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Development of a non-invasive approach for oral squamous cell carcinoma diagnosis

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Abstract

Oral cancer is considered the sixth most common cancer worldwide. It is reported that 90% of the cases corresponds to oral squamous cell carcinoma (OSCC). Despite the progress in cancer research, the five-year survival rate remains low, mostly due to advanced stages of diagnosis and development of loco-regional recurrence. OSCC results from accumulation of numerous genetic and epigenetic changes, followed by clonal expansion. Therefore, one goal of this project was to characterize OSCC genetic and epigenetic profiles in order to find potential biomarkers that can be used to detect OSCC in early stages and also predict the disease progression. Moreover, since biopsy is an invasive and painful method, it is mostly used when there is a suspicion of malignancy. Consequently, it was also aimed to try to validate a non-invasive method for OSCC diagnosis and to follow up the patients. Taking into account the aims of this project, 65 samples from tumour tissues were acquired and analysed by MS-MLPA in order to detect the genetic and epigenetic alterations that can be associated with OSCC initiation and progression. Furthermore, 12 of this 65 samples were also analysed by aCGH. Regarding genetic events, the most frequent copy number variations (CNVs) detected by MS-MLPA were gains at chromosomes 16p and 19p, and losses at 3p, 9p and 11q. Additionally, the main rearrangements detected through aCGH were gains at chromosomes 3q, 8q and Xq, and losses at 3p,18q Yp and Yq. The methylation status of 25 genes was assessed and the results revealed that gene promotor methylation of *WT1, PAX5, GATA5, MSH6* and *RARB* represent good epigenetic biomarkers for OSCC. After the OSCC characterization, 59 samples acquired by scraping the tumour surface from the patients were also analysed by MS-MLPA. The results from this non-invasive method were compared with tumour tissue results and it was found agreement between the two samples in 60% and 72% of the genes analysed, as respects to CNVs and methylation status, respectively. These results were truly promising in an attempt to validate this non-invasive approach for screening the oral cavity and to follow up the patients diagnosed with OSCC.

In order to discovery some potential genetic and epigenetic alterations that can be associated with early stages of disease and risk of develop tumour relapses or metastasis, 49 samples of the surgery resection margin were analysed by MS-MLPA. It was suggested that deletion of *CDKN2A* and methylation of *TP53* and *WT1* are initial alterations in the OSCC carcinogenesis process.

Keywords: OSCC, MS-MLPA, genetic and epigenetic alterations.

Resumo

O cancro oral é considerado a sexta neoplasia mais comum a nível mundial, sendo que mais de 90 % dos casos correspondem a carcinomas do tipo epidermoide. Apesar dos avanços a nível tecnológico e clínico, a taxa de sobrevivência a cinco anos não melhorou significativamente nos últimos anos devido, essencialmente, ao diagnóstico tardio e à elevada taxa de recidivas locais ou metástases. Atualmente, sabe-se que o carcinoma epidermoide da cavidade oral (CECO) resulta da acumulação de alterações genéticas e epigenéticas, seguida de expansão clonal. Assim sendo, este trabalho teve como objetivo a caracterização molecular do CECO na tentativa de encontrar potenciais biomarcardores que possam vir a ser úteis no diagnóstico precoce e na previsão da progressão da doença. Além disso, tendo em conta que a biopsia é um método invasivo e que causa desconforto, apenas é usado quando existe suspeita de malignidade. Consequentemente, através da realização deste projeto, pretendeu-se também validar uma metodologia não invasiva para diagnosticar o CECO e acompanhar os doentes. Tendo em conta os principais objetivos deste estudo foram inicialmente analisadas 65 amostras de tecido tumoral, recorrendo à metodologia MS-MLPA, na tentativa de encontrar as principais alterações genéticas e epigenéticas associadas com o CECO. Além disso, 12 dessas amostras foram também analisadas por aCGH. Considerando as variações genéticas, as alterações mais frequentes detetadas por MS-MLPA dizem respeito a ganhos localizados nos cromossomas 16p e 19p e perdas nos localizadas nos cromossomas 3p, 9p e 11q. Os resultados obtidos com a técnica aCGH demostraram maioritariamente ganhos nos cromossomas 3q, 8q e Xq e perdas nos cromossomas 3p, 18q, Yp e Yq. A metodologia MS-MLPA permitiu inferir o estado de metilação de 25 genes, levando a concluir que a metilação dos genes *WT1, PAX5, GATA5, MSH6 e RARB* podem funcionar como biomarcadores para o CECO. Após a caracterização molecular do tumor foram analisadas, por MS-MLPA, 59 amostras provenientes da raspagem de células na região tumoral. A comparação destes resultados com os obtidos no tecido tumoral revelou que 60% dos genes analisados para o número de variação de cópias eram concordantes entre as duas amostras e, ainda, que o estado de metilação de 72% dos genes analisados também era concordante. Estes resultados demonstram ser promissores na tentativa de validação desta metodologia não invasiva. A análise de 49 amostras de tecido macroscopicamente não tumoral, contíguo ao tumor, permitiu sugerir que a deleção do gene *CDKN2A* e a metilação dos genes *TP53* e *WT1* são alterações iniciais no processo de carcinogénese do CECO.

Palavras-chave: CECO, MS-MLPA, alterações genéticas e epigenéticas.

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List of acronyms and abbreviations

5-FU	5-fluorouracil
aCGH	Array Comparative Genomic Hybridization
AKT	Protein Kinase B
APC	Adenomatous polyposis coli
ASR	Age-standardised rate
ATM	ATM serine/threonine kinase
BCL2	BCL2, apoptosis regulator
BFB	Breakage-fusion-bridges
BRCA1	Breast cancer1
BRCA2	Breast cancer 2
CADM1	Cell adhesion molecule 1
CASR	Calcium-sensing receptor
CCDN1	Cyclin D1
CD44	CD44 molecule
CDH1	Cadherin 1
CDH13	Cadherin 13
CDH2	Cadherin 2
CDK	Cyclin-dependent kinase
CDK6	Cyclin-dependent kinase 6
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cfDNA	Cell free DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CHEK1	Checkpoint kinase 1
CHFR	Checkpoint with forkhead and ring finger domains
cm	Centimeters
CNV	copy number variation
CREM	cAMP responsive element modulator
CSMD1	CUB and Sushi multiple domains 1
DAPK	Death-Associated Protein Kinase 1
DCC	DCC netrin 1 receptor

DCUN1D1	DCN1, defective in cullin neddylation 1, domain containing 1)
DDR	DNA damage responsive
DNMT	DNA methyltransferase
DSB	Double-strand breaks
EBV	Epstein-Barr virus
EGFR	Epidermal Growth Factor
ErbB	Epidermal growth factor receptor
ESR1	Estrogen receptor 1
FDA	Food and Drug Administration
FHIT	Fragile histidine triad
FISH	Fluorescence in Situ Hybridization
GALR1	Galanin receptor 1
GATA4	GATA binding protein 4
GATA5	GATA binding protein 5
GSTP1	Glutathione S-transferase pi 1
H2AFX	H2A histone family, member X
HIF	Hypoxia inducible factor
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinomas
HPV	Human papillomavirus
hsr	homogeneously staining region
IL12A	Interleukin 12A
IL2	Interleukin 2
ING1	Inhibitor of growth family, member 1
JAK	Janus kinase
KLK3	Kallikren-related peptidase 3
KLLN	Killin
LOH	Loss of heterozygosity
LRP12	Low density lipoprotein receptor-related protein 12
MAPK	Mitogen-activated protein kinases
MGMT	O-6-methylguanine-DNA methyltranferase
MLH1	mutL homolog 1

MLH3	mutL homolog 3
MLPA	Multiplex Ligation-dependent Probe Amplification
MME	Membrane metallo-endopeptidase
MRE11A	MRE11 homolog A, double strand break repair nuclease
mRNA	Messenger ribonucleic acid
MSH6	mutS homolg 6
MS-MLPA	Methylation Specific Multiple Ligation-dependent Probe Amplification
MTAP	Methylthioadenosine phosphorylase
mTOR	Mechanistic target of rapamycin
MTUS1	Microtubule associated tumour suppressor 1
MVD	Mevalonate diphospho-decarboxylase
MYC	V-myc avian myelocytomatosis viral oncogene homolog
NOTCH1	notch1
OSCC	Oral squamous cell carcinoma
PAH	Phenylalanine hydroxylase
PARD6G	Par-6 family cell polarity regulator gamma
PAX5	Paired box 5
PAX6	Paired box 6
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PMP22	Peripheral myelin protein 22
pRb	Phosphorylated Retinoblastoma
PTCH1	Patched 1
PTEN	Phosphatase and tensin homolog
PTK2	Protein tyrosine kinase 2
PTPRD	Protein tyrosine phosphatase, receptor type, D
PYCARD	PYD and CARD domain containing
QT	Chemotherapy
RARß	Retinoic acid receptor, beta
RAS	Rat sarcoma viral oncogene
RB1	RB transcriptional corepressor 1

RT	Radiotherapy
SCC	Squamous cell carcinoma
SOX2	Sex determining region Y-box 2
SPSS	Statistical Package for Social Sciences
STAT3	Signal transducer and activator of transcription 3
STK11	Serine/threonine kinase 11
Tblue	Toluidine Blue
THBS1	Thrombospondin 1
TM4SF1	Transmembrane 4 L six family member 1
TNM	Tumour-node-metastasis
TP53	Tumour protein p53
TP63	Tumour protein p63
TP73	Tumor protein p73
TSC2	Tuberous sclerosis 2
TSG	Tumour suppressor gene
TUSC3	Tumour suppressor candidate 3
VHL	von Hippel-Lindau tumor supressor
WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1
WNT1	Wingless-type MMTV integration site family, member 1
WT1	Wilms tumor 1

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1. Introduction

1.1. Cancer

Cancer is one of the leading causes of death worldwide. According to GLOBOCAN, in 2012, 14.1 million new cancer cases were estimated and 8.2 million people died due to this disease (GLOBOCAN, 2012). It is predicted that this number is increasing, mainly, due to the growth and aging of population, allied to some lifestyle behaviours, such as smoking, inappropriate nutrition and physical inactivity (Torre *et al.*, 2015).

