UNIVERSITY OF BELGRADE

FACULTY OF BIOLOGY

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HER2 AND *c-MYC* MUTATIONAL STATUS AND *INK4a/ARF* METHYLATION STATUS IN TUMORS, TUMOR MARGINS AND UNAFFECTED ORAL MUCOSA OF PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA

Doctoral Dissertation

Belgrade, 2018.

UNIVERZITET U BEOGRADU

BIOLOŠKI FAKULTET

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MUTACIONI STATUS *HER2* I *c-MYC* GENA I METILACIONI STATUS *INK4a/ARF* LOKUSA U TUMORU, TUMORSKOJ MARGINI I NEIZMENJENOJ ORALNOJ SLUZOKOŽI PACIJENATA OBOLELIH OD SKVAMOCELULARNOG KARCINOMA USNE DUPLJE

Doktorska disertacija

Beograd, 2018.

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Normal cells bear the seeds of their own destruction in the form of cancer genes. The activities of these genes may represent the final common pathway by which many carcinogens act. Cancer genes may not be unwanted guests but essential constituents of the cell's genetic apparatus, betraying the cell only when their structure or control is disrupted by carcinogens.

Bishop, 1982 (Nobel Prize Winner, 1989)

HER2 AND *c-MYC* MUTATIONAL STATUS AND *INK4a/ARF* METHYLATION STATUS IN TUMORS, TUMOR MARGINS AND UNAFFECTED ORAL MUCOSA OF PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMA

SUMMARY

Introduction: The development of malignant diseases is due to the accumulation of genetic and epigenetic changes. Oral squamous cell carcinoma (OSCC) is an aggressive and very common malignancy of the oral cavity. Patho-histological methods lack sensitivity in terms of the evaluation of the risk of OSCC recurrence and metastases. This issue can potentially be overcome by assessing molecular changes in OSCC and its margins.

Aims: (a) to determine the presence of oncogene amplification (*c-MYC* and *HER2*) and tumor suppressor gene methylation (*P14* and *P16*) in tumor, tumor margin and healthy oral mucosa of patients with OSCC; (b) establish a potential association between molecular and clinical parameters.

Material and methods: DNA was isolated from tumor, margin and oral mucosa tissue of 40 patients with OSCC, operated at the Clinic for Maxillofacial Surgery, School of Dental Medicine. The presence of *C-MYC* and *HER2* gene amplification was determined by real-time PCR, and *P14* and *P16* methylation by methyl-specific PCR. Statistical analysis with SPSS was applied to estimate the association between molecular and clinical findings.

Results: Tumor tissues showed the highest prevalence of alterations and oral mucosa the lowest. Multiple alterations were significantly more frequent in tumors and tumor margins compared to unaffected oral mucosa ($P<0.001$ and $P=0.027$, respectively). *HER2* amplification in margin tissue ($P < 0.001$) and swabs ($P = 0.013$), as well as the existence of three co-alterations in margins and unaffected oral mucosa were correlated with shorter survival ($P = 0.035$ and $P=0.027$, respectively).

Conclusion: *HER2* amplification, as well as the presence of three co-alterations in margins and unaffected oral mucosa proved to be markers of poor outcome in OSCC.

Keywords: amplification, methylation, oncogenes, *HER2*, *c-MYC*, tumor suppressor genes, tumors, tumor margins, healthy mucosa, oral cancer

Scientific field: Biology

Narrower scientific field: Genetics

UDC: 616.31-006.6:577.2(043.3)

MUTACIONI STATUS *HER2* I *c-MYC* GENA I METILACIONI STATUS *INK4a/ARF* LOKUSA U TUMORU, TUMORSKOJ MARGINI I NEIZMENJENOJ ORALNOJ SLUZOKOŽI PACIJENATA OBOLELIH OD SKVAMOCELULARNOG KARCINOMA USNE DUPLJE

SAŽETAK

Uvod: Razviće malignih oboljenja uslovljeno je akumulacijom genetičkih i epigenetičkih promena. Oralni skvamocelularni karcinom (OSCK) je najčešći malignitet usne duplje. Patohistološkim metodama evaluacije rizika od pojave recidiva i metastaza nedostaje senzitivnost, a taj problem može potencijalno da bude prevaziđen analizom molekularnih promena u OSCK-u i njegovim marginama.

Ciljevi: (a) utvrditi prisustvo amplifikacije onkogena (*c-MYC* i *HER2*) i metilacije tumor supresorskih gena (*P14* i *P16*) u tumoru, margini i zdravoj oralnoj sluzokoži pacijenata sa OSCK; (b) ustanoviti postojanje asocijacije između molekularnih i kliničkih parametara.

Materijal i metode. DNK je izolovana iz tkiva tumora, margina i oralne sluzokože 40 pacijenata sa OSCK-om, operisanih na Klinici za maksilofacijalnu hirurgiju Stomatološkog fakulteta. Prisustvo amplifikacije *C-MYC* i *HER2* gena određeno je metodom PCR u realnom vremenu, a *P14* i *P16* metilacije metodom metil-specifičnog PCR-a. Statistička analiza SPSS paketom je primenjena za procenu asocijacije između molekularnih i kliničkih nalaza.

Rezultati: Najveća učestalost promena pokazana je u tumorskom tkivu, a najmanja u zdravoj oralnoj sluzokoži. Višestruke promene (ko-alteracije) su bile znatno češće u tumorima i tumorskim marginama nego u sluzokoži ($P < 0.001$ odnosno $P = 0.027$). Amplifikacija *HER2* gena u tkivu margina ($P < 0.001$) i sluzokoži ($P = 0.013$), kao i postojanje tri ko-alteracije u marginama i neizmenjenoj oralnoj sluzokoži korelisane su sa kraćim preživljavanjem ($P = 0.035$ odnsono $P = 0.027$).

Zaključak: *HER2* amplifikacija, kao i prisustvo tri ko-alteracije u marginama i zdravoj oralnoj sluzokoži pokazale su se kao prediktori lošeg ishoda u OSCK-u.

Ključne reči: amplifikacija, metilacija, onkogeni, *HER2*, *c-MYC*, tumor supresorski geni, tumori, tumorske margine, zdrava sluzokoža, kancer usne duplje

Naučna oblast: Biologija

Uža naučna oblast: Genetika

UDK: 616.31-006.6:577.2(043.3)

CONTENTS

1. Introduction

1.1. Cancer

Cancer is one of the leading causes of morbidity and mortality worldwide. The number of new cases is rising every year and is expected to increase over 70% in the next two decades [1] Cancer is a group of related diseases characterized by abnormal and uncontrolled cell cycle as well as inactivation of apoptotic mechanisms, and the acquisition of metastatic properties i.e. the capability of spreading to the surrounding tissues and to other organs. This abnormal and uncontrolled cell cycle mostly results from the activation of protooncogenes which promote cell growth and proliferation, and the inactivation of tumor suppressor genes which inhibit cell divisions and control its survival [1-4]. Tumor expansion, invasion of tumor cells into neighboring tissues and spreading of tumor cells to other locations can lead to rapid failure of one or multiple organs [5].

Normally, the human cells divide and grow to form new cells as the body needs them; when the cells become damaged or grow old they die and new cells take their place. The cell cycle progression and differentiation is a tightly controlled process with complex mechanisms. If this orderly process is disturbed, the cells will break free from the homeostatic balance between cell proliferation and cell death which lead to cancer development [2, 3,6].

Most adult cells survive on average for 4-6 weeks and then have to be replaced, either by replication of existing cells or from stem cell precursors. Since every cell gets a substantial amount of daily DNA damage and 10^{11} or more of them will replicate each day it means a lot of potential cancer cells. However, cancer is surprisingly rare and this can be explained by the existence of some extraordinarily effective barriers to cancer cell development. Cancer may well originate in a single transformed cell, but is clinically detectable by direct observation or conventional investigations, only when replication has increased the number of cancer cells to around one billion. In other words, by the time a cancer is discovered the original cancer cell has undergone some thirty or more cell divisions and acquired a lot of new molecular aberrations [7]. Many of these molecular alterations which lead to abnormal biological behavior of cancer cells are related to cell cycle regulation systems [8].

Cells need to survive in a tissue environment where they are inter-dependent on each other for functioning and loss of contact with neighboring cells will lead to cell death. Cell proliferation is stimulated by external signals and cell division will end by withdrawing of the stimulating signals and/or by stopping signal. Cell contact is a common stopping signal for terminating cell proliferation (cell-contact inhibition). A tumor cell is independent of other cells for proliferation and even for survival; it can divide without stimulation and this division cannot be stopped by cell-contact. Tumor cells in a malignant tumor have additional properties including the production of matrix metalloproteinase (MMP), acquired mobility, and anoikis-resistance. With the ability to produce MMPs to digest extracellular matrixes (ECMs), the tumor can invade into neighboring tissues. With the anoikis-resistance property, a tumor cell can survive without anchoring to neighbor cells/ECMs, and it can immigrate passively to other organs via blood or actively if it has additionally obtained motility (these differences are illustrated in Table 1). We can summarize the differences between normal cells and malignant cells as follows [5]:

- Normal cells have full differentiation with full efficiency of DNA repair and other functions; whereas tumor cells have a low degree of cell differentiation with functional deficiency of cell repair mechanisms.
- A normal stem cell proliferation is strictly controlled, whereas that of tumor cells is out of control and unlimited.
- The tolerance of a normal cell to a dominant DNA mutation is low since an abnormal phenotype will lead to cell death or apoptosis. Differently, a malignant tumor cell can be tolerant to some DNA mutations because of immaturity on functionality. The surviving chance of a tumor cell from a DNA injury though misrepair of DNA is higher than that of a normal cell.

Table 1: Differences between normal and malignant tumor cells.

Property	Normal cells	Malignant tumor cells
Cell differentiation	High	Low
DNA repair	Full	Defficient
Cell proliferation	Well-controlled	Unlimited
Tolerance to DNA mutations	Low	High

The normal cells are specialized and mature into distinct cell types with a specific function, while cancer cells are less specialized, have no specific function and continue to divide ignoring both cell division stop signals and apoptosis signals [2]. The transformation of a normal cell into a tumor cell is a multistage process called carcinogenesis, which is characterized by accumulation of genetic and epigenetic alterations in cancer-related genes [7, 9, 10].

The cancer evolution is guided by the accumulation of these alterations within a clonal population of cells. The genotypic changes can affect hundreds of genes leading to phenotypic changes in crucial cellular functions, such as reduction of or resistance to cell death, increased proliferation, induction of angiogenesis, and the ability to invade and metastasize. There are many mechanisms which underlie these changes that include but are not limited to, genomic instability through chromosomal rearrangements, gene amplifications, deletions, methylations and point mutations [11].

In other words, genetic and epigenetic alterations allow a normal cell to achieve the "hallmark" features of cancer summarized as [7]:

- Capacity to proliferate despite the lack of exogenous mitogens.
- Refractoriness to growth-inhibitory signals.
- Resistance to apoptosis.
- Unrestricted proliferative potential (immortality).
- Capacity to recruit a vasculature (angiogenesis).
- Ability to invade surrounding tissue and eventually metastasize.

The normal cells go through a series of changes to form cancer cells. At first, there is only an increase in the number of cells in the organ or tissue (hyperplasia); the cells look normal under the microscope and there is no change in the tissue organization. Then in the second stage, the cells that increased in number become abnormal under the microscope (dysplasia); they are still not cancer cells but the tissue organization is changed. The cells of these two stages may or may not become cancer cells. At the last stage, the cells become completely transformed into cancer cells and tissue appears abnormal. These sequential histopathological alterations (Figure 1) are determined by the accumulation of a series of genetic events. In cellular carcinogenesis, various genes interact with each other, leading to multiple alterations that occur in a rather complex way and in different phases of disease progression [2, 12].

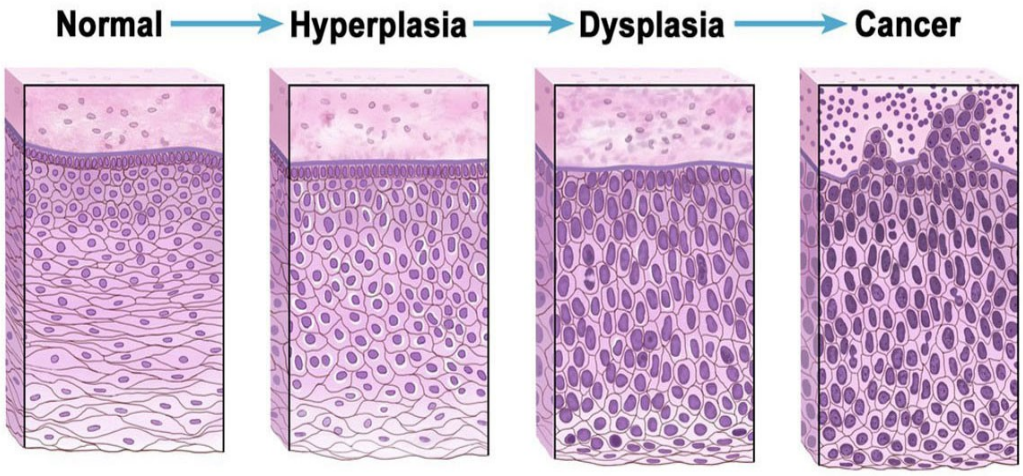


Figure 1: The changes that normal cell/tissue goes through to become a tumor (sourse: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/hyperplasia).

The interactions between various factors which lead to cell transformation are given in Figure 2.

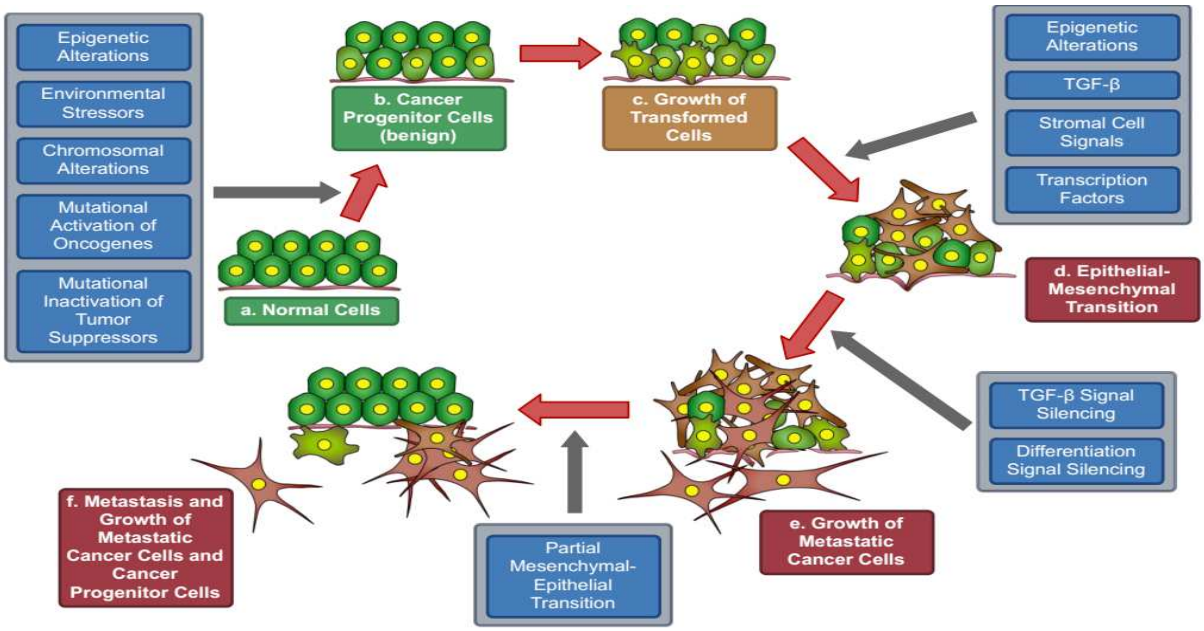


Figure 2: The transformation of normal cells into cancer progenitor cells and progression of metastatic cancer. a: hexagons with yellow dots represent normal cells; b: faded green, distorted hexagons with yellow dots represent cancer progenitor cells; c: progenitor cells are increasing in number; d: star-like brown cells represent the metastatic form of cancer cells, a mixed population

of progenitor and adult cells; e: overgrowth of metastatic cells; f: both metastatic and adult progenitor cells leave site. Progression: Cancer progenitor cells develop from normal cells (a to b); After growth (b to c), they undergo epithelial to mesenchymal transformation-EMT (c to d); Differentiation signals decrease and growth signals increase, producing a combination of progenitor and adult metastatic cancer cells (d to e); After the outgrowth of metastatic cells, translocation to a distant location occurs (e to f) (source: Sarkar S, Horn G, Moulton K, Oza A, Byler S, Kokolus S, Longacre M. Cancer development, progression, and therapy: an epigenetic overview. International journal of molecular sciences. 2013 Oct 21;14(10):21087-113.)

1.2.Risk factors

There is a number of intrinsic (biological) and external factors that influence the development of cancers. The intrinsic factors include the age and hormonal status of the individual, familial history, and genetic predisposition. The external factors include diet and lifestyle, individual's habits like smoking and alcohol use, exposure to toxic chemicals and radiation, some infections, etc. The main risk factors are [1, 2, 12-14]:

- Tobacco use (smoking and chewing).
- Alcohol consumption.
- Environmental and occupational risks.
- Certain infections as the infections with hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) and human papillomavirus (HPV).
- Genetic background

1.3.Carcinogenesis

Carcinogenesis is a multistep process in which many different molecular events lead to the disruption of normal regulatory pathways that control basic cellular functions including cell division, differentiation, and cell death; these molecular events include genetic and epigenetic changes [7, 9, 12-15]. The three major steps in carcinogenesis are:

- **Initiation:** Neoplasia initiation is essentially an irreversible change in an appropriate target somatic cell; it involves one or more stable cellular changes arising spontaneously or induced by exposure to a carcinogen. This is considered to be the first step in carcinogenesis, where the cellular genome undergoes mutations, creating the potential for neoplastic development, which predisposes the affected cell and its progeny to subsequent neoplastic transformation. The initiating mutation creates the stable potential for pre-neoplastic cellular development in cells with proliferative capacity. The transformed cell undergoes continuous division with fidelity to the transformed karyotype and, possibly, with further mutations, before a malignant lesion is manifested.
- **Promotion:** The initiated cell remains harmless unless it is stimulated to undergo and expand by self–proliferation, upsetting the cellular balance, leading to abnormal growth, and further mutations. The subsequent changes of an initiated cell leading to neoplastic transformation may involve more than one step and require repeated and prolonged exposures to promoting stimuli. Expression of the initial mutation will depend on interaction with other oncogenic mutations and also on factors that may temporarily change the patterns of specific gene expression, such as cytokines, lipid metabolites, and certain phorbol esters. This may result in an enhancement of cellular growth potential and/or an uncoupling of the intercellular communication processes.
- **Progression:** in which successive changes in the neoplasm give rise to increasingly malignant sub-populations; the cells also detach from the primary tumor site and invade other tissues and organs forming metastatic growths. The process may be accelerated by repeated exposures to carcinogenic stimuli or by selection pressures favoring the autonomous clonal derivatives.

Multistep process of carcinogenesis from the genetic perspective is shown in Figure 3.

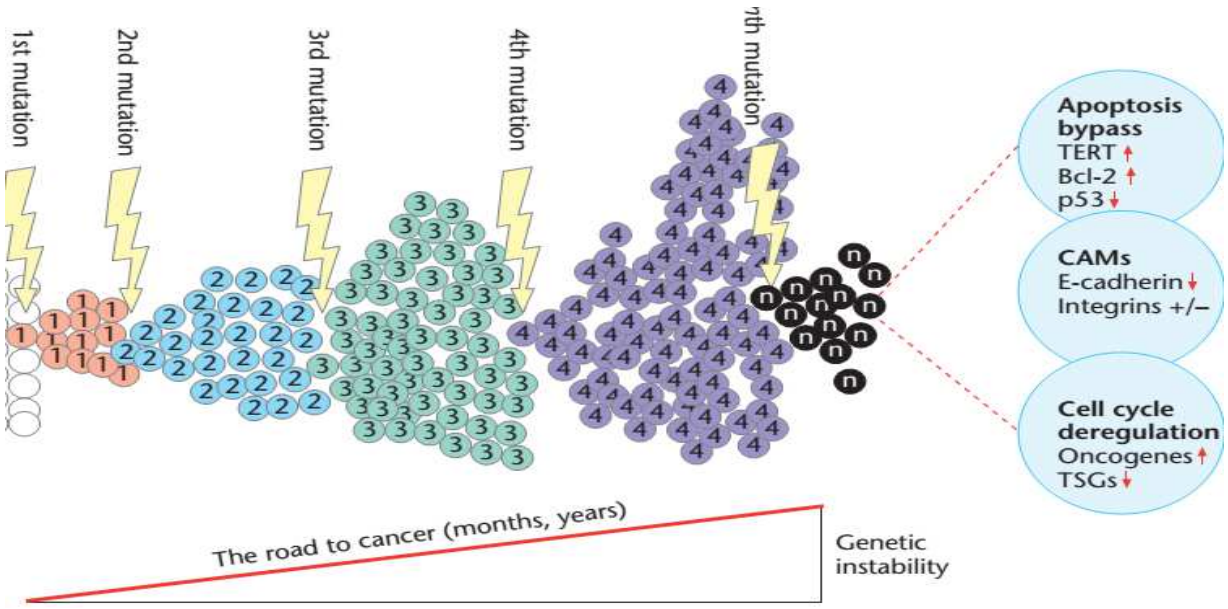


Figure 3: The development of a malignant tumor begins with a mutation in a long-lived cell, probably a stem cell. That mutation gives the cell a growth advantage lead to successive rounds of mutation and clonal expansion until the malignant genotype is acquired. In many cases, one of the first mutations is likely to be in a 'caretaker' gene that maintains genome integrity. The malignant phenotype is likely to be a manifestation of disturbances in the control of cell proliferation, cell death and cell adhesion. CAM=cell adhesion molecule; TERT= telomerase reverse transcriptase. (source: Weinberg R. A. "The Biology of Cancer" 2nd ed. New York, Garland Science, Taylor & Francis group (2014): 34-45, 58-68, 231.)

The causal factors of carcinogenesis, both external and internal, may act together or in sequence, can initiate and promote this multi-step process [15, 16].

1.4.Cancer classification

Tumors are classified into [14, 15, 17, 18]:

Benign tumors: slow-growing expansive masses that compress rather than invade surrounding tissue; they generally do not pose threat, except when growing in a limited space like the skull. They usually can be readily surgically excised.

Malignant tumors or cancers: usually rapidly growing, invading surrounding tissue and, most significantly, colonizing distant organs.

Cancers are classified by their histologic or tissue type into six major categories [17, 18]:

- **Carcinomas:** the most common type, originating from different epithelial cells. Since the epithelium covers the skin, lines the respiratory and alimentary tracts and metabolizes absorbed carcinogens, i.e. because of its exposure to carcinogens, about 90% of cancers occur in epithelia. Adenocarcinoma and squamous cell carcinoma are the main types and characterized by rapid spreading.
- **Sarcomas:** originate in connective and supportive tissues (soft tissues) including muscles, bones, cartilage, and fat.
- **Leukemia:** blood cancers that affect the bone marrow and lead to the production of excessive immature white blood cells.
- **Lymphoma:** cancer of lymphatic system which may affect lymph nodes at specific sites like stomach, brain, etc.
- **Myeloma:** this type of cancer originates in the bone marrow affecting plasma cells.
- **Mixed types:** which have two or more cancer components like the mixed mesodermal tumor, carcinosarcoma, adenosquamous carcinoma, and teratocarcinoma.

Classification by grade: Cancers may be classified also according to grade. The abnormality of the cells with respect to surrounding normal tissues determines the grade of cancer. Increasing abnormality increases the grade, from 1–4. Cells that are well differentiated closely similar to normal specialized cells belong to low-grade tumors. Cells that are undifferentiated are highly abnormal regarding to surrounding tissues are high-grade tumors [18]:

- Grade 1 – well-differentiated cells with slight abnormality.
- Grade 2 – cells are moderately differentiated and slightly more abnormal.
- Grade 3 – cells are poorly differentiated and very abnormal.
- Grade 4 – cells are immature and primitive and undifferentiated.

Classification by stage: Cancers are also classified individually according to their stage. There are several types of staging methods. The most commonly used method uses classification in

terms of tumor size (T), the degree of regional spread or node involvement (N), and distant metastasis (M). This is called the TNM staging system (more details in staging of OSCC) [18].

1.5.Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors; it holds the eighth position in the cancer incidence ranking worldwide, though with considerable epidemiologic variation between different geographic regions due to cultural and behavioral differences (diet, exposure to specific risk factors, etc.) [19-23].

OSCC accounts for more than 90% of all cancers of the oral cavity and causes significant mortality and morbidity worldwide. It arises from the squamous epithelium of oral mucosa [20, 24, 25]. It occurs in equal to slightly higher ratio in males versus females (vary between 1:1 to 1.5:1) and in older people (between 40-65 years), although it has been reported in people younger than 40 and older than 65 years of age [19, 20, 24].

OSCC is a heterogeneous and a multifactorial, but largely preventable disease, with complex molecular abnormalities involving different oncogenes, tumor suppressor genes, and their respective pathways [9, 26-29].

As in other cancers, there are several etiological risk factors of OSCC, but smoking and alcohol consumption remain the main factors. Smoking harmful effect on oral tissues is due to the fact that tobacco smoke contains ~300 carcinogenic compounds that are converted into reactive metabolites. Reactive metabolites are capable of interacting with DNA by the action of oxidative enzymes and the tumor-inducing action of tobacco resulting from the genotoxic effects of carcinogens (nitrosamine, polycyclic hydrocarbons) in tobacco smoke. In addition to the action of carcinogenic substances, continuous exposure to the heat resulting from the tobacco combustion further aggravates the aggression to the oral mucosa. The mechanisms by which alcohol induces carcinogenesis is still unknown, but might be via the genotoxic effect of ethanol metabolite acetaldehyde, the production of reactive oxygen- and nitrogen species, changes in folate metabolism, generation of DNA adducts and inhibition of DNA repair. Also, alcohol could exert its damaging effect directly, either acting as a solvent of carcinogens from tobacco smoke or damaging the oral mucosa, which would enhance the penetration of carcinogens from tobacco

smoke. Acetaldehyde can form adducts with DNA, interfering with DNA synthesis and repair [25, 26, 30-33].The etiological factors of oral cancer are summarized in Table 2 [25]. These predisposing factors may influence the development of cancer, and act individually, in combination with another carcinogen (co-carcinogen), or in combination with other non-carcinogens agents (promoters), that help the carcinogens to mutate or depress cells [24].

Table 2: Etiologic factors of oral cancer.

Factor	Example
Tobacco smoking	Pipes, cigars, cigarettes, bidis, reverse smoking
Smokeless tobacco	snuff dipping, tobacco sachets, tobacco chewing
Chewing habit	Betel chewing, betel quid (pan), areca nut
Alcohol	spirits, wines and beers, alcohol and tobacco synergism
Diet and nutrition	Iron deficiency, vitamin A, E and C deficiencies, nutritional deficiencies and alcoholism
Dental factors	Poor oral hygiene, faulty restorations, sharp edges of teeth, ill-fitting dentures.
Viruses	Herpes simplex virus (HSV), human papillomavirus (HPV), human immunodeficiency virus (HIV).
Chronic infection	Candidiasis, syphilis.
Radiation	Ultraviolet light.
Other as Occupational	Textile workers.

