including sites distant from the primary tumors. Patient 2 developed five additional tumors and patient 3 developed two.

ITH of the primary tumors:

The mutational profiles were used to compare the primary tumors. Although *TP53* was mutated in many tumors, the mutations differed. Only two *PIK3CA* mutations were found in more than one patient (*PIK3CA* p.W1051* in patients 3 and 4; *PIK3CA* p.G1050S in patients 3 and 5).

ITH of nodal metastases and subsequent neoplastic events:

We compared the genetic profiles of metastases and secondary events with those of the primary tumors. In patient 1 (pT3N2cM0), almost all mutations in the primary tumor, except for *BRAF p.T599K*, endured during nodal invasion. However, in patient 2, the primary tumor and the later neoplastic event did not share any mutation. Specifically, although the *KRAS*, *TP53*, and *NOTCH1* genes were mutated in both samples, the loci differed. However, the *KRAS* mutation acquired by the second carcinoma (*KRAS p.A130Q*) persisted in the third to sixth neoplasias. In patient 3, the primary tumor and the second event did not share mutations, and the mutations in the third carcinoma were unique to that event.

ITH of primary tumors

The ITH of all primary tumors was analyzed by comparing different tumor regions. Four samples from patient 1 were analyzed (Table 2). Only four of nine mutations were shared by all samples (5/9 not shared, heterogeneity rate [HR] = 55%). Notably, the *BRAF p.T599K* mutation was evident in only one of the four samples; for *CTNNB1*, multiple mutations (*p.A43S, p.V57G*) were present in the same samples (2 and 3), but only one mutation (*p.V57G*) was found in both samples 1 and 3. In patient 2 (Table 3), 10 mutations were found, but none were shared (HR 100%). Although *TP53, CDKN2A,* and *PTEN* were mutated in all samples, all mutations were unique. *KRAS* was mutated in one sample only, as was *BRAF* (p.V600M in sample 3). In patient 3 (Table 4), three samples contained a total

of seven mutations; none were shared (HR 100%). *NOTCH1* was mutated in all three samples, but the mutations were unique. *KRAS* and *TP53* were mutated in one sample (#1), but not in the others. *PIK3CA* was not mutated in sample #1, but was mutated at different loci in the other two samples (p.G1050S and p.G1051*, respectively). Of five samples from patient 4 analyzed, one (# 5) did not amplify correctly. Six mutations were identified; all were unique (HR 100%). Four samples from patient 5 contained six mutations; all were unique (HR 100%).

ITH of non-neoplastic mucosae and nodal metastases/subsequent neoplastic events

For patient 1, we analyzed two samples from the adjacent non-neoplastic mucosa. All mutations were shared by the primary tumor and the mucosa. The two samples differed only in the absence of the *TP53*_C>T splicing mutation from sample #1 (HR 14%). We analyzed four samples from the nodal metastasis, but DNA amplification failed for sample #3. Four of eight mutations were not shared (HR 37%). We analyzed two non-neoplastic mucosal samples from patient 2; six unique mutations were found (HR 100%). The ITH of four of the five recurrences was studied (one of two samples from the third recurrence did not amplify). The second carcinoma had four mutations (two samples). *KRAS* (p.A130Q) was present in both samples, but mutations in *TP53*, *PTEN*, and *NOTCH1* were identified in only one sample. In the fourth neoplasm, the same *KRAS* mutation (*KRAS p.A130Q*) was present in both samples analyzed. Additional mutations were found in *NOTCH1* of only one sample (HR 50%). In the fifth and sixth carcinomas, *KRAS_p.A130Q* remained present in all samples; this was the only mutation found in the sixth carcinoma. The *HRAS* (*p.G13D*) mutation was shared by all samples from the fifth recurrence. Mutations were also found in *TP53*, *CDKN2A*, *PTEN*, and *NOTCH1*, but (apart from *NOTCH1*; both samples mutated, but in different loci), the mutations were present in only one sample (HR 71% and 0% for fifth and sixth recurrences, respectively).

The non-neoplastic mucosal samples from patient 3 could not be amplified. Patient 3 developed two additional carcinomas with HRs of 100%. Patient 4 yielded only one sample of non-neoplastic adjacent mucosa, in which mutations were found. Patient 5 yielded three samples from non-neoplastic adjacent mucosa; only one sample harbored a mutation (*TP53* p.L194F; HR 100%).

Phylogenetic analysis of mtDNA

D-loop mtDNA analysis was used to integrate the genetic relationships of the multiple samples from different tumor regions(114),(113),(112),(104). For patient 1, clusters of primary 2 with nodal metastases 1, 3, and 4, and of primaries 3 and 4 with nodal metastasis 2 and normal epithelium are shown in Figure 3A. Primary 1 was completely independent. The short secondary branches of the phylogenetic tree indicate that the genetic relationships were close. Patients 2 and 3 yielded different patterns; nodal metastasis did not occur, but local recurrences were analyzed. In patient 2 (Fig. 3B), the samples from non-neoplastic mucosa were (closely) phylogenetically related to the primary tumor. However, although primary tumor samples 1 and 2 were phylogenetically related to each other and to non-neoplastic mucosa, sample 3 belonged to a distinct evolutionary line. Analysis revealed long secondary branches indicating wide genetic distances. A similar pattern was evident in patient 3 (Fig. 3C). Patients 4 (Fig. 3D) and 5 (Fig. 3E) did not develop neoplastic events after removal of the primary tumors. In both patients, mtDNA analysis disclosed the presence of at least two clusters within the tumors and different phylogenetic distances between multiple samples from the tumors and the non-neoplastic adjacent mucosae.

FIG 17. Hematoxylyn and Eosin of primary tumour from patient 1 with related multiple tumor regions.

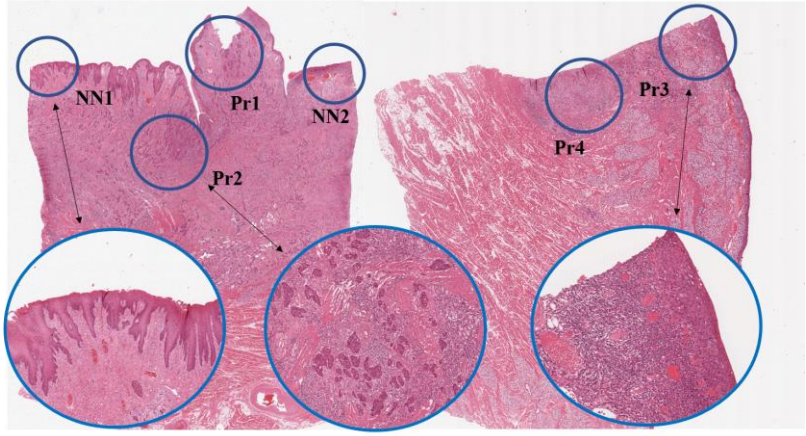


FIG 18. Hematoxylyn and Eosin of lymph node metastasis from patient 1 with related multiple tumor regions.

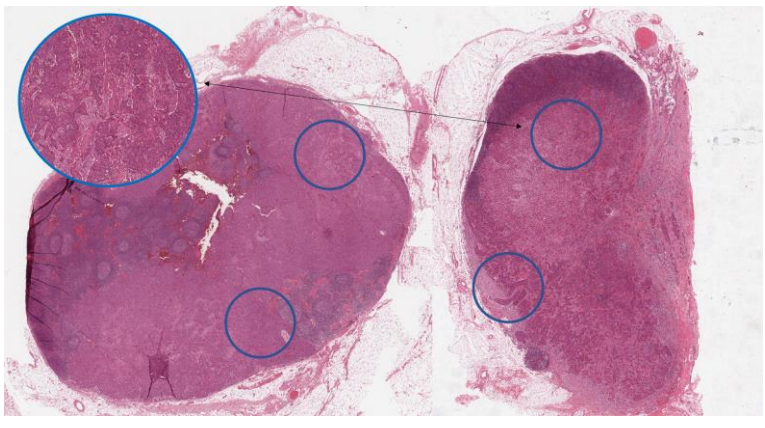


Table 8:Summary of patient characteristics: (M: Male F: Female)

Patient	Sex	Age	Smoke	Site	TNM	Grading	Histology	Site II	Site III	Site IV	Site V	Site VI
1	M	62	No	Tongue	T3N2c	G4	Squamous	Not Occured	Not Occured	Not Occured	Not Occured	Not Occured
2	F	67	No	Floor	T2N0M0	G2	Squamous	Gingiva	Mandibular gingiva	Maxillary gingiva	Lip	Palate
3	M	52	No	Tongue	T4N0M0	G4	Squamous	Tongue	Tongue	Not Occured	Not Occured	Not Occured
4	M	59	No	Tongue	T1N0M0	G1	Squamous	Not Occured	Not Occured	Not Occured	Not Occured	Not Occured
5	F	58	No	Tongue	T2N0M0	G2	Squamous	Not Occured	Not Occured	Not Occured	Not Occured	Not Occured

Table 9: mutational profile of patient 1 considering the various events (VAF: variant allele frequency; NA: not avaliable) and heterogeneity rate; DOC: depth of coverage:

Patient	Sample	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA	Heterogeneity rate
Patient 1	Non Neoplastic 1	WT	WT	WT	WT	WT	rs1800899 VAF: 49%; p.P72R rs1042522 VAF: 21%; p.P36P rs1800370 VAF: 21%;	WT	WT	p.443S VAF:4 p.V57G VAF: 5%	WT	p.D1698D VAF: 52.4%;	WT	14% (1/7)
	Non Neoplastic 2	WT	WT	WT	WT	WT	rs1800899 VAF:63.6% g.7578370C>T splic.Junc VAF: 17%; p.P72R rs1042522 VAF: 50%; p.P36P rs1800370 VAF: 56.7%	WT	WT	p.443S VAF: 2%; p.V57G VAF: 3%;	WT	p.D1698D VAF: 38.3%;	WT	
	Primary 1	WT	WT	WT	WT	WT	rs1800899 VAF: 33%; g.7578370C>T splic.Junc VAF: 10%; p.P72R rs1042522 VAF: 20%; p.P36P rs1800370 VAF: 100%;	WT	WT	p.V57G VAF: 5%;	WT	WT	PIK3CA_K1043Q VAF: 4.5%;	55% (5/9)
	Primary 2	WT	WT	WT	p.T599K VAF: 4.8%;	WT	rs1800899 VAF: 58.6%; g.7578370C>T splic.Junc VAF: 24%;	WT	WT	p.443S VAF: 3%; p.V57G VAF: 5%;	WT	p.D1698D VAF 42%	PIK3CA_K1043Q VAF: 5.3%	
	Primary 3	WT	WT	WT	WT	WT	rs1800899 VAF: 50%; g.7578370C>T splic.Junc VAF: 14%; P72R rs1042522 VAF: 20%; P36P rs1800370 VAF: 60%;	WT	WT	p.V57G VAF: 5%;	WT	p.D1698D VAF: 43.7%	WT	
	Primary 4	WT	WT	WT	WT	WT	rs1800899 VAF: 84.1%; g.7578370C>T splic.Junc VAF: 24%; p.P72R rs1042522 VAF: 0.75%; p.P36Prs1800370 VAF:50%;	WT	WT	p.443S VAF: 2%,p.V57G VAF: 2%;	WT	p.D1698D VAF: 37%;	WT	
	Nodal Metastasis 1	WT	WT	WT	WT	WT	rs1800899 VAF: 50%; p.P72R rs1042522 VAF: 20%	WT	WT	p.V57G VAF: 5%;	WT	p.D1698D VAF: 36.6%;	PIK3CA_K1043Q VAF: 2.81%;	50% (4/8)
	Nodal Metastasis 2	WT	WT	WT	WT	WT	rs1800899 VAF: 55.8%; g.7578370C>T splic.Junc VAF: 42%; p.P72R rs1042522 VAF: 20%; p.P36P rs1800370 VAF: 20%;	WT	WT	p.443S VAF: 2%; p.V57G VAF: 1%;	WT	p.D1698D VAF: 32.2%;	PIK3CA_K1043Q VAF: 4.6%;	
	Nodal Metastasis 3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
	Nodal Metastasis 4	WT	WT	WT	WT	WT	g.7578370C>T splic.Junc VAF: 23%;	WT	WT	p.443S VAF: 5%; p.V57G VAF: 3%;	WT	p.D1698D VAF: 37.7%;	PIK3CA_K1043Q VAF: 3.9%;	

Table 10 : mutational profile of patient 2 considering the various events and heterogeneity rate (VAF: variant allele frequency; NA: not avaliable; DOC: depth of coverage):