Cancer is a disease characterized by genomic instability, known as "disease of the genome" (Garraway and Lander, 2013). This idea was supported by Boveri after observing chromosomal aberrations (Boveri, 2008). Over the years, as regards the genomic instability of cancer, researchers showed that some mutated genes were directly related to cancer, which can be divided in two classes: the oncogenes (that are derived from proto-oncogenes that suffer mutations with dominant gain of function) and the tumour suppressors genes (in which mutations lead to a recessive loss of function) (Garraway and Lander, 2013). The mechanisms that lead to mutated genes involve several genomic alterations such as deletion or nucleotide substitution, alterations in the copy number of chromosomes and DNA rearrangements (Macconaill and Garraway, 2010).

Although there are several types of cancer, it was suggested that all cancer cells shared some characteristics that make them a complex disease. Thus, cancer cells are: insensitive to anti-growth signals; capable of inducing angiogenesis and sustaining proliferative signalling; able to evade apoptosis. Furthermore, they have the potential of invasion and metastasis and possess limitless replicative potential (Hanahan and Weinberg, 2000). More recently, it was suggested that they are able to evade immune responses and to promote local inflammation. It was also suggested that they possess deregulated cell metabolism (Hanahan and Weinberg, 2011) (Figure 1).

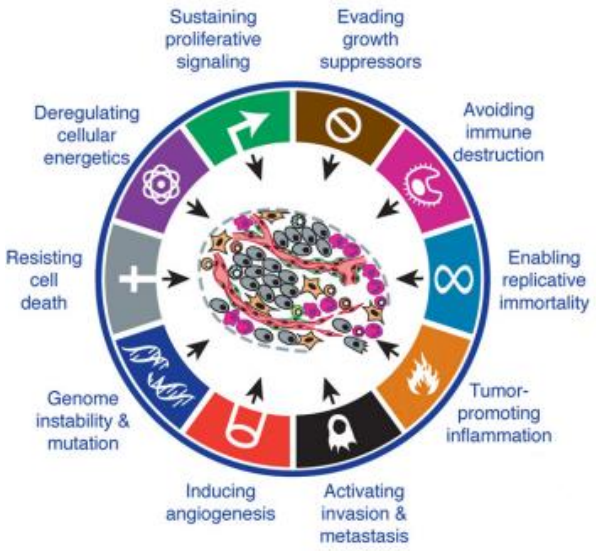

Figure 1- The hallmarks of cancer. (Adapted from Hanahan and Weinberg, 2011).

1.2. Oral cancer

Head and neck cancer (HNC) involves several subtypes of cancer, including tumours that arise from nasal cavity, thyroid, trachea, nasopharynx, oropharynx, hypopharynx, larynx and oral cavity (Stadler *et al.*, 2008).

The oral cavity comprises lip, tongue, floor of the mouth, salivary glands, buccal mucosa, gingiva and palate (Figure 2). Actually, oral cancer refers to tumour malignancies that may arise in any of these anatomic sites (Warnakulasuriya, 2009; Rhodus, 2009), being the tongue cancer the most common (Rhodus, 2009).

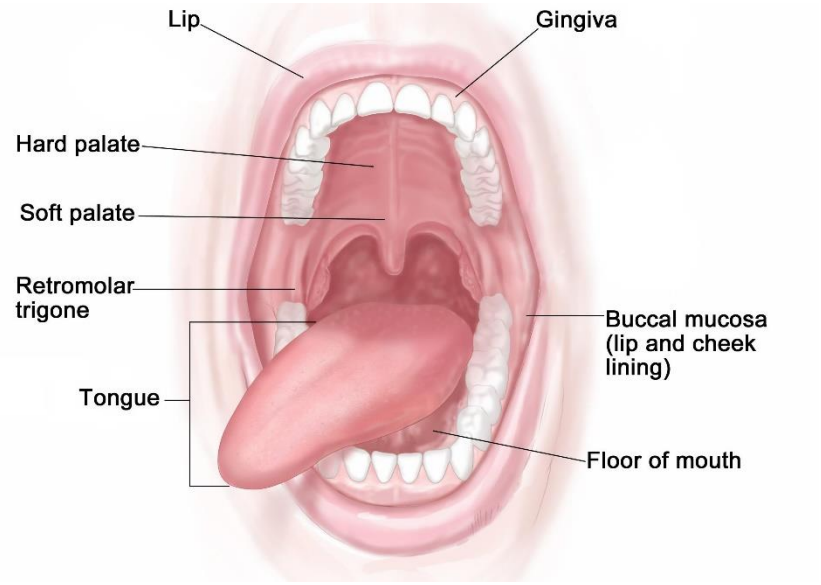

Figure 2- Anatomy of the oral cavity (adapted from Diagram, 2016).

Along with pharyngeal, oral cancer is the six most common cancer worldwide (Warnakulasuriya, 2009). It is suggested that oral cancer is a result of accumulation of numerous genetic and epigenetic alterations, followed by clonal expansion. The development and progression of this disease involves several interacting pathways that are deregulated (Tan *et al.*, 2013).

It is reported that more than 90% of oral malignancies correspond to squamous cell carcinomas (SCC) (Tsantoulis *et al.*, 2007). The oral squamous cell carcinoma (OSCC) is proved to be a heterogeneous disease, meaning that oral tumours are composed by a heterogeneous cell population, like many other cancers (Chiou *et al.*, 2008).

Although therapies to treat oral cancer have been improved, the five-year survival rate did not suffer substantial alterations in the past decades, remaining only about 50% (Noorlag *et al.*, 2014). The main reason for this is, probably, related to the advanced stage of diagnosis and the frequent development of secondary tumours (Smeets *et al.*, 2006). Accordingly, in order to overcome this problem, improved diagnostic methods must be developed and therefore the pathways involved in the development of oral cancer should be better understood (Rajmohan *et al.*, 2012).

1.3. Epidemiology

Oral cavity cancer shows high incidence rates worldwide, especially in some developed countries (Figure 3). In 2012, 300.400 new cases were estimated with an age-standardized rate (ASR) of 4 per 100.000. This disease is more frequently in men than women, (198.975 new cases in men comparatively with 101398 in women) (GLOBOCAN, 2012).

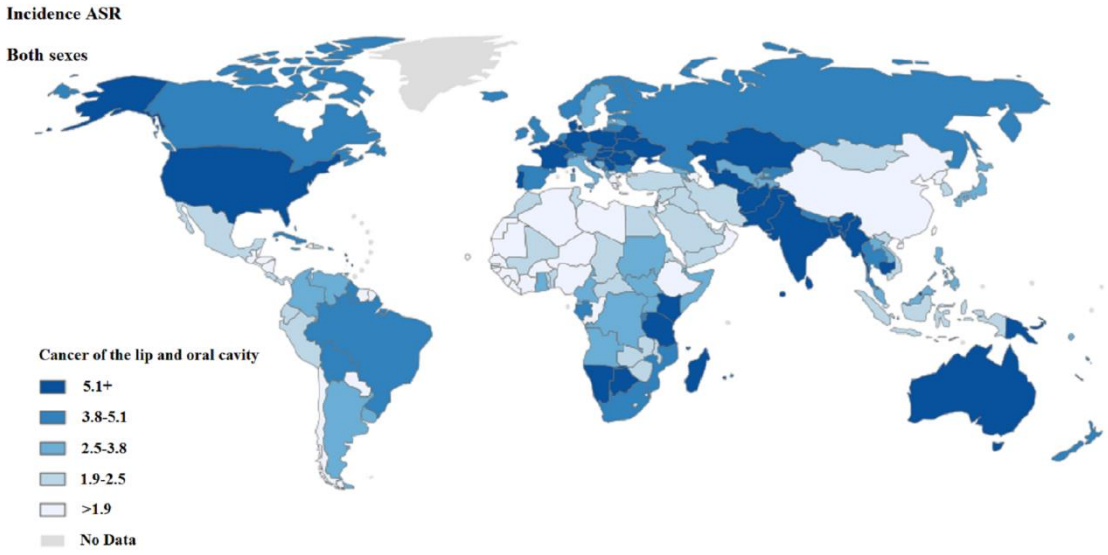

Figure 3- Incidence of lip and oral cavity worldwide, in both genders (Adapted from GLOBOCAN, 2012).

Despite the fact that the incidence of this malignancy is higher in developed countries, India presents the highest rate of oral cancer with 77.003 new cases in 2012, mainly due to the Indian lifestyle (Nagpal and Das, 2003).

Portugal has also a high incidence rate of oral cancer, being reported 1294 new cases in the last GLOBOCAN analysis. As in the great majority of the countries, this disease is also more frequent in males, being considered the 7th neoplasia more common in Portuguese men (GLOBOCAN, 2012).

Oral cancer is an unusual disease in young people (Warnakulasuriya *et al.*, 2007), being the average of detection at 62 years old. However, it is revealed that more than a quarter of patients that develop this disease are younger than 55 (Society, 2015).

1.4. Oral squamous cell carcinoma histology and progression

The oral cavity is lined by a protective mucous membrane, namely oral mucosa. The squamous epithelium that lines the oral cavity is characterized to be stratified and varies in terms of keratinization and thickness (Pai and Westra, 2009).

Oral cancer is considered to be an epithelial neoplasia. The development of a carcinoma is dependent of a multi-step process involving the transition from pre-malignant lesion to metastatic tumour. The progression of neoplasia evolves from benign hyperplasia, to dysplasia to carcinoma *in situ* and, finally, to invasive carcinoma (Rhodus, 2009; Nagpal and Das, 2003) (Figure 4).