The probability of developing OSCC increases with the period of exposure to risk factors and increasing age adds the further dimension of age-related mutagenic and epigenetic changes [34]. As mentioned, the majority of OSCC cases worldwide being linked to consumption of alcohol and tobacco, public health efforts aimed at stopping and discouraging initial use of these addictive substances will have the greatest impact in decreasing the burden of this disease. If trends that have been seen in the United States continue worldwide with public health campaigns, the makeup of OSCC will be intensely different. Namely, additional research efforts into the treatment and prevention of HPV associated oral squamous cell carcinoma will be

necessary, as it will be the prevalent OSCC risk factor [20]. Indeed, the number of oral and oropharyngeal cancers related to HPV has increased dramatically over the past few decades. Even worse, these cancers are becoming more common in younger people with no history of alcohol or tobacco use. The reason for the rising rate of HPV-linked cancers is largely unclear. Prevention is always the best treatment for oral tumors, and knowledge of etiological factors is a prerequisite for any preventive measure. The patient's lifestyle and education are extremely important for preventing this neoplasm. Early detection of the disease is also considered as the most effective prevention of morbidity and mortality [30, 34].

Early stages of the disease are associated with minimal symptoms and because of this or either due to ignorance or inaccessibility of medical care, OSCC in most cases gets detected in the later stages. Thus, there is a need for improvement in early detection of OSCC, because in the initial or early stages, treatment is more effective and the morbidity is minimal. Advanced stages respond poorly to current cancer therapies and have a high incidence of local and regional relapse [31].

Radiotherapy, chemotherapy, and surgery are the cornerstones in the treatment of head and neck cancers. Single modality or multimodality (combined) therapy is used according to the stage of disease [35]. Surgery is the preferred first-line treatment of small, accessible oral SCCs, whereas, advanced-stage oral SCC is usually treated by a combined treatment program of surgery, chemotherapy, and radiotherapy. Despite recent advances in surgical procedures and the significant progress in chemotherapy, radiotherapy, and targeted therapy in the last decades, OSCC long-term survival and prognosis remain poor [34, 36]. A significant percentage of advanced OSCC patients, with histologic documentation of adequate resection margins, still have a poor prognosis, with a high percentage of loco-regional and distant recurrences [37-39]. The prognosis following OSCC recurrence is one of the poorest among recurrent head and neck cancers [40]. The development of loco-regional recurrence at the primary site is correlated with the T-stage and the histopathologic assessment of the margins, and it is the main reason for treatment failure in OSCC. The remaining tumor cells in tumor margins are associated with recurrences. Surgical margins are considered negative based on histologic assessment of the pathological specimen. In spite of the apparently high accuracy of histologic examination, up to 47% of patients with OSCC develop local recurrence, second primary tumors, and regional or distant metastases. Local and loco-regional lymph node recurrence is the most common cause of

treatment failure and accounts for about 60% of all the treatment failure, even when the surgical resection margin is diagnosed as histologically normal mucosa and tumor free. A high risk of local recurrence and new mucosal malignancies of the upper aerodigestive tract is seen in patients treated for oral cancer. The elevated rate of treatment failure in patients with histologically negative margins raises concern about the sensitivity of this method. Obviously it lacks sensitivity to identify cells that already started malignant transformation but have not yet developed a pathologic phenotype. Therefore, the intraoperative diagnosis of histologically negative margins does not eliminate the possibility of the presence of tumor cells contributing to local relapse [38, 41, 42].

The main locations of OSCC are the tongue and the floor of the mouth (worldwide), due to the highest exposure of these areas to carcinogens, while buccal mucosa tumors exceeds the number of tongue tumors in those countries where use of smokeless tobacco is a cultural habit [26, 29].

OSCC is usually painless unless it is secondarily infected. Large lesions may interfere with normal speech, mastication or swallowing. SCC of the tongue, of the floor of the mouth and of the mandibular gingiva often metastasize to regional lymph nodes and are more aggressive with a less favourable prognosis, unlike SCCs of the lip, hard palate and maxillary gingiva which infrequently metastasize to regional lymph nodes, usually run a relatively slow course that causes slightly or no pain and have a relatively favourable prognosis. In general, SCCs of the posterior part of the oral cavity are much more probable to metastasize to regional lymph nodes than are comparable SCCs of the anterior part of the oral cavity [36].

1.5.1. OSCC staging and histological grading

Oral cancer staging is important for establishing proper treatment and determining prognosis. Tumors are staged using TNM system; it provides a reliable basis for patient prognosis and therapeutic planning. T represents the size of the primary tumor; the tumor size (thickness) could influence the prognosis of early oral cancer. In general, median tumor thickness varies between 1.5 and 8 mm for T1 and T2 cancers. Tumor size greater than 4 mm imparts a worse prognosis. Tumor thickness might be the only independent predictor of neck surgery failure. N indicates the status of the regional lymph nodes, and M indicates the presence or absence of distant metastasis. The presence of nodal metastasis is the most important prognostic factor for oral cancers. An approximately 50% reduction in 5-year survival rate is seen with the development of lymph node metastasis in patients with squamous cell carcinoma of the oral cavity. M indicates the presence or absence of distant metastases. Survival of oral and oropharyngeal cancer patients is strongly related to the stage of the disease at diagnosis. The five-year relative survival rate for patients with localized disease is about 80%, the survival rate drops to about 45% for patients with regional spread and to about 20% for those with distant metastasis. The TNM staging system is outlined in Table 3 [17, 44-46].

Table 3. The different TNM system stages of cancer

TNM Staging of Oral Cancer	
Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor 2 cm or less in greatest dimension
T2	Tumor more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumor more than 4 cm in greatest dimension
T4	Tumor invades adjacent structures (e.g., through cortical bone, into maxillary sinus, skin, pterygoid muscle, deep muscle of tongue)
Nodal Involvement (N)	
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
N2	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension.
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension.
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension.
N3	Metastasis in a lymph node more than 6 cm in greatest dimension.
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed.
M0	No distant metastasis.
M1	Distant metastasis.
Stage Grouping	
Stage 0	Tis N0 M0
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0 M0; T1 or T2 or T3 N1 M0
Stage IV	Any T4 lesion, or Any N2 or N3 lesions, or Any M1 lesion

The TNM staging system is not sufficient for optimal prognostic evaluation of OSCC and that is why it must be used with other reliable methods, such as histologic grading. There is consistent evidence of the value of tumor grade in determining prognosis where higher grades equate to a poorer prognosis [45, 47]. The histologic grading follows the descriptions in the World Health Organization classification and is based on the degree of resemblance of the invading carcinoma to the normal epithelium and its ability to form keratinizing islands [48].

The basic biology of initiation and progression of OSCC is still obscure. Screening for potentially malignant changes is typically confounded by difficulty in differentiating between reactive/inflammatory lesions vs. those lesions that are premalignant in nature. Moreover, the histologic diagnosis of dysplasia can be subjective and is thus prone to a considerable range of interpretations. Similarly, no definitive, validated criteria exist for predicting which dysplastic lesions are most likely to develop cancer over time. At present, dysplasia can only be used to indicate that an oral lesion may have an increased risk of malignant transformation. Molecular biomarkers capable of identifying the subset of lesions likely to progress to cancer are required to eliminate clinical diagnostic dilemmas [49]. The study of the carcinogenic process, including continued analysis of new genetic alterations, along with their sequential appearance during initiation, promotion, and progression, will confer great opportunity to identify diagnostic and prognostic markers, which will eventually provide a basis for the application of more rational and efficient treatments [31].

1.6. Molecular alterations and cancer

It is known that the development of cancer is driven by the accumulation of genetic and epigenetic changes within a clonal population of cells; these genotypic changes can affect numerous genes, leading to phenotypic changes in critical cellular functions such as resistance to cell death, increased proliferation, induction of angiogenesis, and the ability to invade and metastasize. There are three types of genes which are mainly altered, thus leading to cancer development: proto-oncogenes (oncogenes), tumor suppressor genes (anti-oncogenes) and caretaker genes such as DNA repair genes. The combination of alterations in these classes of genes produces tumors [2, 7, 49].

1.6.1. Genetic alterations

Genetic alterations may occur in germ cells, resulting in inherited cancer predisposition, or more commonly, they occur in somatic cells, giving rise to sporadic tumors. The first somatic genetic alteration in any of oncogenes or tumor suppressor genes that enables clonal expansion and may be regarded as the initiating insult is not known in the vast majority of human cancers. The tumors progress through the acquisition of further somatic alterations, allowing further rounds of clonal expansion [7]. The main and simplified thought of the genetic basis behind cancer is overexpression of oncogenes and /or the silencing of tumor suppressor genes [50].

The different mechanisms of genetic alterations include large-scale mutations where gain or loss of parts of chromosomes can be the result of an abnormal chromosomal division during cell mitosis, genomic amplification, virus-integration, or chromosome translocation. Small-scale mutations such as point mutations and deletions or insertions of one or two base-pairs may occur in any part of a DNA molecule. In a normal somatic cell large-scale mutations, may lead to cell death. On the contrary, point mutations can survive in a cell and they can accumulate in offspring cells, contributing finally to cell transformation [5]. These frequent genetic alterations resulting in inactivation of multiple tumor suppressor genes and activation of proto-oncogenes in head and neck cancer are summarized in Table 4 [32].

Table 4. Frequent genetic abnormalities in head and neck cancer.

Genetic changes	Locus / gene	Cancer type	Frequency
LOH	*9p21-22/ P16INK4a/P14ARF*	HNSCC OSCC	70–80%
	3p/ RASSF1A, FHIT, RARB2	HNSCC OSCC	30–70%
	17p13/p53	HNSCC	76%
	11q	OSCC	20-33%
	13q14/Rb	HNSCC	68%
	8p	OSCC	53-83%
Mutation	*9p21-22/ P16*	OSCC	70%
	5q21-22/APC	OSCC	50%
	17p13/p53	HNSCC	40-79%
	11p15/H-Ras	OSCC	35-55%
Amplification	*11q13/(PRAD-1/Cyclin D1/hst-1/int-2)*	HNSCC	30–50%
	7p12/EGFR	OSCC	30%

1.6.2. Epigenetic alterations

Some changes occurring during carcinogenesis are not associated with a DNA sequence modification and are designated epigenetic changes [51]. These alterations affect and regulate gene expression. The epigenetic changes include functionally relevant modifications to the genome that act together regulating the genome function by altering the chromatin local structural dynamics. The main epigenetic changes are DNA methylation, histone modifications, and nucleosome remodeling. In general, we can say that epigenetics include all changes in gene expression patterns that do not alter DNA sequence [52-54]. These epigenetic alterations and their biological consequences are illustrated in Table 5 [53].

Table 5. The most common epigenetic alterations.

Epigenetic change	Putative mechanism	Biological consequence
DNA hypomethylation	Activation of cellular oncogenes Activation of transposable element	Increased proliferation, growth advantage, genomic instability, transcriptional noise
DNA hypermethylation	De novo hypermethylation of CpG islands within gene promoters leading to silencing of tumor suppressors and cancer-associated genes	Genomic and chromosomal instability, increased proliferation, growth advantage
Loss of imprinting (LOI)	Reactivation of silent alleles, biallelic expression of imprinted genes	Expansion of precursor cell population
Relaxation of X-chromosome inactivation	Mechanisms is unknown but it appears to be age-related	Altered gene dosage, growth advantage
Histone acetylation	Gain-of-function Loss-of-function	Activation of tumor promoting genes, defects in DNA repair and checkpoints
Histone deacetylation	Silencing of tumor suppressor genes	Genomic instability, increased proliferation
Histone methylation	Loss of heritable patterns of gene expression (“cellular memory”)	Genomic instability, growth advantage
MicroRNAs (miRNAs) amplification in cancer	Function as oncogenes	Neoplastic transformation
MicroRNAs (miRNAs) deletion in cancer	Function as tumor suppressors.	Neoplastic transformation

The epigenetics describes the transmission of the heritable status of gene expression that does not involve sequence change in DNA. Basic epigenetic mechanisms are essential for the development and maintenance of normal states of differentiation and tissue-specific patterns of gene expression within different cell types [55]. The distinct epigenetic mechanisms such as DNA methylation, nucleosome remodeling, histone post-translational modifications, incorporation of histone variants, and non-coding RNA, modulate the chromatin structure at several levels and regulate it [56]. Normally, epigenetic modifications play an important role in the control of gene activity and nuclear architecture [57]. DNA methylation, histone modifications, in addition to posttranscriptional gene regulation by noncoding RNA commonly referred as microRNAs, are the fundamental processes responsible for epigenetic regulation. These mechanisms are critical components in the normal development and growth of cells [54]. Disruption of any of these three distinct and mutually reinforcing epigenetic mechanisms leads to inappropriate gene expression and may initiate genetic instability, resulting in the acquisition of genetic mutations in tumor-suppressor genes and activating genetic mutations in oncogenes [53, 54]. The epigenetic changes, such as promoter hypermethylation of tumor suppressor genes which in many cases are associated with the loss of gene expression and appear to lead to the occurrence of multiple genetic events that are necessary to drive tumor progression, have been observed in many tumor types [58].

The epigenetic changes associated with cancer including oral cancer occur more frequently than gene mutations and may persist for the whole cell life and even for many generations. The hypermethylation and consequent silencing of several tumor suppressor genes have been found to play a role in oral carcinogenesis and to contribute to various phases of neoplastic development and to chemotherapy resistance [53, 58]. Investigations have shown that the number of cancer-related genes that are inactivated by epigenetic modifications equals or even exceeds the number of genes inactivated by mutations [32].

Many key genes may be silenced by epigenetic changes during successive cell differentiation stages during development, and two epigenetic events in particular associated with transcriptional silencing in cancer cells include methylation of CpG islands in gene promoter regions and changes in chromatin conformation involving histone acetylation. More than half of tumor suppressor genes are known to be epigenetically silenced in cancers. Also, the silencing

of DNA mismatch repair genes can cause genetic instability, thus linking epigenetic and genetic factors [7].

Since the epigenetic alterations are heritable but reversible modifications (unlike the genetic which are irreversible alterations) the study of these epigenetic alterations, offers a great potential for the identification of biomarkers that can be used in the diagnosis of the first stages of cancer development and for a more accurate evaluation of the individual risk of developing the disease. In addition to their use for early diagnosis, epigenetic alterations may represent novel prognostic markers as well as potential therapeutic targets in oral cancer. The chance to counteract epigenetically-driven alterations in cancer cells opens an exciting scenario for its possible future fall-out on OSCC patients' care. Namely the use of epigenetic inhibitors in association with traditional anticancer therapeutic agents looks very promising as a tool to improve the chemosensitivity of non-responsive cancers [53, 59, 60]. Therefore, identification of epigenetic biomarkers which are associated with OSCC pathophysiology is urgently needed for early diagnosis, prediction of progression, and application of molecular-targeted therapies with the aim of improving the treatment outcomes [61].

1.7. Oncogenes

In normal, non-transformed cell, oncogenes are known as proto-oncogenes. They are important cellular genes that positively act in normal growth regulatory pathways; they perform physiological functions that are necessary for cellular homeostasis and regulate the processes of growth and proliferation by governing cells signal transduction pathways [9, 12, 62]. Oncogenes acquire the potential to cause neoplastic transformation when they are activated or overexpressed at high levels through point mutations, translocations, deletions, amplification or other genetic mechanisms [63-65]. The discovery of oncogenes has provided more knowledge about the regulation of normal cell proliferation, differentiation, and apoptosis; also it represented an important progress in our understanding of the molecular and genetic basis of cancer [66].

The encoded products of the oncogenes can be classified into six broad groups: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transducers and apoptosis regulators. The activation of oncogenes by chromosomal rearrangements, mutations

and gene amplification gives a growth advantage or increased survival to cells carrying such alterations. All of these three mechanisms cause either an alteration in the oncogene structure or an increase in or deregulation of its expression [63, 66].

The oncogene activation mechanisms are diverse and result in upregulated expression of a normal gene product, expression of a mutant protein with enhanced stability, or altered functionality, altered recruitment, altered subcellular localization of a normal gene product through interaction with an aberrantly expressed or mutant binding partner, etc. The oncogenes show diversity in their pro-neoplastic effects and in the regulatory fail-safes that protect against transformation. Some oncogenes possess a near-complete repertoire of pro-neoplastic properties, requiring less assistance from cooperative mutations. Other oncogenes have a narrow spectrum of biological activity or simultaneously trigger cell proliferation and cell death/senescence and therefore cannot transform a cell without cooperating lesions that decrease or inhibit the intrinsic tumor suppressor mechanisms [62]. Because neoplasia is a multistep process, more than one of these mechanisms usually contributes to the genesis of human cancers by altering a number of cancer-associated genes. In the case of amplification, the gene can be amplified as much as 100-fold, resulting in an excess of normal protein. A similar situation occurs following chromosome rearrangements such as translocations when the transcription of the gene is now regulated by novel regulatory sequences belonging to another gene. In addition, various types of mutations, such as base substitutions, deletions, and insertions are capable of activating proto-oncogenes, base substitutions being the most characteristic oncogene mutations [66].

In summary, the genetic alterations of proto-oncogenes are gain-of-function alterations that stimulate cells to increase their number when they should not; Figure 4 depicts the main mechanisms by which proto-oncogenes are converted into oncogenes.

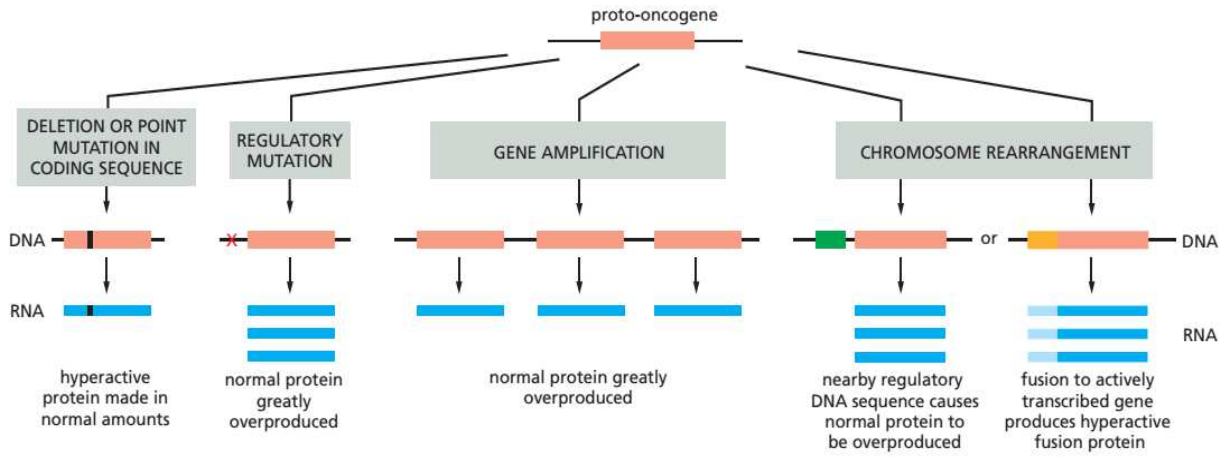


Figure 4: Different mechanisms of oncogene activation (source: Alberts B., Johnson A, Lewis J, Raff M., Roberts K. and Walter P. "Molecular Biology of the Cell" 4th edition New York: Garland Science "Cancer as Microevolutionary Process" (2002): 3398, 3400, 3405, 3423.).

1.7.1. Gene amplification

Gene amplification represents a selective genetic material increase, resulting in its overexpression and conferring a growth advantage to the cell. It is an adaptive mechanism thought to be important for aberrant oncogene expression and contributing to tumorigenesis [64, 68]. It leads to the gain of additional copies of a chromosomal region in the cell. When excessive, amplification may produce karyotype abnormalities called double minutes (DMs) homogeneously staining regions (HRs). DM is an extrachromosomal DNA structure, without centromeres and telomeres, it does not bind the mitotic spindle and replicates autonomously. DMs segregate at random and are not distributed evenly between the daughter cells. The homogeneously staining regions are intrachromosomal segments forming large genomic regions. These repetitions, cytologically visible, follow the same destiny as the rest of chromosomal regions during mitosis. The amplified genomic DNA in DMs and HSRs contains hundreds of copies of one or more genes [69-71]. Both of these abnormalities are seen more frequently in established cell lines than in primary tumors and homogeneously staining regions more frequently in advanced stages of tumors [69]. Such alterations significantly contribute to tumorigenesis and tumor progression [72-75]. In normal cells, replication and recombination are tightly regulated and are less likely to initiate gene amplification, while cancer cells lack control

mechanisms. In addition to that, cellular surveillance systems (checkpoints) that ensure genome integrity at several stages of the cell cycle are impaired in cancer cells and could fail to eliminate cells with extra copies. The growth advantage derived from gene amplification has long been recognized as an important problem for cancer patients. Increased copy numbers of proto-oncogenes, such as *Myc*, and *HER2*, leads to the overexpression of oncogene products that drive abnormal cell proliferation which results in cancer progression and poor patient survival. In addition, the gene amplification is an underlying mechanism for acquired therapy resistance [68].

The etiology of gene amplification is not yet completely understood, but it is considered to be the result of genetic instability. Several studies have shown that many oncogenes, which are amplified in a significant number of human tumors, rarely become amplified alone but present as large amplicons with multiple copies of several genes [69]. The amplified region is called 'amplicon'; it can span several megabases of DNA, and numerous genes are typically included in one amplicon. Co-amplified genes may also contribute to tumorigenesis in concert with oncogenes [71, 72].

Gene amplification is a phenomenon characteristic of many human cancers and has a key role in the mechanism by which a cancer cell activates molecules that confer a selective advantage [76-79]. Oncogenes amplification plays a critical role in several human cancers including OSCC; it is associated with and suggests an aggressive behavior and poor prognosis of the tumor, metastases, resistance to chemotherapy and a decrease in the period during which the patient stays free of the disease, it may be a useful indicator of progression and prognosis in various human cancers [64, 69, 80].The existence of specific regions of the genome that are hotspots for amplification in cancers originating from the same cell suggests that they contain genes relevant for tumor formation and progression [81, 82].

Oncogenes such as *Myc* and *Her-2* have been identified as amplification targets associated with development, progression, or metastasis of cancer [76-79].

1.7.2. *c-MYC* gene

c-MYC is a protooncogene located on the long arm of chromosome 8 (8q24); it is a member of *Myc* gene family, coding for nuclear c-MYC proteins that bind to DNA, facilitating transcription and regulating the activity of other genes involved in cell division [83, 84]. The *MYC* protooncogene family (comprising *c-MYC*, *n-MYC*, and *l-MYC*) ranks among the most exhaustively studied group of genes. The encoded proteins act as master gene regulators; they stimulate virtually all nuclear processes leading to enhanced cell growth and may cancel cell cycle arrest induced by growth-inhibitory pathways [84-86]. *c-MYC* is expressed at low constitutive level in growing cells and is down-regulated in quiescent and differentiating cells [87, 88].

The c-MYC protein is a transcription factor that both activates and represses target genes, using several mechanisms including recruitment of histone acetylases, chromatin modulating proteins, basic transcription factors, DNA methyltransferases, etc. [86, 89]. C-MYC influences the expression of a wide variety of gene families which contribute to the abnormal growth abilities of transformed cells when the c-MYC expression is altered. Its pleiotropic effects occur at the molecular and cellular level and have impact on almost every activity of the cell [90-92]. These effects are summarized in Figure 5.

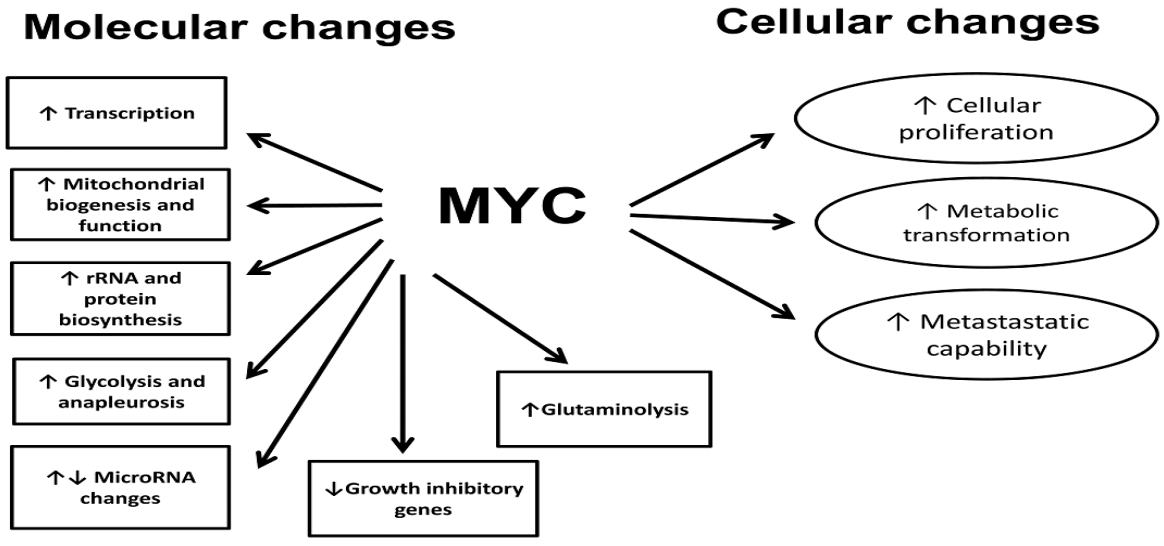


Figure 5: Pleiotropic effects of *c-MYC* expression (source: Miller D. M., Thomas D. S., Islam A., Muench D. and Sedoris K. "c-Myc and Cancer Metabolism" Clin Cancer Res.(2012)18(20): 5546–5553.)

c-MYC is also implicated in the control of apoptotic phenomena, possibly leading to tumor regression depending on cell types, cell interactions, extracellular matrix, and neighboring cells [83]. The effects induced by *c-MYC* can be either primary (when *c-MYC* is activated by amplification or translocation), or as a downstream effect of other activated oncogenes. In either case, it appears that c-MYC plays an important role in sustaining the changes which occur with transformation [90]. Figure 6 summarizes the connections of c-MYC with its target genes and their interactions.

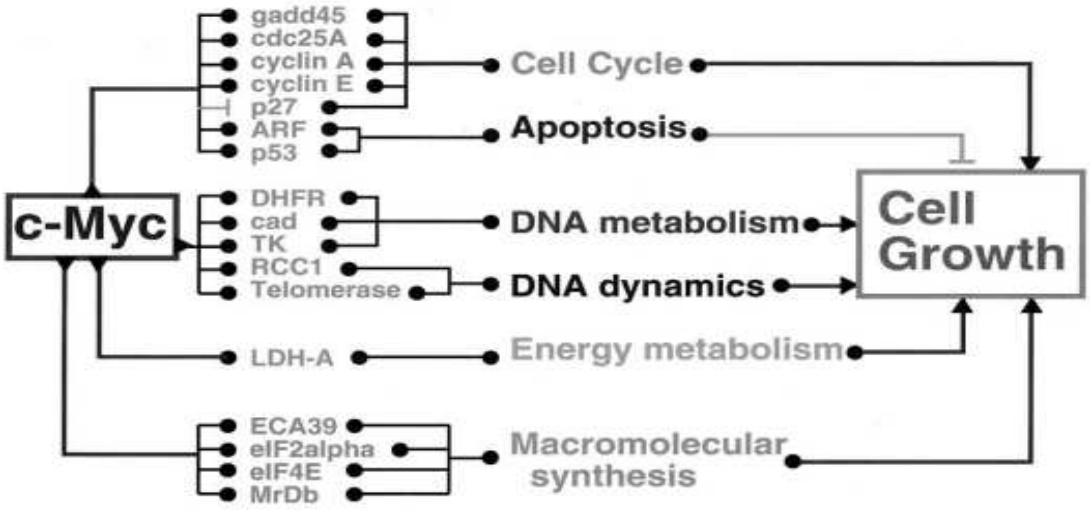


Figure 6: Connections between c-MYC, putative target genes, cellular functions and cell growth (source: Dang C. V. " MINIREVIEW: c-Myc Target Genes Involved in Cell Growth, Apoptosis, and Metabolism" MOL. CELL. BIOL. (1999) 19 (1): 1-11.).