Patient	Sample	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA	Heterogeneity rate
Patient 2	Non Neoplastic 1	p.E31* VAF: 23%	p.G48C VAF: 23%	WT	WT	p.V742I VAF: 24%	WT	WT	NA	WT	WT	WT	WT	100% (6/6)
	Non Neoplastic 2	p.G48V VAF: 22%	p.L56M VAF: 27%	WT	WT	p.V845M VAF: 35%	WT	WT	NA	WT	WT	WT	WT	
	Primary 1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	100% (10/10)
	Primary 2	WT	WT	WT	WT	WT	p.H179I VAF: 18%	p.R29Q VAF: 11%	NA	WT	p.C250Y VAF: 9%	WT	WT	
	Primary 3	p.P34R VAF: 90%	WT	WT	p.V600M VAF: 14%	p.A750V VAF: 14%	p.P295S VAF: 8%	p.A42T VAF: 7%	NA	p.H24Y VAF: 8%	p.L318F VAF: 6%	WT	WT	
	Second 1	p.A130Q VAF: 39%	WT	WT	WT	WT	p.C238Y VAF: 13%	WT	NA	WT	p.V191M VAF: 28%	WT	WT	75%(3/4)
	Second 2	p.A130Q VAF: 61%	WT	WT	WT	WT	WT	WT	NA	WT	WT	p.G1704E VAF5%	WT	
	Third 1	p.A130Q VAF: 51%	WT	WT	WT	WT	WT	WT	NA	WT	WT	p.D1681N VAF23%	WT	NA
	Third 2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
	Fourth 1	p.A130Q VAF: 42%	WT	WT	WT	WT	WT	WT	NA	WT	WT	p.R1594W VAF6%	WT	50%(1/2)
	Fourth 2	p.A130Q VAF: 42%	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT	
	Fith 1	p.A130Q VAF: 45%	WT	p.G13D VAF: 16%	WT	WT	p.R248W VAF: 6%	p.A44Q VAF: 10%	NA	WT	p.E242K VAF: 6%	p.V1599M(VAF10%)	WT	71%(5/7)
	Fith 2	p.A130Q VAF: 35%	WT	p.G13D VAF: 18%	WT	WT	WT	WT	NA	WT	WT	p.C1685Y(VAF6%)	WT	
	Sixth 1	p.A130Q VAF: 50%	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT	0%(0/1)
	Sixth 2	p.A130Q VAF: 45%	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT	

Table 11 : mutational profile of patient 3 considering the various events and heterogeneity rate (VAF: variant allele frequency; NA: not avaliable; DOC: depth of coverage):

Patient	Sample	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA	Heterogeneity rate
Patient 3	Non Neoplastic 1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Non Neoplastic 2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
	Primary 1	p.34L VAF7%	NA	WT	NA	NA	p.A74V VAF9%	NA	NA	NA	WT	p.T1602* VAF15%	WT	100% (7/7)
	Primary 2	WT	WT	WT	NA	NA	WT	NA	NA	NA	WT	p.G1704E VAF11%	p.G1050S VAF7%	
	Primary 3	WT	WT	WT	NA	WT	WT	NA	NA	NA	WT	p.Q1691* VAF8%	p.G1051* VAF6%	
	Second 1	WT	WT	p.G12D VAF8%	WT	WT	p.A189TV AF11%	NA	v	NA	WT	p.L1712F VAF7%	p.E547L VAF7%	100% (4/4)
	Second 2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
	Third 1	WT	WT	WT	WT	WT	WT	NA	NA	NA	WT	WT	p.S541F VAF8%	100% (4/4)
	Third 2	WT	WT	WT	WT	WT	p.G279KV AF35%	NA	NA	NA	WT	WT	WT	
	Third 3	WT	WT	WT	WT	WT	p.H297YV AF18%	NA	NA	NA	WT	p.D1681N VAF27%	WT	

Table 12: mutational profile of patient 4 considering the various events and heterogeneity rate (VAF: variant allele frequency; NA: not avaliable; DOC: depth of coverage):

Patient	Sample	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA	Heterogeneity rate
Patient 4	Non Neoplastic 1	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	NA
	Primary 1	WT	WT	WT	WT	WT	WT	WT	WT	WT	p.A328V VAF22%	WT	p.W1051* VAF22%	100 % (6/6)
	Primary 2	WT	WT	WT	WT	WT	WT	WT	WT	WT	p.R173C VAF29%	WT	WT	
	Primary 3	WT	WT	WT	WT	WT	p.A159W VAF13%	WT	WT	WT	p.S207C VAF9%	WT	WT	
	Primary 4	WT	WT	WT	WT	p.R783C VAF66%	WT	WT	WT	WT	WT	WT	WT	
	Primary 5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	

Table 13: mutational profile of patient 5 considering the various events and heterogeneity rate (VAF: variant allele frequency; NA: not avaliable; DOC: depth of coverage):

Patient	Sample	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA	Heterogeneity rate
Patient 5	Non Neoplastic 1	WT	WT	WT	WT	WT	p.L194F VAF 25%	WT	WT	WT	WT	WT	WT	
	Non Neoplastic 2	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	100% (1/1)
	Non Neoplastic 3	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	
	Primary 1	WT	WT	WT	WT	WT	WT	WT	WT	WT	p.P244L VAF 20%	WT	WT	
	Primary 2	WT	WT	p.R73C VAF 21%	WT	WT	WT	WT	WT	WT	WT	WT	p.P539L VAF6 %	100% (6/6)
	Primary 3	WT	WT	WT	WT	WT	p.P191R VAF 9%	WT	WT	WT	WT	WT	WT	
	Primary 4	p.P140L VAF 6%	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	p.G1050S VAF22 %	

Fig. 19: Phylogenetic trees by sequencing of mtDNA D-loop region using UPGMA:

A: primary 2 clusters with nodal metastases 1, 3, and 4, primaries 3 and 4 with nodal metastasis 2 and normal epithelium. Primary 1 was completely independent. The short secondary branches of the phylogenetic tree indicate that the genetic relationships were close.

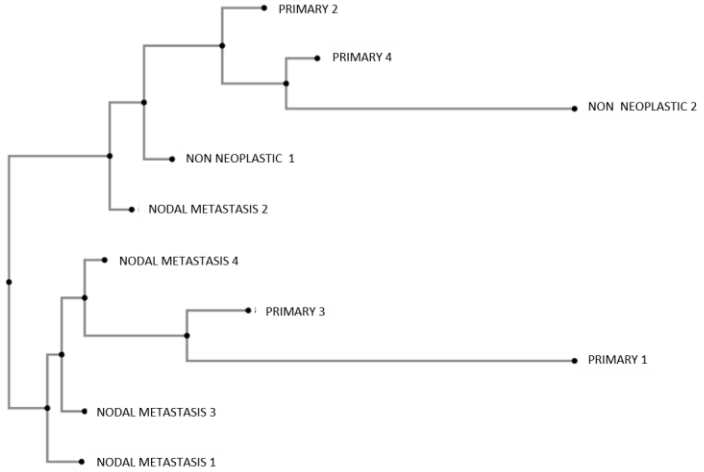
B: Phylogenetic tree of patient 2: the samples from non-neoplastic mucosa were closely phylogenetically related to the primary tumor. Primary tumor samples 1 and 2 were phylogenetically related to each other and to non-neoplastic mucosa, sample 3 belonged to a distinct evolutionary line. Analysis revealed long secondary branches indicating wide genetic distances.

C: Phylogenetic tree of patient 3: primary 1 and 3 were closely related to second 1. The other elements of the tree were less related each other.

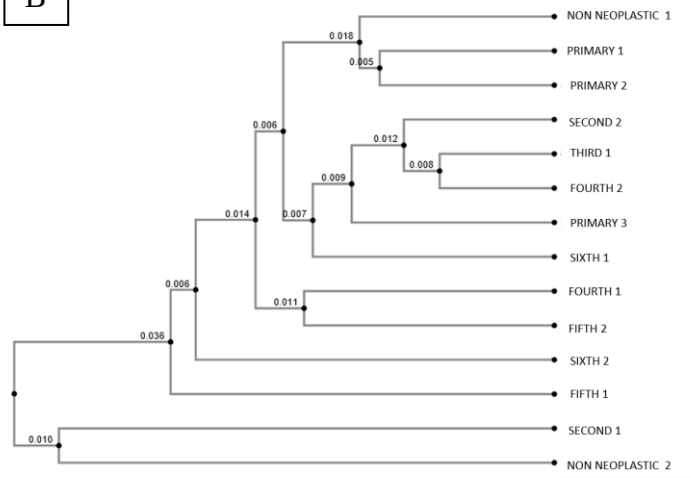
D: Phylogenetic tree of patient 4: all collected primary tumors, except primary 2, were genetically identical.

E: Phylogenetic tree of patient 5: primary 1, 2, 3 clustered together, while primary 4 took place independently.

A



B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PRIMARY 2
SECOND 2
THIRD 1
FOURTH 2
PRIMARY 3
SIXTH 1
FOURTH 1
FIFTH 2
SIXTH 2
FIFTH 1
SECOND 1
NON NEOPLASTIC 2
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0.005
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0.007
0.006
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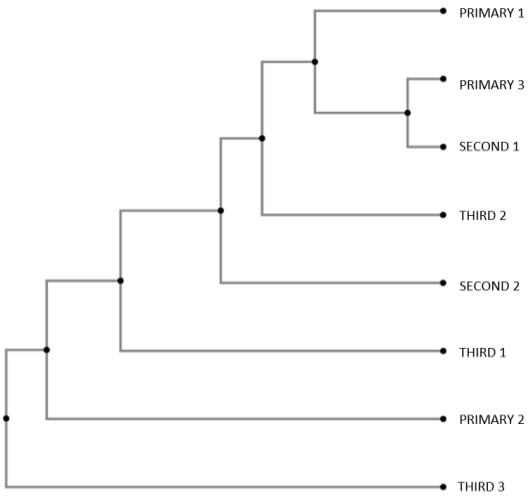
0.010

C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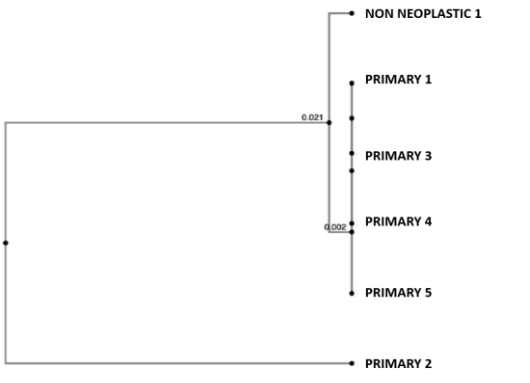
PRIMARY 3
SECOND 1
THIRD 2
SECOND 2
THIRD 1
PRIMARY 2
THIRD 3

D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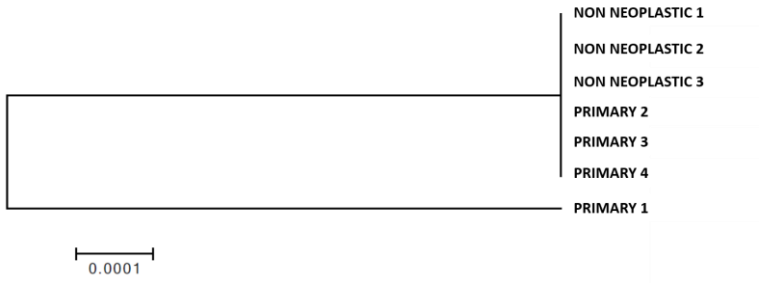
PRIMARY 3
PRIMARY 4
0.002
PRIMARY 5
PRIMARY 2

E



Discussion

OSCC is a locally aggressive tumor of epithelial origin. Despite multimodal treatment (surgery, radiation, and/or chemotherapy), most patients develop multiple local relapses or distant metastases(117). Many cases are diagnosed at advanced stages, which may render prognosis unfavorable(118). Although early diagnosis has improved, neither overall survival nor long-term prognosis has changed significantly in recent years(119). The Slaughter theory of field cancerization explains the development of recurrence(69),(120),(121). Many molecular studies have confirmed genetic abnormalities in regions distant from primary OSCCs, even in the absence of histological or clinical changes(122),(123). Many efforts have been made to identify histological or molecular biomarkers predicting relapse or treatment failure. However, neither the use of biomarkers nor modern drugs (i.e., cetuximab) has improved overall survival or locoregional control(124). ITH may explain the observed variations in clinical behavior and responses to treatment; subcloning may foster tumor aggressiveness(125).

In other anatomical areas, genetic analysis of various tumor regions revealed ITH(126). NGS permits parallel in-depth investigation of genomic hot spots, with work at the single-cell level. Unlike Sanger sequencing or allele-specific qPCR, deep parallel NGS allows quantitative investigation of several genomic targets in a single experiment, commencing with FFPE tissue. Our patients exhibited unique genetic patterns. Although genes such as *TP53* were mutated in all five patients, the mutations were identical in only two cases

(*PIK3CA p.W1051** in patients 3 and 4; *PIK3CA p.G1050S* in patients 3 and 5), confirming high-level heterogeneity among tumors of the same subtype, similar to other studies(127); individualized molecular diagnosis is essential.

Our ITH data confirm that analysis of a single tumor sample underestimates the mutational landscape. As several subclones co-existed in our patients, mutations were evident in a few tumor regions (i.e., the *PTEN* mutations of patient 4 differed in samples 1–3 of the primary tumor). We evaluated ITH quantitatively by calculating HRs(116), which ranged from 55% to 100%. In patient 1 (HR 55%), half of the mutations were not shared by all tumor regions. In patients 2 and 3 (HR 100%), no mutation was shared by all samples. Thus, clinical information from a single biopsy should not be used to drive clinical decisions or reach biological conclusions.

Several attempts have been made to quantify intratumor heterogeneity. Mroz et al. developed a system (MATH)(127) for the scoring of dispersion of mutant allele frequencies from single WES samples. Despite the absence of data from multiple samples, high-level heterogeneity was associated significantly with poor prognoses and treatment responses(128). We found that the ITH of early-stage and advanced tumors was high. High ITH rates were evident even in the tumors of patients who were free of neoplastic events during post-surgical follow-up, as well as in patients with less favorable clinical courses. However, the prognostic implications of our analysis should be tested in a larger patient population.