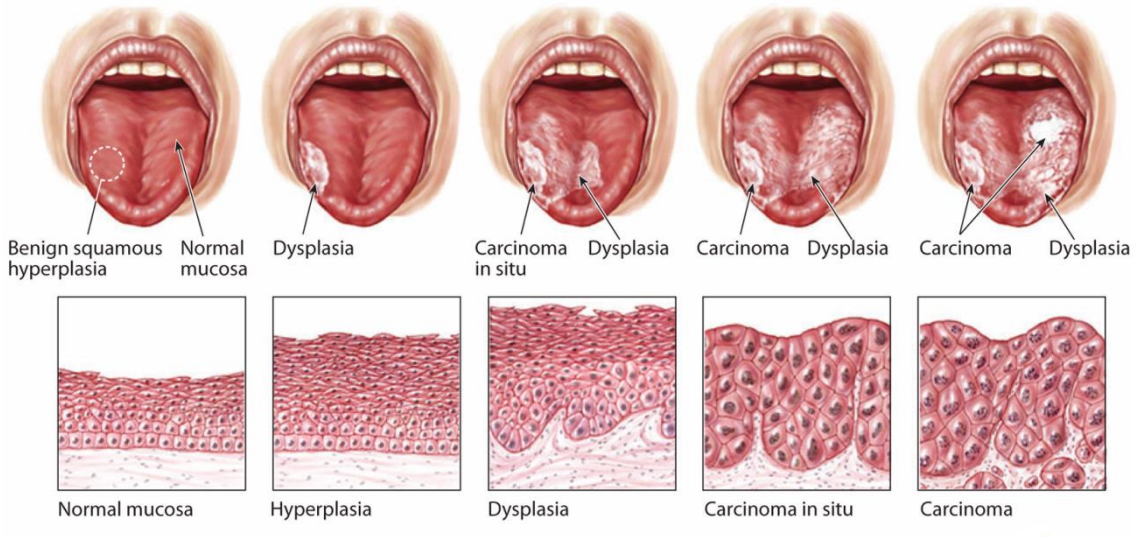

Figure 4- Genetic progression model of OSCC tumourigenesis. Clinical and histologic progression from benign squamous hyperplasia through more advanced stages of squamous dysplasia to invasive squamous cell carcinoma *in situ*. (Adapted from Pai and Westra, 2009).

There are four oral lesions that play a distinct role in oral cancer, such as leukoplakia, erythroplasia, lichen planus and erythroleukoplakia (Rhodus, 2009; Tsantoulis *et al.*, 2007). The leukoplakia is the most common oral lesion and it is phenotypically characterized by white patches or plaques. This lesion, associated with some risk factors, increases the possibility of developing a tumour (Rhodus, 2009). It is suggested that this process starts with the overgrowth of deregulated stem cells, present in the basement membrane. Thus, these cells replace the normal epithelium by expanding upward and laterally (Nagpal and Das, 2003).

The neoplastic alterations on the epithelium surface, termed dysplasia, involve abnormal cellular organization, increased mitotic activity and nuclear expansion with pleomorphism. In order to distinguish the severity of the cellular atypia, a three-degree scale was developed. Although the classification depends on the pathologist, when the atypia is limited only to 1/3 of the epithelium, the dysplasia is considered *mild*, if the atypia riches 2/3 of the epithelium, the dysplasia is referred as *moderate* and finally, the atypia that involves the entire epithelium is considered a *severe dysplasia* (Pai and Westra, 2009).

Although in some cases the patients diagnosed with oral carcinoma did not show dysplasia in previous biopsies, the existence of epithelial dysplasia is considered to be the main condition for carcinoma development (Reibel, 2003). With the progression of dysplasia, the carcinoma *in situ* can invade the basement membrane and infiltrate in subepithelial connective tissues through nests and cords (Pai and Westra, 2009). At least, in more advanced stages, though lymphatic spaces and perineural invasion, the nests and cords can invade skeletal muscle, craniofacial bones and facial skin (Thompson, 2006).

In terms of histology, at microscopy level, there is no significant differences between SCC that arise from oral cavity and those localized in other head and neck sites (Thompson, 2006).

1.5. Staging

Staging cancers is essential to determine the prognosis of the patient and to establish which treatment should be applied. The anatomic staging of oral cancer is provided by the TNM (tumour-node-metastasis) system. T represents the size of the primary tumour, N expresses the status of the regional lymph nodes and M indicates the presence or absence of distant metastasis (Neville and Day, 2002).The TNM system is showed in Table 1.

Table 1- TNM Staging of oral cancer. Adapted from Neville *et al.*, 2002 (Neville and Day, 2002).

Primary tumour (T)	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour 2 cm or less in greatest dimension
T2	Tumour more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumour more than 4 cm in greatest dimension
T4	Tumour invades adjacent structures
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
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

The T, N, M categories can be combined in 32 possible ways, which are grouped in 5 different stages: 0, I, II, III or IV. Stage IV itself is divided into four different stages (Trotta *et al.*, 2011; Neville and Day, 2002) (Table 2).

Table 2- Oral cavity stages based on TNM classification. Adapted from Neville *et al.*, 2002 (Neville and Day, 2002).

Cancer stage	T category	N category	M category
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N1	M0
III	T1,T2,T3	N0,N1	M0
IV	T1,T2,T3 T4 Any T Any T	N2 N0,N1,N2 N3 Any N	M0 M0 M0 M1

1.6. Risk factors

Taking the oral cancer etiology into account, it seems that several risk factors are involved in this malignancy, such as carcinogen exposure, poor / lack of oral hygiene, eating habits, family history, chronic inflammation and viruses (Pai and Westra, 2009; Rhodus, 2009).

1.6.1. Tobacco

The main risk factor associated with oral cancer development is tobacco smoking, especially if combined with alcohol consumption (Hashibe *et al.*, 2000). A study demonstrated the risk of smoking, revealing that in 400 oral cancer patients, 72% smoke and from those, more than 50% consume more than one pack per day (Rhodus, 2009). The exposure to tobacco smoking, in a passive way, also appears to increase the probability of developing oral cancer (Pai and Westra, 2009).

Tobacco has carcinogenic properties as nitrosamines and polycyclic hydrocarbons that may lead to genotoxic effects (Pai and Westra, 2009). Tobacco smoking is correlated with mutations on the *TP53* (tumour protein p53) gene that is associated with resistance to radiotherapy (RT). Furthermore, smoking promotes tumour hypoxia that also affects the efficacy of RT. The carbon monoxide derived from smoking binds with hemoglobin, forming a carboxyhemoglobin complex that

increases the dose of radiation needed for the treatment (Kawakita *et al.*, 2012). Studies demonstrate that the cessation of tobacco smoking reduces, but does not eliminate, the risk of developing cancer (Pai and Westra, 2009). Moreover, generally, patients who stop smoking during RT, have better prognosis (Kawakita *et al.*, 2012).

1.6.2. Alcohol

Alcohol is already considered to be a risk factor for oral cancer and its effect increases substantially when conjugated in a synergetic manner with tobacco smoking (Dal Maso *et al.*, 2015). Alcohol is known to be a chemical solvent and consequently it is responsible for an increasing and longer exposure of the oral mucosa to tobacco carcinogens (Pai and Westra, 2009). Although alcohol is not treated as carcinogen, its direct metabolite, acetaldehyde, has carcinogenic properties that can affect the mechanisms of DNA synthesis and repair (Pai and Westra, 2009; Sciubba, 2001).

1.6.3. Nutritional factors and other lifestyle behaviours

It is reported that nutrition plays a critical role in oral cancer development, suggesting that a poor diet, deficient in fruits, vegetables and carotenoids increases the probability of having this disease (Foulkes, 2013; Petti, 2009; Ram *et al.*, 2011). Several studies suggest that fruits and vegetables are composed by substances containing antioxidant and anti-carcinogenic properties as vitamins (A, C and E), fibers, folates, carotenoids and flavonoids that can reduce the risk of oral cancer development (Petti, 2009; Foulkes, 2013). Additionally, these substances can also have a counterbalance role by decreasing the effects of carcinogenic factors (Petti, 2009).

Studies revealed that there are other factors that are implicated in oral cancer incidence, such as oral hygiene and sunlight exposure (Ram *et al.*, 2011). Poor oral hygiene practices and prolonged irritation of teeth revealed to have a role in the development of oral cancer. Besides that, it is believed that poor oral hygiene can stimulate the carcinogenic potential of tobacco (Ram *et al.*, 2011). Moreover, it was already verified that prolonged exposure to sunlight may be a risk factor of oral cancer, mainly for lip cancer. In this regard, a study revealed that in Greece, amongst oral cancer patients, nearly 60% had lip cancer, probably due to prolonged exposure to the sunlight (Foulkes, 2013).

1.6.4. Virus

The number of oral cancer cases related to smoking and alcohol consumption has been declining over the past 30 years, since smoking habits are lower. In turn, the number of young people diagnosed with OSCC, who do not consume tobacco or alcohol, has been increasing (Young *et al.*, 2015). These cases are known to be related to virus infection and according to the data, the patients tend to be younger (Young *et al.*, 2015; Chawla *et al.*, 2015; Gupta and Gupta, 2015).

1.6.4.1. Human papillomavirus

Human papillomavirus (HPV) infection can be responsible for tumour initiation, since it was already discovered that viral genomic DNA is integrated in the genome of more than 90% of cervical tumours and in 70% of oropharyngeal tumours (Pai and Westra, 2009).

So far, more than 50 HPV are recognized to infect the human mucosa. Among them, the HPV 16 is found in more than 90% of HPV-related HNC cases (Tornesello *et al.*, 2014). The HPV infection invades epithelium basal cells of skin or mucosa. After the invasion, infected cells can proliferate and spread laterally. Then, early viral genes are expressed, in particularly the *E6* and *E7* oncogenes. These two oncoproteins interact with the tumour suppressor genes *TP53* and *RB1* (RB transcriptional corepressor 1), respectively. When the E6 oncoprotein interacts with *TP53*, it promotes its degradation by an ubiquitin-dependent manner, inhibiting p53-mediated apoptosis. The E7 oncoprotein forms a complex with *RB1* causing its inactivation. This inactivation leads to E2factor (E2F) free accumulation which originates increase of *CDKN2A/p16* (cyclin-dependent kinase inhibitor 2A) and, subsequently, abnormal cell proliferation (Agrawal *et al.*, 2013; Leemans *et al.*, 2011).

Studies revealed that HNC HPV-related had histologic and molecular differences from HNC HPV-negative (Pai and Westra, 2009). Furthermore, HPV-positive patients have a better prognosis compared with HPV-negative (Gupta and Gupta, 2015).