It is amplified and found to be overexpressed in various human cancers and contribute to at least 40% of tumors [84, 90, 92]. It is also found amplified in 20-40% of oral cancers [93]. In several types of cancer, *c-MYC* amplification is frequently associated with more aggressive tumors [69, 94]. *c-MYC* overexpression to some extent correlates with gene amplification but not in all tumors; some tumors with *c-MYC* amplification display increased level of the oncoprotein and some other tumors reveal oncoprotein overexpression without gene amplification. It is usually associated with poorly differentiated oral carcinomas. *c-MYC* is considered as an important

marker in oral dysplasia and OSCC and its presence is correlated with the stepwise progression of dysplasia changes and tumor grade [95, 96].

1.7.3. *ERbB2* gene

ErbB2 or ***HER2*** is a proto-oncogene located on the long arm of chromosome 17 (17q12). It codes for a transmembrane tyrosine kinase receptor *HER2*, it is a member of the epidermal growth factor receptor (EGFR) family along with 3 other receptors (ErbB1, ErbB3, and ErbB4) [97, 98]. These receptors are considered as potent mediators of cell growth and development. The ErbB protein is composed of three domains: an extracellular domain that can bind different growth factors and act as an input layer of membrane receptors and their ligands to trigger the signal coming from outside the cell, a single hydrophobic transmembrane segment which is a core system processing layer of protein kinases transmitting the signal to the nucleus, and finally, an intracellular domain which has a protein kinase domain and represents an output layer of transcription factors regulating genes that affect various cellular functions. The intracellular protein kinase activity is activated by the binding of extracellular ligands or dimerization of the receptor with another ErbB family member [99-101]. ErbB2 biological effects are mediated by kinase activity resulting in phosphorylation of tyrosine residues in the cytoplasmic portion of the receptor molecule, leading to activation of downstream growth-promoting pathways [102]. The ErbB2 physiological role is to serve as a co-receptor in the context of ErbB ligand signaling. It appears to be the preferred partner of other ligand bound ErbBs [103].

ErbB2 is involved in many cell activities, including growth, development, and differentiation. It is a key signal transduction molecule that is overexpressed in a variety of human cancers [104, 105]. ErbB2 has no known direct activating ligand and may be in an activated state constitutively or become active upon heterodimerization with other family members such as HER1 and HER3. Homo or heterodimerization results in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiates a variety of signaling pathways, principally the mitogen-activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and protein kinase C (PKC) resulting in cell proliferation, survival, differentiation, angiogenesis, and invasion [97, 105, 106].

In normal cells, ErbB2 signaling is controlled by the EGF-related ligands through the obligate formation of heterodimers with other ErbBs [103]. The activation of ErbB2 and the other ErbBs can enhance the malignant potential of the cells, and the overexpression of these receptors correlates with poor prognosis [28]. The ErbB2 is frequently upregulated in human cancers where it plays an important role. It can promote tumorigenesis and tumor progression [107, 108].

ErbB2 amplification and overexpression of its protein have been reported in several human malignant neoplasms; it is frequent in squamous cell carcinomas including OSCC [69, 108, 110]. The ErbB2 high expression level, frequently associated with gene amplification, has been correlated with increased tumor invasion, progression, metastasis, resistance to chemo-radiotherapy, suggesting that this gene may represent an important indicator of poor prognosis [108, 111].

The detrimental consequences of ErbB2 overexpression are summarized in Figure 7.

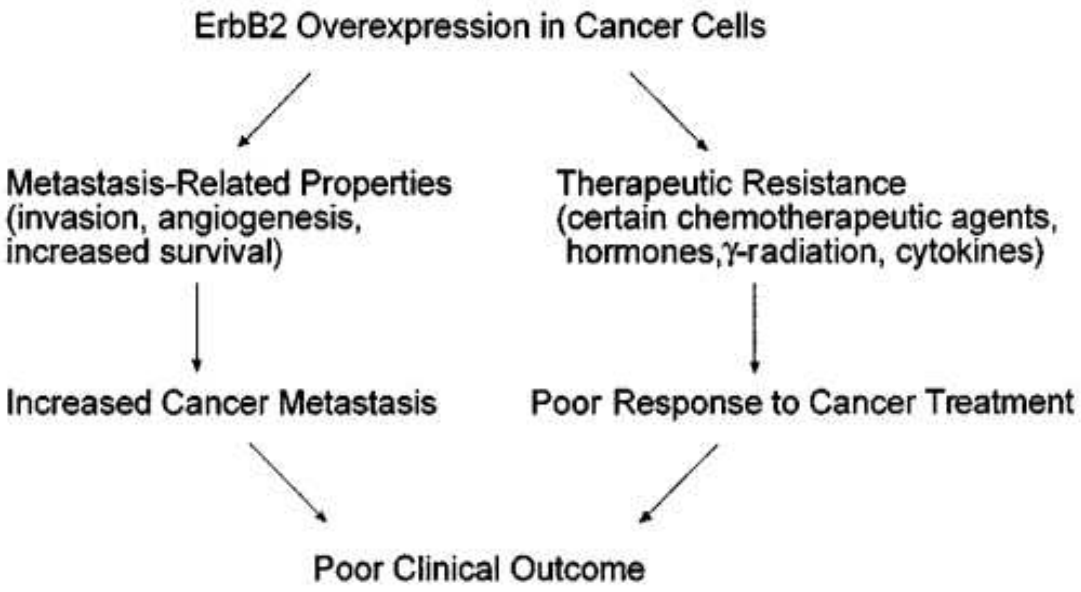


Figure 7: The detrimental outcome of ErbB2 overexpression in cancer cells (source: Yu D. and Hung M-C. "Overexpression of ErbB2 in cancer and ErbB2-targeting strategies" Oncogene (2000) 19: 6115 – 6121.).

Although in some studies ErbB2 overexpression has been found to correlate with poor survival of patients with OSCC, generally speaking the results are still controversial and so remains the role of ErbB2 in OSCC [112-115].

1.8. Tumor suppressor genes

Tumor suppressor genes (TSGs) are normal cellular genes that slow down cell divisions, repair DNA damages and control apoptosis [116]. These genes encode proteins that either have a damping or repressive effect on the regulation of the cell cycle or promote apoptosis and sometimes do both. Tumor suppressor genes regulate cellular activities including cell cycle checkpoint responses, mitogenic signaling, detection of DNA damage and repair, protein degradation and ubiquitination, cell specification, differentiation and migration, carcinogen detoxification, senescence and tumor angiogenesis [59, 117]. We can classify tumor suppressor genes functions into the following categories [117]:

- Repression of genes which are essential for continuing the cell cycle.
- Coupling the cell cycle to DNA damage, stopping cell division if there is DNA damage for repair.
- Initiation of apoptosis to remove the threat it poses, when the DNA damage cannot be repaired.
- Blocking loss of contact inhibition,
- Involvement in cell adhesion to prevent tumor cells from dispersing, and inhibition of metastasis [118, 119].
- DNA damage repair (DNA repair proteins are usually classified as tumor suppressors) [117, 120].

Alterations in these genes cause reduction or loss in their function and the cells can progress to cancer, usually in combination with other alterations. The inactivation of these genes cripples growth-inhibitory pathways and may be more important than the activation of oncogenes for the development and formation of cancer cells [15, 121]. The loss-of-function alterations of TSG relieve cells of inhibitions that normally help to hold their numbers in check [67]. The tumor suppressor genes are inactivated mainly by mutations, loss of heterozygosity or DNA

methylation [24, 122]. Deletions, mutations and hypermethylation of TSG are the major molecular determinants of most common human cancers [59, 123]. Inherited abnormalities of tumor suppressor genes have been found in some family cancer syndromes. They cause certain types of cancer to be hereditary. However, most tumor suppressor gene abnormalities are acquired [116].

TSG cancer-preventive effects usually require the presence of only one functional allele, i.e. TSG mutations have a recessive character and consequently for TSGs to be inactivated alterations of both alleles are necessary [67, 117].

Both genetic and epigenetic alterations play an important role in the initiation and progression of OSCC.

Different molecular alterations involved in oral carcinogenesis are illustrated in Figure 8.

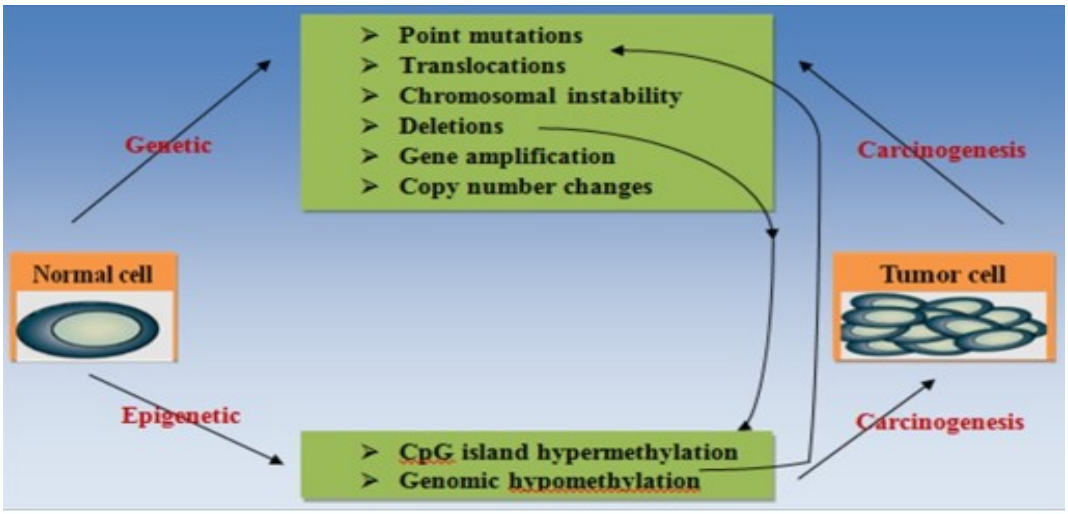


Figure 8: Genetic and epigenetic mechanisms in carcinogenesis of human oral cancer (source: Bhatia V., Goel M. M. and Makker A. " DNA Methylation: An Epigenetic mechanism in oral squamous cell carcinoma" South Asian J Exp Biol. (2014) 4 (2): 33-41.)

As previously mentioned DNA hypermethylation may be an alternative mechanism to mutations or deletions in disrupting tumor suppressor gene function [57].

1.8.1. Tumor suppressor gene methylation

DNA methylation is the process by which the DNA molecules are chemically altered through an addition (reversible addition) of methyl groups to specific nucleotide (cytosine) on the chain of the millions of base pairs of nucleotides that compose DNA [125-127]. DNA methylation is the main epigenetic factor influencing gene activities; it is a potent mechanism for silencing gene expression and maintaining genome stability in the face of a vast quantity of repetitive DNA, which can otherwise mediate illegitimate recombination events and cause transcriptional deregulation of nearby genes [128, 129]. The DNA methylation affects the binding of proteins to their cognate DNA sequences, and prevents the binding of basal transcriptional machinery and ubiquitous transcription factors; it contributes to epigenetic inheritance, allele-specific expression, inactivation of the X chromosome, genomic stability and embryonic development. Through these pathways; progressive DNA methylation is thought to be an agent both of normal development and aging, as well as of neoplastic transformation [56, 130].

DNA methylation involves direct chemical modification to the DNA by the transfer of a methyl group onto the C5 position of the cytosine base to form 5-methylcytosine (5mc). DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs) that transfer a methyl group from S-adenyl methionine (SAM) to the fifth carbon of a cytosine residue to form 5mc. Three main DNMTs have been identified. DNMT1 maintains the existing methylation patterns following DNA replication, whereas DNMT3A and DNMT3B are responsible for *de novo* methylation patterns. The overexpression of these DNMTs that leads to CpG island hypermethylation of TSGs, has been observed in cancers and is associated with TSG expression silencing.

Approximately 45% of the mammalian genome consists of transposable and viral elements that are silenced by bulk methylation. The vast majority of these elements are inactivated by DNA methylation or by mutations acquired over time as the result of the deamination of 5-methylcytosine (5mC). If expressed, these elements are potentially harmful as their replication and insertion can lead to gene disruption [129]. In mammalian cells, DNA methylation is a relatively stable modification that occurs in the context of CpG dinucleotides, the presence of these CpG dinucleotide sites being irregular, with some regions containing a high frequency of CpG dinucleotides known as (CpG islands), in contrast to areas where these dinucleotides are

underrepresented. CpG rich regions are often situated in promoters that are proximal to the transcription start sites of many genes while the remainder of the genome is relatively CpG poor.

Two mechanisms are proposed through which DNA methylation leads to tumor suppressor genes silencing: (1) DNA methylation may directly block the specific binding sites of transcription factors, (2) methyl-CpG-binding proteins (MBDPs) which are regarded as important "translators" between DNA methylation and transcriptional silencing recognize m5CpG sequencies and silence transcription [59]. So DNA methylation represses transcription directly by inhibiting the binding of specific transcription factors and indirectly by recruiting methyl-CpG-binding proteins and their associated repressive chromatin remodeling activities. The significance of DNA methylation is emphasized by the growing number of human diseases that are known to occur when this epigenetic mechanism is altered, and there is increasing interest in developing ways of pharmacologically reversing such epigenetic abnormalities [128].

Abnormal DNA methylation could stand for less (hypomethylation) or more (hypermethylation) than in normal or typical DNA [52, 127, 134].

DNA methylation is an important factor in the development of cancer; it may represent an early and fundamental step in the pathway by which normal tissue undergoes neoplastic transformation. The disruption of normal DNA methylation patterns is one of the most common features of transformed cells and it is an early event in the tumorigenesis process. In tumor cells, the normal regulation of the DNA methylation machinery is severely disrupted, such that the regional specificity of methylation patterns begins to be reversed, resulting in *de novo* methylation of CpG islands and hypomethylation of repetitive DNA. The assessment of the methylation profiles within the neoplastic tissue may provide key information for enhancing the diagnosis, predicting the clinical behavior, and designing specific treatment plans for individual patients. In addition to the inhibition of genes expression, DNA methylation can also increase the probability that affected genes undergo a mutational event. Abnormal methylation patterns can also indirectly affect gene activity with the disruption of the transcription-translation process by increasing the probability for a mutational event to take place and reducing overall chromosomal stability. Methylated cytosine has a greater propensity to undergo spontaneous deamination and the formation of thymine. If this does occur on a tumor suppressor gene, then a point mutation develops and loss of control of cell proliferation can occur [10, 137- 139].

During the process of carcinogenesis contradictory changes in DNA methylation patterns occurs, with simultaneous global hypomethylation and regional hypermethylation changes. Global DNA hypomethylation was the first epigenetic alteration found in human cancer. Gain of DNA methylation "hypermethylation" in normally unmethylated promoter regions is the most widely studied epigenetic abnormality in carcinogenesis and regarded as the major epigenetic alteration that leads to transcriptional silencing of tumor suppressor genes [10, 32, 59].

Many tumor suppressor genes are susceptible to promoter hypermethylation; these genes are distributed in all cellular pathways connected to tumor development [59, 122]. Both abnormal DNA methylation patterns, hypermethylation and hypomethylation, have been associated with a large number of human malignancies. Figure 9 illustrates the different DNA methylation pattern alterations in tumorigenesis.

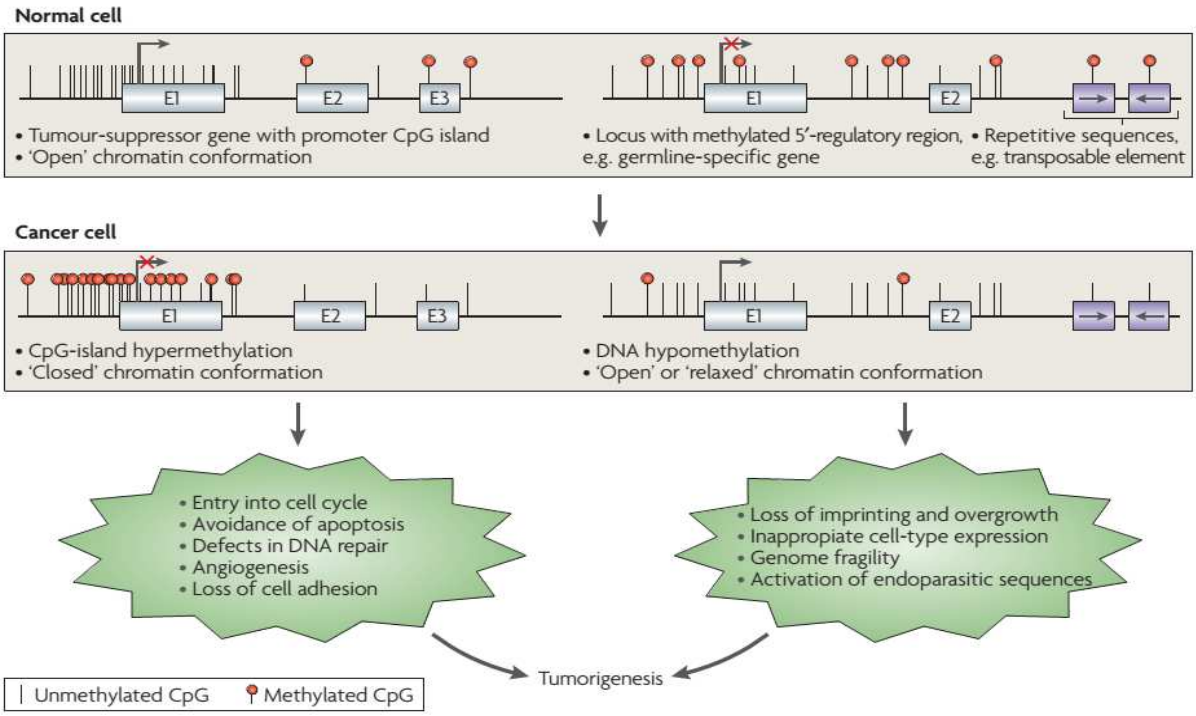


Figure 9: Altered DNA-methylation patterns in tumorigenesis (source: Esteller M. "Cancer epigenomics: DNA methylation and histone-modification maps" Nature Reviews Genetics (2007) 8 (4): 286-298.).

The relative timing of global and repeat sequence hypomethylation and hypermethylation of individual CpG islands varies in different cancers [54]. Figure 10 shows a summary of DNA methylation patterns changes during cancer and aging, and the different molecular activities and cellular functions of DNA methyltransferases (DNMTs).

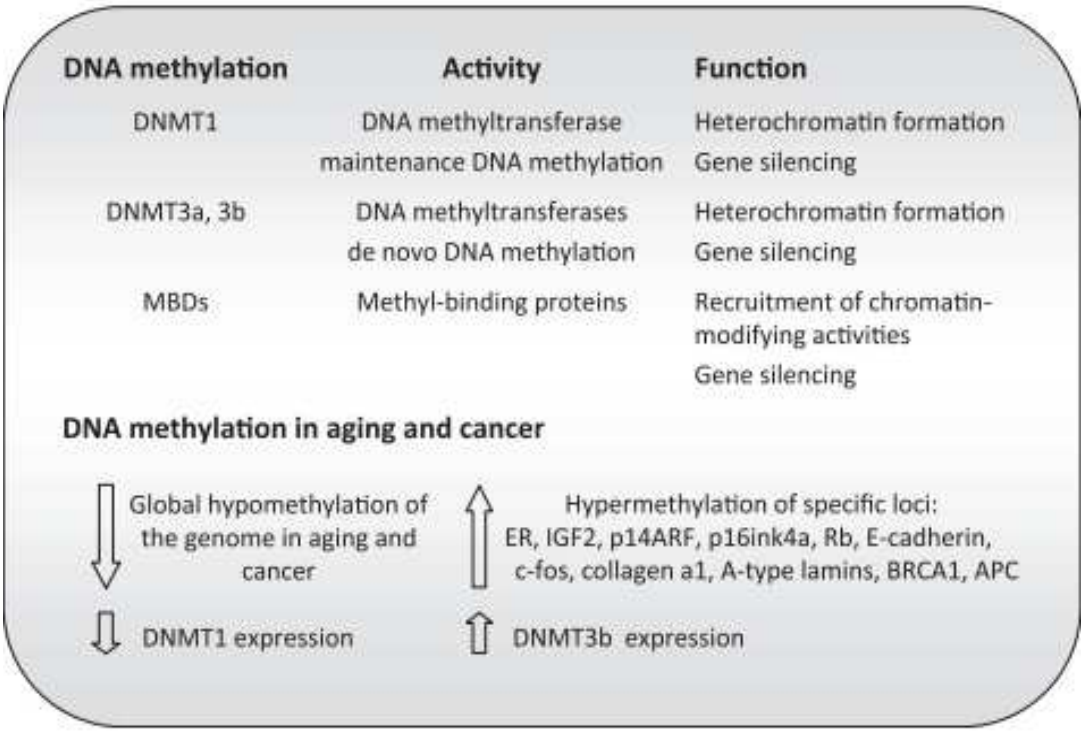


Figure 10: DNA methylation patterns changes during cancer and aging, and molecular activities and cellular functions of DNMTs (source: Gonzalo S. "Epigenetic alterations in aging" Journal of Applied Physiology(2010) 109 (2):586–597.).

The hypermethylation of tumor suppressor genes has been detected in OSCC and is a hallmark of many other cancers; it is mediated by the enzyme DNA methyltransferase and results in stable transcriptional silencing of tumor suppressor activity of these genes [49, 127].

DNA methylation plays a significant role in the development and progression of OSCC. More frequent and higher levels of promoter DNA methylation of several genes have been observed in OSCC tissues and precancerous oral lesions in comparison to corresponding normal tissues [58, 124]. In the last decade, aberrant promoter hypermethylation of many genes has been observed in oral and oropharyngeal cancer tissue as well as in premalignant oral lesions and histologically

healthy mucosa surrounding the tumor. Thus, methylation could be considered as an early marker of malignant progression. Hypermethylation of many tumor suppressor genes including *P14* and *P16* has been seen in dysplasias and in histologically normal-appearing margins of OSCC resections [10, 52, 53]. Table 6 shows a summary of sixteen genes with substantial evidence for hypermethylated promoter region in OSCC and their reported clinicopathological associations [52].

Table 6. Candidate genes frequently silenced by promoter hypermethylation in OSCC tumor tissue.

Mechanism	Gene	Gene function	Clinicopathological association*
Cell cycle regulation	*CYCA1*	Cell cycle	Lower histological grade
	CHFR	Early G2/M checkpoint	Higher T status
	P14ARF	Proapoptosis	LNM**, T status (T2-3), advanced stage, reduced recurrence rate, favourable prognosis
	p15 *P16INK4A*	Cyclin-dependent kinase inhibitor 2B Regulates cell cycle G1 progression	Anatomic site (tongue SCC), alcohol and tobacco use Larger tumor size, LNM, advanced stage Younger age, increased recurrence rate, poor prognosis
DNA repair	*hMSH1/hMSH2*	DNA mismatch repair	—
	MGMT	Guanine alkylation repair	Reduced overall and disease-free survival
Signal transduction	*EDNRB* *RUNX3* *SFRP1*	Endothelin receptor type B Wnt pathway antagonist Wnt pathway antagonist	Alcohol and tobacco use LNM, advanced stage, poor differentiation Male gender
Tissue invasion/ metastasis	*ECAD*	Calcium-dependent cell-cell adhesion glycoprotein	LNM, increased metastatic potential, reduced disease-free survival
Tumor suppression	*HIN1*	Inhibitor Ras pathway	Reduced disease-free survival
	DAPK1	Proapoptosis	LNM
	DCC	Proapoptosis	Invasion of bone and deep tongue, reduced survival
	RASSF1A/RASSF2	Negative RAS effector,proapoptotic, microtubule stabilization	Decreased disease-free survival, radioresistance
Other	*KIF1A*	Cell division and microtubule-dependent intracellular organelle transport	Malignant histology

*Reported significant associations and trends.

**Lymph node metastasis

1.8.1.1. *P14^{ARF}* gene

P14^{ARF} gene belongs to the *INK4b-ARF-INK4a* locus on the short arm of chromosome 9 (9p21). It codes for the P14^{ARF} protein which is a nuclear protein that exhibits tumor suppressive functions (tumor suppressor). P14 has many biological functions. Its chief function is to suppress aberrant cell growth by inducing the p53 pathway but also displays p53 independent activities. The suppression of aberrant cell growth in response to oncogene activation by activating the transcription factor p53 that initiates the expression of many apoptosis inducers and cell cycle inhibitory genes is one of the most well-defined functions of P14. P14 is thought to stabilize and stimulate p53 activity by neutralizing the inhibitory effect of two ubiquitin ligases; murine double minute 2 (MDM2), and ARF-binding protein1/Mcl1-ubiquitin ligase E3 (ARF-BP1/Mule). Both proteins are specific ubiquitin ligases for p53 and can inhibit its tumor suppressor function [52, 123, 145]. As a result of MDM2 function inhibition and stabilization of p53 by P14, the stabilized p53 can induce temporary and permanent growth arrest, DNA repair, terminal differentiation or apoptosis in response to oncogenic signals and DNA damage [145, 146]. Elevated expression of ARF counteracts the negative control of MDM2 on p53, leading to stabilization of p53 and activation of a p53-dependent transcriptional program that potentiates apoptosis or induces cell cycle arrest according to tissue type and activating signals. ARF also functions independently of p53 to inhibit cell growth, notably by attenuating the transactivating activity of growth-promoting genes such as *E2F1* and *c-MYC*. DNA damage caused by various cellular stresses stimulates the p53 pathway through activation of the ataxia–telangiectasia mutated (ATM) and/or ataxia–telangiectasia and Rad3–related (ATR) kinases depending on the nature of the inducing signal. These enzymes increase the transcriptional activity of p53 by promoting its phosphorylation by C-terminal Src kinase-homologous kinase (CHK) kinases. The ability of ARF to inhibit MDM2 can modify the p53-dependent DNA damage response. Some forms of DNA damage such as UV and cytotoxic drugs can directly stimulate the expression of ARF which impinges on ATM and/or ATR signaling by mechanisms that do not involve the p53 pathways but modify the activity of the ATM/ATR enzymes either directly or indirectly. Besides, there is also some evidence that ARF contributes to some DNA repair pathways and to chromosomal stability independently of p53; The pathways stimulated by ARF that are

significant in maintaining genomic integrity and play a role in genome stability are represented in Figure 11.

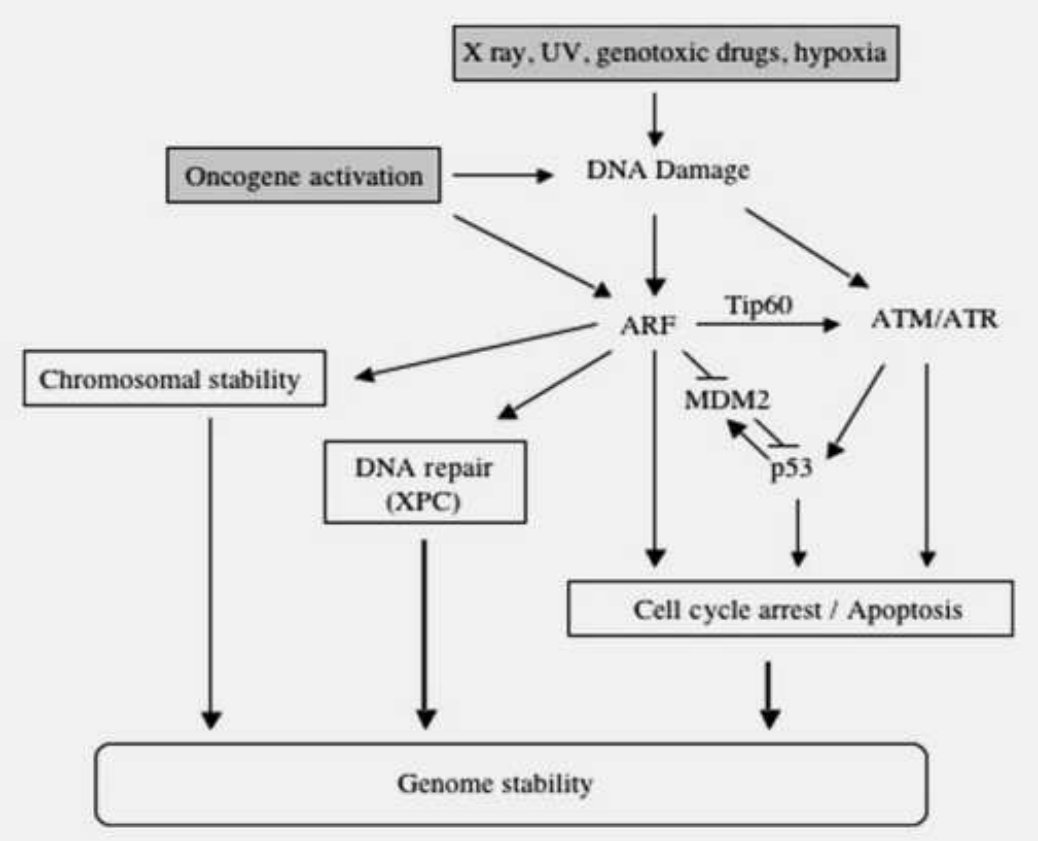


Figure 11: A model for the role of ARF in tumor suppression (source: Ozenne P., Eymin B., Brambilla E. and Gazzeri S. "The ARF tumor suppressor: structure, functions and status in cancer" Int. J. Cancer (2010) 127: 2239–2247.).