Multiregion tumor sampling yields useful clinical and prognostic information in terms of individualized diagnoses and can be used to track tumor evolution. Mutations present at one stage of tumor evolution may later be lost. For example, *BRAF* mutations were found only in single samples of the primary tumors of patients 1 and 2, and were subsequently lost, perhaps because the mutations did not afford a proliferative advantage. However, certain mutations persisted during tumor evolution. Interestingly, in patient 2, the second OSCC acquired a *KRAS* mutation retained in all subsequent relapses by all samples; a dominant subclone controlled tumor evolution. Patient 3 exhibited extreme heterogeneity; early subclones were replaced by new subclones, reflecting genetic instability(129). Overall, ITH analyses of primary tumors and subsequent neoplastic events revealed different patterns of

disease evolution, enriching our understanding of OSCC biology and informing future treatment strategies.

Previous studies have used mtDNA analysis to distinguish second primary tumors from local recurrences(130)(104)(114)(113). mtDNA is more abundant than nuclear DNA, overcoming amplification failure from formalin-fixed paraffin-embedded tissues that may have been over-fixed or contain little DNA. Moreover, mtDNA is non-coding and generally accumulates neutral mutations; mtDNA does not influence cell proliferation. For patient 1, sample 3 of the nodal metastasis lacked adequate DNA for analysis, but phylogenetic relationships could be derived based on mtDNA. In this case, the primary tumor and the metastases were closely related (Fig. 3A), in agreement with the low level of tumor heterogeneity evident on driver gene analysis. This is also in accordance to a particular study by Wen B. et al whose conclusions state that genetically clonal tumor cells are predominantly responsible for the composition of metastatic primary tumors and their paired lymph node metastases(133). In contrast, the phylogenetic trees of patients 2, 3, and 5 revealed that tumor evolution was accompanied by high-level heterogeneity; the between-sample genetic distances were large, as evidenced by the lengths of the secondary branches. However, patient 4 expressed four closely related subclones (primaries 1, 3, 4, and 5) and only one independent subclone (primary 2).

Non-neoplastic mucosal analysis in terms of driver genes and mtDNA indicated that field cancerization was present in almost all cases. In patient 1, many mutations were common to the non-neoplastic surrounding mucosa and the primary tumor. In patients 2 and 5, different mutational patterns were evident in adjacent, morphologically normal tissue; all genes evaluated from patient 4 were wild type. Thus, from a phylogenetic viewpoint, case 2 showed that normal mucosa could cluster with different tumor subclones, indicating the presence of a “second field tumor,” first described by Braakhuis et al.(131) and confirmed by Gissi et al.(104) This tumor arises from the same genetically altered mucosal field as the primary OSCC, but the tumors share only some (not all) genetic alterations. Interestingly, some heterogeneity was also evident among multiple samples of non-neoplastic mucosa; this constitutes the first evidence of intrafield heterogeneity. A heterogeneous pre-neoplastic field may affect prognosis and treatment. Indeed, different tumor areas may vary in terms of aggressiveness, requiring different surgical approaches and adjuvant or neoadjuvant treatment.

In conclusion, even though the sample size is very small and heterogeneous with regard to anatomic site, our study of tumor heterogeneity revealed a complex OSCC landscape. This report may be considered a proof of principle which demonstrates that genetic analysis of tumors is important to develop a mutational profile of disease, which differs for every patient. Molecular study of a single biopsy does not yield information that is representative of the disease or useful when planning individualized surgical or medical therapy. Multiregion tumor analysis detects clonal mutations that persist during tumor evolution. Molecular heterogeneity involves not only the primary tumor and local recurrences, but also the surrounding field. The surgical implications of this fact remain to be explored.

PROJECT 4: INVESTIGATION OVER INTRATUMOR HETEROGENEITY AS A PROGNOSTIC FACTOR IN PREDICTING DISEASE RELAPSE IN ORAL SQUAMOUS CELL CARCINOMA

AIMS:

Limited clinical value of a single sample for the assessment of molecular profiles of tumours is a direct consequence of intratumour heterogeneity (ITH).(132) Therapeutic strategies based on lacking molecular diagnosis expose clinicians to the risk of errors and distortions. Therefore, the study of intratumor heterogeneity is the goal of future precision medicine. Prognostic implications of intratumour heterogeneity are not fully understood. Aim of this project was to investigate the relationship between ITH and local progression of OSCC.

METHODS:

Patients selection:

Patients operated for OSCC and attending regular follow up for disease relapse at the Unit of Oral Medicine and Maxillofacial Surgery S.Orsola Hospital, University of Bologna were recruited for the study.

Inclusion criteria:

- A diagnosis of primary tumor of T2-T4 according to the p-TNM classification of tumours(Amin MB, Edge SB, Greene FL, et al, eds. AJCC Cancer Staging Manual. 8th ed. New York: Springer; 2017).
- Absence of Nodal involvement
- Absence of lichenoid inflammation in tumour microenvironment
- Surgical margin of resection free from neoplasia
- Follow up ≥ 3 years after surgical resection

If during the follow up interval the patient had experienced disease progression, he/she was included in group 1 (recurrent OSCCs), otherwise, if no relapses were recorded, the patient was included in group 2 (non-recurrent OSCCs). Recruitments of both cohorts ended when numerosity reached the 5 units for a total of 10 studied patients.

Selection of the sampled areas:

Multiple samples were obtained from each tumour and from related adjacent non neoplastic mucosa. In particular, with respect to primary tumours, histological slides were reviewed with the help of expert pathologists and two areas ≥0.5 cm apart exhibiting 70% of tumor cells were selected for analysis. At least five 10-µm-thick sections of the same areas were macrodissected manually (using a scalpel) to collect about 1 cm^2 tissue. The study was approved by our Institutional Ethics Committee (S. Orsola Hospital, project mtDNA01, approval code 020/2013/U/Tess) and informed consent was obtained from all patients. Two additional samples were collected from mucosae surrounding tumors; again, all samples were ≥0.5 cm apart. Using the same protocol, two samples from surgical specimens of secondary manifestations were collected and analyzed. DNA purification from each sample was performed as described in project 3. In brief, macrodissected tissue was digested at 56°C for 3 h using the solution of the Quick Extract^{TM} FFPE DNA extraction kit (Epicentre, Madison, WI, USA). After denaturation at 95°C for 5 min, each suspension was centrifuged at 10,000 x *g* at 4°C for 5 min to pellet undigested tissue and solidify floating paraffin. DNA for PCR was collected from the interphase and stored at 4°C.

Next Generation Sequencing

We subjected the following genes (selected by DriverDBv2(105)) involved in head and neck squamous cell carcinogenesis(106) to deep sequencing: *KRAS* (exons 2–4), *NRAS* (exons 2–4), *HRAS* (exons 2, 3), *BRAF* (exon 15), *PIK3CA* (exons 10, 21), *TP53* (exons 4–9), *NOTCH1* (exons 4, 6, 11, 26, 27), *PTEN* (exons 5–8), *CDKN2A* (exons 1, 2), *EGFR* (exons

18–21). Locus-specific amplicon libraries with tagged primers were generated using overhang adapters based on the 5′ Nextera sequences; these were recognized during the second round of short PCR, which added P5/P7 sequencing adapters (Illumina, San Diego, CA, USA) and sample-specific indices. In the first round of enrichment PCR, seven multiplex PCR tubes were used for parallel amplification of all of the above DNA regions using Phusion U (Thermo Fisher, Waltham, MA, USA) as the proofreading enzyme. The amplification products were mixed and purified using MagSi-NGSPREP-Plus beads (Magnamedics, Geleen, the Netherlands), quantified with the aid of a Quantus fluorometer (Promega, Madison, WI, USA), and employed as templates (100 ng) in the second (barcoding) PCR step (eight cycles). The amplicons were purified using MagSi-NGSPREP beads, quantified employing the fluorometer, pooled, and loaded onto a MiSEQ platform (Illumina). FASTQ files trimmed in terms of the multiplex identifiers were used for quality control. Only reads of PHRED quality score > Q30 and length > 100 bp were retained for mapping and variant analysis in a Galaxy Project environment(107). Raw reads were mapped to the hg19 human reference genome sequence, and read alignment files in BAM format were generated with Bowtie2 mapping, GATK local realignment, HaplotypeCaller and Picard MarkDuplicates. The BAM files were analyzed using an Integrative Genomic Viewer (IGV)(108) to manually identify all mutations of a clinical sensitivity threshold > 5%; only bidirectional variant calls with more than 10 reads were reported(109). The depth of coverage was assessed manually using the IGV, and regions with less than 100 reads were discarded. To test the reliability of variant allele frequencies (VAFs), three cell lines with known mutations (SW620: *KRAS pG12V*; CAL62: *KRAS p.G12R*; OCUT: *BRAF p.V600E*) were spiked into a background of wild-type DNA (DNA female pool, code G1521; Promega) at different concentrations, as described previously(110). ASLNAqPCR (an orthogonal method) was used to derive sensitivities and specificities for detection of *KRAS* codons 12–13 and *BRAF* codon 600(110)(111).

Evaluation of intratumor heterogeneity (ITH)

The reliability of a single sample to reflect the genetic profile of the tumor was assessed in both groups comparing the number of somatic mutations obtained from one sample with respect to multiple samples investigation.

Similarly, the occurrence of a WT/Mutated dicrepancy among samples was analyzed to investigate the implications of a targeted therapy guided by a single tumor sample. In particular if one sample exhibited mutations in a studied gene but the other was wild type patient was considered discrepant. On the contrary, if both samples were mutated or both wild type no discrepancies were recorded.

The degree of ITH for OSCC somatic mutations was derived by calculating the heterogeneity rates (HR) of all affected genes and dividing that sum by the number of affected genes not shared by all tumor regions, as described previously(116). These values were used to compare the ITH in the group of recurrent and non recurrent OSCCs. The same protocol was used in the group of recurrent OSCCs for secondary neoplastic events to compare ITH values during tumour evolution. ITH was also calculated in both groups for non-neoplastic mucosae surrounding primary tumors, to explore field cancerization.

Tracking Tumor Evolution:

OSCC genetic profiles obtained from multiple samples were used to identify mutations that persisted during tumor evolution and in particular from adjacent non neoplastic filed.

In addition, the investigation of genetic relationship among samples was enriched by mtDNA analysis. Phylogenetic trees were constructed as in project 3. D-loop sequencing featured amplification of four segments covering the region from positions 15,995 to 700 of human mitochondrial DNA (NC_012920 gi:251831106; MITOMAP: a Human Mitochondrial

Genome Database, http://www.mitomap.org). Locus-specific amplicon libraries with tagged primers were generated using overhang adapters based on the 5′ Nextera sequences, as described above for OSCC driver genes. FASTQ files were filtered in terms of PHRED score > Q30 and read length > 100 bp, as above, and converted to FASTA format. Four FASTA files representative of each of the four D-loop fragments were created by Perl and processed by Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand) to allow for multiple sequence alignment to identify heteroplasmies. The four consensus sequences were then joined and used to construct phylogenetic trees employing MAFFT (https://mafft.cbrc.jp/alignment/server/), with UPGMA/Jukes-Cantor serving as the substitute model(51).

RESULTS:

Patients Characteristics:

In the group of 5 Recurrent OSCC 2 patients were female and 3 patients were female (mean age 53±12,9). In the group of 5 non recurrent OSCC 4 patients were male and 1 patient was female (mean age 53±9.2). Affected areas in the group of recurrent OSCC included (cheek, gingiva, tongue, floor of the mouth) whereas in the group of non-recurrent OSCCs arose in tongue and gingiva.

All ten tumours exhibited histological evidence of submucosal infiltration. The degree of differentiation in the group of recurrent OSCCs was 2/5 well differentiated, 2/5 moderately differentiated and 1/5 scarcely differentiated. In the group of non-recurrent OSCCs 2/5 were moderately differentiated and 3/5 well differentiated.

In the group of recurrent OSCC 3/5 developed 2 more neoplastic events after surgical excision, 1/5 one subsequent event and 1/5 5 neoplastic events following primary tumor.

Mutational Analysis:

We investigated the effect of Intratumoral heterogeneity on the representativeness of single samples comparing the number of mutations found in the tumour whether one or more samples were analysed.

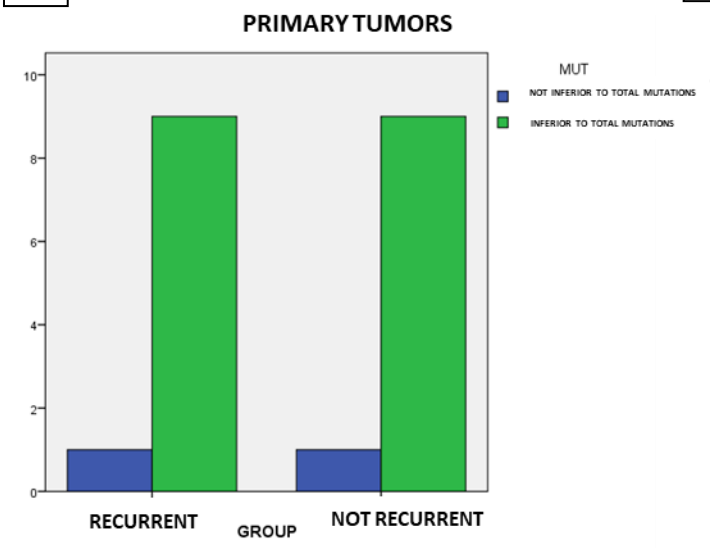
In primary tumours of both groups of OSCC 9 out of 10 (90%) single samples yielded a number of somatic mutations which was lower than the number of mutations derived from multiple samples analysis.(Fig.20A)

In non neoplastic adjacent mucosae, mutational analysis based on a single sample was less informative than the multi samples approach in 7 out of 8 cases in the group of recurrent OSCC (samples from patient 5 couldn't be amplified). (Fig.20C) By contrast, only 3 out of 10 samples from the group of non recurrent OSCC were less informative than the combination of two samples for the analysis of mutational profile of the tumour surrounding field.