1.6.4.2. Epstein - Barr virus

Another virus that can be associated with oral cancer development is Epstein - Barr virus (EBV) (Shimakage *et al.*, 2002). EBV is a γ-Herpes virus, which creates a latent infection in lymphocytes (Chawla *et al.*, 2015). It is proved that this virus endures and can replicate in the epithelial cells of oral mucosa (Shimakage *et al.*, 2002). Taking this into account, Shimakage *et al.* (2002) showed that EBV virus can infect epithelial cells, being responsible for some types of oral

cancers (Shimakage *et al.*, 2002). Studies confirmed the presence of mRNA and proteins of EBV virus in a high percentage of OSCC cases, suggesting that, in these cases, EBV is responsible for this malignancy (Chawla *et al.*, 2015; Cruz *et al.*, 1997; Gupta and Metgud, 2013).

It is suggested that OSCC patients infected with EBV have better prognosis comparatively with those that are not infected with this virus (Llewellyn *et al.*, 2001).

1.7. Detection and diagnosis

As for the majority of the cancers, the early detection of oral cancer is crucial for treatment and survival of patients (Kugimoto *et al.*, 2012). Although oral cavity is a region that can be visually examined due to its easy access, the OSCC are often diagnosed at advanced stages (III or IV) (Hassona *et al.*, 2015; van der Waal *et al.*, 2011). Besides that, the majority of oral cancers, in particular OSCC, are diagnosed when signs and symptoms are already present (van der Waal *et al.*, 2011).

Oral cancer screening can be performed by a physical examination that consists in detection of nodules, mucosa alterations (as changes in colour or texture), swellings and unexplained lymph adenopathy. The physical examination can also be performed by examination (Kao and Lim, 2015). Nonetheless, this method is controversial because it depends on the experience and knowledge of the operator (Awan, 2014). Therefore, a suspicion of a positive finding must be confirmed by biopsy (Kao and Lim, 2015). Nowadays, biopsy represents the main method used for oral cancer detection and diagnosis. This method is followed by a histopathological evaluation performed by a pathologist. Even though biopsy is considered to be the gold standard for oral cancer diagnosis, it is expensive, invasive and also a lengthy process. So, other techniques can be used in order to diagnose this disease in early stages (Awan, 2014).

Toluidine Blue (Tblue) is a metachromatic dye that has affinity to cancer cells, since it stains the acidic tissue components used in pre cancer and cancer detection (Awan, 2014; Kao and Lim, 2015). Nonetheless, the positive results should be confirmed by biopsy (Kao and Lim, 2015).

Another method of screening is the use of optical technologies that is advantageous, because it provides a real time assessment in a minimal invasive manner.

Autofluorescence is the optical technology most frequently used. This technique provides real time results, is easy to perform and cost effective. Although this method allows the determination of the difference between diseased oral mucosa and “normal” oral mucosa, it does not always provide the type of oral lesion. Moreover, the positive results for oral lesion must be confirmed by

biopsy (Awan, 2014). Optical technologies also include Raman spectroscopy, confocal laser endomicroscopy, confocal reflectance microscopy and narrow band imaging (Davies *et al.*, 2015).

Exfoliative cytology is another diagnostic method, which consists in tissue collection from mucosal surfaces by scrapping or brushing to detect cytological alterations. This technique is advantageous, because it is painless, non-invasive and simple to perform. Additionally, cell sample collection can be repeated numerous times to diagnose and follow-up the patients (Verma *et al.*, 2015; Bremmer *et al.*, 2005). A study revealed that Tblue staining and exfoliative cytology used in combination is a sensitive and specific method for oral lesions detection (Gupta *et al.*, 2007). Bremmer *et al.* (2009) analysed the DNA from exfoliated cells samples from 25 patients with oral lesions and compared the results with biopsy results from the same patients. Their results showed that the non-invasive assay had a high sensitive rate and a positive predictive value of 100%. Thus, they inferred that this non-invasive technique could be promising to detect oral lesions, including, oral cancer (Bremmer *et al.*, 2009). However, up to now, this technique is still limited because some false-positive and false-negative results were reported in a few studies (Kao and Lim, 2015).

The promising methods of oral cancer detection consist in the use of biomarkers present in biofluids such as saliva or plasma (Kao and Lim, 2015). Blood analyses are a minimally invasive technique that has been gained clinical value to cancer diagnosis. In plasma/serum of several cancer patients, the presence of cell-free DNA (cfDNA) from tumour cells has been suggested to have diagnostic value. It was already discovered that cfDNA contains genetic and epigenetic alterations that are related with cancer initiation, progression and resistance such as loss of heterozygosity (LOH) and mutations on tumour suppressor genes/oncogenes (Schwarzenbach *et al.*, 2011). Saliva is an oral fluid that is easily accessed by a non-invasive approach. The use of this biofluid has been considered a promising method to diagnose several diseases, including cancer. Although more studies should been done in this area, it is already described that salivary biomarkers can be used to OSCC diagnosis, especially in early stages of the disease (Lee and Wong, 2009; Guerra *et al.*, 2015).

Despite of the advances in diagnostic techniques, the detection of oral cancer, in an early stage, remains insufficient. Definitely, more accurate techniques should be implemented in order to do a diagnosis of oral cancer at early stages, promoting better prognosis. Besides that, instead of invasive techniques, which is the case of the biopsy, non-invasive or less invasive diagnosis methods should be applied.

1.8. Therapy for oral cancer

The treatment applied to oral cancer patients depends on the staging and the location of the tumour (Foulkes, 2013). Factors as nutritional status, general health, consumption of tobacco and alcohol must also be taken into account (Rhodus, 2009). Moreover, the choice of the appropriated treatment requires a sense of balance between tumour eradication and the anatomic and function preservation of the organs involved (Prince *et al.*, 2010).

The main types of oral cancer treatments are local surgery, radiotherapy (RT) and chemotherapy (QT), which can be applied in combination (Rhodus, 2009; Foulkes, 2013). Tumours detected at early stages (I or II) can be curable by surgery or RT by itself. In contrast, tumours at advanced stages (III or IV) are normally treated with surgery followed by RT or QT (Tsantoulis *et al.*, 2007). However, in advanced stages, with the presence of metastasis, the QT is only used as a palliative treatment (Gold *et al.*, 2009).

1.8.1. Surgery

The purpose of surgery is the excision of the tumour. Normally, some portion of normal cells are also removed to prevent tumour recurrence and to eliminate lymph nodes that can be affected by the cancer. Surgery is considered the only curative option for oral cancer. After tumour removal, a reconstructive operation can be done in order to restore the anatomic form and function of the affected areas (Foulkes, 2013). In patients who had oral cancer at advanced stage, the surgery *per se,* is not sufficient to treat, so, the use of RT as an adjuvant is needed (Ko and Citrin, 2009).

1.8.2. Radiotherapy

RT uses high energy radiation (x-rays) to destroy cancer cells or to inhibit DNA replication. RT is used in different situations, such as:

- small primary tumours as an alternative of surgery;

- larger tumours in order to try to reduce the initial tumour before surgery;

- after surgery, that allows the elimination of some cancer cells that are not removed from surgery or in areas that through surgery it is difficult to achieve;

- incurable cancers. In these cases, RT is used as a palliative treatment, trying to reduce pain and bleeding and control tumour growth (Foulkes, 2013).

This treatment can affect the normal tissues that may become inflamed and in some cases may fall apart (Foulkes, 2013).

1.8.3. Chemotherapy

QT is based on the use of anti-cancer drugs that can be administered into vein or by mouth. The classic drugs more used in oral cancer QT are Cisplatin, Carboplatin, 5-fluorouracil (5-FU), Paclitaxel and Docetaxel, which can be used alone or in a combination of two. This kind of treatment has shown to be effective, especially when combined with RT or surgery (Foulkes, 2013). However, QT has several side effects, since these drugs target cells in rapid proliferation. Therefore, normal cells can also be affected, causing secondary effects that alter the quality of life (Gerber, 2008).

1.8.4. Target therapy

In the last years, oncobiologists focus their research in the molecular mechanisms involved in carcinogenesis, which allows the development of new therapeutic approaches. Target therapies are based on the use of agents that target specific molecules responsible for tumour initiation and growth. It is intended for this kind of treatment to be less aggressive for the patients, since the targeted molecules are expressed at high concentrations or even only in cancer cells. The agents used in target therapies are divided in two main types: monoclonal antibodies and small molecule inhibitors (Gerber, 2008).

In oral cancer, molecular agents targeting epidermal growth factor receptor (EGFR) are frequently used, since EGFR is highly expressed in this cancer. Up to now, Cetuximab is the only EGFR-target agent approved by Food and Drug Administration (FDA) for oral cancer treatment (Boeckx *et al.*, 2013).

Cetuximab (commercially available as Erbitux®, Merck KGaA, Germany) is a human/murine chimeric monoclonal antibody that inhibits tumour development by binding to the EGFR extracellular domain and, consequently, inhibiting EGFR dimerization and activation (Blick and Scott, 2007). Therefore, Cetuximab prevents proliferation, angiogenesis, anti-apoptotic signalling, invasion and metastasis (Burtness, 2005). Cetuximab can be used alone or in combination with RT or even in combination with classic QT (Boeckx *et al.*, 2013).

The use of Cetuximab may lead to secondary effects as a result of the EGFR being also expressed in normal tissues. The more common side effects reported are cutaneous rash, nausea, diarrhea and vomiting (Gerber, 2008).

1.9. Genetic and cytogenetic alterations in oral squamous cell carcinoma

The initiation and development of cancer results from changes in several pathways due to accumulation of genetic *loci* alterations that include point mutations, insertions, deletions, amplifications and chromosomal rearrangements (Tan *et al.*, 2013). The most common altered genes in OSCC are divided in two main groups: oncogenes and tumour suppressor genes (Tsantoulis *et al.*, 2007; Tan *et al.*, 2013; Cha *et al.*, 2011).