In addition, P14 attenuates ribosomal RNA synthesis and processing by binding to nucleophosmin (NPM) [145, 147, 148]. NPM is a nucleolar phosphoprotein, and its overexpression level correlates directly with the proliferative state of a cell. It is involved in diverse cellular processes including ribosome biogenesis. P14 induces the SUMOylation of its partner binding proteins like Mdm2 and NPM. The effects of this SUMOylation are diverse and can control protein trafficking and stability, ubiquitination, transcription factors activities, DNA repair and centromeric cohesion. P14 also promotes autophagy. The major functions of P14 are summarized in Table 7 [145, 148].

Table 7. Major cellular functions of $P14^{ARF}$

Cellular functions	Mechanisms
Tumor suppression:	
Cell growth control	Activation of p53 leading to cell cycle arrest or apoptosis
Ribosome biogenesis	p53-independent cell cycle arrest or apoptosis
DNA damage response	Decreased rRNA transcription and processing
	Activation of p53 pathways
	Activation of ATM/ATR/CHK pathways
	Activation of DNA repair pathways
	Maintenance of chromosomal stability
Other functions:	
Autophagy	Alteration of the mitochondrial membrane potential
Sumoylation	Association with UBC9 (E2). Biological impact unknown

Alterations of *INK-4bARF-INK4a* locus which lead to the inactivation of P14 are frequently identified in human cancers [149, 150]. These alterations can be homozygous deletions, frameshift microdeletions/insertions, and nucleotide substitution, as well as promoter CpG islands hypermethylation- the main epigenetic alteration and a significant mean of P14 transcriptional silencing in a variety of tumor types. The frequency of *P14* promoter hypermethylation varies in different tumor types. Previous studies have reported that epigenetic alterations of the *P14* gene are important events in head and neck carcinogenesis and in the development of benign tumors [153].

P14 hypermethylation results in loss of p53 function and deactivation of p21-induced cell proliferation. Studies of *P14* hypermethylation in oral cancers are somewhat conflicting. It has been associated with increased tumor size and tumor stage and nodal metastasis [141], but it has also been shown that $P14^{ARF}$ hypermethylation in late-stage tumors, including oral cancer, is

associated with a lower recurrence rate and a better clinical outcome compared with patients with tumors that were not *P14*^{ARF}-hypermethylated. Its expression is an independent predictor of both relapse and survival in squamous cell carcinomas of the anterior tongue [141, 145]. In addition, it has been found that in patients who are under radiotherapy, *P14* hypermethylation might render tumor cells more sensitive to radiation, improving the prognosis [154].

1.8.1.2. *P16*^{INK4a} gene

P16^{INK4a}***gene*** also belongs to the *INK4b-ARF-INK4a* locus on the short arm of chromosome 9 (9p21), known with different names (*P16*, *CDKN2A, pl6INK4, CDK4I* and *MTS1*)[155, 156]. It encodes another tumor suppressor - p16 protein which acts as cell cycle inhibitor that blocks abnormal cell growth and proliferation by binding to cyclin-dependent kinases (CDK) 4 and 6, and cyclin D [52, 155].

P16 function is primarily related to cell cycle as a negative regulator of retinoblastoma tumor suppressor protein-E2F transcription factor pathway (pRb-E2F pathway) [157, 158]. At G1-to-S transition, p16 specifically inhibits cyclin-dependent -CDK4 and 6-mediated phosphorylation of pRb that prevents the release of transcription factors. Sequestering E2F factors as a component of pRb-E2F complexes will consequently block cell cycle progression. Altered or inactivated P16 is unable to form these complexes and therefore cannot inhibit cell progression through abnormal mitotic division [52, 145, 158-160]. P16 also contributes to cell cycle progression through alternate and independent regulatory pathways as phosphorylation of the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II, which is an essential regulatory event in transcription [158, 161-163]. P16 is involved in cellular senescence and aging; its expression level increases remarkably with aging in both healthy and disease states. The elevated P16 level induces cellular senescence and aging in various progenitor cells and premalignant tumor cells [158, 164, 165].

The P16 tumor suppressor functions through protein-protein interactions with diverse target proteins. Any proteins that are able to influence these interactions between P16 and its targets could contribute to the regulation of P16, either positively or negatively. They modulate P16-

CDK4 association as well as subsequent CDK4-mediated phosphorylation of pRb, most of which are related to human cancers [158].

P16 is one of the main factors to avert tumor formation and its alterations are among the most frequent alterations in human cancers. Its inactivation is observed in close to 50% of all human cancers [158, 166]. The estimated frequencies of P16 inactivation vary between different types of tumors and ranges from 20% in breast cancer, 30% in bladder cancer to 60% in head and neck squamous cell carcinoma and 50-70% in melanoma [158].

Four types of alterations inactivate *P16* gene: homozygous deletion, loss of heterozygosity (LOH), point mutations and hypermethylation. Homozygous deletion and promoter hypermethylation usually constitute the majority of *P16* alterations. While homologous deletions and aberrant methylation-mediated silencing usually lead to complete loss of *P16* function in the cell, point mutations may only impair the structure and function of *P16*. An elevated level of *P16* induced by oncogenes, DNA damage response, or aging can trigger and accelerate cellular senescence. While the overexpression of *P16* is associated with poor prognosis for many cancers including oral cancers and is used as a diagnostic tool, its overexpression has also been implicated in apoptosis, cell invasion, and angiogenesis. The downregulation of *P16* contributes to cancer progression by promoting aberrant cell proliferation, and the loss of its expression leads to cell immortalization [156, 158, 167]. The altered expression of this gene has previously been observed in head and neck squamous cell carcinomas (HNSCC) as one of the genetic alterations in the histologically- free resection margins that are adjacent to the primary tumor site and may indicate an early malignant change [42]. Figure 12 shows the interactions of $P16^{INK4A}$ and $P14^{ARF}$ in the cell cycle.

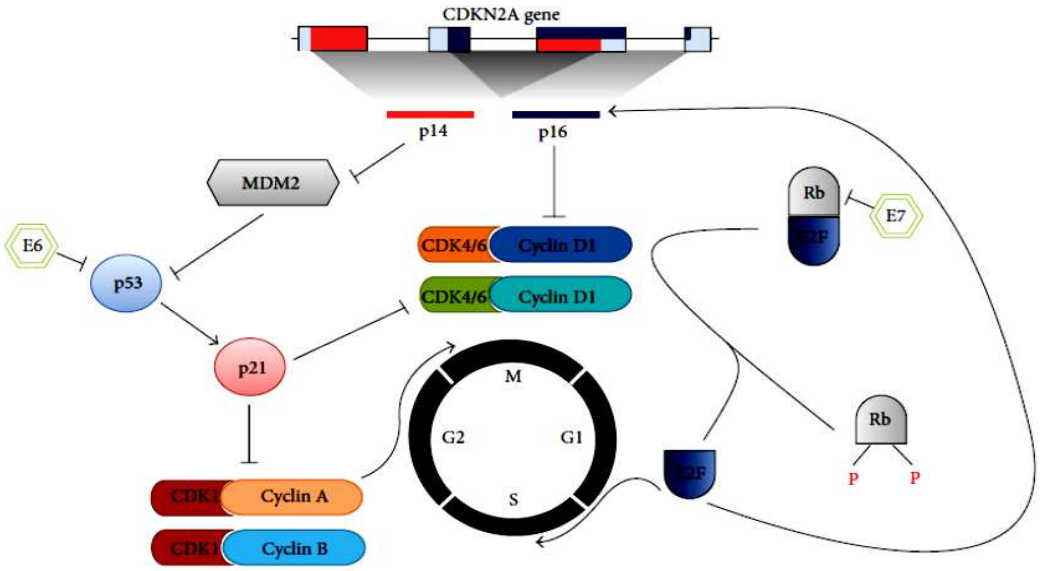


Figure 12: The interactions of *P16*INK4a and *P14*ARF in cell cycle arrest, The *P16*INK4a protein inhibits the CDK4/6-cyclin D1 complexes, keeping the retinoblastoma (Rb) proteins in a dephosphorylated state, and enables binding and inactivating the E2F transcription factors. Free E2F ensures the transcription of various proteins, most of them are necessary for progression to S phase. *P16*INK4a is also upregulated by E2F. In contrast, *P14ARF* stabilizes and thus activates the tumor suppressor gene p53 by inhibiting MDM2, which inactivates p53 by ubiquitin-mediated degradation. Active p53 induces the expression of p21, a negative cell cycle regulator which is an inhibitor of the CDK1-cyclin A/B complexes, thereby preventing the progression from G2 phase to metaphase. The human papilloma virus oncoproteins E6 and E7 interfere in the Rb pathway and in the p53 pathway, in order to bypass the cell cycle checkpoints. The E7 oncoprotein promotes the progression to S phase. It binds the Rb proteins and thereby releases the E2F transcription factors. The E6 protein targets p53 and induces loss of function by degradation (source: Alkaabi A., vanBockel L. W., Pothen A. J., and Willems S. M. "p16INK4A and p14ARF Gene Promoter Hypermethylation as Prognostic Biomarker in Oral and Oropharyngeal Squamous Cell Carcinoma: A Review". Disease Markers Volume 2014 (Article ID 260549).).

2. Rationale of the study

A significant percentage of OSCC patients have a poor prognosis. The disease is also characterized by a relatively high percentage of recurrences and loco-regional and distant metastases. Histological assessment of the patient's tumor and tumor resection margins is currently the main diagnostic and prognostic tool and is still considered as the gold standard for tumor behavior prediction. However, it appears that histological status does not always predict tumor progression and recurrences. This is especially the case when the tumor margins are described as "histologically negative", i.e. tumor-free. Obviously histology lacks sensitivity for the identification of cells that have already undergone some molecular changes but have not yet developed a pathologic phenotype, either within the zone of the primary tumor, or even in other part of the oral cavity of OSCC patients, seemingly unaffected. Therefore, the study of the molecular changes in the tumor, free surgical margins and normal buccal mucosa of the OSCC patients may assist in the identification of genetic/epigenetic alteration potentially useful as predictors of tumor behavior.

In other words, considering that genetic alterations come before phenotypic changes of the epithelium, molecular assessment of tumor, but mostly surgical margins and healthy oral cavity mucosa, could constitute a more sensitive approach to detect the signs of early malignant transformation.

3. Aims

The aims of the present study were to:

1. Determine the frequency and level of amplification of oncogenes *HER2* and *c-MYC* in tumor tissue, histologically negative margins and healthy buccal mucosa of patients with oral squamous cell carcinoma;

2. Determine the frequency of methylation of the *CDKN2A* locus ($P16^{INK4a}$ and $P14^{ARF}$) in tumor tissue, histologically negative margins and healthy buccal mucosa of patients with oral squamous cell carcinoma;

3. Analyze the association between molecular alterations in oral squamous cellular carcinoma and histological and clinical parameters (stage/grade, recurrences and survival);

4. Analyze the association between molecular alterations in OSCC and epidemiological factors;

5. Determine the copy number variation (CNV) in tumor tissue and histologically negative margins, compared to reference DNA on a small number of selected OSCC patients.

4. Material and Methods

4.1.Material

4.1.1. Patients and sampling

A group of 40 OSCC diagnosed patients (38% female and 62% male, aged 65.31±10.50 years; 56% of them were smokers, and 45% were alcohol consumers), who underwent surgery at the Clinic of Maxillofacial Surgery, School of Dental Medicine, University of Belgrade, during the period from March 2014 to May 2016, were included in this study. Tumor tissues, tumor free (histologically negative) margins tissues, and normal buccal mucosa swabs were collected from each participant and frozen at -20°C pending further analyses.

The histopathological diagnosis of OSCC was established in accordance with the World Health Organization (WHO) guidelines, and the tumor staging was performed using the TNM classification. All tumors were reviewed by a pathologist and were primary tumors with an infiltrative pattern of invasion. The locations of tumors were as follows: lip (n = 11), mandibular mucosa (n = 3), buccal mucosa (n = 1), floor of the mouth (n = 16), anterior tongue (n = 1), and oropharynx (n = 8). Three samples were taken from each patient: tumor, tumor margin, and swab. Margin samples were taken at least 5 mm from the edges of the surgical defects after primary tumor excision, and the absence of neoplastic cells was afterwards histologically confirmed. Buccal swab samples, considered as an acceptable source for OSCC biomarker detection, were taken contra-laterally of the tumor localization from the healthy buccal mucosa 2 days after tumor resection and after careful mouth disinfection.

The study was performed according to the ethical principles governing medical research and human subjects as laid down in the Helsinki Declaration (2013 version), and with the approval of the Ethics Committee of the study institution in Belgrade (n^o 36/12). All study participants were informed of the procedures and signed a written consent form.

4.1.2. DNA extraction

DNA from frozen tumor and margin tissue was extracted according to the standard phenol/chloroform extraction procedure described by Sambrook and colleagues after proteinase K digestion, (***see supplementary1.1***). Commercial kit (Invitrogen, Carlsbad, CA) was used for DNA extraction from swabs. All manufacturers' recommendations were followed for reaction mixes and profiles (***see supplementary 1.2, 1.2.1 and 1.2.2***).

The concentrations of the extracted DNA were assessed spectrophotometrically. Absorbance was measured on wave length of 260nm (A260) and 280nm (A280) and the A260/A280 ratio was between 1.8 and 2.0. The isolated DNA was stored at -20 °C until further analyses.

4.2.Methodology

Methylation status of *P14* and *P16* gene promoters was assessed using methylation-specific PCR (MSP). A quantitative real-time polymerase chain reaction (qPCR) and comparative cycle threshold (Ct) (ΔΔCt) method of quantitation of *HER2* and *c-MYC* was performed.

4.2.1. Polymerase chain reaction

Polymerase chain reaction (PCR) is a widely used technique by which a single or a few copies of a DNA segment are amplified to produce thousands to millions of copies of a particular DNA sequence. It is a very quick, relatively inexpensive, reliable and simple technique. By this technique, specific DNA fragments from minute quantities of biological material may be amplified, even when DNA source is of relatively poor quality. The basic PCR principle is a chain reaction in which one DNA molecule is used to produce two copies, then four, then eight and so forth. This reaction is accomplished by polymerases, which are thermostable enzymes that string DNA building blocks, so a supply source (dNTPs) of these blocks (four deoxyribonucleotides) is needed together with two short single strand fragments of DNA (oligonucleotides) that initiate DNA synthesis and serve as primers in new strands synthesis [169-171].

The main components for the reaction are water, PCR buffer, $MgCl_2$, dNTPs, forward primer, reverse primer, target DNA, and the enzyme Taq polymerase. These requirements and steps of amplification cycles (denaturation, annealing, and extension) in a polymerase chain reaction are illustrated in Figure 13.

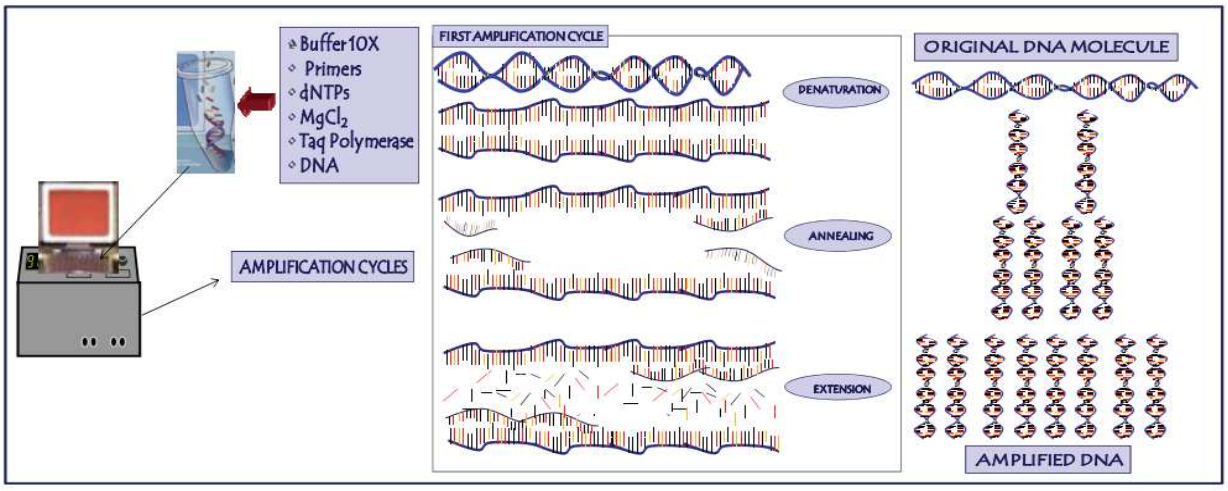


Figure 13: The requirements and steps of amplification cycles (denaturation, annealing and extension) in a polymerase chain reaction (PCR) (source: Hernandez-Rodriguez P. and Arlen Gomez Ramirez A. G. "Polymerase Chain Reaction: Types, Utilities and Limitations" Polymerase Chain Reaction, Dr Patricia Hernandez-Rodriguez (Ed.), InTech (2012), DOI: 10.5772/37450.)

The PCR technique involves three major steps: the first step is *denaturation* in which the DNA is denatured at high temperatures (between 90-97°C). The second step is *annealing* where the primers anneal to the DNA template strands to initiate the extension, this occurs at a lower temperature (50-60°C). The third step is *extension* in which primer elongation occurs at approximately 72°C to create a complementary copy strand of DNA [169, 170, 172].There are many types of PCR-based laboratory techniques such as *real-time PCR,* also called quantitative real time polymerase chain reaction (qPCR) which is used to amplify and simultaneously quantify a targeted DNA molecule; *inverse PCR* is a variant of the PCR that is used to amplify DNA with only one known sequence; *nested PCR* is a type of PCR designated to reduce the product contamination due to the amplification of unexpected primer binding sites; *multiplex PCR* which simultaneously amplifies several DNA sequences; *semiquantitative PCR* which allows an approximation to the relative amount of nucleic acids present in a sample; *reverse transcriptase PCR (RT-PCR)* which generates cDNA (DNA complementary to RNA) that is then

amplified by PCR, and *Touchdown PCR* is one these PCR types by which primers will avoid amplification of nonspecific sequences [169, 170]. There is also *methylation-specific PCR* which is used to determine the DNA methylation status [173]. Nowadays, PCR is widely used in the investigations and diagnosis of an increasing number of diseases; it became the standard method for research on nucleic acids, and it has been considered as an essential tool in molecular biology that allows amplification of nucleic acid sequences (DNA and RNA) through repetitive cycles. In microbiology and molecular biology, it is used as research procedure for DNA cloning, Southern blotting, DNA sequencing, and recombinant DNA technology. It is used for the diagnosis of microbial infections and epidemiological studies, it is used in the identification and characterization of viral, bacterial, parasitic and fungal agents. PCR is also used in forensic laboratories and for diagnostic tests including those for genetic diseases, cancers [169-172].

4.2.2. Bisulfite modification of DNA and methylation-specific PCR

To study DNA methylation status, the extracted genomic DNA has to be modified by bisulfite treatment, the so-called bisulfite conversion that involves the conversion of cytosine to uracil while leaving 5-methylcytosine (5-mC) intact. Unmethylated cytosine residues are deaminated to uracil and methylated cytosine (5-mC) residues remain unaffected, enabling PCR amplification to recognize uracils as thymines and 5-mC or 5-hmC as cytosines. This allows discrimination between methylated and unmethylated cytosine residues, offering single-nucleotide resolution information about the methylated areas of DNA [174, 175]. Figure 14 illustrates the DNA modification. Beside DNA samples from tissues specimens, controls for totally methylated and totally unmethylated human DNAs were always included in the MSP [174]. Our samples were converted using EZ DNA Methylation™ Kit ***(see supplementary 2)***.

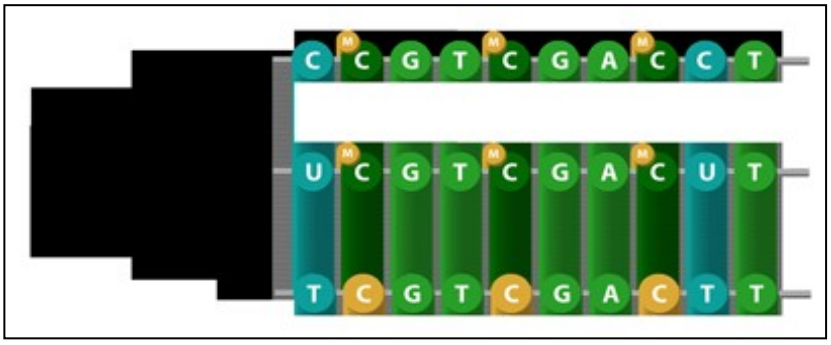


Figure 14: The bisulfite modification of DNA (source: https://www.epigentek.com/catalog/dna-bisulfite-conversion-c-75_21_47.html)

Several methods have been developed to study and analyze DNA methylation, but MSP is one of the most commonly used. The important advantage of MSP over other methods is its simplicity. MSP requires a much lower amount of DNA, isotope use is usually unnecessary, and any CpGs, regardless of the sequence around, can be evaluated. Also, interpretation of the results is quite simple, which is ideal for a large number of clinical samples analysis. Indeed it has been widely used to detect aberrant hypermethylation and inactivation of tumor suppressor genes in cell lines and tumor samples, to evaluate methylation status of any DNA sequences, such as viral genes, and imprinted X-linked and autosomal genes [173, 176]. MSP is a qualitative technique used to detect the presence of methylation in bisulfite-converted DNA; it is a rapid and cost-effective, sensitive, and specific method for determining the methylation status

of virtually any CpG rich region. It facilitates the detection of low numbers of methylated alleles and the study of DNA from small samples [177, 178].

An adaptation of the MSP protocol suggested by Herman et al in 1996 was implemented [179]: two separate PCR reactions were performed for each gene, a set of four primers per each gene was used: one pair for the detection of methylated CpG-cytosine and the other two for the identification of unmethylated CpG-cytosine. The primer sequences and annealing temperatures are listed in Table 8.

Table 8. Primer sequences, amplicon lengths and annealing temperatures for the primers used.

Gene	Primer sequence (5' - 3')	Amplicon length (bp)	Annealing temperature (°C)
ErbB2 Fwd ErbB2 Rev	CCTCTGACGTCCATCCT ATCTTCTGCTGCCGTCGTT	98	55
c-MYC Fwd *c-MYC* Rev	GCTCCAAGACGTTGTGTGTTCG GGAAGGACTATCCTGCTGCCAA	158	55
D2R Fwd D2R Rev	CCACTGAATCTGTCCTGGTATG GTGTGGCATAGTAGTTGTAGTGG	112	55
P14 UI *P14* U2	TTTTTGGTGTTAAAGGGTGGTGTAGT CACAAAAACCCTCACTCACAACAA	132	53
P14 M1 *P14* M2	GTGTTAAAGGGCGGCGTAGC AAAACCCTCACTCGCGACGA	122	53
P16 U1 *P16* U2	TTATTAGAGGGTGGGGTGGATTGT CAACCCCAAACCACAACCATAA	151	60
P16 M1 *P16* M2	TTATTAGAGGGTGGGGCGGATCGC GACCCCGAACCGCGACCGTAA	150	65
HPV 16 Fwd HPV 16 Rev	TCAAAAGCCACTGTGTCCTG CGTGTTCTTGATGATCTGCA	120	53

Fwd - forward primer; Rev – reverse primer; U1 – unmethylated forward primer; U2 – unmethylated reverse primer; M1 – methylated forward primer; M2 – methylated reverse primer

The modified genomic DNA samples were PCR amplified in a total volume of 50 µl, and the reaction mix contained 1 × PCR Buffer (10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100), 8 mM $MgCl_2$, 1.25 mM dNTPs, 0.6 µM primers (Invitrogen, Life Technologies, Carlsbad, CA, USA), 0.4 µg/µl BSA, 5% DMSO, 1.5U Dream Taq Green DNA Polymerase (Thermo Fisher Scientific) and 3 µl of bisulfite treated DNA template. Reactions were carried out in Gene AmpR PCR System PeqStar 96 Universal thermal cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) (Figure 15) and performed under the following conditions: initial denaturation at 95°C

for 5 minutes, followed by 40 cycles of *95°C* denaturation for 30 seconds and annealing for 30 seconds, then *72°C* extensions for 30 seconds, and final extension at *72°C* for 4 min. Moreover, to detect the presence of human papillomavirus type 16 (HPV16) in the tumor samples, we use the primers for the E6 gene (shown in Table 8) and Dream Taq Green DNA Polymerase. The reaction performed following the manufacturer's recommendations as follows: denaturation at *95°C* for 2 minutes, followed by 35 cycles of denaturation at *95°C* for 30 seconds, annealing at 53*°C* for 30 seconds, and extension at *72°C* for 1 minute, with final extension at *72°C* for 7 minutes.

Figure 15: PeqStar 96 Universal thermal cycler.

The PCR products were loaded on 8% polyacrylamide gels (PAA) for electrophoresis, then stained with ethidium bromide and visualized under UV light; the PEQlab electrophoretic unit is shown in Figure 16.

Figure 16: Gel loading and the electrophoretic unit

4.2.3. Gel electrophoresis

The term electrophoresis refers to the migration of charged molecules in an electrical field in stabilized media such as gels; molecule migration velocity depends on the field strength, on the net charge, size and shape of the molecules, and also on the ionic strength, viscosity, and temperature of the medium in which they are moving. The electrophoresis as a general term covers all applications regardless of the material being studied and the medium being used [180-183]. The nucleic acid DNA has a net negative charge and when subjected to an electrical field it will migrate toward the positive pole in a predictable and reproducible mode that can be accounted as a negative exponential function of length; shorter molecules will migrate faster and longer molecules will migrate slower [181, 182]. There are various types of gel electrophoresis that include differences in gel type, i.e. in size, shape, and porousness. The three common media used for gel electrophoresis are starch, polyacrylamide, and agarose. The typical method used for separation, identification, and purification of nucleic acids is the electrophoresis through an agarose or polyacrylamide gels. Even very small molecules of nucleic acids (i.e., oligonucleotides) are easily separated in an electrical field by one or the other medium (through an agarose or polyacrylamide gels). The fundamental principles for choosing polyacrylamide or agarose gel electrophoresis are the length and whether or not the nucleic acid is single-stranded

or double-stranded. Short, single-stranded DNAs like oligonucleotides require polyacrylamide gels whereas long, double stranded DNAs are best resolved on agarose gels [181-184]. Agarose gels are popularly used for the separation of medium and large-sized nucleic acids and have a wide separation range, but a relatively low separating power. The main advantages of using agarose gels are: they are non-toxic, good for separating large DNA molecules, quick and easy to cast, can recover samples by melting the gel, and digesting with an enzyme or treating with chaotropic salts [184]. In contrast to these advantages, there are some disadvantages of using agarose gels including the high cost of agarose, fuzzy bands and poor separation of low molecular weight samples [184]. Polyacrylamide gels are formed by the polymerization of acrylamide with a cross-linking agent commonly N,N,N',N'-tetramethylethylenediamine (TEMED) as the catalyst. They have much more resolving power than agarose, can accommodate larger quantities of DNA without significant loss in resolution and polyacrylamide gels recovered DNA is extremely pure. In addition, the pore sizes in polyacrylamide gels are easily altered by changing the concentrations of the two monomers. The main advantages of using polyacrylamide gel are: a stable chemically cross-linked gel, sharp bands, and good for separation of low molecular weight fragments, while the main disadvantage of using polyacrylamide gels is the toxicity of monomers [184].