In recurrent local events,8 out of 10 (80%) single samples from the second event yielded a number of somatic mutations which was inferior to multiple samples analysis whereas the same trend was observed in 4 out of 6 samples from the third event developed after primary tumour. Only one patients developed further events. (Fig.20B)

FIG.20 The figure illustrates the reliability of a single sample in describing mutational profile in primary tumors (A), secondary adverse events (B) and adjacent mucosa (C). Green columns refer to samples in which the number of DNA somatic mutations in the ten studied genes were quantitatively inferior to the number of mutations derived from multiple samples analysis. Conversely, Blue columns refer to samples in which the number of somatic mutations was superimposable to somatic mutations calculated with multiple samples.

A



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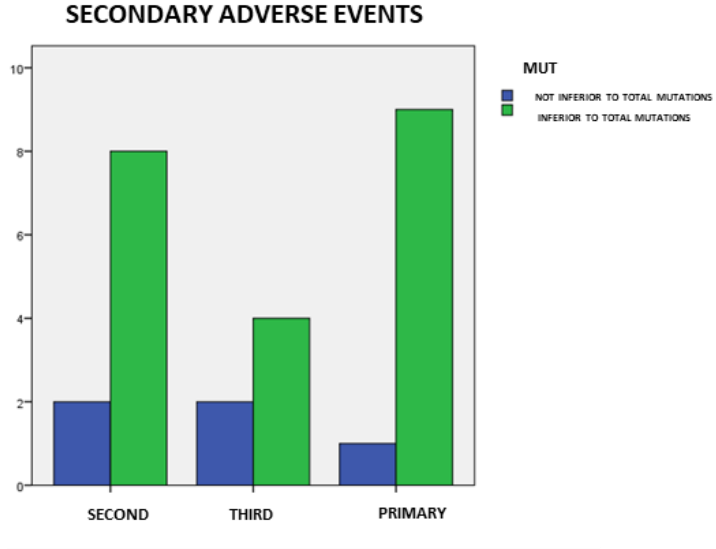

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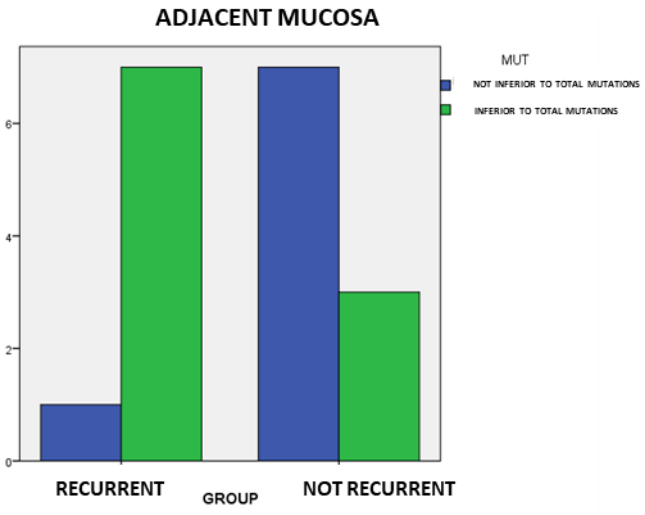
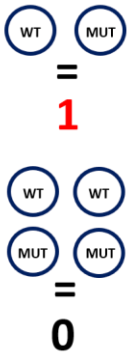


The occurrence of a WT/Mutated discrepancy among samples was analysed in all OSCC cases and for all 10 studied genes, to investigate one of major risks of molecular misdiagnosis that may lead to inappropriate targeted therapies : *KRAS* (2/10) *NRAS*(3/10) *HRAS*(3/10) *BRAF*(1/10) *EGFR*(2/10) *TP53*(4/10) *CDKN2A*(3/10) *PTEN*(1/10) *NOTCH1*(3/10) *PIK3CA*(1/10).(Table 14)

Table 14 The occurrence of WT/Mutated discrepancy between samples may reflect a source of bias for targeted molecular therapy. 1= presence of discrepancy. One out of 2 samples harbored genetic mutations in a studied genes while the other sample from a distant area resulted wild type (WT). 0= absence of discrepancy. Both samples mutated or both WT

PZ	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	PTEN	NOTCH1	PIK3CA
1	0	1	0	0	0	0	1	0	0	0
2	0	0	0	0	1	0	1	0	0	0
3	0	0	1	0	0	0	0	0	0	0
4	1	0	0	1	1	0	0	0	0	0
5	1	0	0	0	0	1	0	0	0	1
6	0	0	1	0	0	0	0	0	0	0
7	0	0	0	0	0	1	1	0	0	0
8	0	1	0	0	0	1	0	0	1	0
9	0	0	0	0	0	1	0	0	1	0
10	0	1	1	0	0	0	0	1	1	0



Values of Heterogeneity Rate (HR) in primary tumours and adjacent mucosa:

The degree of ITH for studied primary OSCCs was derived by calculating the heterogeneity rates (HR) of all affected genes and dividing that sum by the number of affected genes not shared by all tumor regions.(116)

HR values between groups were then statistically compared using the non parametric U-Mann-Whitney test for independent samples (IBM® SPSS Software v.21). P value scored

0,095 and did not reach significance indicating the absence of a statistical difference in the degree of intratumor heterogeneity among the groups of recurrent and non recurrent OSCC. By contrast, U-Mann-Whitney test performed on samples from non neoplastic adjacent mucosa disclosed that non recurrent OSCC tends to exhibit statistically lower values of HR if compared to recurrent OSCCs (p= 0,032).

A statistical relation was also investigated between HR and clinical site among the ten studied tumours. Sites were divided in two groups; one involving tongue and the other comprising sites other than tongue. No statistically significant relations were found (p=0,114).(Table 16-17-18)

Values of Heterogeneity Rate (HR) and local progression of the disease:

HR was calculated also for neoplastic events following primary tumour. Among second events, 3 out 5 cases exhibited HR values that were lower/equal if compared to related primary tumour. None of the three events developed after second tumour had HR values that were greater than its corresponding predecessors. Only one patient developed several events following the third. In particular, fourth and third events had superimposable values of HR, fifth event had HR values greater than its predecessor while in sixth event HR was lower than in the fifth. More in general, if secondary neoplastic manifestations were analysed together, 8 out of 11 cases exhibited HR values that were lower/equal if compared to the previous event.(Table 19)

Analysis of tumor evolution through mutational analysis:

Somatic mutations derived from multi samples analysis of tumours were analyzed during disease progression. In particular, we found 9 mutations that showed the ability to be transferred to future adverse events. Mutations involved the genes *KRAS*(p.A130Q)

NRAS(p.G138R) *HRAS*(p.H27H) *EGFR*(p.Q787Q and p.R836R) *TP53*(p.Y236* and p.R273C) *NOTCH1*(p.D168D and p.G212D).

6 out of 9 mutations with the ability to persist during OSCC evolution were detectable at early stage of tumorigenesis in the non neoplastic surrounding field. Interestingly, 1 out of 6 (NRASp.G138R) in patient 1 was not detectable in primary tumour but was transferred directly to second neoplastic event in some sort of evolutionary jump.

3 out 9 persistent mutations were not found early in non neoplastic adjacent mucosae (*TP53* p.R273C, *NOTCH1* p.G212D, *KRAS* p.A130Q) but developed at later stages of tumorigenesis. In particular *TP53* p.R273C in patient 1 was found in primary tumour, it was transferred to second event but was not found in the third OSCC. By contrast, in patient 2 *NOTCH1* p.G212D arose late in OSCC evolution and was found only in second neoplastic manifestation from which it was transferred to the third. Interestingly, in patient 4 *KRAS* p.A130Q arose after primary tumour but was transferred from second event to all 4 subsequent neoplastic relapses.

In only one patient (Patient 5) persistent mutations could not be identified. (Table 20)

Analysis of tumor evolution mtDNA phylogenetic trees:

The analysis of phylogenetic trees, integrated data from mutational analysis and helped to visualize the evolutionary connections among samples in the progression from non neoplastic mucosae to recurrent events in OSCC.

Interestingly, in 3 out of 4 patients from the group of recurrent OSCCs samples from non neoplastic mucosa were displayed on different branches. Heterogeneity was confirmed as an early process in tumorigenesis and not restricted only to tumours. In particular, in patient 4 samples seemed to belong to distinct genetic clusters as their position on the tree was considerably distant.

mtDNA analysis of multiple tumour samples tracked the evolution of subclones during disease progression.

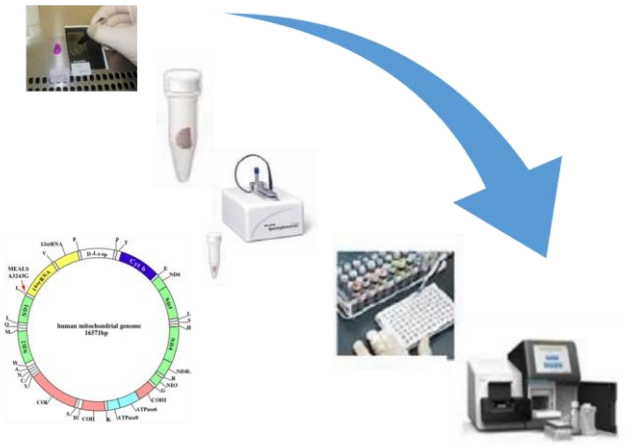
In patient 1 only one sample of primary tumour was located in proximity to samples from second and third adverse event, suggesting a common evolutionary line.

In patient 3 samples from non neoplastic mucosae clustered in mutual proximity but their MtDNA profiles appeared evolutionarily more similar to samples from secondary adverse events rather than primary tumour. This pattern suggested a pivotal role of the field in tumour progressions and in the formation of second field tumours. (Fig.22 A,B,C,D,E)

Table15. Summary of patients characteristics (M: Male F: Female nM : months)

PATIENT	SEX	AGE	SITE	TNM	HISTOLOGY	RECURRENT EVENTS
1	F	32	CHEEK	T4N0M0	EPIDERMOIDAL WELL DIFFERENTIATED	2(24 M-12Y)
2	F	57	GUM	T4N0M0	SQUAMOUS MODERATELY DIFFERENTIATED	2(1 M -18M)
3	M	57	TONGUE	T3N0M0	SQUAMOUS MODERATELY DIFFERENTIATED	1(12M)
4	F	67	FOM	T2N0M0	SQUAMOUS POORLY DIFFERENTIATED	5(10M-5M-20M-10M-10M)
5	M	52	TONGUE	T4N0M0	SQUAMOUS WELL DIFFERENTIATED	2(6M-110M)
6	M	59	GUM	T3N0M0	SQUAMOUS MODERATELY DIFFERENTIATED	NO
7	M	46	TONGUE	T3N0M0	SQUAMOUS MODERATELY DIFFERENTIATED	NO
8	F	55	GUM	T4N0M0	SQUAMOUS WELL DIFFERENTIATED	NO
9	M	43	TONGUE	T4N0M0	SQUAMOUS WELL DIFFERENTIATED	NO
10	M	66	TONGUE	T3N0M0	SQUAMOUS WELL DIFFERENTIATED	NO

FIG.21 The figure illustrates the workflow for samples processing and data analysis: from FFPE blocks, selected areas were manually macrodissessected and processed for DNA and mtDNA extraction. Amplicon libraries for studied genes were then generated for DNA sequencing through MiSEQ platform (Illumina).



	Sample Mutations	Tumour Mutations	Shared
PT1 N1	21	46	1
PT1 N2	24		
PT1 PR1	33	53	2
PT1 PR2	20		
PT1 2.1	4	5	2
PT1 2.2	3		
PT1 3.1	5	10	1
PT1 3.2	6		
PT2 N1	3	7	3
PT2 N2	7		
PT2 PR1	1	3	1
PT2 PR2	3		
PT2 2.1	6	9	1
PT2 2.2	4		
PT2 3.1	3	3	1
PT2 3.2	1		
PT3 N1	5	8	2
PT3 N2	5		
PT3 PR1	2	4	1
PT3 PR2	3		
PT3 2.1	7	10	2
PT3 2.2	5		
PT4 N1	3	6	0
PT4 N2	3		
PT4 PR1	3	10	0
PT4 PR2	7		
PT4 2.1	3	4	1
PT4 2.2	2		
PT4 3.1	2	2	1
PT4 3.2	1		
PT4 4.1	2	2	1
PT4 4.2	1		
PT4 5.1	6	7	2
PT4 5.2	3		
PT4 6.1	1	1	1
PT4 6.2	1		
PT5 N1	NA	NA	NA
PT5 N2			
PT5 PR1	3	5	0
PT5 PR2	2		
PT5 2.1	4	4	0
PT5 2.2	0		

Table 16: Mutational analysis in the group of recurrent OSCC. For each tumour the number of mutations found through NGS, the number of mutations found in each sample and the number of shared mutations among samples are indicated.