Detection of cytogenetic abnormalities has been revealed as useful markers for diagnosis and prognosis of tumours, including for OSCC (Gollin, 2001; Ribeiro *et al.*, 2014b). Although some genetic abnormalities associated with HNSCC have been detected, cytogenetic analyses of solid tumours can be difficult due to numerous factors, such as low mitotic index or small specimen size. It is reported that only 30% of HNSCC can grow in culture and yield metaphase spreads can be analysed (Gollin, 2001).

It is revealed that structural chromosome alterations are frequent in HNSCC. The most common alterations that have been reported are deletions, translocations, homogeneously staining regions (hsr) and isochromosomes. Nevertheless, other alterations less frequent have been also described, including duplications, insertions, inversions, endoreduplication, ring chromosomes and dicentric chromosomes (Gollin, 2014).

Despite the complexity of OSCC, its high frequency of near-triploid and its composition of multiple clonal numerical and structural chromosome abnormalities, chromosomal gains and losses in OSCC have been described (Gollin, 2014). Martin *et al.* (2008) reported chromosomal losses at 3p, 4p, 8p, 11q, and 8q and chromosomal gains at 3q, 5p, 7p/q, 8q, 9q, 11q, 14q, 19q, and 20q in 31 cell lines of OSCC (Martin *et al.*, 2008). A more recent study, developed by Ribeiro *et al.* (2014), identified the highest rates of gains at chromosomes 8 and 11 and chromosomal region 3q and the highest rates of losses at 3p and 8p, in OSCC samples (Ribeiro *et al.*, 2014b). Besides these alterations, other chromosomal abnormalities have been detected in HNSCC (Tan *et al.*, 2013; Gollin, 2014; Gollin, 2001).

The genes that have been reported as the most involved in HNSCC initiation and development are represented in Table 3 with the respective chromosome locations. The alterations associated with these genes will be further discussed.

Table 3- Most frequent altered genes in OSCC.

Region	Genes	Gene name	Alteration	References
3p	*FHIT*	Fragile histidine triad	Loss	(Gollin, 2014; Ribeiro *et al.*, 2014b)
	RARB	Retinoic acid receptor, beta	Loss	(Ribeiro *et al.*, 2014b)
3q	*TP63*	Tumour protein p63	Gain	(Gollin, 2014)
	PIK3CA	Posphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha	Gain	(Gollin, 2014)
7p	*EGFR*	Epidermal growth factor	Gain	(Gollin, 2001; Ribeiro *et al.*, 2014b)
8p	*CSMD1*	CUB and Sushi multiple domains 1	Loss	(Gollin, 2014)
	WHSC1L1	Wolf-Hirschhorn syndrome candidate 1-like 1	Gain	(Gollin, 2014)
8q	MYC	v-myc avian Myelocytomatosis viral oncogene homolog	Gain	(Ribeiro *et al.*, 2014b; Gollin, 2014)
	PTK2	Protein tyrosine kinase 2	Gain	(Gollin, 2014)
9p	PTPRD	Protein tyrosine phosphatase, receptor type, D	Loss	(Ribeiro *et al.*, 2014b)
	CDKN2A	Cyclin-dependent kinase inhibitor 2A	Loss	(Gollin, 2014)
9q	*NOTCH1*	Notch 1	Gain	(Tan *et al.*, 2013)
11q	*CCND1*	cyclin D1	Gain	(Gollin, 2014; Gollin, 2001; Martin *et al.*, 2008)
	ATM	ATM serine/threonine kinase	Loss	(Gollin, 2014)
13q	RB1	RB transcriptional corepressor 1	Loss	(Gollin, 2014)
	ING1	Inhibitor of growth family, member 1	Loss	(Koontongkaew *et al.*, 2000; Pande *et al.*, 1998)
17p	*TP53*	Tumour protein p53	Loss	(Guo and Califano, 2015; Agrawal *et al.*, 2011; Stransky *et al.*, 2011; Cancer Genome Atlas, 2015)
18q	*GALR1*	Galanin receptor 1	Loss	(Gollin, 2014)
	PARD6G	Par-6 family cell polarity regulator gamma	Loss	(Gollin, 2014)

1.9.1. Region 3p

Losses of 3p chromosomal region can be detected in 56% to 78% of oral dysplasias and in more than 90 % of OSCC patients. It is suggested that the loss of 3p is related with early stages of OSCC. The loss of this arm results from isochromosome formation or chromosome breakage (Gollin, 2014).

The *FHIT* (fragile histidine triad) gene, mapped at 3p14 is the most frequent and reported loss at this region, in OSCC. It is reported that loss of the protein coded by *FHIT* leads to DNA damage, genetic instability and consequently to OSCC development and progression (Gollin, 2014; Ribeiro *et al.*, 2014b). *RARB* (retinoic acid receptor, beta) is another gene mapped at this region that has been reported as involved in OSCC carcinogenesis. *RARB* encodes for the retinoic acid receptor beta, which participates in embryonic morphogenesis, cell growth and differentiation through binding to retinoic acid. The loss of this gene can induce carcinogenesis by lack of response to retinoids (Table 3) (Ribeiro *et al.*, 2014b).

1.9.2. Region 3q

It is believed that gains in the long arm of chromosome 3 are the most common chromosomal alteration in HNSCC (Gollin, 2014; Ribeiro *et al.*, 2014b).

Gains in this region seem to be involved with overexpression of cancer-related genes such as *PIK3CA* (phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha) and *TP63* (tumour protein p63) (Gollin, 2014; Sherr and Roberts, 1999) (Table 3).

The *PIK3CA* is encoded at 3q26.3 and its increased copy number is identified in numerous cancer cases (Gollin, 2014). Overexpression of this gene leads to uncontrolled cell growth and survival, transformation and drug resistance. This oncogene is mutated in 6% to 29% of HNSCC (Murugan *et al.*, 2008). The amplification of *PIK3CA* is related with tumour relapse and poor prognosis (Gollin, 2014) (Table 3).

TP63 is mapped at 3q28 and its overexpression and/or its amplification is associated with invasive HSCC and poor survival rates (Rothenberg and Ellisen, 2012; Gollin, 2014).

Snijders *et al.* (2005) demonstrated that the gene *TM4SF1* (transmembrane 4 L six family member 1) at 3q24-25 is amplified in OSCC (Snijders *et al.*, 2005). Moreover, other studies identified *MME* (membrane metallo-endopeptidase), *IL12A* (interleukin 12A), *DCUN1D1* (DCN1, defective in cullin neddylation 1, domain containing 1) and *SOX2* (sex determining region Y-box 2) as genes that

have extra copy number in OSCC (Ribeiro *et al.*, 2014b; Martin *et al.*, 2008; Gollin, 2001). In general, it is reported that gains in 3q25-29 are related to poor survival rates (Gollin, 2014).

1.9.3. Region 7p

Gains of 7p region are reported in HNSCC cell lines, specifically 7p12-22 bands. 7p12 mapped for the *EGFR* gene, which is overexpressed in around 90% of OSCC cases (Gollin, 2001; Gollin, 2014; Martin *et al.*, 2008) (Table 3). The overexpression of *EGFR* is caused by gene copy number, gene amplification, increased mRNA synthesis, decreased downregulation or expression of EGFRvIII (Gollin, 2014). EGFR is a receptor tyrosine kinase of the ErbB (epidermal growth factor receptor) family of cell surface receptors. After activation by ligand binding, EGFR forms a dimer and activates downstream pathways such as PI3K (phosphoinositide 3-kinase), AKT, JAK/STAT (janus kinase/signal transducer and activator of transcription) and Ras (Table 3). These pathways are involved in proliferation, evasion of apoptosis, invasion, angiogenesis and metastasis (Kalyankrishna and Grandis, 2006). The overexpression of this oncogene is associated with poor prognosis and with increased local recurrence (Guo and Califano, 2015).

1.9.4. Region 8p

Loss of 8p region is detected in 58% of HNSCC, most frequent at 8p21 and 8p22-p23. *CSMD1* (CUB and Sushi multiple domains 1) is mapped at 8p23 and its expression is abnormal in several HNSCC as a consequence of deletion, epigenetic silencing or aberrant splicing (Table 3) (Gollin, 2014). Other genes, mapped at this region, including *GATA4* (GATA binding protein 4), *MTUS1* (microtubule associated tumour suppressor 1) and *TUSC3* (tumour suppressor candidate 3) are also usually deleted in HNSCC (Ribeiro *et al.*, 2014a; Ribeiro *et al.*, 2014b).

Gains of 8p region have also been detected, particularly at 8p11.2 band. *WHSC1L1* (Wolf-Hirschhorn syndrome candidate 1-like 1) is a gene mapped at this band and is overexpressed in several HNSCC (Table 3) (Gollin, 2014).

1.9.5. Region 8q

Studies proved that gains of 8q regions, especially bands 8q23.1-8q24.22, are involved in numerous HNSCC cases. Gains of this arm are essentially due to isochromosome formation (Gollin, 2014). The oncogene *MYC* (v-myc avian myelocytomatosis viral oncogene homolog) is an important gene mapped at 8q region that is overexpressed in several OSCC, due to gene amplification or copy

number gain. *MYC* overexpression is related with poor prognosis (Table 3) (Gollin, 2014; Ribeiro *et al.*, 2014b). The gene *PTK2* (protein tyrosine kinase 2) mapped at 8q24 is another gene that is overexpressed in several HNSCC (Table 3). The overexpression of *PTK2* is related with invasiveness (Gollin, 2014). Other genes mapped at 8q as *LRP12* (low density lipoprotein receptor-related protein 12) and *WNT1* (wingless-type MMTV integration site family, member 1) are also overexpressed in OSCC (Gollin, 2014; Ribeiro *et al.*, 2014b; Ribeiro *et al.*, 2014a).

1.9.6. Region 9p

The short arm of chromosome 9 is lost in a large number of HNSCC. Loss of 9p regions is associated with early stages of the disease (Ribeiro *et al.*, 2014b; Gollin, 2014). Isochromosome formation and deletions of variable sizes are the main causes of 9p losses. At this region, there are important genes that are reported to be lost or mutated in OSCC as *PTPRD* (protein tyrosine phosphatase, receptor type, D) at 9p23-24 and *CDKN2A* at 9p21.3 (Table 3). (Gollin, 2014; Ribeiro *et al.*, 2014b).