The exact position of DNA bands within the gel can be determined by staining with low concentrations of the intercalating fluorescent ethidium bromide dye and visualization under ultraviolet light [185].

4.2.4. Real Time Quantitative PCR

Real-time polymerase chain reaction (qPCR) is a modification of PCR, introduced by Higuchi and coworkers in 1992. Real-time PCR enables exact quantification of specific nucleic acids in a complex mixture even if the starting amount of material is at a very low concentration. It has become widely used for the quantification of specific sequences in complex mixtures, for instance in genotyping analyses, the quantification of viral load in patients, and the estimation of gene copy number in cancer tissue. However, this technology is most commonly used to study gene expression levels by coupling it with the reverse transcription-PCR (RT-PCR) [186-188]. The fundamental purpose of real-time PCR is to precisely differentiate and measure specific

nucleic acid sequences in a sample. Real-time PCR amplifies the specific target sequence and monitors the amplification reaction progress using fluorescent technology. The qPCR reaction basic principle is that short DNA sequences are copied and the amount of DNA in the reaction should double at each cycle, resulting in an exponential amplification of the initial target DNA during the early cycles when the PCR components are largely available compared to the target sequence. But, as product accumulates, the substrates are reduced, resulting in the inhibition of the reaction [186, 187, 189]. The PCR reaction can be broken into three distinct phases: *exponential, linear*, and *plateau***.** The first phase of the reaction is the *exponential phase***,** where the reaction is progressing with 100% efficiency and with product doubling at each cycle. Sometimes attaining 100% efficiency is not possible and careful optimization of PCR conditions must be conducted to ensure that reactions are proceeding as efficiently as possible [186, 189]. While the amplicon continuously accumulates, the PCR ingredients are consumed, the primer starts competing with amplicon reannealing to itself, and the reaction capability decreases. Over time, the reaction slows down and enters the second phase, *linear phase* ***or*** the *non-exponential phase* where there is no longer near doubling of the amplicon. Because of substrates reduction and product inhibition, the reaction will slow down and stop, and enter the *plateau phase***.** Compounded variation during the linear phase can lead to large differences in the final amount of product and each replicate reaction can plateau at different points due to different reaction kinetics unique to each sample. Figure 17 represents the three real-time PCR reaction phases and the variations of these phases for each sample.

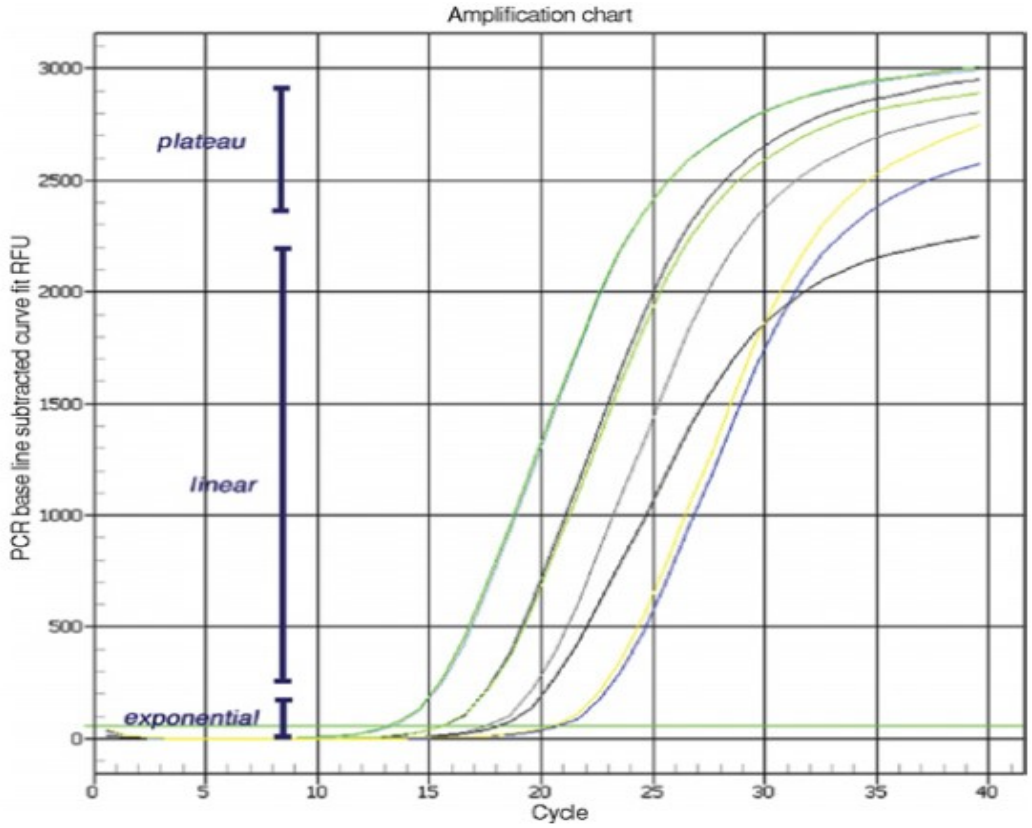


Figure 17: PCR reaction phases, the x-axis: cycle number, y-axis: amount of DNA (source: Fraga D., Tea Meulia T., and Fenster S. "Real-Time PCR" Curr. Protoc. Essential Lab. Tech. (2008)10.3.1-10.3.34.).

Real-time PCR measures the amount of amplicon produced during each amplification cycle using fluorescence-based technology and can quantify amplicon production at the exponential phase of the PCR reaction in contrast to measuring the amount of product at the end-point of the reaction. The amplicon is monitored by labeling the accumulating product with a fluorescently tagged substrate during the amplification procedure. In real-time PCR reaction, a fluorescent reporter molecule such as a double-stranded DNA- binding dye is used to monitor the progress of the amplification reaction. With each amplification cycle, the increase in fluorescence intensity is proportional to the increase in amplicon concentration; with the qPCR instrument system, the data for each sample during each PCR cycle will be collected. The resulting plots of fluorescence vs. cycle number for all the samples are then set with their background fluorescence at a common starting point (a process known as baseline correction). Then, a threshold level of fluorescence is set above the background but still within the linear phase of amplification for all the plots. The cycle number where the amplification plot crosses this threshold, the fluorescence

level is called the "Ct" or threshold cycle. This Ct value can be directly related to the starting target concentration of the sample. The greater initial DNA template amount in the sample, the earlier Ct value for that sample [186, 189, 190]. DNA binding dyes, like SYBR Green I and EvaGreen® dyes, these dyes are cost-effective and easy to use. When such dyes are free in solution, they display comparatively low fluorescence, but when bound to double-stranded DNA, their fluorescence increases by over 1000- fold. As the double-stranded DNA increases, the dye binding sites will increase. This dye property provides the mechanism that allows it to be used to track the PCR product accumulation. As the target DNA is amplified, it's increasing concentration in the solution directly proportion to the fluorescence, it can be directly measured by the increase in fluorescence signal; SYBR® Green I is 1000-fold more fluorescent in the bound state than in the unbound state. As PCR amplification increases the amount of dsDNA present, the fluorescence signal increases proportionately. Figure 18 represents bounded and unbounded SYBR Green I detection mechanism.

Figure 18: SYBR Green I detection mechanism (source: Agilent Technologies "Introduction to Quantitative PCR: Methods and Applications Guide" (2016).)

The results are calculated as "relative quantity to the calibrator", where the calibrator sample is assigned an arbitrary quantity of "1" and all the other samples are expressed in terms of their fold difference to this sample. The earliest approximation method of comparative quantitation ΔΔCt is used for this purpose; it utilizes the equation [189, 191]:

Relative quantity to the calibrator = $2^{-\Delta\Delta Ct}$

Where $\Delta\Delta Ct = (Ct_{GOI} - Ct_{norm})$ unknown $- (Ct_{GOI} - Ct_{norm})$ calibrator

GOI refers to the gene of interest (tested gene), norm refers to the reference gene

The ΔCt values calculation is illustrated in Figure 19.

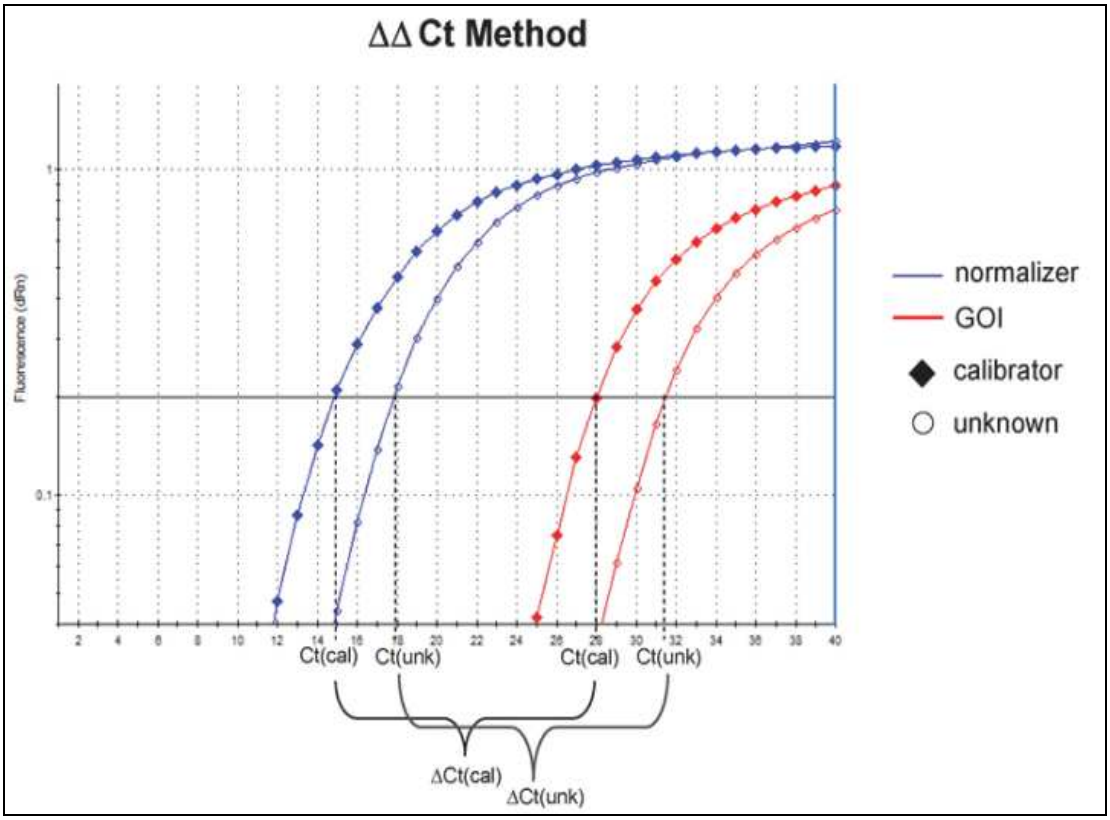


Figure 19: The Calculation of ΔCt using ΔΔCt method, where ΔCt is determined by calculating the difference between the Ct of the normalizer and Ct of the GOI for each sample (source: Agilent Technologies "Introduction to Quantitative PCR: Methods and Applications Guide" (2016).).

A quantitative real time polymerase chain reaction and comparative cycle threshold (Ct) method was performed for *ErbB2* and *c-MYC* quantitation [192]. Maxima SYBER Green qPCR Master Mix (Thermo-Fisher Scientific, Waltham, MA, USA) was used following the manufacturer's instructions and 20ng of DNA was added as a template in each reaction. All qPCR experiments were performed in duplicate. The primer sequences, amplicon lengths and annealing temperatures are listed in Table 8. To confirm the specificity of the amplified products, a melting curve analysis was performed in each case and the Ct value was calculated for each sample and the amplification levels were calculated as $2^{-\Delta\Delta Ct}$. A single copy gene encoding the dopamine D2

receptor (*D2R*) was used as a reference gene, to normalize the amplification levels of *ErbB2* and *c-MYC*. In order to avoid any false positives, a gene dose greater than 3.5 was considered as amplification.

4.2.5. Array comparative genomic hybridization analysis

In the present study, array comparative genomic hybridization (CGH) analysis was performed commercially in the Institute of Medical Genetics, Academic Hospital of Udine, Italy. In brief, CGH is a molecular cytogenetic method for analysing copy number variations (CNVs) in the DNA of a test sample compared to a reference sample. This is achieved through the use of competitive fluorescence in situ hybridization and involves the isolation of DNA from the two sources to be compared, and a reference source. Each DNA sample is labeled with fuorescent molecules of different colours, usually red and green, followed by the denaturation of the DNA, and the hybridization of the two resultant samples in a 1:1 ratio to a normal metaphase spread of chromosomes, to which the labeled DNA samples will bind. Using fluorescence microscope and computer software, the differentially coloured fluorescent signals are then compared along the length of each chromosome for identification of chromosomal differences between the two sources. A higher intensity of the test sample colour in a specific region of a chromosome indicates the gain of material of that region in the corresponding source sample, while a higher intensity of the reference sample colour indicates the loss of material in the test sample in that specific region. A neutral colour (yellow if the fluorophores are red and green) indicates no difference between the two samples in that location.

In array CGH, a sensitive, fast and highthroughput technique, the metaphase chromosomes are replaced by cloned DNA fragments (+100–200 kb) of which the exact chromosomal location is known, thus allowing the detection of CNV in more detail and making possible the detection of copy number changes of 5-10 kb. It has been successfully applied in the identification of new and recurrent microdeletions and duplications in birth defects, but also in cancer.

Array CGH is based on the same principle as conventional CGH. DNA from a control sample and DNA from a patient sample are differentially labelled with two different fluorophores (Cy3

and Cy5, usually) and equal quantities of the two DNA samples are cohybridized competitively onto oligonucleotide targets. This is followed by digital capturing and quantification of the relative fluorescence intensities of each of the hybridized fluorophores. If there is an altered Cy3:Cy5 ratio this indicates a loss or a gain of the patient DNA at that specific genomic region (Figure 20).

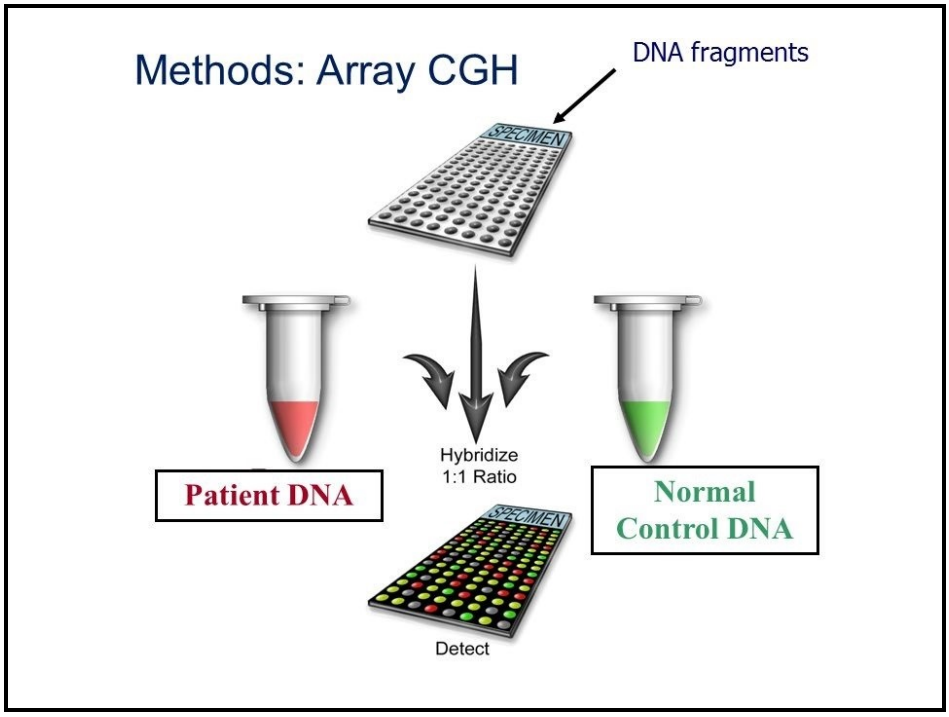


Figure 20: Array CGH analysis; hybridization of differentially labeled test (patient) and reference (normal control) DNA. (source: http://www.utoronto.ca/cancyto).

CGH analysis was performed on 5 patients only. Two samples from each patient were used for DNA extraction - a tumor and a margin sample. Genomic DNA from a healthy donor was used as reference.

4.3.Statistical analysis

Statistical Package for Social Science (SPSS software package, version 17.0; SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Pearson's chi-squared test (χ^2) and Fisher's exact test were performed for association studies. Kaplan–Meier analysis was used for survival estimation. Survival curves were compared by log-rank test. All statistical tests were two-sided and P values of <0.05 were considered statistically significant.

5. Results

5.1. Distribution of genetic and epigenetic alterations in tumors, tumor margins and healthy mucosa

Our results show a statistically significant difference in gene alteration distribution between tumors, tumor margins, and swabs for all markers analyzed, with tumors showing the highest prevalence of alterations and swabs the lowest. All the main data are summarized in Table 9.

Table 9. Frequency of alterations in tumors, margins and swabs

	N	*HER2* amplification	P	*c-MYC* amplification	P	*P14* methylation	P	*P16* methylation	P
Tumor	40	10		9		36		31	
Margin	40	4	0.034	4	0.023	32	0.004	28	<0.001
Swab	40	3		2		25		14	

When considering all the three types of samples together, the most frequent alteration was *P14* methylation. *P14* was methylated in 36 samples (90%) of tumors, in 32 samples (80%) of margins, and in 25 samples (62.5%) of healthy mucosa. The average incidence of *P14* methylation was 77.5%. There was a significant difference in *P14* methylation frequency between tumors and healthy oral mucosa (Figure 21).

P16 methylation was the following most frequent molecular event. *P16* was methylated in 31 samples (77.5%) of tumor tissues, in 28 samples (70%) of margin tissues, and in 14 samples (35%) of healthy mucosa swabs. The average frequency of *P16* methylation was 61.7%. There

was a statistically significant difference in *P16* methylation percentage between tumor and margins, and tumors and healthy mucosa (Figure 21).

HER2 amplification was found in 10 samples (25%) of tumors, in 4 samples (10%) of margins, and in 3 samples (7.5%) of healthy mucosa; its average frequency was 14.16%. There was a statistically significant difference between *HER2* amplification in tumors and mucosa swabs (Figure 21).

Finally, the least frequent molecular alteration was *c-MYC* amplification. *c-MYC* was amplified in 9 samples (22.5%) of tumors, in 4 samples (10%) of marginal tissue, and in 2 samples (5%) of normal mucosa, the average frequency of amplification being 12.5%. The difference in percentage of *c-MYC* amplification between tumors and mucosa samples was statistically significant (Figure 21).

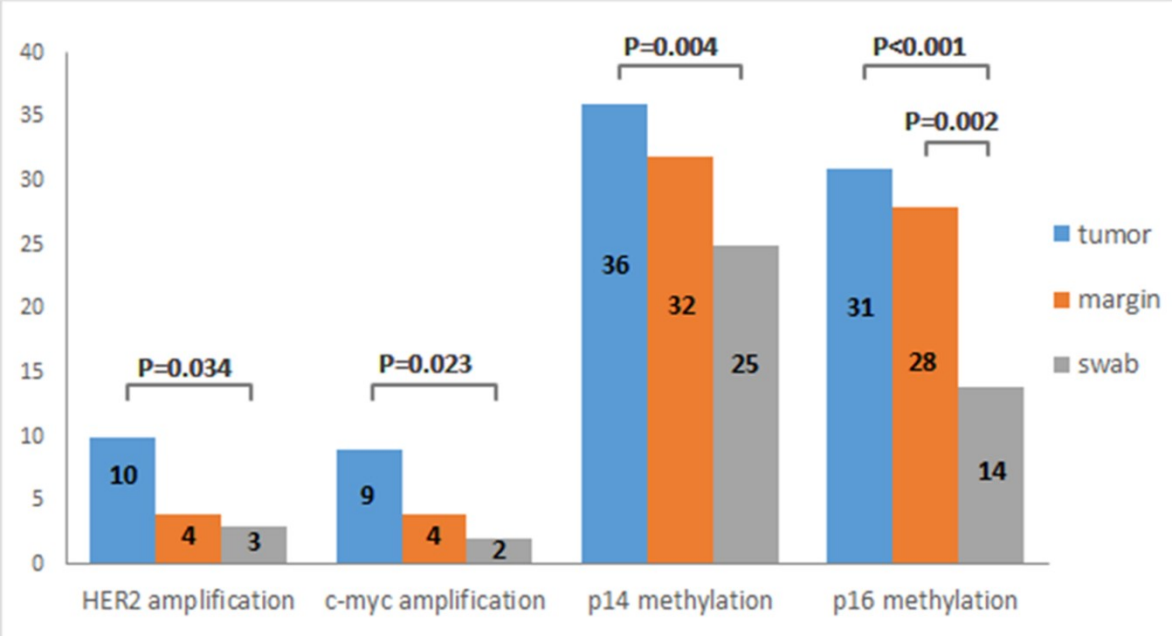


Figure 21: Distribution of genetic/epigenetic alterations in tumor, margin and swab samples.

5.2. Co-alterations in tumors, tumor margins, and swab samples

Molecular co-alterations in tumor, margin and mucosa swab samples were observed. The average co-alterations number in tumors was 2.15 per sample; 10 samples (25%) were with no or one alteration while 30 samples (75%) were with two or more alterations. The average co-

alterations number in margins was 1.70 per sample; 17 samples (42%) were with no or one alteration while 23 samples (58%) were with two or more alterations. Finally, in normal mucosa swab samples the average co-alterations number was 1.13 per sample; 30 samples (75%) were with no or one alteration, while 10 samples (25%) were with two or more alterations. The differences between tumors and margins, as well as between tumors and swabs were statistically significant (P<0.001 and P=0.027, respectively). The co-alterations in the three tissue types are illustrated in Figure 22. Five patients had all four alterations in their tumor samples, while none of the swabs harbored all of these genetic/epigenetic alterations.

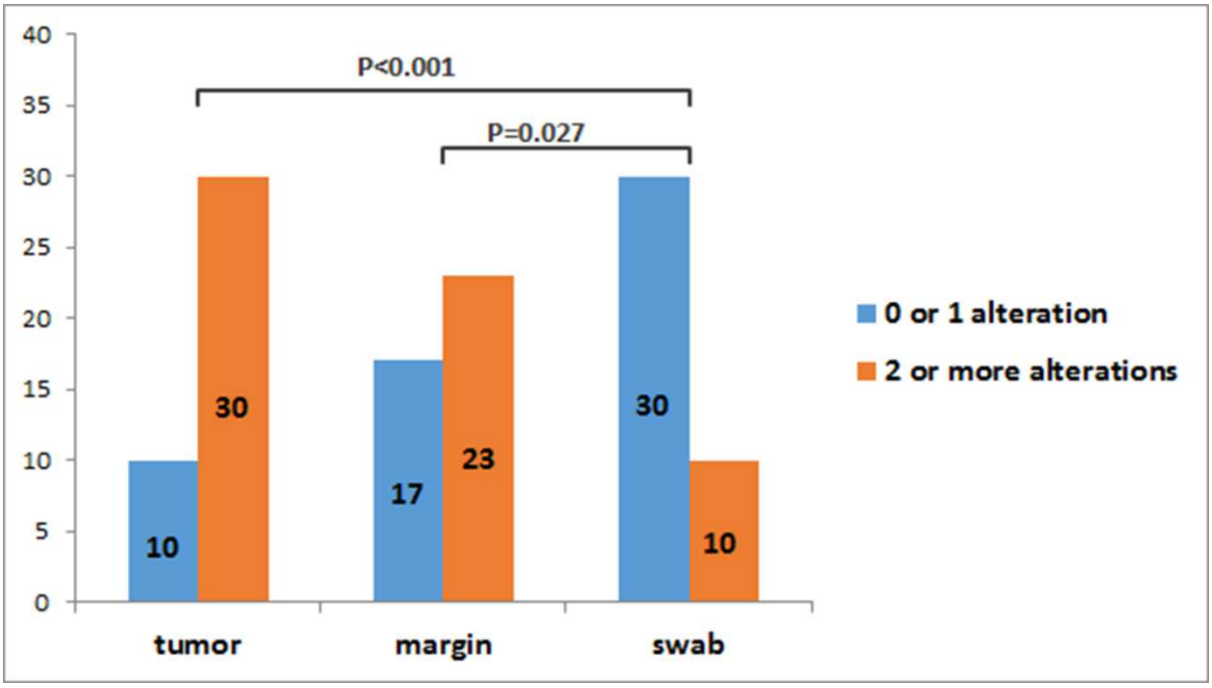


Figure 22: Co-alterations in tumor, margin and swab samples.

When considering the alterations simultaneously in tumor, tumor margin and healthy mucosa samples, *P14* methylation was found most frequently; methylation was found in the three tissues (tumors, margins, and mucosa) of 25 patients (62.5%); methylation in tumors and margins was found in 7 patients (17.5) and methylation only in the tumor tissues was found in 4 patients (10%). Four patients (10%) had no *P14* methylation in any of the tissues.

P16 methylation was found in the three tissues (tumors, margins and mucosa) of 14 patients (35%); methylation in the tumor and margin tissues was found in 14 patients (35%), and in the

tumor tissue only in 3 patients (7.5%). In 9 patients (22.5%) no methylation in any of the tissues could be detected.

HER2 amplification was found in all three tissues of 3 patients only (7.5%). *HER2* was amplified in the tumor tissue of 6 patients (15%), in the tumor and margin tissues of one patient (2.5%). There was no *HER2* amplification in the tissues of 30 patients (75%).

c-MYC amplification was found in the three tissues of 2 patients (5%), in the tumor and margin tissues of 2 patients (5%) and only in the tumor tissues of 5 patients (12.5%). Thirty one patients (77.5%) had no *c-MYC* amplification in any of the tissues. The distribution of these alterations in the three tissue types is given in Figure 23.

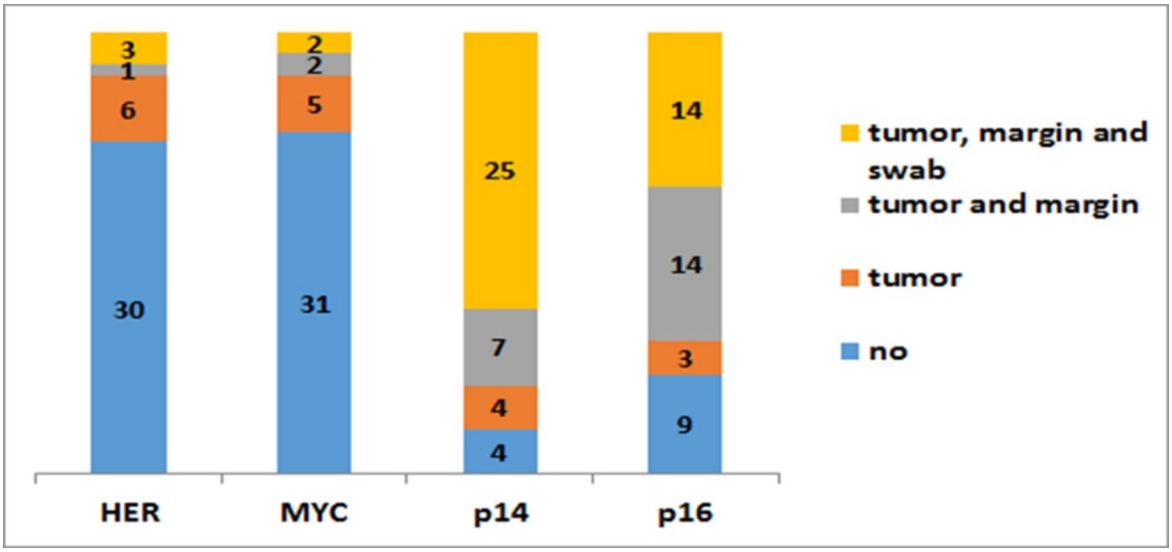


Figure 23: The distribution of the gene alterations in the three tissue types.