	Sample mutations	Tumour mutation	Shared
PT6 N1	8	12	3
PT6 N2	7		
PT6 1.1	4	6	2
PT6 1.2	4		
PT7 N1	2	2	2
PT7 N2	2		
PT7 1.1	6	6	2
PT7 1.2	2		
PT8 N1	3	4	3
PT8 N2	4		
PT8 1.1	7	20	2
PT8 1.2	15		
PT9 N.1	2	2	2
PT9 N.2	2		
PT9 1.1	2	4	0
PT9 1.2	2		
PT10 N1	2	2	2
PT10 N2	2		
PT10 1.1	2	14	0
PT10 1.2	12		

Table 17 Mutational analysis in the group of non recurrent OSCC.

PATIENT	REC/NREC	HR PR	HRN
1	1	96	97
2	1	60	57
3	1	75	75
4	1	100	100
5	1	100	
6	2	66	75
7	2	66	0
8	2	90	25
9	2	100	0
10	2	100	0

Table 18 Heterogeneity Rate (HR) values in primary tumors and related adjacent mucosa. Patients 1-5 (Recurrent OSCC) Patients 5-10 (Non recurrent OSCC). Mann-Whitney U test for independent samples was used to compare HR values in primary tumour and adjacent mucosa of the two groups (recurrent and non recurrent OSCC).

	Null Hypothesis	TEST	P value (sig. 0.05)	Decision
1	Distribution of Heterogeneity Rate (HR) values is similar in primary tumours of recurrent and non recurrent OSCCs	Mann-Whitney U test for indipendent samples	0,095	Keep null hypothesis
2	Distribution of Heterogeneity Rate (HR) values is similar in adjacent mucosa of recurrent and non recurrent OSCCs	Mann-Whitney U test for indipendent samples	0,032	Reject null hypothesis

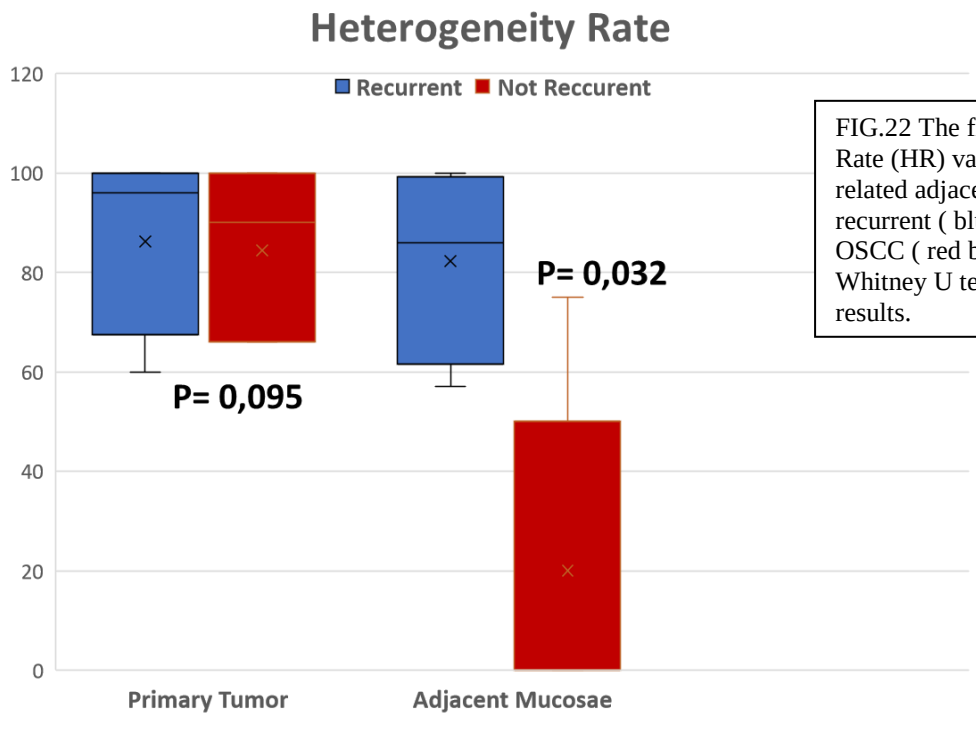


FIG.22 The figure illustrates Heterogeneity Rate (HR) values in primary tumors and related adjacent mucosa in both groups of recurrent (blue boxes) and not recurrent OSCC (red boxes). P values refer to Mann-Whitney U test for independent samples results.

PATIENT	HR I	HR II	HR III	HR IV	HR V	HR VI
1	96	60				
2	60	88	66			
3	75	80				
4	100	75	50	50	71	0
5	100	100	100			

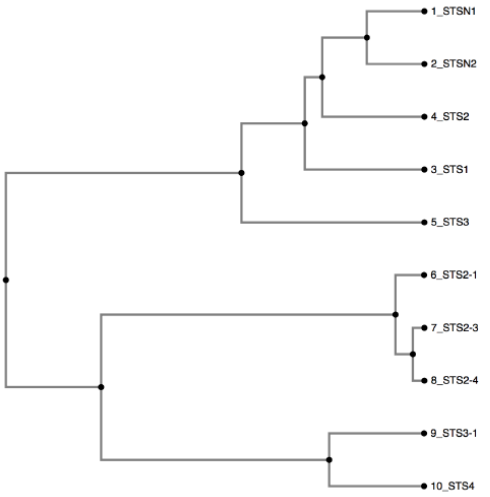
Table 19 Heterogeneity Rate (HR) values in second events (II-VI) after primary tumour (I) from the group of recurrent OSCC.

TAB 20 Tracking tumour evolution through somatic mutations analysis. For each patient (1-10) mutations that persisted from mucosal field (N) to neoplastic events (I-VI) are highlighted

	N	I	II	III	IV	V	VI
1	NRAS p.G138R EGFRp.R836R	EGFRp.R836R TP53 p.R273C	NRAS p.G138R EGFR p.R836R TP53 p.R273C	NRAS p.G138R EGFRp.R836R			
2	EGFRp.Q787Q NOTCH1p.D1698D	EGFRp.Q787Q NOTCH1p.D1698D	EGFRp.Q787Q NOTCH1p.D1698D NOTCH1p.G212D	EGFRp.Q787Q NOTCH1p.D1698D NOTCH1p.G212D			
3	NOTCH1p.D1698D	NOTCH1p.D1698D	NOTCH1p.D1698D				
4			KRASp.A130Q	KRASp.A130Q	KRASp.A130Q	KRASp.A130Q	KRASp.A130Q
5							
6	HRASp.H27H EGFRp.Q787Q NOTCH1p.D1698D	HRASp.H27H EGFRp.Q787Q NOTCH1p.D1698D					
7	EGFRp.Q787Q NOTCH1p.D1698D	EGFRp.Q787Q NOTCH1p.D1698D					
8	HRASp.H27H EGFRp.Q787Q NOTCH1p.D1698D	HRASp.H27H EGFRp.Q787Q NOTCH1p.D1698D					
9	EGFRp.Q787Q NOTCH1p.D1698D	EGFRp.Q787Q NOTCH1p.D1698D					
10	EGFR p.R836R TP53p.Y236*	EGFR p.R836R TP53p.Y236*					

Fig. 23: Phylogenetic trees by sequencing of mtDNA D-loop region using UPGMA for recurrent OSCC:
A: Patient 1: only one sample of primary tumour was located in proximity to samples from second and third adverse event, suggesting a common evolutionary line.
B: Patient 2: samples from non neoplastic mucosa were displayed on different branches. Heterogeneity was confirmed as an early process in tumorigenesis
C: Patient 3: samples from non neoplastic mucosae clustered in mutual proximity but their MtDNA profiles appeared evolutionarily more similar to samples from secondary adverse events rather than primary tumour
D: Patient 4: samples from non neoplastic mucosae seemed to belong to distinct genetic clusters as their position on the tree was considerably distant
E: Patient 5: Mutational analysis did not exhibit common mutations among samples. MtDNA analysis integrated data from mutational analysis and helped to visualize the evolutionary connections among samples.

A



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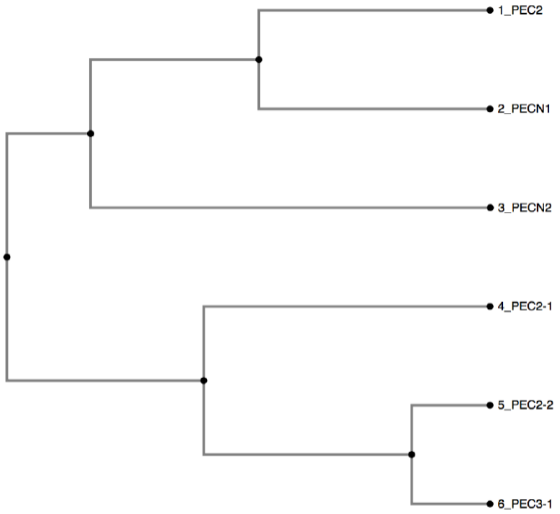

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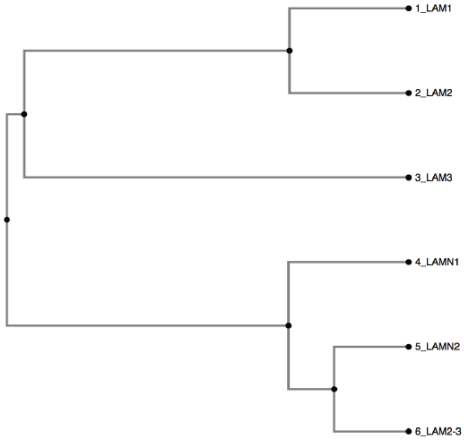

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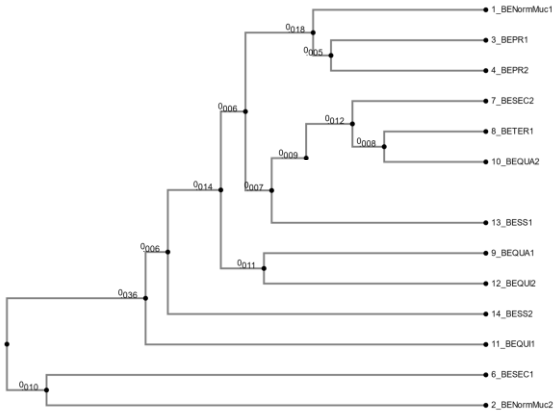

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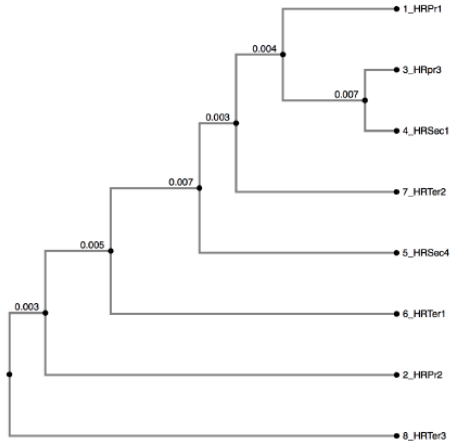

DISCUSSION:

Despite recent advances in OSCC treatment (radical surgery, chemotherapy and molecular therapy with anti-EGFR monoclonal antibodies) mortality rates did not improve significantly.(117) Unfavourable prognosis of OSCC frequently depends on scarce loco regional control. After resective surgery patients operated for OSCC tend to develop distant metastasis or new oral carcinomas.(96)

The theory of field cancerization explains this aggressive behaviour. Although tumour adjacent epithelium may appear clinically or histologically unaffected, unseen genetic errors may be responsable for disease progression.(5) Next Generation Sequencing(NGS) improved our understanding of molecular biology of OSCC and confirmed the theory of field cancerization. A genetic classification of secondary OSCCs was proposed by Braakhuis et al. and distinguished between Local Recurrence, Second Primary Tumour and Second Field Tumour, whether second events were genetically proximal to index tumour, to the adjacent field or were genetically independent.(49)

However, deep coverage of NGS also revealed that genetic differences may exist among tumour cells.(56) Intratumour Heterogeneity theory states that during tumour evolution, under the effect of Darwinian-like pressures, cancer cells may follow distinct evolutionary lines.(58) Consequently, genetically heterogeneous subclones may coexist within the tumour.

In many cases genetic investigations on tumours rely on single biopsy samples. Due to intratumoral heterogeneity, a single sample may offer a narrow insight on tumour genetics depending on the area the specimen is taken from.(132)

In the present study we compared genetic profiles of OSCCs obtained from one or multiple samples. Results showed that the number of mutations yielded by a single sample was in most cases inferior to the profile revealed by two samples. In particular in both groups of recurrent and non recurrent OSCC 90% of samples was less informative than the combination of two samples.

The limited perspective offered by a single sample was confirmed also for secondary events. However, 80% of samples from the second events and 66.6% of samples from the third events were inferior in terms of number of mutations to the analysis of multiple samples. What apparently suggests an increase in homogeneity during disease progression may be the effect of selective pressures supporting clones that better survive in oral field microenvironment.

In project 3 it was demonstrated that cellular heterogeneity applies not only to tumours but also to adjacent field. Indeed, one sample was less informative than two samples also in almost all cases of non neoplastic adjacent mucosa from recurrent OSCC. Curiously, similar results were not observed in the group of non recurrent OSCC where mutations yielded by samples of non neoplastic adjacent mucosa resulted more homogeneous. In particular, only 3 out of 10 samples were less informative than the combination of two samples.

Being molecular targeted therapy deeply influenced by genetic profiles of tumours, intratumour heterogeneity represents a source of distortion that may misguide therapeutic strategies.