The protein coded by *PTPRD* is a tyrosine phosphatase receptor type D that plays important roles in cellular signalling, including in dephosphorylation of *STAT3* (signal transducer and activator of transcription 3), inhibiting tumour cell growth. Therefore, *PTPRD* mutations or deletions can directly lead to tumour growth due to hyperactivation of *STAT3*.

CDKN2A is a gene that encodes for p16 protein. This protein is important to cell cycle regulation due to its interaction with Rb (retinoblastoma) protein. The p16 inhibits cyclin-dependent kinases (CDK) 4 and 6, facilitating the phosphorylation of Rb (once CDK 4,6 inhibit Rb phosphorylation) (Tan *et al.*, 2013). *CDKN2A* gene is often altered in HNSCC (9% to 12% of HNSCC cases) (Gollin, 2014). Although loss of heterozygosity of this gene is the most frequent alteration detected in HNSCC, *CDKN2A* can also be inactivated by point mutations or methylation of the 5' CpG region (Leemans *et al.*, 2011; Tsantoulis *et al.*, 2007; Guo and Califano, 2015). In oral cancers, loss of p16 protein expression were found in more than 83% of cases (Tsantoulis *et al.*, 2007). Studies revealed that loss of p16 in HNSCC patients is related to poor prognosis (Tsantoulis *et al.*, 2007; Park *et al.*, 2007).

1.9.7. Region 9q

At 9q region, specific at 9q34.3, is mapped the *NOTCH1* gene that is overexpressed in numerous OSCC cases (Table 3) (Tan *et al.*, 2013). It is reported that NOTCH1 alterations were

found in from 14% to 15% of HNSCC, being the second most altered gene in this malignancy (Sun *et al.*, 2014). *NOTCH1* plays crucial roles in normal cells differentiation, lineage commitment and embryonic development (Tan *et al.*, 2013). This gene encodes for a protein that is a transmembrane ligand receptor. It is suggested that in HNSCC, *NOTCH1* acts as a tumour suppressor gene based in the position and characteristics of *NOTCH1* mutations and the inactivation of both alleles. In contrast, it is also suggested that in some oral cancer cases, *NOTCH1* may act as an oncogene (Sun *et al.*, 2014; Tan *et al.*, 2013; Guo and Califano, 2015).

1.9.8. Region 11q

The amplification of 11q13 band is the chromosomal alteration more studied of chromosome 11 in HNSCC. The 11q13 amplification is, normally, a result of BFB (breakage-fusion-bridges) cycles initiated by a break at the common chromosomal fragile site FRA11F. It is suggested that this amplification occurs at early stages of the disease, being essential for the transition from moderate to severe dysplasia. Almost all of the 13-14 genes mapped at 11q13 are overexpressed in HNSCC. The gene considered to be the most important of this band is the *CCND1* (cyclin D1) oncogene (Table 3), which plays an important role in G1/S transition. Accordingly, overexpression of this gene leads to a faster transition from G1 to S. The overexpression of cyclin D1 is present in 36% to 66% of oral cancer and potentially malignant lesions. The amplification of the *CCND1* leads to accumulation of cyclin D1, which is related to a bad prognosis and with increased probability of cervical lymph node metastasis occultation, mainly in low stages tumours. (Gollin, 2001; Gollin, 2014; Martin *et al.*, 2008).

Besides the amplification at this region, loss of the long arm of chromosome 11 has been also detected, distal to 11q13 amplification (Gollin, 2014). It is suggested that in HNSCC, 11q distal losses are the first step in 11q13 amplification process. Distal 11q contains a couple of DNA damage responsive (DDR) genes, including *MRE11A* (MRE11 homolog A, double strand break repair nuclease) (11q21), *ATM* (ATM serine/threonine kinase) (11q23.3), *H2AFX* (H2A histone family, member X) (11q23.3) and *CHEK1* (checkpoint kinase 1) (11q24.2) (Gollin, 2014; Gollin, 2001; Martin *et al.*, 2008).

ATM is one of the most important genes involved in DDR. *ATM* is phosphorylated in responses to DSB (DNA double-strand breaks) caused by ionizing radiation. After *ATM* phosphorylation, proteins involved in DDR are also phosphorylated leading to cell cycle arrest,

apoptosis and DNA repair. Loss of *ATM* is associated with OSCC development and resistance to RT (Gollin, 2014).

1.9.9. Region 13q

The long arm of chromosome 13 is lost in a large number of HNSCC cases, especially the 13q12.11 and 13q14.2 bands. One gene included in 13q is the *RB1* that plays crucial roles in cell cycle control and its loss is associated with development of tumours, including OSCC (Table 3) (Gollin, 2014). *RB1* encodes for Rb protein, which, in a hypo-phosphorylated state, binds and inactivates the E2F transcription factor. This transcription factor is responsible for cell cycle progression from G to S phase (Tsantoulis *et al.*, 2007). A study developed by Soni *et al.* showed that around 90% of OSCC cases and 84% of potentially malignant lesions exhibits altered expression of one or more compounds of Rb network (Soni *et al.*, 2005). Studies revealed that absence of phosphorylated Rb (pRb) expression was about 70% in oral cancers and 64% in potentially malignant lesions (Koontongkaew *et al.*, 2000; Pande *et al.*, 1998).

ING1 (inhibitor of growth family, member 1) is mapped at 13q.34 and it is suggested that mutations on this gene leads to uncontrolled cell growth that may be associated with tumour development, including OSCC (Table3) (Szyfter *et al.*, 2014).

1.9.10. Region 17p

Loss of 17p13 region is the chromosomal alteration more relevant of chromosome 17 since the tumour suppressor gene *TP53* is mapped at this region (17p13.1). *TP53* encodes for p53 protein, which is considered the "guardian of the genome" (Lane, 1992). This protein accumulates in response to DNA damage as well as to other stress factors. p53 accumulation leads to cell cycle arrest or apoptosis, depending on whether the DNA damages are repaired or not (Guo and Califano, 2015). The *TP53* is the most frequently altered gene in HNSCC as well as in potentially malignant lesions (Agrawal *et al.*, 2011; Stransky *et al.*, 2011; Cancer Genome Atlas, 2015). In HNSCC, the *TP53* alterations more frequent are missense mutations (50% to 60%). Missense mutations in *TP53* can have two consequences: a stable protein that loses its key binding ability or a protein that acts as a dominant negative inactivating the wild-type TP53 (Guo and Califano, 2015). Studies revealed that *TP53* mutations are related to poor prognosis, decreased rates of survival and increased risk of locoregional recurrence (Guo and Califano, 2015; Tan *et al.*, 2013). Furthermore, mutations in this

gene are also related to resistance to therapies as cisplatin, fluorouracil and RT (Table 3) (Tan *et al.*, 2013).

1.9.11. Region 18q

Loss of 18q is common in HNSCC, especially loss of 18q23. This event is related to advanced stages of tumour and poor prognosis. Two of the 18 genes affected by the loss of this arm are *GALR1* (galanin receptor 1) and *PARD6G* (par-6 family cell polarity regulator gamma) (Table 3). *GALR1* mapped at 18q23 is lost in HNSCC as a result of promoter methylation. *GALR1* encoded to a G-protein coupled receptor that is important to inhibit proliferation in keratinocytes through the inactivation of the MAPK (mitogen-activated protein kinases) pathway (Gollin, 2014).

It is demonstrated that *PARD6G* deletions affect ciliogenesis, interphase and spindle microtubule organization and it also leads to defects in the centrosome organization and function (Gollin, 2014).

1.10. Epigenetic alterations

Epigenetics is described as mitotically and/or meiotically heritable changes in gene expression without alterations in DNA sequence (Jones and Baylin, 2007). This kind of alterations are important in many physiological processes as differentiation, embryogenesis, genomic imprinting and chromosomal domains inactivation.

As in many other biological processes, epigenetic mechanisms can be dysregulated. Epigenetic abnormalities are relevant in some diseases, including cancer (Taby and Issa, 2010; Jones and Baylin, 2007).

Epigenetic changes include three main mechanisms: DNA methylation, histone modification and microRNA or interfering RNA expression (Taby and Issa, 2010).

1.10.1. DNA methylation

DNA methylation is a covalent modification that consists in the addition of a methyl group to the carbon 5 of a cytosine at CpG islands. This reaction is catalysed by DNA methyltransferases (DNMTs) (Das and Singal, 2004). DNA methylation represents the most frequent and best characterized epigenetic event in carcinogenesis, including in oral cancer (Noorlag *et al.*, 2014).

This epigenetic event can affect gene expression though TSGs hypermethylation, genome hypomethylation or direct mutagenesis (Stirzaker *et al.*, 2014) (Figure 5).

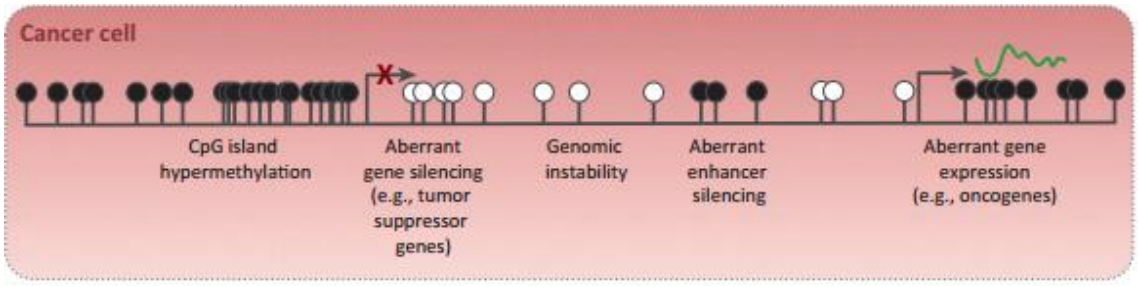

Figure 5- DNA methylation in cancer. CpG islands hypermethylated, resultant in gene silencing and hypomethylation of CpG-poor oncogene promoters, resultant in genomic instability and abnormal gene expression. (Black circle - methylated CpG; White circle - unmethylated CpG). (Adapted from Stirzaker *et al.*, 2014).