5.3. Molecular alterations and clinical and histopathological data

According to our results, a clear trend of an increasing number of alterations with increasing clinical stage and histological grade was observed for all the studied markers except *P14*, and in all three types of patient specimens, but without reaching statistical significance (Table 10). Interestingly, the only exception was the association between *P14* methylation and lower histological grades and clinical stages. *P14* methylation of mucosa samples of OSCC shows an association with lower clinical stages (p=0.033).

Table 10. The association between the presence of gene amplification (*HER2, c-MYC*) and methylation (*P14* and *P16*) and the clinical stage and histological grade

		Low stage (%)	High stage (%)	P value	Low grade (%)	High grade (%)	P value
TUMOR	*HER2*	22.7	27.8	0.497	16.7	28.6	0.355
	c-MYC	13.6	33.3	0.135	16.7	25	0.447
	P14	90.9	88.9	0.617	100	85.7	0.224
	P16	68.2	88.9	0.118	66.7	82.1	0.249
MARGIN	*HER2*	4.5	16.7	0.230	8.3	10.7	0.654
	c-MYC	9.1	11.1	0.617	8.3	10.7	0.654
	P14	90.9	66.7	0.065	91.7	75	0.225
	P16	68.2	72.2	0.529	66.7	75	0.521
SWAB	*HER2*	4.5	11.1	0.423	8.3	7.1	0.668
	c-MYC	0	11.1	0.196	0	7.1	0.485
	P14	77.3	44.4	0.035*	66.7	60.7	0.505
	P16	31.8	38.9	0.446	16.7	42.8	0.108

Low stage - stage I and II; high stage – III and IV, low grade – grade I; high grade – grade II and III

5.4. The association of molecular alterations and overall survival

The evaluation of 3-year survival rates with Kaplan – Meier analysis and log-rank test, showed a statistically significant association between multiple alterations in swabs and patient's survival. It was shown that patients with 3 alterations in swab samples had a significantly shorter survival (*P*=0.027). The Kaplan-Meier survival curves for patients with multiple alterations are shown in Figure 24.

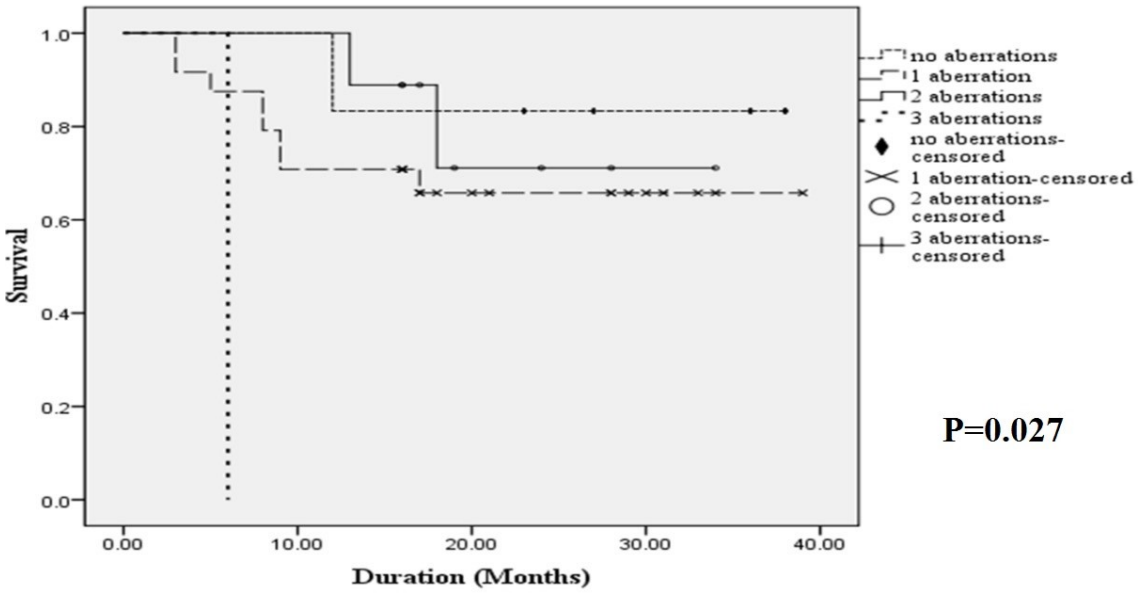


Figure 24. The association between multiple alterations in swab samples and survival.

There was no association between survival and multiple alterations in tumors and margins (*P*=0.635 and *P*=0.158, respectively). The association of survival with multiple alterations in tumors and margins is shown in Figure 25a and Figure 25b.

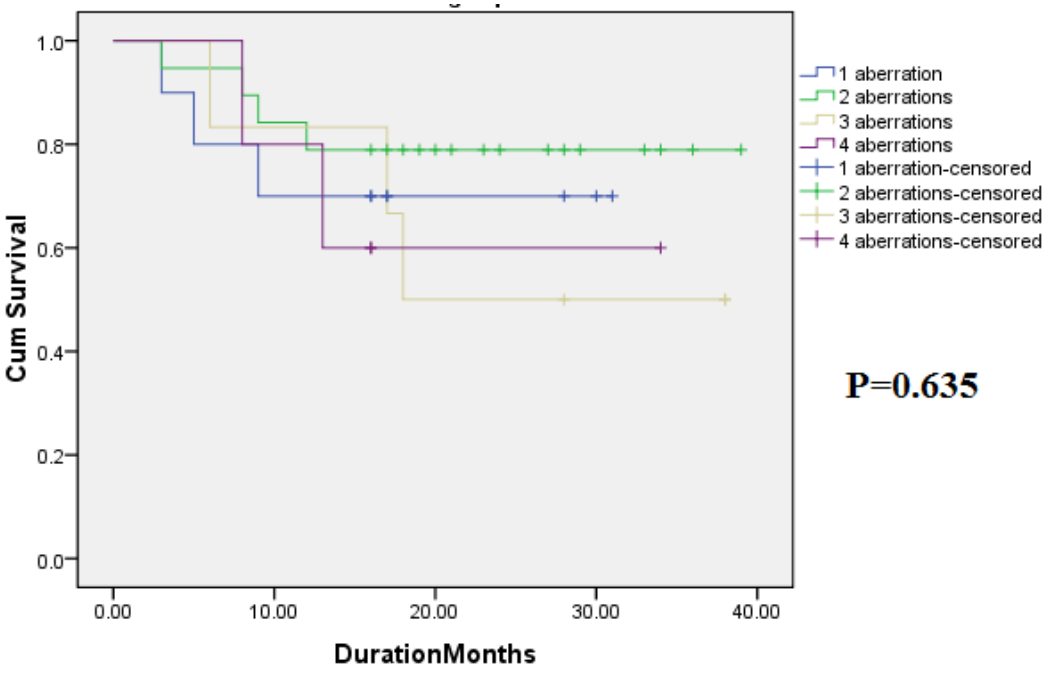


Figure 25a: The association between multiple alterations in tumor samples and survival.

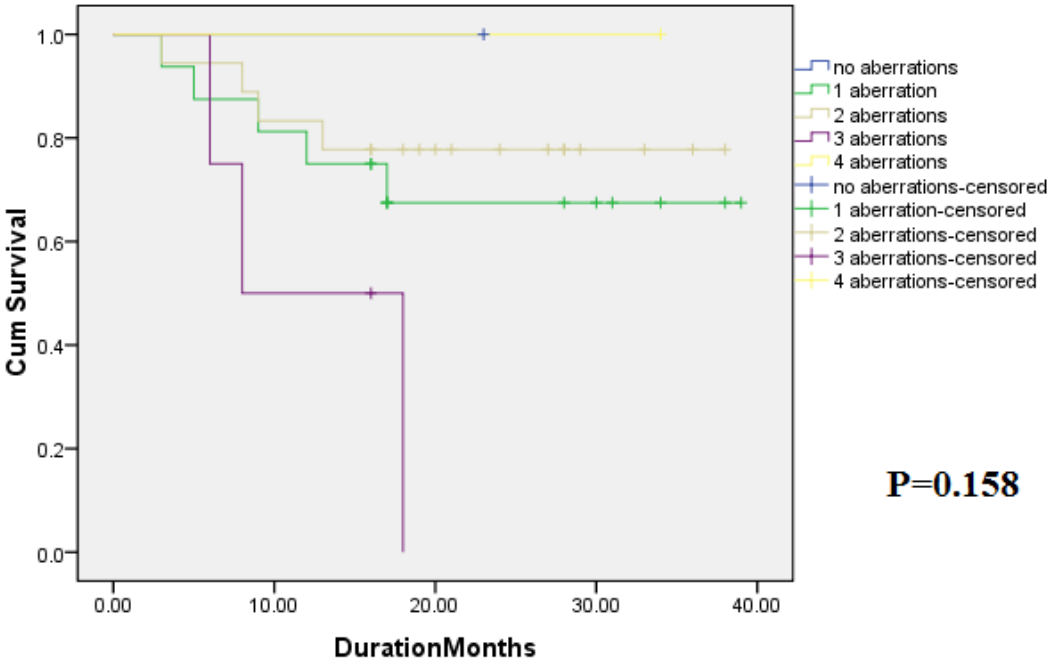


Figure 25b: The association between multiple alterations in margin samples and survival.

The same analysis was used to evaluate the association between survival and the alterations of the studied markers in the three tissue types. Taken altogether, there was no significant association between survival and the alterations of *c-MYC*, *HER2*, *P14* and *P16*. *P* values were 0.235, 0.078, 0.341, and 0.238 respectively. The association between survival and the alterations of the *c-MYC*, *HER2*, *P14*, and *P16* in all the tissue samples are given respectively in Figure 26a, Figure 26b, Figure 26c and Figure 26d.

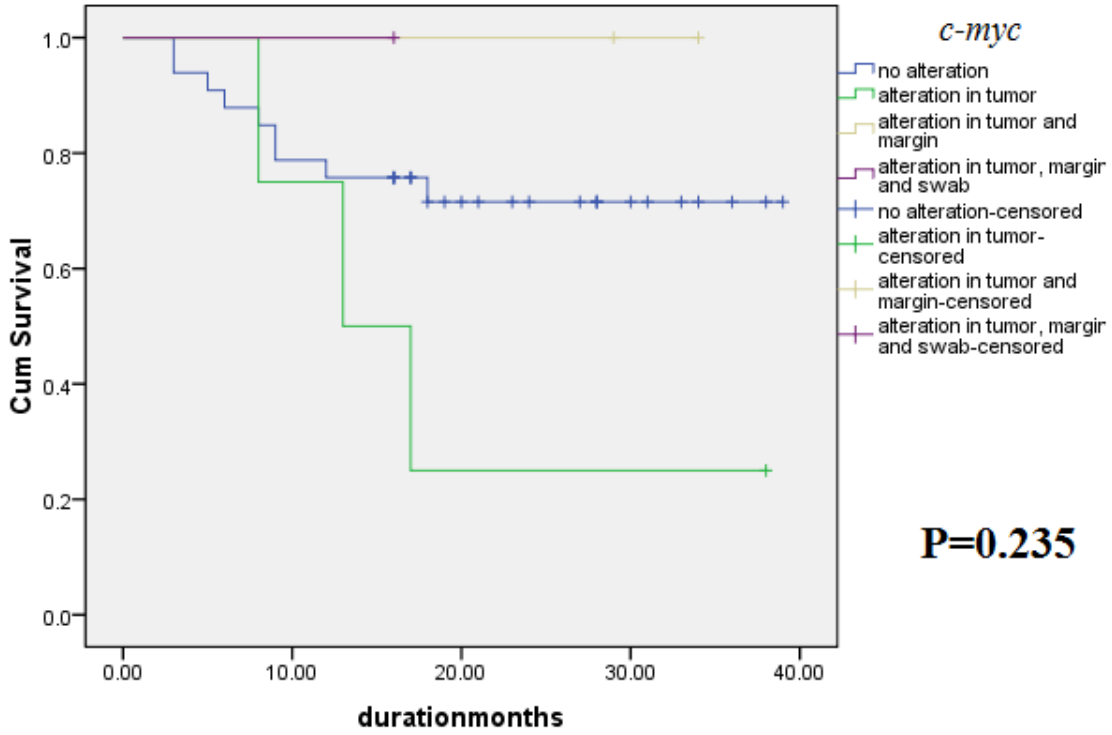


Figure 26a: The association between survival and the alterations of *c-MYC* in the tissue samples.

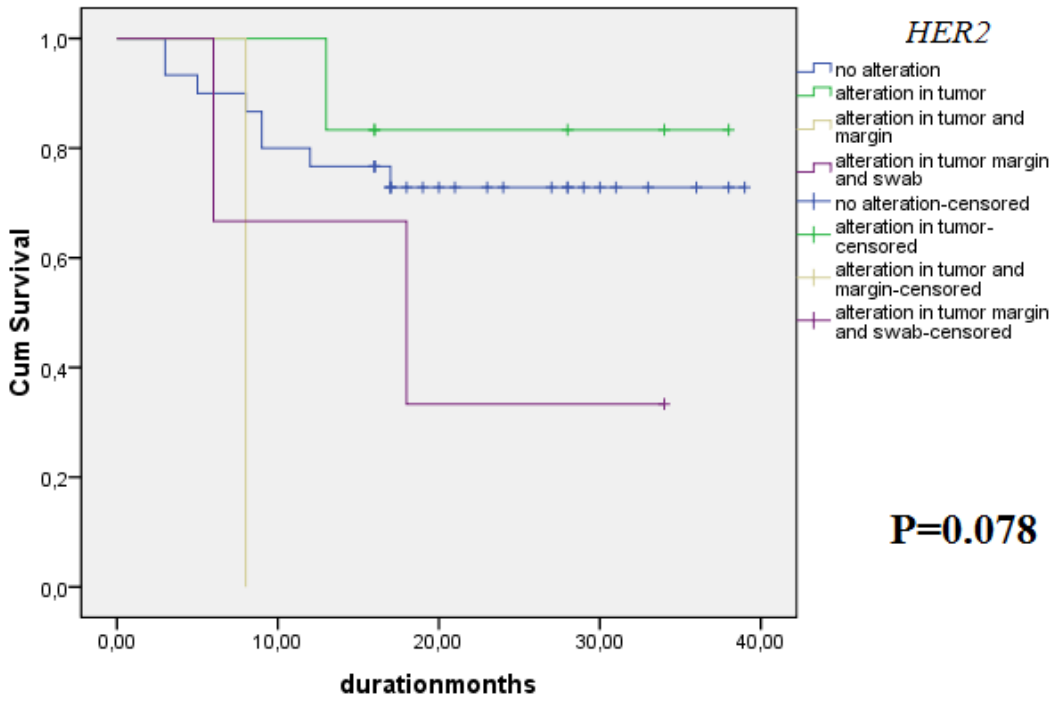


Figure 26b The association between survival and the alterations of *HER2* in the tissue samples.

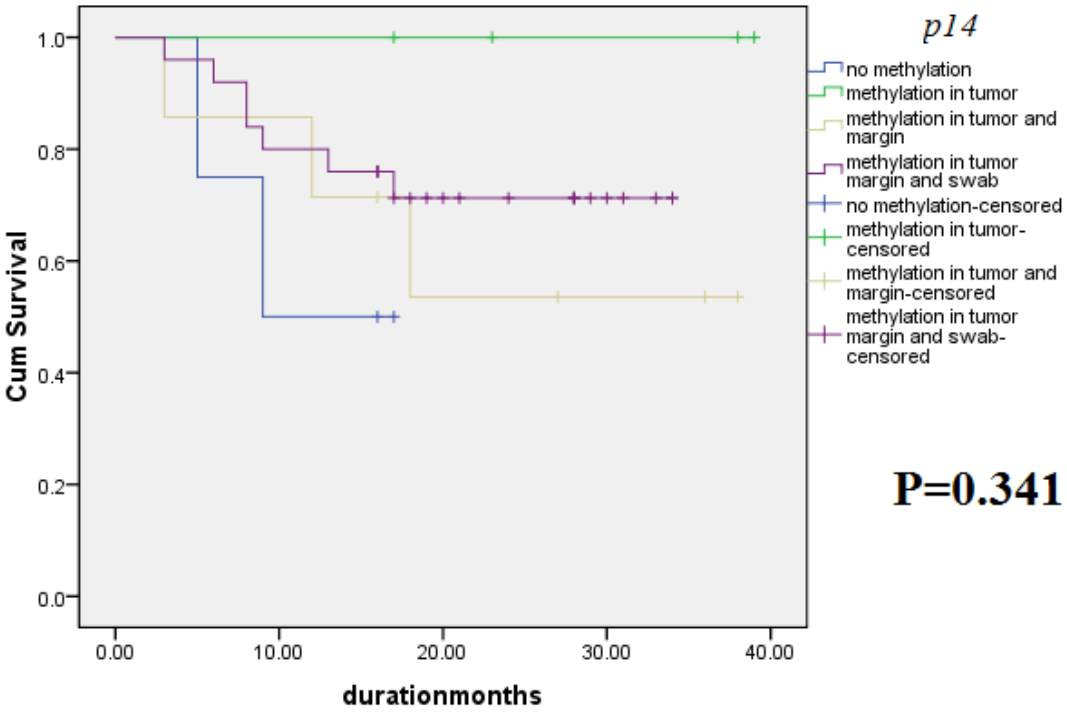


Figure 26c the association between survival and the methylation of *P14* in the tissue samples.

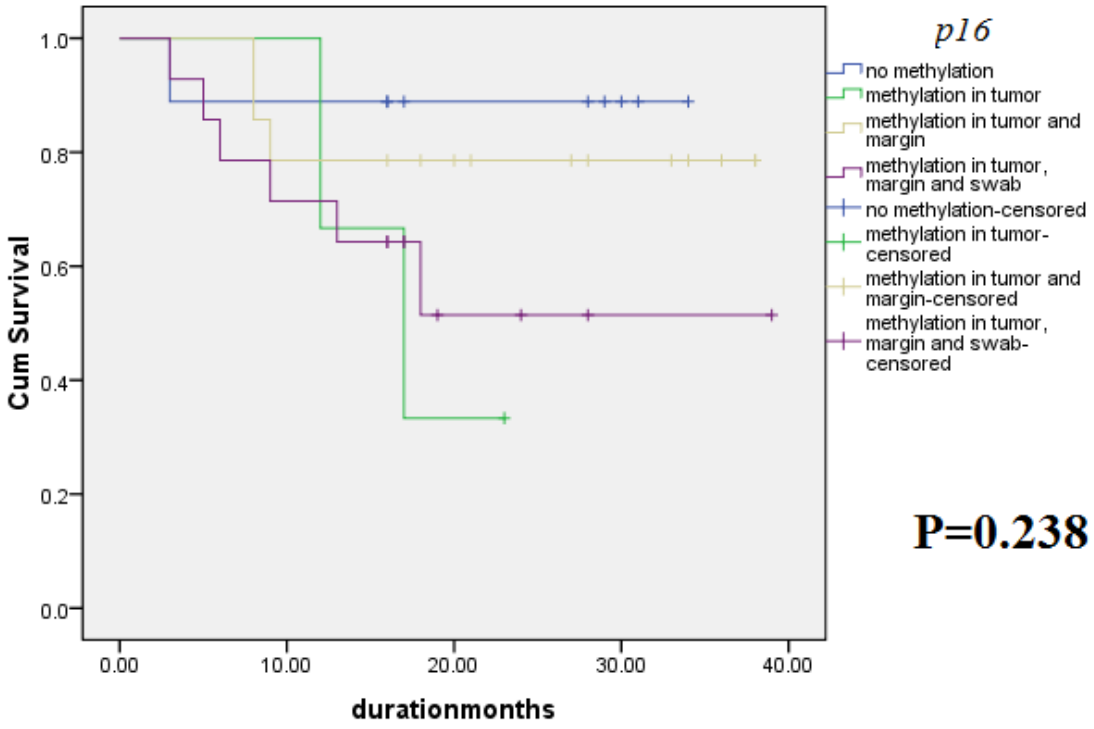


Figure 26d: The association between survival and the methylation of *P16* in the tissue samples.

However, when considering separately different genes and different types of tissues, a significant association between the amplification of *HER2* in margin samples and decreased survival was observed ($P = 0.035$) (Figure 27).

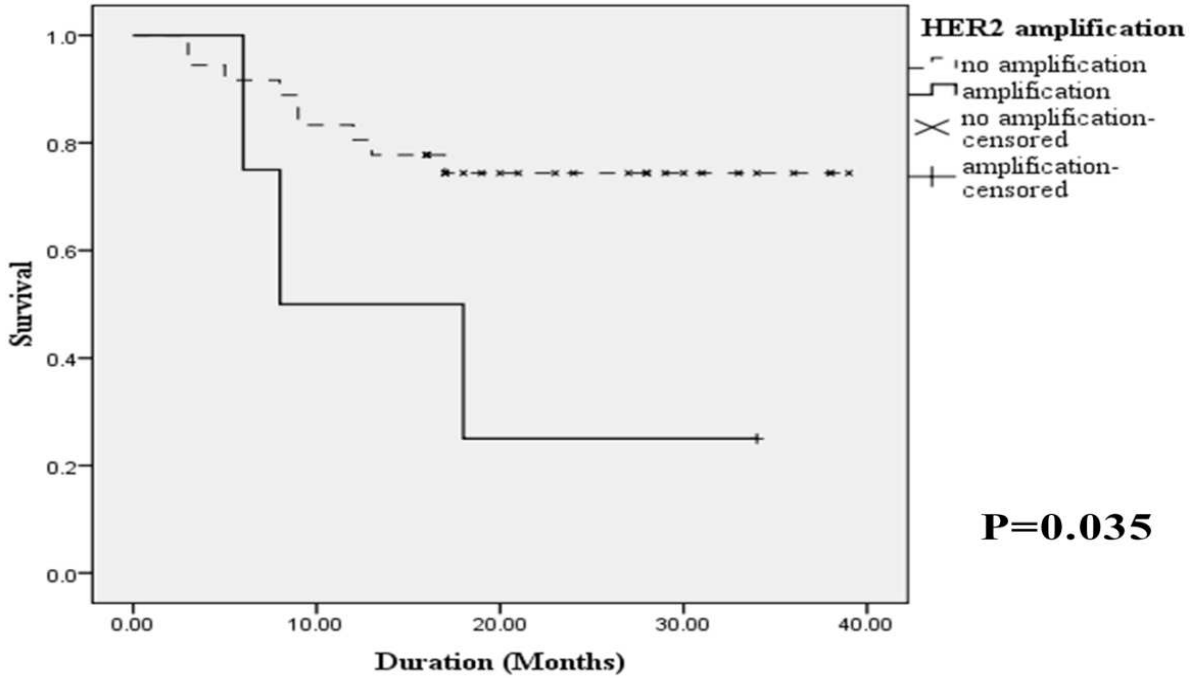


Figure 27: The association between *HER2* amplification in margin samples and decreased survival

There was no association between survival and *HER2* amplification in tumor and swab samples (*P*=0.478, and *P*=0.187, respectively).

Also, there was no significant association between survival and *c-MYC* amplification in tumor, margin and swab samples (*P*=0.841, *P*=0.223, and *P*=0.436, respectively)

There was no significant association between survival and *P14* methylation in tumor, margin, and swab samples (*P*=0.219, *P*=0.811, and *P*=0.708, respectively)**.** Similarly, no association of *P16* methylation in the tumor, margin, and swab samples with survival could be established neither (*P*=0.220, *P*=0.652, and *P*=0.163, respectively).

5.5. Molecular alterations and disease specific survival

Using Kaplan-Meier analysis with long-rank test, the 3-year disease-specific survival rates were also calculated and a highly significant association between shorter survival and *HER2* amplification in margin samples (P<0.001) and swabs (P=0.013) was found.

Simultaneous *HER2* amplification in all three patients' samples was also correlated with poor survival (P < 0.001), as showed in **Figure 28a**, **Figure 28b** and **Figure 28c**.

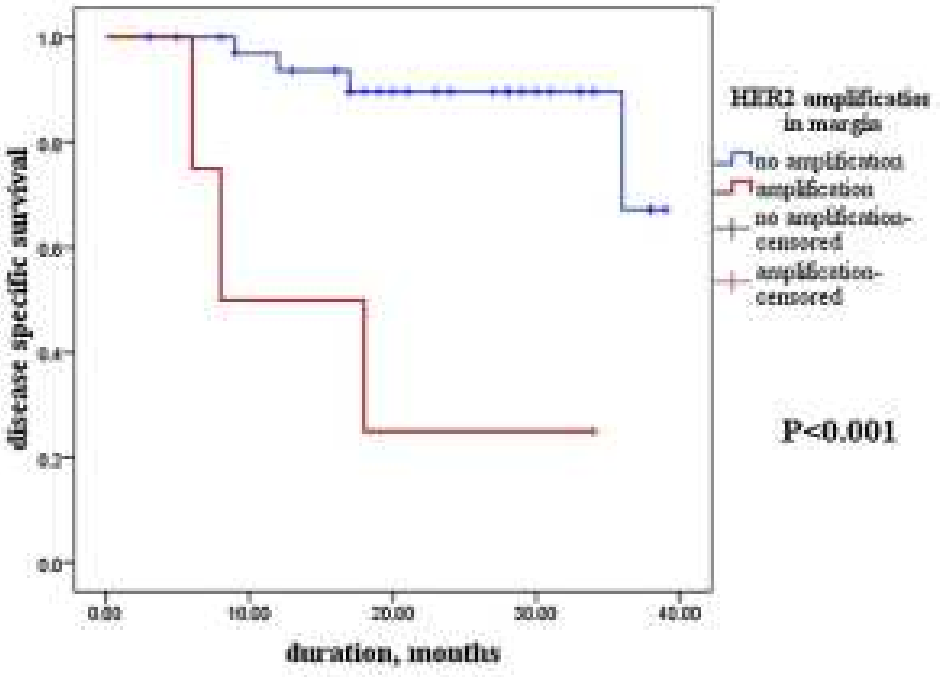


Figure 28a: The association of disease specific survival with *HER2* amplification in tumor margin samples.

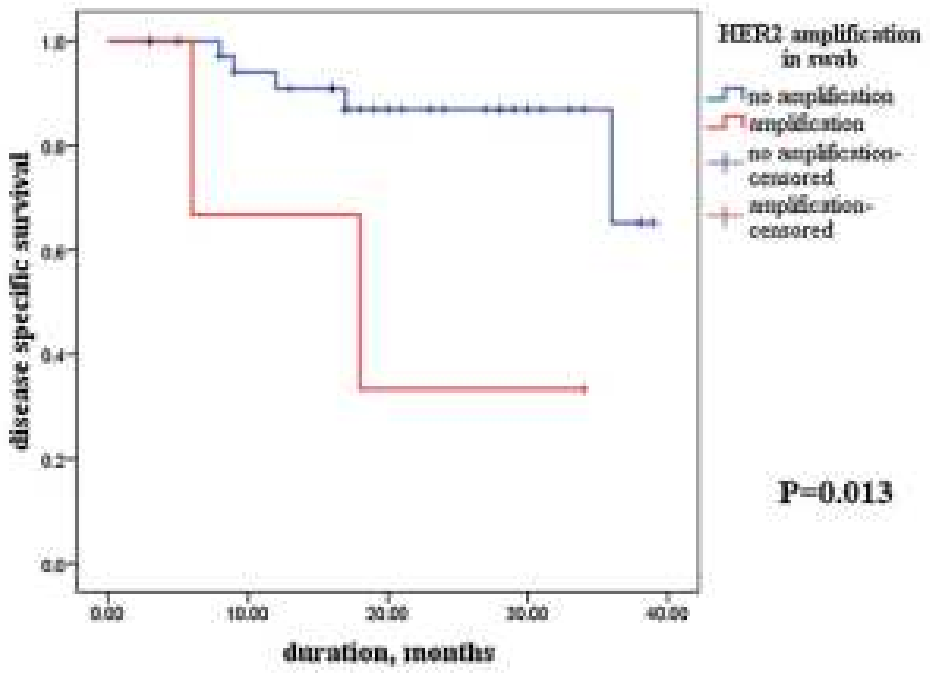


Figure 28b: The association of disease specific survival with *HER2* amplification in healthy mucosa samples.