Indeed, underdiagnosis derived from the investigation of a limited area of the tumour may occult mutations potentially targetable by molecular drugs.

We investigated the occurrence of a WT/Mutated discrepancies and we found that such eventuality could involve all studied genes, independently from clinical behaviour.

However, since mutations may differ among subclones a measure of ITH was essential. As described previously(116) we obtained a value representative of the degree of ITH by calculating the heterogeneity rates (HR) of all affected genes and dividing that sum by the number of affected genes not shared by all tumor regions.

HR values of primary tumors in two groups of recurrent and non recurrent OSCCs were statistically compared to investigate prognostic implications of ITH. No statistical significant difference was recorded among groups. According to our results no particular degree of heterogeneity was able to distinguish OSCC with aggressive local behaviors. Genetic instability in tumours may produce heterogeneous subclones but not all subclones may reflect aggressive behaviors. This finding is in disagreement with Mroz et al.(127,128) who found a relation between high intra-tumor genetic heterogeneity and worse clinical prognosis. Mroz et al. however, used MATH values to calculate intratumour heterogeneity, a measure based on whole-exome sequencing of tumors but applied to single biopsy specimen. (128)Differences in the methodology may explain divergent results especially if a multi region approach to the study of intratumour heterogeneity is not considered.

Interestingly, ITH analysis of non neoplastic adjacent mucosa disclosed that non recurrent OSCC tends to exhibit statistically lower values of HR if compared to recurrent OSCCs (p= 0,032). Fields of less aggressive OSCCs are thus more homogeneous.

A prognostic implication of genetic heterogeneity of pre neoplastic field is here documented for the first time. Hypothetically, genetic homogeneity around primary tumour could reflect less genetic instability of surrounding mucosae and related lower ability to harbour the development of aggressive mutations.

By contrast, ITH investigated in secondary events showed that both increase or decrease in the degree of HR could occur during disease progression. Nevertheless, more than 50% of studied local relapses had ITH levels lower than related pre-existing tumour.

Although clonal selection of more aggressive mutations could genetically explain decreasing values of heterogeneity in recurrent OSCC the studied population is too limited and further studies are encouraged.

Genetic relationships between the field and neoplastic events were also investigated through mtDNA based phylogenetic trees and the identification of persisting mutation.

9 mutations exhibited the ability to be transferred to chronologically subsequent events. 6 out of 9 mutations were early detectable in pre neoplastic field whereas 3 developed at later stages.

Evidence of genetic links among samples from pre neoplastic field and chronologically subsequent events was also suggested by mtDNA analysis(i.e patient 1). This finding confirms Braakhuis classification of second field tumours. However, the use of mutational analysis in tracking tumour evolution combined with mtDNA phylogenetic trees suggested a more complex landscape. In particular, in patient 1 linear persistence of many mutations from pre neoplastic field to later events seemed to suggest Braakhuis second field tumours but NRASp.G138R “jumped” primary tumour and was transferred directly to second neoplastic events. By contrast, in patient 5 mutational analysis did not identify persistent mutations as for Braakhuis second primary tumours but mtDNA phylogenetic tree exhibited apparent genetic continuity among samples from subsequent events as in local recurrences/second field tumours. These findings confirm that during tumour evolution

continuing shaping of mutational profile of both fields and tumours takes place under microenvironment selective pressures. In a similar context, the study of intratumour heterogeneity through multi region analysis is a good approach for the observation of complex interactions between OSCC and the field.

In conclusion, the degree of genetic heterogeneity of pre malignant field seems to have an impact on loco regional prognosis of patients operated of OSCC. Under the effect of genetic instability, high heterogenous fields could more easily host the development of aggressive mutations responsible for disease relapse. On the contrary increasing homogeneity during tumor progression could reflect clonal selection under the pressure of tumour-field microenvironment. However, due to small size of studied population and evidence of different patterns of tumor evolution future studies confirming our results are strongly encouraged. In addition, the analysis of multiple areas instead of a single specimen confirmed to exhibit higher reliability in describing mutational profile and should be acknowledged for future research, diagnostic and therapeutic strategies in the management of OSCC.

SUPPLEMENTRY MATERIALS PROJECT 4:

Table 21 Somatic mutations profiles in recurrent OSCCs. For each sample, related somatic mutationst in the ten studied genes are illustrated. (N: adjacent Normal Mucosa Pr. Primary tumour VAF Variant allele frequency)

	NoCaso	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA
STS N1		p.G115E VAF 10% Pd57N VAF 6% p.G10 R 6%		p.G13D VAF8% C p.V14M VAF 9% NC p.G75G VAF 5% C	p.Q609 * VAF 9% c	p.F712F VAF 15% NC p.R836R VAF 18 %	p.A189A VAF 13% C p.P191L VAF 12% C p.I255I VAF 28% NC	p.A86V VAF 70% p.P61S 14% NC	NA	NA	WT	p.N214F VAF 41% NC p.E330L VAF 21% NC p.E334L 21% p.G597D VAF 68% NC p.E606L VAF68% C p.D1698D VAF 14%Cp	WT
STS N2		p.G12S VAF 11% C p.H27Y VAF 12% C	p.M1I VAF 19% C p.G10E VAF 11% C p.G138R vaf 8% c	p.H27H VAF 84% C p.S65R VAF 5% nc	p.Q609K vaf 11%	p.E697K vaf 10% nc p.P694T vaf 8% nc p.P 699P vaf 10% p.L703L vaf 10% p.Q787Q vaf 43%	p.G117R vaf 35% C p.S215N VAF 10% C	p.P40S (TCC) vaf 6% p.L78F VAF 10% NC	NA	NA	p.H196H(CAT) VAF 14% NC p.P204S (TCA)VAF7% C p.R233Q(CAA) VAF 9% NC	p.D582D VAF 25% NC p.R1586(TGC) vaf 5% p.D1698D VAF 51 % C	p.N526SVAF 21% NC
STS PR1		p.V14I vaf7% C	p.E3K VAF19% NC p.R123R NC VAF 9% p.F141K VAF 24% NC	p.V7M VAF 5% NC p.G15C VAF7% p.T20I VAF 6% C p.E49E VAF7%NC p.A59V vaf 11% p.S65N vaf 5% p.E76E VAF 7% NC	WT	p.E711K vaf 16% p.H835H vaf 46% p.R836R vaf 56% nc p.D837N vaf 10% p.V851I 11% p.R841R vaf 10%	p.N239N vaf 13% c p.L252F vaf 13% c p.D259D 15% p.R273C vaf 17%	wt	na	na	p.N184L(TTG) vaf 8% p.H196M(ATG) vaf 8% p.V222V(GTA) vaf 11% nc p.K223K(AAA) vaf 6% nc p.P244L(CTT) vaf 5% p.V249HV (GTA) vaf8% nc	p.A208V vaf 60% p.G326D vaf 26% p.S333N vaf 14% p.D338(AAC) vaf 12% p.A340T vaf 26%p.A585V vaf 13% p.T602I vaf 13% p.S1695N vaf 7%	WT
STS PR4		p.D30D vaf 6% p.E62K vaf 11%	wt	p.T20I vaf 11%	wt	p.R836R vaf 39% p.L838L(CTA) vaf 22%	p.L206L vaf 10% p.G244S vaf 18% p.R248Q vaf 10% p.P322T vaf 5% p.T329I vaf 59%	p.L47R vaf 7% p.R51K vaf 21% p.R62K vaf 18%	na	na	p.Q171*(tag) vaf 13% p.H196M(ATG) vaf 7% p.S229L(TTA) vaf 7% p.V249V(GTA) vaf 5% p.G251D(GAT) vaf 7%	p.H316H vaf 84% p.G597D vaf 18%	WT
common		0	0	0	0	1	0	0			1	0	0
STS 2.3		WT	p.G138R vaf 6%	wt	wt	p.R836R(CGT) vaf 57%	p.R273C vaf 17%	WT	na	na	WT	p.p.C339Y vaf 5% p.S341N vaf 5%	WT
STS 2.4		WT	WT	p.I24V vaf 5%	NA	p.R836R(CGT) vaf 46%	p.R273C vaf 14%	wt	na	na	wt	wt	wt
COMMON			1			1	1						
STS 3.1		WT	p.G138R vaf 5%	wt	wt	p.R836R(CGT) vaf 46%	p.R290C vaf 5%	wt	na	na	WT	p.V324V vaf 5% p.S1589S vaf 5%	
STS 3.3		p.L23L vaf 25%	WT	WT	NA	p.R836(CGT) vaf 90%	p.G245S vaf 29% p.R248S vaf 31% p.C275Y vaf 91%p.K291R vaf 6%	na	na	NA	WT	wt	WT
COMUNE		0	0	0	0	1	0	0			0	0	0
	NoCaso	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA
PECN1		wt	wt	wt	na	p.Q787Q(CAA) vaf 51%	wt	wt			wt	p.G310G vaf:10% p.D1698D vaf:44%	wt
PECN2		wt	p.V112M vaf.10% p.L133L vaf:30% p.H131N vaf:5%	wt	wt	p.Q787Q(CAA)vaf 37%	wt	p.P75P vaf.5%	na	na	wt	p.G310G vaf 18% p.D1698D vaf.40%	wt
COMUNE		0	0	0	0	1(1/1)	0	0			0	2	0
PEC1		wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	p.D1698D vaf:37%	wt
PEC2		wt	wt	wt	na	p.Q787Q(CAA) vaf 58%	wt	p.E33G vaf:20%	na	na	wt	p.D1698D vaf:37%	wt
COMUNE		0	0	0	0	0	0	0	0	0	0	0	0
PEC 2.1		wt	wt	wt	na	p.Q787Q(CAA) vaf 46%	p.Q192* vaf 67%	wt			wt	p.G212D vaf 6% p.C339Y vaf 6% p.A340T vaf 6% p.D1698D vaf 28%	wt
PEC 2.2		WT	wt	WT	WT	p.Q787Q(CAA) vaf 72%	p.G240E vaf 17%	wt	na	na	WT	p.T211(ATT) vaf 5% p.D337(aat) VAF 5%	wt
COMUNE		0	0		0	1	0	0	0	0	0	0	
PEC 3.1		WT	WT	WT	WT	p.Q787Q(CAA) vaf 38%	WT	WT	WT	WT	WT	p.G212D vaf 6% p.D1698D vaf 33%	WT
Pec 3.2		WT	WT	WT	WT	p.Q787Q(CAA) vaf 38%	WT	WT	WT	WT	WT	WT	WT
COMUNE		0	0	0	0	1(1/1)	0	0		0		0	
	NoCaso	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA
LAM N1		WT	WT	WT	WT	p.Q787Q(CAA) vaf 100 %	WT	p.P81P vaf 7%	NA	NA	WT	p.P213L vaf 7% p.G326S vaf 6% p.D1698D vaf 41%	WT
LAM N2		WT	WT	WT	WT	p.Q787Q(CAA) vaf 100 % p.L718L(TTG) vaf 5%	WT	p.R54C vaf 6%	NA	NA	WT	p.T1697I vaf 8% p.D1698D vaf 57%	WT
COMUNE		0	0	0	0	1(1/2)		0			0	1	
LAM 1.2		WT	WT	WT	WT	p.Q787Q(CAA)	WT	wt	NA	NA	WT	p.D1698D vaf 31%	WT
LAM 1.3		WT	WT	p.S65S vaf 6% p.D69D vaf 6%	NA	p.Q787Q(CAA)	WT	WT	NA	NA	WT	wt	WT
COMUNE		0	0	0	0	1(1/1)	0	0	0		0	0	
LAM 2.1		G13D vaf 15%	WT	WT	NA	p.Q787Q(CAA) vaf 100%	p.R290C vaf 11%	wt	NA	NA	WT	p.S341N vaf 9% p.F1593F vaf 5% p.L1601L vaf 5% p.D1698D vaf 32%	WT
LAM 2.2		p.T127T vaf 14%	WT	p.M67I vaf 5%	NA	p.Q787Q((CAA) vaf 100%	WT	NA	NA	NA	WT	p.S341N vaf 17% p.D1698D vaf 42%	WT
COMUNE		0	0	0	0	1(1/1)	0	0			0	1	
	NoCaso	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA
BE N1		p.E31* VAF:23%	p.G48C VAF:23%	WT	WT	p.V742I VAF: 24%	WT	WT	NA	NA	WT	WT	WT
BE N2		p.G48V VAF: 22%	p.L56M VAF: 27%	WT	WT	p.V845M VAF: 35%	WT	WT	NA	NA	WT	WT	WT
BE 1		WT	WT	WT	WT	WT	p.H179I VAF: 18%	p.R29Q VAF:11%	NA	WT	p.C250Y VAF:9%	WT	WT
BE 2		p.P34R VAF: 90%	WT	WT	p.V600M VAF: 14%	p.A750V VAF: 14%	p.P295S VAF: 8%	p.A42T VAF: 7%	NA	p.H24Y VAF:8%	p.L318F VAF: 6%	WT	WT
BE 2.1		p.A130Q VAF: 39%	WT	WT	WT	WT	p.C238Y VAF:13%	WT	NA	WT	p.V191M VAF: 28%	WT	WT
BE 2.2		p.A130Q VAF: 61%	WT	WT	WT	WT	WT	WT	NA	WT	WT	p.G1704E VAF5%	WT
BE 3.1		p.A130Q VAF: 51%	WT	WT	WT	WT	WT	WT	NA	WT	WT	p.D1681N VAF23%	WT
BE 3.2		p.A130Q VAF: 51%	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
BE 4.1		p.A130Q VAF: 42%	WT	WT	WT	WT	WT	WT	NA	WT	WT	p.R1594W VAF6%	WT
BE 4.2		p.A130Q VAF: 42%	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT
BE 5.1		p.A130Q VAF: 45%	WT	p.G13D VAF:16%	WT	WT	p.R248W VAF:6%	p.A44Q VAF: 10%	NA	WT	p.E242K VAF:6%	p.V1599M(VAF10%)	WT
BE 5.2		p.A130Q VAF: 35%	WT	p.G13D VAF:18%	WT	WT	WT	WT	NA	WT	WT	p.C1685Y(VAF6%)	WT
BE 6.1		p.A130Q VAF: 50%	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT
BE 6.2		p.A130Q VAF: 45%	WT	WT	WT	WT	WT	WT	NA	WT	WT	WT	WT
	NoCaso	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	AKT1	CTNNB1	PTEN	NOTCH1	PIK3CA
MR N1		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MR N2		NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
MR 1		p.P34L VAF7%	NA	WT	NA	NA	p.A74V VAF9%	NA	NA	NA	WT	p.T1602* VAF15%	WT
MR 2		WT	WT	WT	NA	NA	WT	NA	NA	NA	WT	p.G1704E VAF11%	p.G1050S VAF7%
MR 2.1		WT	WT	p.G12DVAF8%	WT	WT	p.A189TVAF11%	na	na	na	WT	p.L1712F VAF7%	p.E547L VAF7%
MR 2.2		WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
MR 3.1		WT	WT	WT	WT	WT	WT	na	na	na	WT	WT	p.S541F VAF8%
MR 3.2		WT	WT	WT	WT	WT	p.G279KVAF35%	na	na	na	WT	WT	WT