Promoter hypermethylation of TSGs is considered the main mechanism involved in carcinogenesis. Although DNA sequence is maintained, promoter hypermethylation leads to a closed chromatin configuration and, consequently, TSGs silencing. (Ha and Califano, 2006; Noorlag *et al.*, 2014).

Promoter hypermethylation of TSGs has been widely studied in OSCC, allowing the knowledge of several genes that are hypermethylated in this malignancy. Some of these genes are represented in Table 4.

Epigenetic changes, particularly, DNA methylation are early events in the carcinogenesis process, being responsible for tumour initiation and progression and, consequently, predisposing cells to accumulation of genetic abnormalities (Taby and Issa, 2010). Therefore, it is important to identify epigenetic alterations in order to find biomarkers that can be used not only to detect OSCC in early stages, but also to assess the disease's progression (Li *et al.*, 2015).

Contrary to genetic alterations, DNA methylation is reversible. This fact represents an attractive target for developing new therapeutic approaches using DNMT inhibitors that can reactivate the transcription of methylated TSGs (Noorlag *et al.*, 2014)

Table 4- Frequent hypermethylated genes in OSCC.

GENE	GENE NAME	LOCATION	FUNCTION	REFERENCES
APC	Adenomatous Polyposis Coli	5q21-q22	Tumour Suppression	(Noorlag *et al.*, 2014)
CDH1	Cadherin 1	16p22.1	Cell adhesion	(Kulkarni and Saranath, 2004; Arantes *et al.*, 2015)
CDH13	Cadherin 13	16q23.3	Cell adhesion	(Noorlag *et al.*, 2014)
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A	9p21.3	Tumour Suppression	(Maruya *et al.*, 2004; Noorlag *et al.*, 2014; Arantes *et al.*, 2015; Sanchez-Cespedes *et al.*, 2000)
CDKN2B	Cyclin-Dependent Kinase Inhibitor 2B	9p21	Tumour Suppression	(Noorlag *et al.*, 2014)
CHFR	Checkpoint with Forkhead and Ring finger domains	12q24.33	Mitotic Checkpoint	(Noorlag *et al.*, 2014)
DAPK	Death-Associated Protein Kinase 1	9q34.1	Apoptosis	(Maruya *et al.*, 2004; Noorlag *et al.*, 2014; Arantes *et al.*, 2015; Sanchez-Cespedes *et al.*, 2000)
ESR1	Estrogen Receptor 1	6q24-q27	Activation of transcription; Metastasis-suppressor properties	(Noorlag *et al.*, 2014)
FHIT	Fragile Histidine Triad	3p14.2	Tumour Suppressor	(Chang *et al.*, 2002; Lin *et al.*, 2015)
GSTP1	Glutathione S-Transferase Pi 1	11q13.2	Protection against DNA damage caused by glutathione	(Sanchez-Cespedes *et al.*, 2000)
MGMT	O-6-methylguanine-DNA methyltransferase	10q26	DNA Repair	(Maruya *et al.*, 2004; Sanchez-Cespedes *et al.*, 2000)
MLH1	mutL homolog 1	3p21.3	DNA repair	(Arantes *et al.*, 2015)
RARß	Retinoic Acid Receptor, beta	3p24	Tumour Suppression	(Maruya *et al.*, 2004; Noorlag *et al.*, 2014)
TP73	Tumour Protein p73	1p36.3	Pro-apoptotic and anti-apoptotic properties	(Arantes *et al.*, 2015; Maruya *et al.*, 2004)

1.11. Laboratory techniques

To investigate the genetic and epigenetic alterations previous referred, several laboratory techniques can be used such as array Comparative Genomic Hybridization (aCGH) and Methylation Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA).

1.11.1. Array Comparative Genomic Hybridization

aCGH is a high-resolution technique that allows detection of genomic imbalances, namely copy number variations (CNVs) by screening the entire genome (Shinawi and Cheung, 2008). In this technique, two different genomic DNA samples, test and reference, are differentially labelled and hybridized to DNA targets arrayed on a solid support (a glass slide) (Bejjani and Shaffer, 2006).

The first step underlying the mechanism of aCGH is DNA extraction from the two different samples, reference (control) and test. After that, an equal amount of DNA from the test and reference are differently labelled with cyanine 3 (cy3) and cyanine 5 (cy5). The labelled DNA is co-hybridized into an array containing DNA targets that have been spotted on a slide. The slides are then scanned into images by using a microarray scanner. The ratio between the two probes, which represents the ratio of DNA quantity in the test and reference is measured in each spot. So, equal intensity of both dies means that the test and reference have the same amount of DNA. However, an alteration in cy3:cy5 ratio indicates loss or gain of DNA in the test sample when compared with the reference (Figure 6) (Bejjani and Shaffer, 2006; Shinawi and Cheung, 2008; Pinkel and Albertson, 2005).

The main advantages of this technique are high resolution, automation, simplicity and the possibility of mapping different imbalances through all the genome. Furthermore, compared with cytogenetic techniques, aCGH does not require previous cell culture. Normally, this technique only requires a small amount of DNA. Even though, aCGH has also some limitations, since it does not allow the detection of balanced rearrangements such as inversions, insertions and/or translocations or polyploidy (Shinawi and Cheung, 2008).

aCGH represents a powerful tool used in clinical diagnosis and also in research. Regarding research applications, this methodology is important to gene discovery and cancer profiling (Bejjani and Shaffer, 2006).

Since genetic and epigenetic instability are the main characteristics of cancer, aCGH can be used in order to find CNVs that can be associated with each cancer type, providing cancer profiles knowledge, which can be important to cancer diagnosis and patients follow-up (Pinkel and Albertson, 2005; Shinawi and Cheung, 2008).

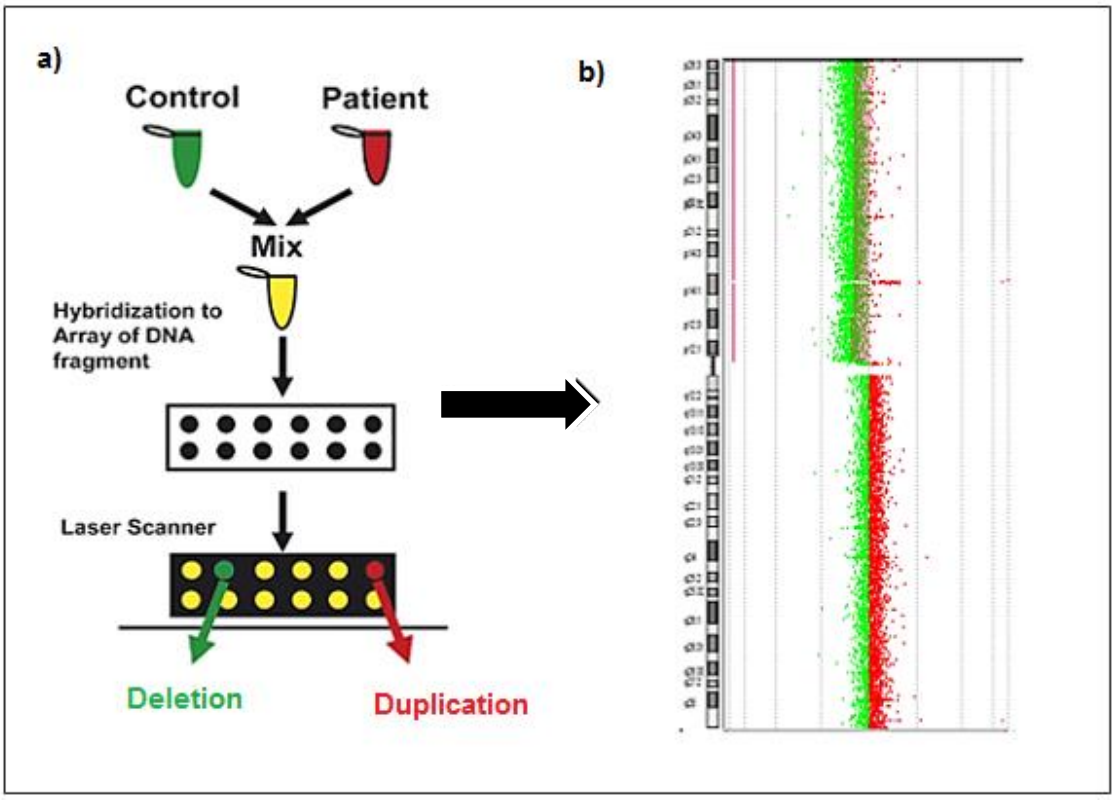

Figure 6- Principles of aCGH. **a)** Reference DNA is labelled with a green fluorescence dye (Cy3) and the DNA of the sample to be tested is labelled with red (Cy5). The two samples are mixed and co-hybridized to an array containing genomic DNA targets. The fluorescence intensity is measured and it is proportional to the ratio of the copy numbers of DNA sequences in the test and reference genomes. The green areas on the slides correspond to less DNA in the test sample (deletion) and red areas indicate extra DNA copies in the test sample (duplication) A Scanner is responsible for the conversion of the results into images. **b)** Example of an array profile. (Adapted from Shinawi & Cheung, 2008).

1.11.2. Methylation Specific Multiple Ligation-dependent Probe Amplification

Multiplex Ligation-dependent Probe Amplification (MLPA) is a multiplex Polymerase Chain Reaction (PCR) method that detects CNVs of up to 50 DNA sequences, in a single reaction. The main characteristic that distinguish this method from others, like common PCR, is the fact that in MLPA, instead of target sequence amplification, it is the MLPA probes that are amplified. Besides that, only a single PCR primer pair is needed for MLPA amplification (MRC-Holland; Schouten *et al.*, 2002).

MLPA comprises five main steps: Denaturation and hybridization; Ligation; PCR amplification; Capillary electrophoresis and fragment analysis. After DNA denaturation, a MLPA probe mixture is added to overnight hybridization. Each MLPA probe is composed by one short synthetic oligonucleotide and one long probe oligonucleotide, derived from phage M13. After hybridization is

completed, ligase is added and, consequently, the probe can be amplified by PCR. Finally, the amplification products are separated by capillary electrophoresis and data can be analysed (MRC-Holland).