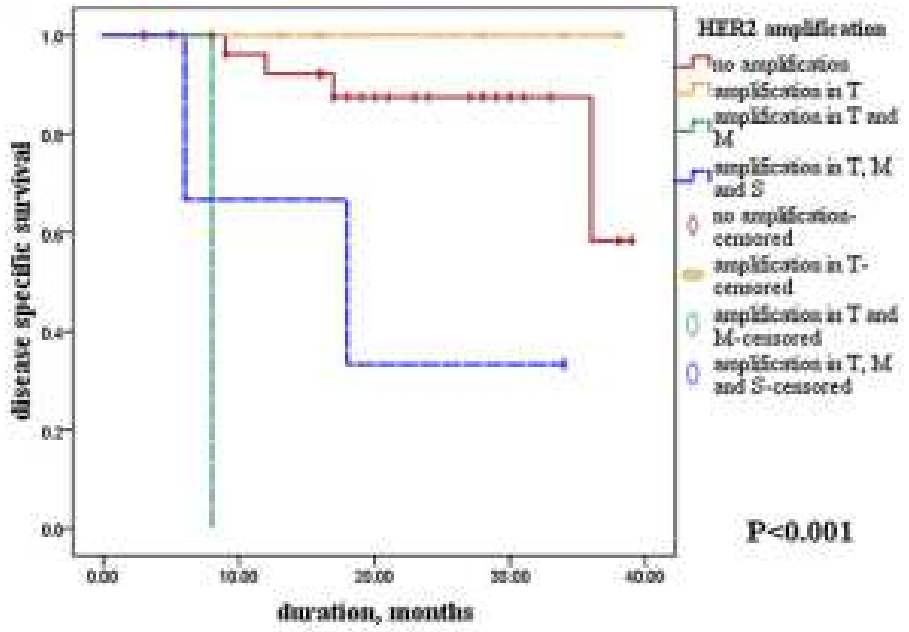


Figure 28c: The association of disease specific survival with the simultaneous *HER2* amplification in all three samples.

No significant disease-specific survival decrease was observed in association with *c-MYC* amplification, *P14* or *P16* methylation.

However, there was a significant association between decreased survival and the presence of more than two alterations in margin (P=0.001) and swab samples (P<0.001). These findings are given in Figure 29a and Figure 29b.

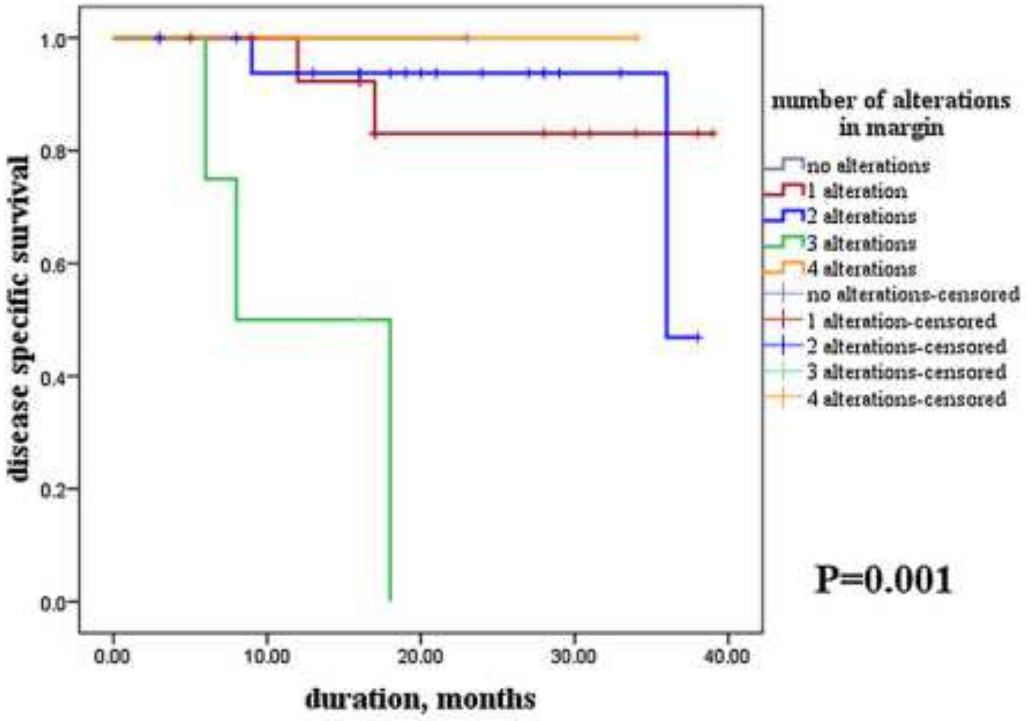


Figure 29a: The relation of disease-specific survival with the presence of more than two alterations in tumor margin.

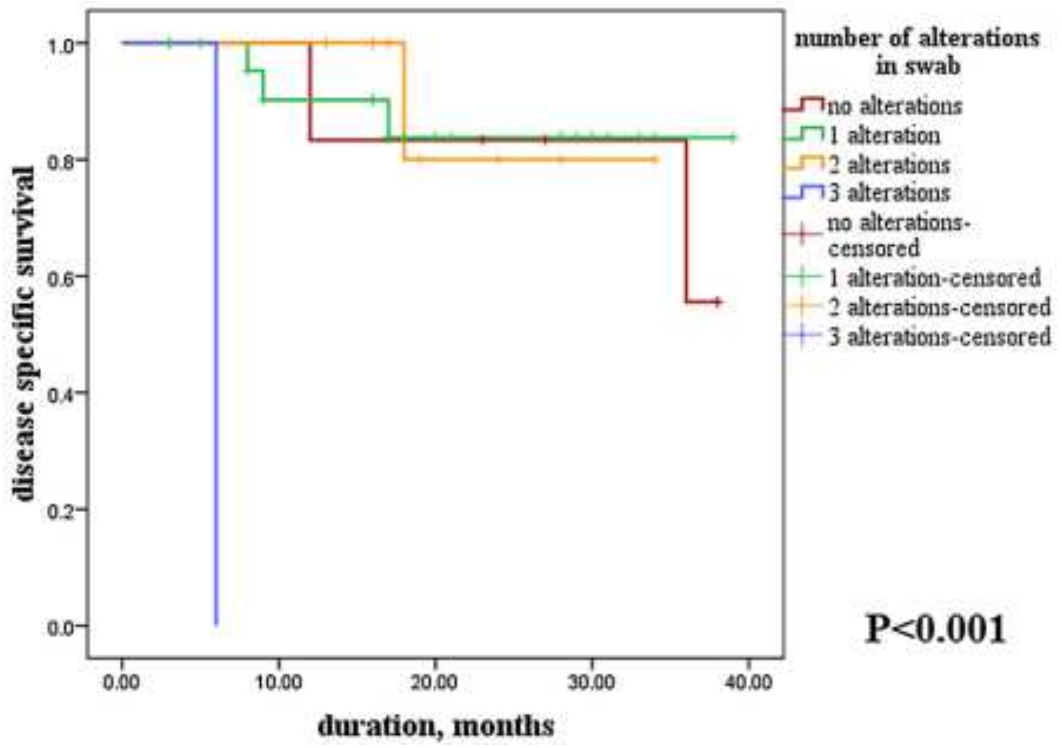


Figure 29b: The relation of disease-specific survival with the presence of more than two alterations in swab samples.

5.6. Molecular alterations and epidemiological and etiological factors

Interestingly, we were not able to establish a significant association between various epidemiological factors (age, sex, smoking and drinking status) and molecular findings. The lack of a strong relationship between these parameters and molecular changes is probably due to a relatively small number of patients, but also potential inaccuracy of the data obtained during patients interviewing and charting (denial of smoking or alcohol drinking, for instance).

As one of recently uncovered risk factors, with relatively high impact on OSCC development in the younger population human papilloma virus type 16 was also tested. Out of 40 samples, HPV16 was detected in eight samples (20%). Four of them were lip tumors and four were oropharyngeal tumors. In these eight samples, *HER2* amplification was found in two cases, and *c-MYC* amplification was found in one case only. *P14* and *P16* were hypermethylated in the majority of HPV-positive cases. Six out of eight HPV positive patients showed *P14* and *P16* hypermethylation at the same time. There was no statistically significant association between HPV infection and any of the molecular alterations ($P>0.05$).

5.7. Array CGH analysis

Deletions and duplications were found in all the analyzed samples. A high heterogeneity was also observed. Namely, most aberrations were present in single patients. Though significant differences between tumor and margin were present, several variants seemed to be characteristic for both the tumor and the corresponding margin. The differences between the genomes originating from tumor and from margin are illustrated in Figure 30a, Figure 30b and Figure 31.

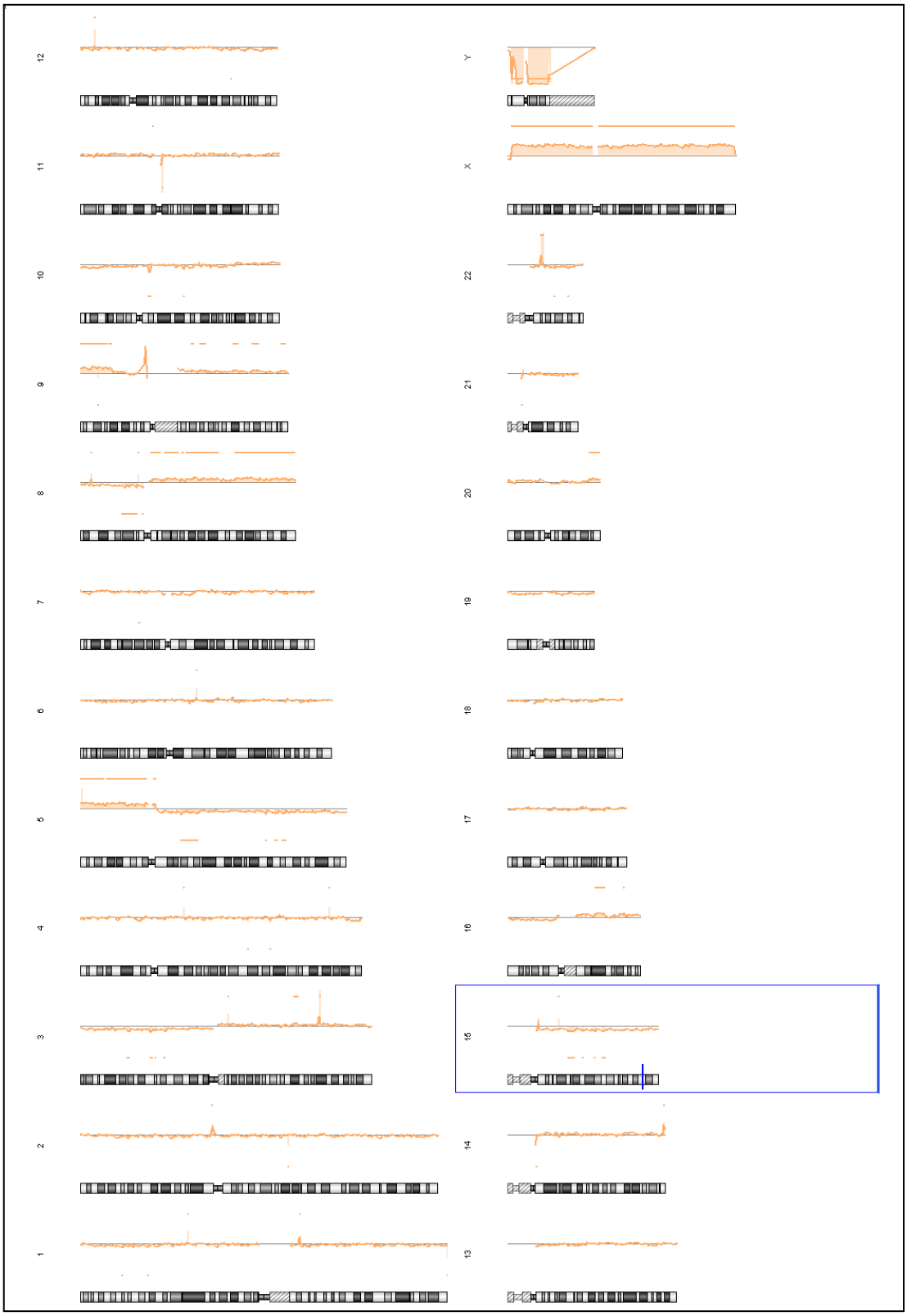


Figure 30a: Comparative representations of deletions and duplications in margin tissues versus a normal reference DNA.

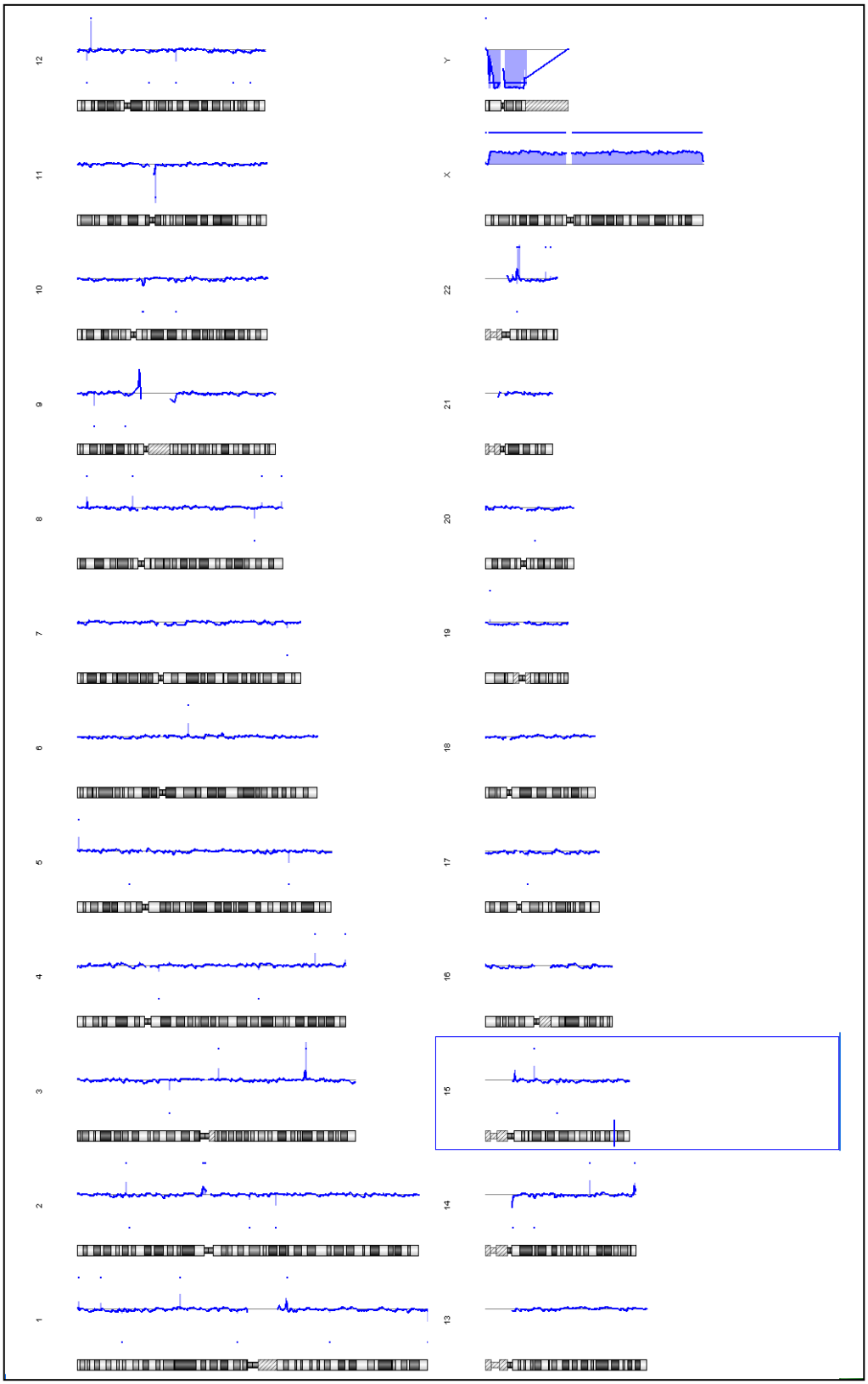
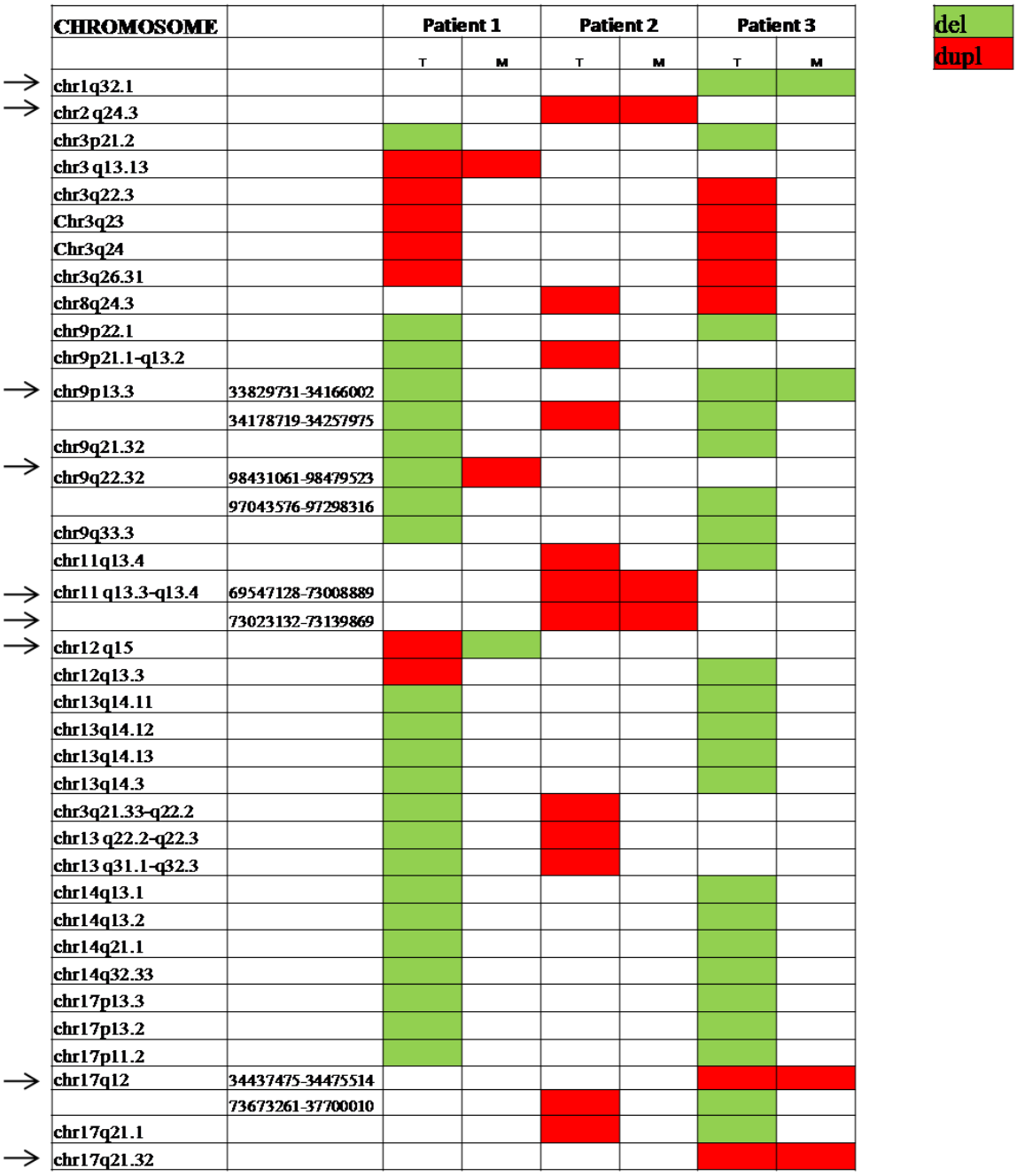


Figure 30b: Comparative representations of deletions and duplications in tumor tissues versus a normal reference DNA.



	CHROMOSOME		Patient 1		Patient 2		Patient 3	
			T	M	T	M	T	M
→	chr1q32.1							
→	chr2 q24.3							
	chr3p21.2							
	chr3 q13.13							
	chr3q22.3							
	Chr3q23							
	Chr3q24							
	chr3q26.31							
	chr8q24.3							
	chr9p22.1							
	chr9p21.1-q13.2							
→	chr9p13.3	33829731-34166002						
		34178719-34257975						
	chr9q21.32							
→	chr9q22.32	98431061-98479523						
		97043576-97298316						
	chr9q33.3							
	chr11q13.4							
→	chr11 q13.3-q13.4	69547128-73008889						
→		73023132-73139869						
→	chr12 q15							
	chr12q13.3							
	chr13q14.11							
	chr13q14.12							
	chr13q14.13							
	chr13q14.3							
	chr3q21.33-q22.2							
	chr13 q22.2-q22.3							
	chr13 q31.1-q32.3							
	chr14q13.1							
	chr14q13.2							
	chr14q21.1							
	chr14q32.33							
	chr17p13.3							
	chr17p13.2							
	chr17p11.2							
→	chr17q12	34437475-34475514						
		73673261-37700010						
	chr17q21.1							
→	chr17q21.32							

Figure 31: Comparison of anomalies in tumor and margin tissues in three patients. Red colour indicates the duplications and the green colours shows deletions. Only anomalies common between tumor and margin and anomalies common in tumors of at least tow different patients are presented.

In nine regions in total, margin and tumor tissues showed significant similarities in terms of detected anomalies. The regions and the genes of potential interest within those regions are given in Table 11.

Table 11. Nine common regions between tumors and margins and the genes within the regions

Chromosomes	**Genes**
Chr1q32.1	TIMM17A; ERT
Chr2q24.3	GRB14; COBLL1; SLC38A11; SCN3A; SCN2A; CSRNP3; GALNT3; TTC21B; SCN1A; SCN9A; SCN7A; XIRP2; B3GALT1; STK39;
Chr3q13.13	unknown
Chr9p13.3	UBE2R2; UBAP2; DCAF12;
Chr9q22.32	unknown
Chr11q13.3-q13.4	FGF4; FGF3; ANO1; FADD; PPFIA1; CTTN; SHANK2; DHCR7; NADSYN1; KRTAP5-9; KRTAP5-10; RNF121; IL18BP; NUMA1; LRTOMT; FOLR3; FOLR2; INPPL1; PHOX2A; CLPB; PDE2A; ARAP1; STARD10; ATG16L2; FCHSD2; P2RY2; P2RY6; ARHGEF17; RELT; FAM168A
Chr12q15	unknown
Chr17q12	cDNA: FLJ21341 fis, clone COL02653
Chr17q21.32	HOXB2; HOXB3; HOXB4; HOXB7; HOXB8; HOXB9

6. Discussion

For decades, scientists have put enormous effort in the study of the origins of human cancer including oral cancer, and the relative roles of genetic versus epigenetic abnormalities have been extensively discussed. An explosion of data showing the importance of both genetic and epigenetic processes has led to the understanding that genetics and epigenetics cooperate at all stages of cancer development [193].

Genetic and epigenetic biomarkers are useful in diagnosis, estimation of the risk of developing cancer, prediction of the treatment efficacy and clinical outcome of different human malignancies. Moreover, some of these markers are expressed during early stages of the tumor development and as a result, provide an opportunity to develop timely intervention and treatment strategies. Attempts are being made to validate cancer biomarkers in histologically tumor-free samples. Once validated, these markers could be used both in clinical settings, and in screenings with the aim of identifying at risk populations. Currently, there are no accurate markers that could be used to recognize with absolute certainty which patients are likely to be at high risk for aggressive tumors. Discovery of such biomarkers is important for better management of patients and their stratification for treatment purposes.

The oral cavity is a very fertile soil for tumor development. The increased opportunity for tumor development at this site is mainly due to its exposure to various environmental mutagens (carcinogens). Mutagens create fields with genetically/epigenetically altered cells that are at increased risk of undergoing malignant transformation. Indeed, oral cancer appears to be an anaplastic predisposition of numerous cells, resulting in multifocal development of neoplasia, rather than a process locally affecting a restrained number of cells [194].

Our understanding and knowledge of the molecular biology of oral squamous cell carcinoma have progressed significantly over the past decade and yet this profound increase in basic science knowledge has not affected the ability to control OSCC or provide new tools to improve patients' outcome. The main challenges providing comprehensive management for patients with oral cancer which include difficulty in predicting the capricious clinical behavior of oral cancer, recurrence at the primary site after resection, cervical and distant metastasis, and the development of second primary oral cancers have remained the same. These clinical challenges and solutions to them have a molecular basis. Furthermore, molecular approaches are clearly

going to be used to predict clinical behavior, determine prognosis, guide surgical treatment, and assist with tumor surveillance.

Tumor recurrence and second primary tumors, even when surgical margins are histopathologically normal (tumor-free), support the concept of field cancerization, i.e. the formation of multiple patches of premalignant disease. Both local recurrences and second primary tumors are considered to be a poor prognostic sign. Local recurrences occur in 10-30%, while the incidence rate of second primary tumors is 10-35% of patients with surgical margins diagnosed as histopathologically tumor-free, depending on both the localization of the first primary tumor and the age of patients with oral squamous cell carcinoma [195].

The debate on surgical margins in oral cancer patients in terms of their importance for the recurrence risk assessment is never-ending, yet it appears that their histological status may not always be a reliable predictor of patient's fate. Histopathology lacks sensitivity in identifying cells that have already started the process of malignant transformation but have not yet developed a fully neoplastic phenotype. Therefore, the analysis of molecular changes in the tumor tissue, tumor margins tissue and normal oral mucosa of the OSCC patients might assist in identification of biological markers predictors of recurrences. It would also help to better classify patients according to the level of risk of loco-regional recurrence and development of a second primary tumors.

The present study analyzed a particular group of genes (*c-MYC* and *HER2* oncogenes, and *P14* and *P16* tumor suppressors) involved in the regulation of the cell cycle, simultaneously in tumors, tumor margins and unaffected oral mucosa of patients with OSCC. The results suggest that neither "negative" margins nor "normal" mucosa could be considered as such. Namely, alterations were found not only in tumor tissue, but also in margins and oral swabs. The finding of cells in the tumour margins and normal mucosa harboring multiple genetic alterations that can potentially lead to a neoplastic transformation in tumor margins and normal mucosa is one more contribution to the theory of oral field cancerization [196].

As expected, the highest incidence of alterations was found in the tumor tissues. The next highest incidence was in the tumor margin tissues and, again quite expectedly, the lowest levels were detected in 'normal' oral mucosa of our OSCC patients.

Interestingly, epigenetic changes were significantly more frequent with an average 69.6% (77.5% and 61.7% in *P14* and *P16* respectively) than mutations which were found with an average frequency of 13.34% (14.17% and 12.5% in *HER2* and *c-MYC* respectively). Co-alterations (concurrent molecular events) were more prevalent in tumors with an average 2.15 alterations per sample and margins with an average 1.70 alterations per sample, than in 'normal' mucosa which was with an average 1.13 alterations per sample. A significantly lower incidence of co-alterations was found in swabs compared to both tumors and margins. None of the swab samples harbored all 4 alterations, but OSCC patients with 3 alterations in the 'normal' mucosa had a statistically significant shorter survival.