Table 22 Somatic mutations analysis in non recurrent OSCCs. For each sample mutations in the ten studied genes are illustrated. (N: adjacent Normal Mucosa VAF Variant allele frequency)

NoCaso	KRAS	NRAS	HRAS	BRAF	EGFR	TP53	CDKN2A	PTEN	NOTCH1	PIK3CA
SEG N1	WT	WT	p.H27H VAF 50%	WT	p.Q787Q VAF 100% p.794S VAF 7%	p.R156L VAF 49% p.L289L VAF 16%	WT	WT	p.V1686V 14% p.d1698D VAF 33% p.S1711N 14%	WT
SEG N2	WT	WT	p.G13R VAF9%) p.H27H VAF 50%	WT	P.778L VAF 6% p.Q787Q VAF 100% p.L798L VAF 8%	WT	WT	WT	p.d1698D VAF 37%	WT
SEG 1.1	WT	WT	p.H27H VAF 54%	WT	p.Q787Q VAF 100%	WT	WT	WT	p.d1698D VAF 24%p.G1710S vaf 5%	WT
SEG 1.2	WT	WT	WT	WT	p.Q787Q VAF 100% p.791Q VAF 5%	WT	WT	WT	p.C215C vaf 6% p.D1698D VAF 58%	WT
CAS N1	WT	WT	WT	WT	p.Q787Q VAF 100%	WT	WT	WT	p.D1698D VAF 55%	WT
CAS N2	WT	WT	WT	WT	p.Q787Q VAF 100%	WT	WT	WT	p.D1698D VAF 53%	WT
CAS 1.1	WT	WT	WT	WT	p.Q787Q VAF 46%	p.Q192Q vaf 2% p.E198K vaf 5%	p.D84N vaf 5%	WT	p.d1698D VAF 47 p.S1708L vaf 6%	WT
CAS 1.2	WT	WT	WT	WT	p.Q787Q VAF 49%	WT	WT	WT	p.D1698D) VAF 60%	WT
FAL N1	WT	WT	p.H27H VAF 55%	WT	p.Q787Q VAF 45%	WT	WT	WT	p.D1698D VAF 61%	WT
FAL N2	WT	WT	p.H27H VAF 51% p.R68Q VAF 7%	WT	p.Q787Q VAF 43%	WT	WT	WT	p.d1698D VAF 55%	WT
FAL 1.1	p.R123K VAF 10% p.A147T VAF 10%	p.E63E VAF 38%	p.H27H VAF 60%	WT	p.Q787Q VAF 90%	WT	WT	p.R233Q VAF 42%	WT	p.T544I VAF 11%
FAL 1.2	p.p140S VAF 30%	WT	p.H27H) VAF 60% p.V7M VAF 10%	WT	p.F712F VAF 96% P.771N VAF 96%	p.G279R VAF 46% p.R282Q VAF46% p.S314F VAF 94%	WT	p.P169L VAF 29% p.L185L VAF 17%	p.D1698D VAF 55% p.A1705A VAF 49% p.T596T VAF 96%	p.R537* VAF 17% p.T544I VAF 11%
BAG N.1	WT	WT	WT	WT	p.Q787Q VAF 100%	WT	WT	WT	p.D1698D VAF 49%	WT
BAG N.2	WT	WT	WT	WT	p.Q787Q VAF 100%	WT	WT	WT	p.D1698D VAF 51%	WT
BAG 1.1	WT	WT	WT	WT	p.Q787Q VAF 100%	WT	WT	WT	p.D1698D VAF 47%	WT
BAG 1.2	WT	WT	WT	WT	p.R836R VAF 45%	p.Y236* VAF 18%	WT	WT	WT	WT
FEM N1	WT	WT	WT	WT	p.R836R VAF 45%	p.Y236* VAF 27%	WT	WT	WT	WT
FEM N2	WT	WT	WT	WT	p.R836R VAF 41%	p.Y236(TAA) VAF 31%	WT	WT	WT	WT
FEM 1.1	WT	WT	WT	WT	p.R836R VAF 40%	p.Y236* VAF 51%	WT	WT	WT	WT
FEM 1.2	WT	p.A59V VAF 38%	p.H27H VAF 74%	WT	p.V689V VAF 19% p.G696E VAF 7% p.L703F VAF 19% P.A755T VAF 16% p.Q787Q VAF 66%	p.M243* VAF 100%	WT	p.S179S VAF 51% p.S229S VAF 32%	p.D1698D VAF 69% p.A208A VAF 88%	WT

Table 22 COSMIC (cancer.sanger.ac.uk/cosmic) analysis for biologic effects somatic mutations found in studied OSCCs and related adverse events. RIC found in Recurrent OSCC) NRIC: found in non recurrent OSCC)

KRAS			
p.G115E VAF 10%	G>A	Pathogenic (score 0.98)	RIC
PD57N VAF 6%	c.169G>A	Pathogenic (score 0.98)	RIC
p.G10 R 6%	c.28G>A	Pathogenic (score 0.98)	RIC
p.G12S VAF 11% C	c.34G>A	Pathogenic (score 0.98)	RIC
p.H27Y VAF 12% C	C>T	Pathogenic (score 0.98)	RIC
p.V14I vaf7% C	G>A	UNKNOWN	RIC
p.V7V vaf 5%	n.c	UNKNOWN	RIC
p.D30D vaf 6%	N.C	UNKNOWN	RIC
p.E62K vaf 11%	G>A	Pathogenic (score 0.98)	RIC
p.L23L vaf 25%	A>G	UNKNOWN	RIC
p.T50A vaf 5%	c.148A>G	Pathogenic (score 0.98)	RIC
p.T124I vaf 7%	G>A	Pathogenic (score 0.98)	RIC
p.G138E vaf 7%	G>A	Pathogenic (score 0.98)	RIC
p.V114I vaf 34%	G>A	Pathogenic (score 0.98)	RIC
G13D vaf 15%	c.38G>A	Pathogenic (score 0.98)	RIC
p.T127T vaf 14%	n.c	UNKNOWN	RIC
p.V7M vaf:5%	G>A	Pathogenic (score 0.98)	RIC
p.E31* VAF:23%	n.c	UNKNOWN	RIC
p.G48V VAF: 22%	n.c.	UNKNOWN	RIC
p.P34R VAF: 90%	C>G	Pathogenic (score 0.99)	RIC
p.A130Q VAF: 39%	n.c.	UNKNOWN	RIC
p.P34L VAF7%	C>T	Pathogenic (score 0.99)	RIC
p.R123K VAF 10%	n.c.	UNKNOWN	NRIC
p.A147T VAF 10%	n.c.	UNKNOWN	NRIC
p.P140S VAF 30%	C>T	Pathogenic (score 0.99)	RIC

HRAS			
p.G13D VAF8% C	G>A	Pathogenic (score 0.97)	RIC
p.V14M VAF 9% NC	NC	UNKNOWN	RIC
p.G75G VAF 5% C	G>A	Neutral	RIC
p.H27H VAF 84% C	T>C	Neutral	RIC-NRIC
p.S65R VAF 5% nc	NC	UNKNOWN	RIC
p.V7M VAF 5% NC	NC	UNKNOWN	RIC-NRIC
p.G15C VAF7%	NC	UNKNOWN	RIC
p.T20I VAF 6% C	C>T	Pathogenic (score 1)	RIC
p.E49E VAF7%NC	NC	UNKNOWN	RIC
p.A59V vaf 11%	NC	UNKNOWN	RIC
p.S65N vaf 5%	NC	UNKNOWN	RIC
p.E76E VAF 7% NC	NC	NEUTRAL	RIC
p.I24V vaf 5%	NC	UNKNOWN	RIC
p.S65S vaf 6%	NC	UNKNOWN	RIC
p.D69D vaf 6% (ok)	NC	UNKNOWN	RIC
p.M67I vaf 5%	NC	UNKNOWN	RIC
p.G12DVAF8%	G>A	Pathogenic (score 0.99)	RIC
p.R68Q VAF 7%	G>A	Pathogenic (score 0.97)	NRIC
p.G13R VAF9%	G>C	Pathogenic (score 0.99)	NRIC
p.V600M VAF: 14%	G>A	Pathogenic (score 0.98)	RIC

NRAS			
p.E132K VAF 16% C	G>A	Pathogenic (score 0.98)	RIC
p.M1I VAF 19%	G>C	Pathogenic (score 0.97)	RIC
p.G10E VAF 11% C	G>A	Pathogenic (score 0.91)	RIC
p.G138R vaf 8% c	G>A	Pathogenic (score 0.91)	RIC
p.E3K VAF19% NC	NC	UNKNOWN	RIC
p.R123R NC VAF 9%	NC	UNKNOWN	RIC
p.F141K VAF 24% N	NC	UNKNOWN	RIC
p.G138R vaf 6%	G>A	Pathogenic (score 0.98)	RIC
p.V112M vaf.10%	G>A	Pathogenic (score 0.98)	RIC
p.L133L vaf:30%	NC	UNKNOWN	RIC
p.H131N vaf:5%	NC	UNKNOWN	RIC
p.G48C VAF:23%	n.c.	UNKNOWN	RIC
p.L56M VAF: 27%	n.c.	UNKNOWN	RIC
p.E63E VAF 38%	n.c.	UNKNOWN	NRIC
p.A59V VAF 38%	n.c.	UNKNOWN	NRIC

EGFR			
p.F712F VAF 15%	C>T	NEUTRAL	RIC
p.R836R VAF 18 %	C>T	NEUTRAL	RIC-NRIC
p.P694T	n.c	UNKNOWN	RIC
p.E697K vaf 10%	n.c	UNKNOWN	RIC
p.P699P vaf 9%		NEUTRAL	RIC
p.L703L vaf 10%		NEUTRAL	RIC
p.E711K vaf 16%	G>A	Pathogenic (score 0.97)	RIC
p.Q787Q vaf 43%	G>A	Pathogenic (score 0.97)	RIC-NRIC
p.H835H vaf 46%		NEUTRAL	RIC
p.D837N vaf 10%	G>A	Pathogenic (score 0.99)	RIC
p.V851I vaf 11%	G>A	Pathogenic (score 0.99)	RIC
p.R841R vaf 10% nc	G>A	NEUTRAL	RIC
p.L838L vaf 22%	NC	UNKNOWN	RIC
p.V742I VAF: 24%	G>A	Pathogenic (score 1.00)	RIC
p.V845M VAF: 35%	G>A	Pathogenic (score 0.99)	RIC
p.A750V VAF: 14%	n.c.	UNKNOWN	RIC
p.794S VAF 7%	n.c.	UNKNOWN	NRIC
p.P778L VAF 6%	G>T	Pathogenic (score 0.89)	NRIC
p.L798L VAF 8%	n.c.	UNKNOWN	NRIC
p.791Q VAF 5%	n.c.	UNKNOWN	NRIC
p.F712F	n.c	UNKNOWN	NRIC
P.771N VAF 96%	n.c.	UNKNOWN	NRIC
p.V689V VAF 19%	n.c	UNKNOWN	NRIC
p.G696E VAF 7%	G>A	Pathogenic (score 0.98)	NRIC
p.L703F VAF 19%	n.c	UNKNOWN	NRIC
p.A755T VAF 16%	G>A	Pathogenic (score 0.99)	NRIC