Oncogenes have been shown to undergo amplification during cancer development; this activation is a hallmark of nearly all advanced tumors. The amplified genes represent attractive targets for diagnostics, prognostics, and therapy. Recently, it has become evident that molecular classification of tumors gives fundamental knowledge about the mechanisms of carcinogenesis and guides clinical practices and the development of targeted therapies [81, 82]. Among the most studied oncogenes in human solid tumors, which are activated by amplification are *c-MYC* and *HER2* [83]. The *c-MYC* oncogene contributes to at least 40% of tumors [90] and has been implicated in their progression [197]. It is amplified in different cancer types at various frequencies: in human breast cancer, it was found to be amplified in the range of 10-50% [198,199], in gastric cancers in the range 15-30% [200], in about 30% of ovarian cancers [201], in up to 50% of hepatocellular carcinomas [197], and in up to 70% of colorectal adenocarcinomas [202]. In the present study, *c-MYC* amplification was the least frequent alteration with an average frequency of 12.5% (22.5% of tumors, 10% of tumor margins, and 5% of swabs harbored the amplification). An increase in the incidence of *c-MYC* amplification with increasing clinical stage and histological grade was registered in tumors, margins, and normal mucosa, but without statistical significance, possibly due to the relatively small sample size. The *c-MYC* amplification in the margins is lower than the results of a previous study on OSCC by our study group which showed a relatively high percentage of cases with *c-MYC* amplification (30%) [203].

However, it must be underlined that this is the first study of *c-MYC* amplification in normal mucosa of patients with OSCC. Since being unique, the findings could not be compared to the findings of other studies in this respect. In the case of oral tumors, previous studies correlated

c-MYC alterations with tumor stage and found that it was a clear indicator of poor prognosis [86]. Usually it was associated with poorly differentiated tumors [93]. *c-MYC* is considered as an important marker in oral dysplasia and OSCC. Its presence was correlated with the progression of dysplastic changes and with tumor grade [95]. The *c-MYC* amplification has been described in OSCC at various frequencies, ranging from 8.8% to 81.3%, depending mainly on the methodology used for the detection of gene amplification. A previous study of this group showed a relatively high percentage of cases with *c-MYC* amplification but no association was found between *c-MYC* amplification and OSCC stages and grades. Some studies have shown a correlation of *c-MYC* alterations (gene amplification and/or protein overexpression) with advanced OSCCs [196, 204, 205], while others have not made a clear correlation [206, 207]. This could be explained by numerous factors modulating the expression and function of *c-MYC* such as cell type, tumor location and the interaction between cells and extracellular environment [83]**.** In conclusion, it must be emphasized that the results of different studies dealing with the importance of *c-MYC* amplification in OSCC are quite inconsistent and often conflicting.

Generally, *HER2* is amplified and overexpressed in a significant number of human tumors; its amplification has frequently been detected in tumors of various tissues such as breast, ovary, bladder, stomach, lung, gastric and salivary glands [69, 208]. It is not uncommon to encounter a huge variation in the frequencies of reported amplification of *HER2* in different studies and for different tissues. For instance, *HER2* over-expression/amplification in ovarian carcinomas shows considerable variation ranging from 8- 66% [209]. Similarly, in OSCC, *HER2* amplification/aberrant expression has been frequently observed, but the reported results are controversial because of their wide range (between 0% and 88%) [112]. Our results showed that *HER2* amplification was a relatively frequent event; it was amplified with an average 14.16% (22.5% of tumors, 10% of tumor margins, and 5% of swabs).The same trend in gene amplification with higher clinical stage and histological grade was also noted for *HER2* as it was the case with *c-MYC* amplification, but without statistical significance. However, importantly, the presence of *HER2* amplification in tumor margins and swabs was significantly correlated with higher disease specific mortality. Our findings are in line with the results of a meta-analysis that demonstrated a significantly higher 5-year mortality rate in *HER2*-positive oesophageal SCC [210]. It is also in line with the results of a previous study performed at the School of Dental Medicine, University of Belgrade, showing *HER2* amplification in OSCC margins to be a

predictor of a poor outcome in OSCC patients [203]. On the other hand, in another study, Hanken et al did not find association of *HER2* amplification with survival of OSCC patients [104]. In breast and lung tumors, *HER2* amplification has been associated with a poor prognosis, while overexpression in gastric tumors was related to the presence of metastases [69]. Two reports on carcinoma ex-pleomorphic adenoma have shown an association of *HER2* amplification with shorter survival as well [208, 211], and with worse prognosis [179].

Once more, we must emphasize, that there are no previous studies dealing with *HER2* amplification in healthy mucosa of OSCC patients, and thus no comparison with literature data could be done in this regard.

As we can see, our results showed that the amplification of both genes is observed in the three tissue types (tumor, tumor margins and normal buccal mucosa) at various frequencies, suggesting that this genetic alteration, to some extent, is an early event in the development of oral cancer. The detection of amplification in normal buccal mucosa could be used as a predictor of disease outcome in OSCC patients.

Recent studies have described epigenetic aberrations and their critical role in cancer progression and prognosis; most of these studies have focused on aberrant DNA methylation status of the promoter regions in TSGs and their silencing effects. Epigenetic alterations in cancer, including oral cancer, affect or inactivate the functions of genes without altering their structure or their DNA sequence [51]. DNA methylation is the most common epigenetic alteration and considered as an early event in oral carcinogenesis [32, 50]. Knowledge of the hypermethylation of certain genes may contribute to a deeper understanding of cancer; the hypermethylation of tumor suppressor gene (TSG) promoters which is regarded as the major epigenetic change of cancer is a powerful mechanism of transcription silencing and can be found in almost all types of cancers. Assessing the methylation status of tumor suppressor genes represents a powerful tool for early diagnosis of various cancer types [51, 59, 179]. The hypermethylation is found in cancer lesions, cancer precursor lesions and in healthy mucosa, but at different percentages and that is why it has been proposed as a diagnostic and prognostic molecular marker to assist in better identification of lesions at risk of malignant transformation [51]. The presence of methylation in specific tumour suppressor genes could modify their function and alter cell cycle control, so the patients could have an increased risk of developing cancer and also a more aggressive

malignancy [51]. Promoter hypermethylation of multiple genes has been previously described in OSCC [52].

Aberrant promoter hypermethylation of *P14* has been observed in many cancer types; it has been observed in lung cancer, breast cancer, gastric cancer, ovarian cancer, uterine cancer, colorectal carcinoma, colon cancer, and in oral cancer as well. In different cancer types it was hypermethylated at various frequencies. For instance, its promoter hypermethylation was observed in about 30% of lung cancers, 28% of colorectal carcinomas, 51% of colon cancers, 24% of breast cancers, 24% of gastric cancers, in 16% of uterus cancers and in 5% of ovarian cancers [52,145,152]. In one study of our group on hypermethylation of *P14* promoter in mucoepidermoid carcinoma (MEC), the most remarkable finding was that 100% of MECS were with *P14* promoter hypermethylation [179]. There are not many studies about the prevalence of *P14* promoter hypermethylation in OSCC. In addition, there are no such studies in margins and normal mucosa of oral cancer patients. The frequency of the reported hypermethylation of the promoter regions of *P14* in OSCC patients ranges from 3.8 to 44% in tumor samples (the only tissue that was tested) [141, 149], which is significantly lower than in our study. Differences in methylation frequencies may, among others, be attributed also to environmental, geographical, ethnical etc. factors [196]. Interestingly, in our study *P14* methylation showed a statistically significant association with lower histological grade tumors (p=0.35), which is in agreement with some previous reports on the association of *P14* methylation with lower recurrence rate and good prognosis [154, 217]. On the other hand, it is in contrast with some other studies. For instance, *P14* hypermethylation has been observed as a late event in carcinogenesis, associated with increased tumor size, tumor stage and nodal metastasis, although in these late-stage tumors it appeared as a predictor of lower recurrence rate and better clinical outcome [52,141].

The *P16* gene was also found to be hypermethylated in different cancer types at various frequencies; its promoter hypermethylation was observed in about 31% of lung cancers, 48% of lymphomas, 37% of colon cancers, 17% of breast cancers, 36% of gastric cancers, and in 18% of ovarian cancer [152].

The loss of *P16* activation by methylation has also been observed in OSCC, and it is considered to be one of the initial events in oral carcinogenesis [213]. There are no many studies dealing with *P16* methylation in OSCC and the results are often conflicting. The percentages of promoter

methylation show also huge variations: between 9% [214] and 87% in tumor tissue [215], 5.7% [216] and 43% in non-neoplastic oral mucosa and tumour-free margins [215], and up to 70% in the blood of OSCC patients [217].

In the present study, similarly to *P14* methylation, *P16* methylation appeared to be quite a frequent event - on average 61.7%. *P16* was methylated in 31 tumor samples (77.5%), in 28 margin samples (70%) and in 14 swab samples (35%). For both genes, the hypermethylation rate was significantly higher in the tumor tissues than in the tumor margins and the normal mucosa. *P16* methylation, as well as *P14*, did not appear to be significantly correlated with tumor phenotype, i.e. with clinico-pathological parameters (with the exception of *P14* methylation in lower grade tumors). However, their high frequency in the analyzed specimens points to the importance of this epigenetic event in oral carcinogenesis. A similar finding was reported in mucoepidermoid carcinomas of the salivary glands in Serbian patients [179].

The hypermethylation of both *P14* and *P16* is detected in the three tissue types- tumor, tumor margins and normal buccal mucosa, suggesting that this epigenetic alteration is an early event in the development of cancer, a view that is in accordance with the concept of "field cancerization" in oral cancer [52]. The detection of aberrant methylation of *P14* and *P16* emerged as a potential biomarker for early detection of various carcinomas, including early detection and treatment of OSCC [32]. Some reports have stated that *P16* methylation was not correlated with tumor stage or grade and has no prognostic significance [218], which is in accordance with our results. However, other studies have found a predictive value of *P16* methylation for advanced OSCCs, earlier tumor recurrence, lymph node involvement and shorter survival [52, 214].

The inclusion of more rigorous treatment and more intensive surveillance during follow-up in patients with methylation changes detected in surgical margins may provide an enhanced overall survival. Further studies of larger patients groups and additional quantitative validation are needed to confirm our findings, along with their therapeutic potential.

Human papilloma virus (HPV) infection is correlated with many cancers, the most well known being its correlation with cervical carcinoma. HPV infection is considered as an etiological factor in the development of OSCC too. Reported incidence of high-risk HPVs in oral carcinoma patients varied from 0% to 100% [32]. HPV DNA has been identified in approximately 24 % of OSCC, with HPV-16 and HPV-18, the most common types, accounting for almost 70 % and 8 %

of HPV positive OSCC, respectively. HPV positive head and neck cancer is associated with poorly differentiated, non-keratinizing, basaloid morphology, wild type p53, overexpression of *P16*, and inactivation of cyclin D1 and Rb. Furthermore Mendenhall et al. reported that high-risk HPV related OSCC is associated with the over expression of *P16* [213]. Our results showed that HPV16 DNA was detected in eight of the 40 tumor samples (20%). No statistically significant association was found between HPV infection and molecular changes, i.e. the present study could establish no specific relationship between HPV and epigenetic silencing of *P16* or *P14*.

In conclusion, the molecular mechanisms of carcinogenesis, tumor progression and metastasis of OSCC are now better understood thanks to the recent advances in molecular biology. However, many unanswered questions remain. The molecular pathological approach is becoming the mainstream in current pathological research.

The presence of histologically normal cells with multiple genetic/epigenetic alterations in tissues surrounding the tumor, and the fact that adequate surgical resection is a key step in the local control and prognosis of OSCC, point to the need for more accurate assessment methods than classical histopathology. Determining the molecular status of tumors, tumor margins and unaffected areas of the oral cavity mucosa, along with the inclusion of a panel of different cell cycle regulating genes involved in the development of cancer as biomarkers, would yield more prognostic information. In the present study, a predictive factor previously determined by our group was confirmed one more time (*HER2* amplification in tumor margins as a factor of shorter survival). In addition, new prognostic parameters have emerged: *HER2* amplification in 'normal' oral mucosa, and multiple alterations in margin and 'normal' oral mucosa as factors of shorter survival. Therefore, the analysis of oncogene amplification and tumor suppressor gene methylation status proved to be a useful approach in the evaluation of the biological characteristics of OSCCs and an additional indicator of patient prognosis and survival.

7. Conclusions

1. Oncogene amplification, although not negligible, was a significantly less frequent molecular event (13%) compared to tumor suppressor gene methylation (70%).

2. Oncogene amplification:
    - *HER2* oncogene was amplified in 25% of tumors, 10% of tumor margins and 8% of normal mucosa. The level of amplification ranged between 3.5 and 80 fold
    - *c-MYC* was amplified in 22% of tumors, 10% of tumor margins and 5% of normal mucosa. The level of amplification ranged between 3.5 and 13 fold

3. Tumor suppressor gene promoter methylation:
    - *P14* was methylated in 90 % of tumors, 80% of tumor margins and 62% of normal mucosa.
    - *P16* was methylated in 78% of tumors, 70% of tumor margins and 35% of normal mucosa

4. *HER2* and *c-MYC* amplification and *P16* methylation did not show statistically significant association with histological grade nor clinical stage. *P14* methylation was associated with lower tumor stages.

5. HPV 16 was detected in 20% of OSCC but the virus did not appear to influence clinical characteristics. No association could be established between molecular alterations and gender, smoking, alcohol consumption and presence of HPV 16.

6. The total number of alterations and *HER2* status in the margins and normal mucosa of OSCC patients had strong impact on survival. *HER2* amplification in tumor margins and normal mucosa was associated with significantly shorter survival. The presence of multiple alterations in these tissues was also associated with shorter survival.

7. Array CGH analysis showed a number of recurrent aberrations involving interesting candidate genes some previously implicated in OSCC and some not, both in tumors and margins.

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Supplementary Materials

1. DNA extraction

1.1. DNA extraction using phenol/chloroform protocol (tissues)

Day 1

1. Cut the tissues into small pieces (as small as possible) in 1.5 ml microcentrifuge tube, add 500µl of NE buffer, 10 µl of 10% Sodium Dodecyl Sulfate (SDS) and 25 µl of proteinase K (20mg/ml)
2. Incubate for 12 to 16 hours at 50°C.

Day 2

1. Add 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1), mix well by inverting the tube, centrifuge for 3minutes at 12000 RPM without cooling.
2. Transfer the upper phase (water supernatant) into new tube, do not disturb the interphase which contains proteins.
3. Add 500 µl of chloroform/isoamyl alcohol (24:1), mix well by inverting the tube, centrifuge for 3minutes at 12000 RPM without cooling.
4. Transfer the upper phase (water supernatant) into new tube, do not disturb the interphase which contains proteins.
5. Add 1/10 of the water phase volume 30-50 µl) of 4M NaCl and 1-5 volume of water phase (about750 µl) of cold 96% ethanol (from -20°C), mix well by inverting the tube, DNA should be seen as white string.
6. (If DNA after step 5 cannot be seen or there is a need for a break, put the tubes in the freezer at -20°C for an hour or until the break is over).
7. Centrifuge the tubes for 3 minutes at 12000 RPM without cooling.
8. Remove the ethanol by pipetting without disturbing the pellet.
9. Add 10 µl of 3M Na acetate and 300 µl of cold 96% ethanol (from -20°C).
10. Mix by inverting the tubes and centrifuge for 3 minutes at 12000 RPM without cooling.
11. Remove the ethanol by pipetting (without disturbing the pellet) and add 1ml of cold 70% ethanol (from -20°C).
12. Centrifuge for 15 seconds (short spin).

13. Remove all the ethanol by pipetting without disturbing the pellet and leave the tubes open to dry at room temperature (they can be left overnight).
14. Dissolve the DNA pellets in water, adding 30 to 100 µl of water, depending on the pellet size.
15. Store the extracted DNA at –20°C or use DNA for the desired downstream application.

1.2. DNA extraction with Invitrogen, Carlsbad, CA (*the PureLink® Genomic DNA*) Kit (swabs)

1.2.1. Manufacturers' recommendations:

Follow the listed recommendations to obtain the best results:

- Maintain a sterile environment when handling DNA to avoid any contamination from DNases
- Ensure that no DNases are introduced into the sterile solutions of the kit
- Make sure all equipment that comes in contact with DNA is sterile including pipette tips and microcentrifuge tubes.
- Do not vortex the samples for more than 5–10 seconds at each vortexing step to avoid extensive shearing of DNA.
- To minimize DNA degradation, perform lysate preparation steps quickly, and avoid repeated freezing and thawing of DNA samples.

1.2.2. Swabs genomic DNA extraction protocol

1. Set a water bath or heat block at 55°C.
2. Place the buccal swab in a sterile, 2-mL microcentrifuge tube. Add 400 µL (for cotton and Dacron swab) or 600 µl (for Omni Swab) PBS to the sample.
3. Add 20 µl Proteinase K into a sterile microcentrifuge tube capable of holding three times the volume of lysate (for example, if you plan to process 600 µl lysate, use a microcentrifuge tube capable of holding 1800 µl).
4. Transfer 200–600 µl swab lysate to the microcentrifuge tube containing Proteinase K (Step 3). Mix well by pipetting.

5. Add an equal volume of PureLink® Genomic Lysis/Binding Buffer to the lysate and mix well by brief vortexing. For example, if you are processing 200 µl lysate, add 200 µl PureLink® Genomic Lysis/Binding Buffer.
6. Incubate at 55°C for at least 10 minutes.
7. Centrifuge briefly to collect any lysate from the tube caps.
8. Add 200 of µl 96–100% ethanol to the tube. Mix well by vortexing for 5 seconds to yield a homogenous solution.
9. Remove a PureLink® Spin Column in a Collection Tube from the package.
10. Add the lysate (~640 µl) prepared with PureLink® Genomic Lysis/Binding Buffer and ethanol to the PureLink® Spin Column.
11. Centrifuge the column at 10,000 × *g* for 1 minute at room temperature. **Note:** If you are processing >200 µl starting material such as blood, buccal swabs, or Oragene™ preserved saliva, you need to perform multiple loading of the lysate by transferring any remaining lysate to the same PureLink® Spin Column (above) and centrifuge at 10,000 × *g* for 1 minute.
12. Discard the collection tube and place the spin column into a clean PureLink® Collection Tube supplied with the kit.
13. Add 500 µl Wash Buffer 1* prepared with ethanol to the column.
14. Centrifuge column at room temperature at 10,000 × *g* for 1 minute.
15. Discard the collection tube and place the spin column into a clean PureLink® collection tube supplied with the kit.
16. Add 500 µl Wash Buffer 2* prepared with ethanol to the column.
17. Centrifuge the column at maximum speed for 3 minutes at room temperature. Discard collection tube.
18. Place the spin column in a sterile 1.5-mL microcentrifuge tube.
19. Add 25–200 µl of PureLink® Genomic Elution Buffer to the column.
20. Incubate at room temperature for 1 minute. Centrifuge the column at maximum speed for 1 minute at room temperature.
21. To recover more DNA, perform a second elution step using the same elution buffer volume as first elution in another sterile, 1.5-mL microcentrifuge tube.
22. Centrifuge the column at maximum speed for 1.5 minutes at room temperature. Remove and discard the column.

23. Store the tubes containing the purified DNA at –20°C or use DNA for the desired downstream application.

1. For long-term storage, store the purified DNA in PureLink® Genomic Elution Buffer at –20°C as DNA stored in water is subject to acid hydrolysis.
2. To avoid repeated freezing and thawing of DNA, store the purified DNA at 4°C for immediate use or aliquot the DNA and store at –20°C for long-term storage.

*-Add 96–100% ethanol to PureLink® Genomic Wash Buffer 1 and PureLink® Genomic Wash Buffer 2 according to instructions on each label. Mix well. Mark on the labels that ethanol is added. Store both wash buffers with ethanol at room temperature.

2. Bisulfite modification of DNA with EZ DNA Methylation™ Kit

- **Reagent preparation**:

• Preparation of CT Conversion Reagent The CT Conversion Reagent supplied within this kit is a solid mixture and must be prepared prior to first use. Prepare as follows:

1. Add 750 µl of water and 210 µl of M-Dilution Buffer to a tube of CT Conversion Reagent.

2. Mix at room temperature with frequent vortexing or shaking for 10 minutes.

Note: It is normal to see trace amounts of undissolved reagent in the CT Conversion Reagent. Each tube of CT Conversion Reagent is designed for 10 separate DNA treatments. Storage: The CT Conversion Reagent is light sensitive, so minimize its exposure to light. For best results, the CT Conversion Reagent should be used immediately following preparation. If not used immediately, the CT Conversion Reagent solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored CT Conversion Reagent solution must be warmed to 37°C, then vortexed prior to use.

- **Preparation of M-Wash Buffer:** Add 24 ml of 100% ethanol to the 6 ml M-Wash Bufferconcentrate (D5001) or 96 ml of 100% ethanol to the 24 ml M-Wash Bufferconcentrate (D5002) before use.

- **Amount of DNA Required for Bisulfite Conversion:** The minimal amount genomic DNA required for bisulfite treatment and subsequent PCR amplification is 100 pg. The optimal amount of DNA per bisulfite treatment is 200 to 500 ng. Although, up to 2 µg of DNA can be processed, it should be noted that high input levels of DNA may result in incomplete bisulfite conversion for some GC-rich regions.

2.1. Protocol:

1. Add 5 µl of M-Dilution Buffer to the DNA sample and adjust the total volume to 50 µl with water. Mix the sample by flicking or pipetting up and down.
   Example: For 14 µl of a DNA sample add 5 µl M-Dilution Buffer and 31 µl water.
2. Incubate the sample at 37°C for 15 minutes. (to insure complete C to T conversion increase temperature to 42°C and extend the incubation time to 30 minutes)

3. After the above incubation, add 100 µl of the prepared CT Conversion Reagent to each sample and mix.
4. Incubate the sample in the dark at 50°C for 12-16 hours.
5. Incubate the sample at 0-4°C (e.g., on ice) for 10 minutes.
6. Add 400 µl of M-Binding Buffer to a Zymo-Spin™ IC Column and place the column into a provided Collection Tube.
7. Load the sample (from Step 5) into the Zymo-Spin™ IC Column containing the M Binding Buffer. Close the cap and mix by inverting the column several times.
8. Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.
9. Add 100 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.
10. Add 200 µl of M-Desulphonation Buffer to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.
11. Add 200 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 µl of M-Wash Buffer and centrifuge for an additional 30 seconds.
12. Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl* of M-Elution Buffer directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

***NB:** The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at or below -70°C. We recommend using 1-4 µl of eluted DNA for each PCR, however, up to 10 µl can be used if necessary. The elution volume can be > 10 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

BIOGRAPHY WITH BIBLIOGRAPHY

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B1. *In extenso* publications:

1. Jelovac DB, Tepavčević Z, Nikolić N, Ilić B, Eljabo N, Popović B, Čarkić J, Konstantinović V, Vukadinović M, Miličić B, Milašin J. The amplification of c-erb-B2 in cancer-free surgical margins is a predictor of poor outcome in oral squamous cell carcinoma. Int J Oral Maxillofac Surg. 2016;45(6):700-5. (M22)

2. Nikolic N, Carkic J, Dimitrijevic II, Eljabo N, Radunovic M, Anicic B, Tanic N, Falk M, Milasin J. P14 methylation: an epigenetic signature of salivary gland mucoepidermoid carcinoma in the Serbian population. Oral surgery, oral medicine, oral pathology and oral radiology. 2018;125(1):52-8. (M22)

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B2. Congress participations:

1. Eljabo NM, Loncar S, Jelovac D, Nikolic N, Dedovic-Tanic N, Carkic J, Toljic B, Vukadinovic M, Milasin J. TP53 mutation and p14-p16 methylation analysis in tumor tissue, tumor-free margins and non-affected oral mucosa in patients with oral cancer-pilot study, V Congress of Serbian Genetic Society, 28. September-3. October 2014, Belgrade, Serbia.

2. Loncaric D, Ilic-Dimitrijevic I, Nikolic N, Zivkovic M, Loncar S, Eljabo NM, Dedovic-Tanic N, Konstantinovic V, Vukadinovic M, Milasin J. The role of p14 and p16 promoter methylation in mucoepidermoid carcinoma pathogenesis, V Congress of Serbian Genetic Society, 28. September-3. October 2014, Belgrade, Serbia.

3. Eljabo N, Nikolic N, Carkic J, Lazarevic M, Jelovac D, Anicic B, Milasin J. p14 and p16 methylation in oral cancer. First Congress of Molecular Biologists of Serbia, 20 – 22 September 2017.

4. Jelovac D, Eljabo N, Gnan C, Allegri L, Baldan F, Mio C, Franzoni A, Milasin J. Comparative genomic hybridization analysis of tumor and tumor margin in patients with oral cancer – a pilot study. First Congress of Molecular Biologists of Serbia, 20 – 22 September 2017.

Изјава о ауторству

Име и презиме аутора Najeeb Mohamed Mohamed Aljabu

Број индекса Б3013/2013

Изјављујем

да је докторска дисертација под насловом

„*HER2* and *c-MYC* mutational status and *INK4a/ARF* methylation status in tumors, tumor margins and unaffected oral mucosa of patients with oral squamous cell carcinoma“

„Mutacioni status *HER2* i *c-MYC* gena i metilacioni status *INK4a/ARF* lokusa u tumoru, tumorskoj margini i neizmenjenoj oralnoj sluzokoži pacijenata obolelih od skvamocelularnog karcinoma usne duplje”

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Потпис аутора

У Београду, 22.06.2018.

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Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Najeeb Mohamed Mohamed Aljabu

Број индекса Б3013/2013

Студијски програм Биологија

Наслов рада **„*HER2* and *c-MYC* mutational status and *INK4a/ARF* methylation status in tumors, tumor margins and unaffected oral mucosa of patients with oral squamous cell carcinoma“**

„Mutacioni status *HER2* i *c-MYC* gena i metilacioni status *INK4a/ARF* lokusa u tumoru, tumorskoj margini i neizmenjenoj oralnoj sluzokoži pacijenata obolelih od skvamocelularnog karcinoma usne duplje”

Ментори Проф. др Јелена Милашин, Проф. др Марина Стаменковић-Радак

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

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Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

„*HER2* and *c-MYC* mutational status and *INK4a/ARF* methylation status in tumors, tumor margins and unaffected oral mucosa of patients with oral squamous cell carcinoma“

„Mutacioni status *HER2* i *c-MYC* gena i metilacioni status *INK4a/ARF* lokusa u tumoru, tumorskoj margini i neizmenjenoj oralnoj sluzokoži pacijenata obolelih od skvamocelularnog karcinoma usne duplje”

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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У Београду, 22.06.2018.

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1. **Ауторство**. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце, чак и у комерцијалне сврхе. Ово је најслободнија од свих лиценци.

2. **Ауторство – некомерцијално**. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела.

3. **Ауторство – некомерцијално – без прерада**. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца не дозвољава комерцијалну употребу дела. У односу на све остале лиценце, овом лиценцом се ограничава највећи обим права коришћења дела.

4. **Ауторство – некомерцијално – делити под истим условима**. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца не дозвољава комерцијалну употребу дела и прерада.

5. **Ауторство – без прерада**. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, без промена, преобликовања или употребе дела у свом делу, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце. Ова лиценца дозвољава комерцијалну употребу дела.

6. **Ауторство – делити под истим условима**. Дозвољавате умножавање, дистрибуцију и јавно саопштавање дела, и прераде, ако се наведе име аутора на начин одређен од стране аутора или даваоца лиценце и ако се прерада дистрибуира под истом или сличном лиценцом. Ова лиценца дозвољава комерцијалну употребу дела и прерада. Слична је софтверским лиценцама, односно лиценцама отвореног кода.