TP53			
p.A189A vaf 10%	C>T	NEUTRAL	RIC
p.P191L vaf 12%	C>T	Pathogenic (score 0.99)	RIC
p.I255I VAF 28%	C>A	Pathogenic (score 0.95)	RIC
p.G117R vaf 35%	G>C	Pathogenic (score 0.85)	RIC
p.S215N VAF 10%	G>A	Pathogenic (score 0.99)	RIC
p.N239N vaf 13%	C>T	Pathogenic (score 0.95)	RIC
p.L252F vaf 13%	C>T	Pathogenic (score 0.95)	RIC
p.D259D 15%	C>T	Pathogenic (score 0.95)	RIC
p.L206L vaf 10%	G>A	NEUTRAL	RIC
p.G244S vaf 18%	G>A	Pathogenic (score 0.99)	RIC
p.R248Q vaf 10%	G>A	Pathogenic (score 0.98)	RIC
p.P322T vaf 5%		UNKNOWN	RIC
p.T329I vaf 59%	C>T	Pathogenic (score 0.98)	RIC
p.R290C vaf 5%	C>T	Pathogenic (score 0.98)	RIC
p.G245S vaf 29%	G>A	Pathogenic (score 0.98)	RIC
p.R248S vaf 31%		UNKNOWN	RIC
p.C275Y vaf 91%	G>A	Pathogenic (score 0.98)	RIC
p.K291R vaf 6%	G>A	Pathogenic (score 0.98)	RIC
p.Q192* vaf 67%	C>T	Pathogenic (score 0.98)	RIC
p.G240E vaf 17%	nc	UNKNOWN	RIC
p.R290C vaf 11%	C>T	Pathogenic (score 0.98)	RIC
p.H179I VAF: 18%	n.c	UNKNOWN	RIC
p.P295S VAF: 8%	C>T	NEUTRAL	RIC
p.C238Y VAF:13%	G>A	Pathogenic (score 0.99)	RIC
p.R248W VAF:6%	C>T	Pathogenic (score 0.94)	RIC
p.A74V VAF9%	C>T	NEUTRAL	RIC
p.A189TVAF11%	G>A	Pathogenic (score 0.99)	RIC
p.G279KVAF35%	n.c.	UNKNOWN	RIC
p.R156L VAF 49%	G>T	NEUTRAL	NON RIC
p.L289LVAF 16%	C>T	Pathogenic (score 0.97)	NON RIC
p.Q192Q vaf 2%	G>A	UNKNOWN	NON RIC
p.E198K vaf 5%	G>A	Pathogenic (score 0.99)	NON RIC
p.G279R VAF 46%	G>A	Pathogenic (score 1.00)	NON RIC
p.R282Q VAF46%	G>A	Pathogenic (score 0.98)	NON RIC
p.S314F VAF 94%	C>T	Pathogenic (score 0.85)	NON RIC
p.Y236* VAF 18%	C>A	Pathogenic (score 0.96)	NON RIC
p.M243I VAF 100%	G>A	Pathogenic (score 0.97)	NON RIC

NOTCH1			
p.N214F	NC	UNKNOWN	RIC
p.E330L	NC	UNKNOWN	RIC
p.E334L	NC	UNKNOWN	RIC
p.G597D	NC	UNKNOWN	RIC
p.E606L	NC	UNKNOWN	RIC
p.D1698D	C>T	NEUTRAL	RIC_NRIC
p.D582D	NC	UNKNOWN	RIC
p.R1586C	C>T	Pathogenic (score 0.88)	RIC
p.A208V	NC	UNKNOWN	RIC
p.G326D vaf 26%	G>A	Pathogenic (score 0.97)	RIC
p.S333N	NC	UNKNOWN	RIC
p.D338N vaf 12%	NC	UNKNOWN	RIC
p.A340T vaf 26% NC	NC	UNKNOWN	RIC
p.A585V vaf 13% NC	NC	UNKNOWN	RIC
p.T602I vaf 13%	C>T	Pathogenic (score 0.85)	RIC
p.S1695N vaf 7% NC	NC	UNKNOWN	RIC
p.H316H vaf 84% NC	NC	UNKNOWN	RIC
p.G597D vaf 18% NC	NC	UNKNOWN	RIC
p.C339Y vaf 5% NC	NC	UNKNOWN	RIC
p.S341N vaf 5% NC	NC	UNKNOWN	RIC
p.V324V	NC	UNKNOWN	RIC
p.S1589S vaf 5% NC	NC	UNKNOWN	RIC
p.G310G vaf:10% NC	NC	UNKNOWN	RIC
.G212D(GAC) vaf 6%	G>T	Pathogenic (score 0.90)	RIC
p.C339Y vaf 6% NC	NC	UNKNOWN	RIC
p.A340T vaf 6% NC	NC	UNKNOWN	RIC
p.T211 vaf 5% NC	NC	UNKNOWN	RIC
p.D337N VAF 5%	A>T	Pathogenic (score 0.99)	RIC
p.G212D vaf 6%	NC	UNKNOWN	RIC
p.P213L vaf 7%	C>T	UNKNOWN	RIC
p.G326S vaf 6%	G>A	UNKNOWN	RIC
p.S341N vaf 17%	NC	UNKNOWN	RIC
p.G1704E VAF5%	n.c.	UNKNOWN	RIC
p.D1681N VAF23%	n.c.	UNKNOWN	RIC
p.R1594W VAF6%	n.c.	UNKNOWN	RIC
p.V1599M(VAF10%)	n.c.	UNKNOWN	RIC
p.C1685Y(VAF6%)	G>A	Pathogenic (score 0.94)	RIC
p.T1602* VAF15%	n.c.	UNKNOWN	RIC
p.G1704E VAF11%	n.c.	UNKNOWN	RIC
p.L1712F VAF7%	n.c.	UNKNOWN	RIC
p.T1697I vaf 8%	n.c.	UNKNOWN	RIC
p.F1593F vaf 5%	n.c.	UNKNOWN	RIC
p.L1601L vaf 5%	n.c.	UNKNOWN	RIC
p.A1705A VAF 49%	G>A	NEUTRAL	NON RIC
p.T596T VAF 96%	n.c.	UNKNOWN	NON RIC
p.A208A VAF 88%	n.c.	UNKNOWN	NON RIC
p.V1686V 14%	NC	UNKNOWN	NON RIC
p.S1711N 14%	NC	UNKNOWN	NON RIC
p.G1710S vaf 5%	NC	UNKNOWN	NON RIC
p.C215C vaf 6%	NC	UNKNOWN	NON RIC
p.S1708L vaf 6%	NC	UNKNOWN	NON RIC

PIK3CA			
p.N526S vaf 21%	A>G	UNKNOWN	RIC
p.G1050S VAF7%	G>A	Pathogenic (score 0.97)	RIC
p.E547L VAF7%	nc	UNKNOWN	RIC
p.S541F VAF8%	c>T	Pathogenic (score 0.95)	RIC
p.T544I VAF 11%	c>T	Pathogenic (score 0.97)	NON RIC
p.R537* VAF 17%	C>T	Pathogenic (score 0.87)	NON RIC

CDKN2A			
p.A86V VAF 70%	C>T	Pathogenic (score 0.99)	RIC
p.P61S 14%	NC	UNKNOWN	RIC
p.P40S	NC	UNKNOWN	RIC
p.L78F	NC	UNKNOWN	RIC
p.L47R vaf 7%	NC	UNKNOWN	RIC
p.R51K vaf 21%	NC	UNKNOWN	RIC
p.R62K vaf 18%	NC	UNKNOWN	RIC
p.P75P vaf.5%	C>T	NEUTRAL	RIC
p.E33G vaf:20%	NC	UNKNOWN	RIC
p.P81P vaf 7%	C>G	NEUTRAL	RIC
p.R54C vaf 6%	NC	UNKNOWN	RIC
p.R29Q VAF:11%	G>A	NEUTRAL	RIC
p.A42T VAF: 7%	n.c.	UNKNOWN	RIC
p.A44Q VAF: 10%	n.c.	UNKNOWN	RIC
PTEN			
p.H196H VAF 14%	NC	UNKNOWN	RIC
p.P204S VAF7%	C>T	Pathogenic (score 0.99)	RIC
p.R233Q VAF 9% NC	G>A	Pathogenic (score 0.99)	RIC
p.N184L vaf 8%	NC	UNKNOWN	RIC
p.H196M vaf 8%	NC	UNKNOWN	RIC
p.V222V vaf 11% nc	NC	UNKNOWN	RIC
p.K223K vaf 6% nc	NC	UNKNOWN	RIC
p.P244L vaf 5%	NC	UNKNOWN	RIC
p.V249HV vaf8% nc	NC	UNKNOWN	RIC
p.Q171* vaf 13%	C>T	Pathogenic (score 0.99)	RIC
p.H196M vaf 7%	NC	UNKNOWN	RIC
p.S229L vaf 7%	NC	UNKNOWN	RIC
p.V249V vaf 5%	NC	UNKNOWN	RIC
p.G251D vaf 7%	G>A	Pathogenic (score 0.99)	RIC
p.C250Y VAF:9%	G>A	Pathogenic (score 0.99)	RIC
p.L318F VAF: 6%	C>T	Pathogenic (score 0.99)	RIC
p.V191M VAF: 28%	G>A	Pathogenic (score 0.97)	RIC
p.E242K VAF:6%	G>A	Pathogenic (score 0.99)	RIC
p.R233Q VAF 42%	G>A	Pathogenic (score 0.97)	NON RIC
p.P169L VAF 29%	C>T	Pathogenic (score 0.95)	NON RIC
p.L185L VAF 17%	n.c.	UNKNOWN	NON RIC
p.S179S VAF 51%	n.c.	UNKNOWN	NON RIC
p.S229S VAF 32%	n.c.	UNKNOWN	NON RIC

SUMMARY:

ITH in Head and Neck Squamous Cell carcinoma (HNSCC) was firstly described by Slaugther et al. in 1953 when he proposed the theory of field cancerization in oral mucosa Slaughter et al. reported that within each OSCC tumour mass areas with different morphological features can be observed.

This brilliant observation changed our way of studying Squamous cell carcinoma of the Head and Neck . The strict follow up applied to patients after surgery and all the strategies adopted to find useful biomarkers for disease progression are directly linked to Skaughter's intuition.

Neither molecular biology nor NGS technology have denied the theory of field cancerition. On the contrary, DNA sequencing have confirmed that Slaughter's intuitions were correct and have paved the way to new landscapes of research.

Studies in this PhD project have focused on Slaughter's field cancerization theory and have investigated OSCC and related mucosal field with sophisticated biomolecular techniques.

PROJECT 1: Ki67 labelling index was investigated in the index tumour and in the contralateral cheek, opposite to tumour resection. Finding a high Ki67 labeling index distant mucosa of patients who experienced a poor loco regional control raised two important concusions. Firstrly, altered field may progress extensively beyond the surgical marginal. Secondly, the ability of detecting the field is pivotal in order to compensate what naked eye cannot see. Indeed molecular study of the field may help the prognosis of the patients. At least, for early diagnosis of tumour recurrences.

PROJECT2: Consequently, Tumour recurrences were the object of further investigation. A genetic approach to Slaughter's theory of field cancerization made clear that the knowledge of the biological nature of tumour recurrences is essential. In particular, significant differences may exist between a local recurrence generated by incomplete surgical resection and a second field tumour, a new neoplasia raised from the accumulation of new mutations in a mutated field. mtDNA analysis was used to investigate the phylogenetic relationship between adverse events. More deeply, the genetic distance between the tumours (index tumour and recurrences) and two areas of the field was studied to distinguish between Second Primary Tumour, Second Field Tumour and local Recurrence. According to prognostic outcome of the studied population it could be noted that Second Field Tumour had the most favourable prognosis with respect to Second Primary Tumour and Local Recurrences. In addition, discrepancy between clinical classification of second events and results from mtDNA analysis was recorded. Results demonstrated that a

biomolecular classification of tumour is important to cope with the biological complexity of field cancerization.

PROJECT 3 and 4: Nevertheless analysis were carried out using only a single tumour sample. As reported in the present thesis, from recent genetic studies it emerged that in many cancers, differences among tumour cells may be revealed if different areas of the tumour are studied. Genetic divergences are the effect of different evolutionary lines adopted by tumour subclones during tumour evolution. Therefore multiple regions from both the index tumour, the adjacent mucosa and second events were studied.

It emerged that Intratumour Heterogeneity (genetic differences among tumour cells) can be observed also for Oral squamous Cell Carcinoma. The analysis of mtDNA also permitted to track subclones that persisted from adjacent mucosa to metastasis and/or second events. Noteworthy, it was reported for the first time that genetic heterogeneity can be observed also in adjacent non neoplastic mucosa. The term Field Heterogeneity could be therefore proposed.

Consequently, the prognostic impact of genetic heterogeneity was investigated, with particular interest for local recurrences. It emerged that low grade of heterogeneity in adjacent mucosa was associated with lower risk of developing a second local neoplasia.

The results obtained during this PhD have confirmed the theory of Slaughter for field cancerization effect. As clinical phenomenon it can't be ignored for further diagnostic and therapeutic effect. As far as the use of Ki67 is concerned the small number of the studied population does not permit to support the use of Ki67 for routine practice but the low cost and easy application include it among the promising biomarkers to be validated at larger levels. Also the mtDNA analysis should benefit from larger studies. In fact it permits to track

the phylogenesis of different subsequent events. However, the bias of genetic heterogeneity should not be underestimated. In fact, a single tumour sample risks of being of limited use.

Despite the prognostic effect of the study of Field Heterogeneity seems intriguing the results should be taken carefully due to the limited studied population.

Nevertheless,we believe in the future, new perspectives may emerge if the result exposed in the present report will be integrated with information from epigenetics and/or HPV+ status.. For this reasons further studies are encouraged and we hope that our results,despite the limits, will help to unveil the complexity of OSCC biology suggested by Slaughter in 1953.

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