In order to detected methylation status of some genes, a MLPA variant was developed: MS-MLPA. In this variant, the main difference is the use of a restriction enzyme, namely, *HhaI.* The process is similar to MLPA, however, in MS-MLPA, after hybridization step, the samples are divided in two reactions: one is carried out as in MLPA and to the other, *HhaI* is added. The restriction enzyme will digest the probes that hybridized with unmethylated DNA. On the other hand, the probes hybridized to methylated DNA remain undigested and therefore, will be amplified by PCR. The amplification products are separated by capillary electrophoresis (MRC-Holland) (Figure 7).

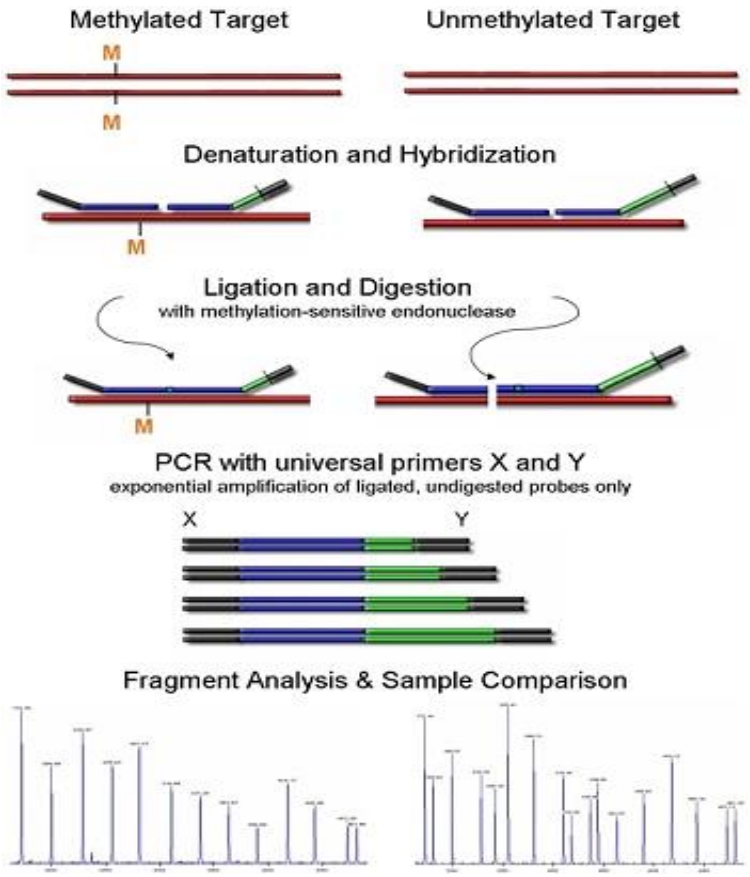

Figure 7- Schematic representation of MS-MLPA assay (Adapted from MRC-Holland, 2016).

As the major of lab techniques, MS-MLPA has several advantages but also some limitations. MS-MLPA allows the detection of genetic and epigenetics, such as CNVs and methylation, in the same reaction and also the detection of small rearrangements. This is advantageous since it saves time consumption and it also reduces the amount of sample needed. Furthermore, compared with other techniques, as karyotyping and Fluorescence in Situ Hybridization (FISH) that are performed

with intact cells, MS-MLPA only requires extracted DNA. Moreover, the results can be obtained in less than 24 hours and the procedure is easy to perform. MS-MLPA is considered to be a low cost procedure when compared with CGH array, for example. As major disadvantages, this technique cannot detect unknown point mutations and copy neutral loss of heterozygosity or distinguish polyploidy from diploidy or haploid. Furthermore, methylation detection is restricted to sites that contains the specific restriction site for HhaI enzyme. In case of tumour samples, their contamination with normal cells can be a great limitation: deletions and duplications can only be unfailingly detected with a percentage of tumour cells greater or equal to 20% or 40%, respectively (Stuppia *et al.*, 2012; Homig-Holzel and Savola, 2012).

Although with some limitations, MS-MLPA has been consider the gold standard for molecular analysis of disorders resultants from genetic and epigenetic alterations, as is the case of cancer (Stuppia *et al.*, 2012).

2. Aims

Oral cancer is considered the 6th most common cancer worldwide. It is already accepted that OSCC results from accumulation of numerous genetic and epigenetic changes, followed by clonal expansion. Despite the progress in cancer research, the five-year survival rate remains low, mostly due to advanced stages of diagnosis and development of loco-regional recurrence. Thus, it is important to characterize the OSCC molecular profiles in order to find potential biomarkers that can be used to detect OSCC in early stages and also predict the disease progression. Therefore, the main goal of this project is the genetic and epigenetic characterization of tumour samples acquired from patients diagnosed with OSCC. It is also our aim to analyse non-tumour tissue acquired from cancer patients in order to discovery potential genetic and epigenetic alterations that can be associated with metastasis or cancer recurrence.

Up to now, biopsy is the main and more accurate technique used to detect OSSC. However, as biopsy is an invasive and also expensive method, it is mostly used when there is a suspicion of malignancy. That is why it is so important to improve diagnostic methods for early detection as well as monitor the state of patients after treatment. Taking this into account, another goal of this project is the molecular analysis of samples, collected by scrapping the tumour surface. This is a non-invasive method and the objective is to evaluate its accuracy in detection of genetic and epigenetic alterations characteristics of OSCC.

3. Materials and Methods

3.1. Samples and patients

The samples analysed in this study were obtained from 65 patients, diagnosed with OSCC in the Maxillofacial Surgery and Stomatology Unit of the Coimbra Hospital and University Centre, CHUC. The samples were collected between 2010 and 2016 with the informed consent of all patients. It should be noted that this study was approved by the Ethics Committee of the Faculty of Medicine of the University of Coimbra. A tumour sample obtained from biopsy or removed at the time of the resection surgery was collected from each patient. Moreover, exfoliated cells from the tumour surface and a sample from the surgery resection margin (macroscopically tumour-free tissue) were also collected from 59 and 48 of the 65 patients, respectively.

The clinical-pathologic features of each patient were obtained, including sex, age at the moment of diagnosis, tumour staging, histological differentiation, tumour localization, type of treatment and the history of tobacco and/or alcohol consumption. The patients were followed up from the time of diagnosis to January, 2016 and presence/absence of metastasis was registered. These features are summarized in Table 5.

Gingival tissues from 8 healthy donors subjected to wisdom teeth removal at Maxillofacial Surgery and Stomatology Unit of the Coimbra Hospital and University Centre, CHUC, EPE were used as tissue controls. Cells acquired by scrapping the oral surface of 11 healthy donors were used as exfoliated cells controls.

After being collected, the samples were processed at cytogenetics and Genomics Laboratory, Faculty of Medicine, University of Coimbra. Formerly, the samples were registered and identified. Subsequently, fresh tumour and non- tumour samples were frozen by immersion in liquid nitrogen and stored as -80^{o}C until DNA extraction, while the exfoliated cells were stored at 4^{o}C until DNA extraction.

Table 5- Clinical-pathologic features of the patients. NA- not available RT-Radiotherapy, QT-Chemotherapy

		Number of patients
Sex	♂	53
	♀	12
Age	<60	32
	≥60	33
Tumour localization	Tongue	31
	Floor of the mouth	18
	Other	16
Stage	I	12
	II	17
	III	9
	IV	26
	NA	1
Metastasis/ relapse (follow-up)	Yes	22
	No	43
Tobacco	Yes	43
	No	17
	NA	5
Alcohol	Yes	42
	No	16
	NA	7
Treatment	Surgery alone	18
	Surgery + RT	31
	Surgery + QT + RT	5
	QT + RT	6
	NA	5

3.3. DNA extraction

Genomic DNA from tumour samples, exfoliated cells and surgery resection margins were extracted using High Pure PCR Template Preparation Kit (Roche GmbH, Mannheim, Germany), according to the manufacture's recommendations (Roche, Accessed: 18-06-2016).

3.4. DNA quantification and assessment of DNA purity

After extraction, DNA was quantified (ng/μL) and its purity was measured by spectrophotometer NanoDrop-1000 (Thermo Fisher Scientific, Wilmington, USA), using 2μL of each sample. DNA purity was evaluated through the values of ratio absorbance at 260nm and 280nm (A260/A280) and also ratio absorbance at 260nm and 230nm (A260/A230). DNA is considered to be pure when the value of the ratio A260/A280 is approximately 1.8 and the value of the ratio A260/A230 is between 1.8 and 2.

3.5. Array Comparative Genomic Hybridization

CNVs of 12 OSCC tissue samples were evaluated through *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis* (Agilent Technologies, Santa Clara, California, USA) according to the manufacture's recommendations (Agilent, 2015). The aCGH was carried out using an Agilent SurePrint G3 Human Genome microarray 180 K (Agilent Technologies, Santa Clara, CA, USA), an oligonucleotide microarray containing approximately 180.000 60-mer probes with a 17-kb average probe spacing.

For each aCGH experiment, 1100ng of DNA were used in a total volume of 26μL. Tumour samples were labelled with Cy5 and controls were labelled with Cy3. After labelling, the DNA was purified and the degree of labelling was accessed by NanoDrop-1000 (Thermo Scientific, Wilmington, USA). The next step was the clean -up that allowed the removal of primers and nucleotides in excess with Amicon 30-kDA individual filters (Millipore, Billerica, MA, USA). After that, the tumour samples DNA and the corresponding controls were combined with Human Cot-1 DNA (Kreatech Diagnostics, Amsterdam, Netherlands), treated with Agilent blocking agent and 2x Hi-RPM buffer and hybridized in a 4x180K oligonucleotide slide. The hybridization occurred at 65°C for 24h in a hybridization oven (Agilent Technologies) at a constant rotation of 20 rpm.

The hybridized slides were scanned with scanner (scanner C, Agilent) and the data was processed with the Feature Extraction software v10.7. The data from the slide images were analysed using an aberration calling algorithm, AMD-2, and a threshold filter that requires at least three contiguous probes. Finally, the results were analysed using Agilent Genomic Workbench v6.5.

3.6. Methylation-Specific Multiplex Ligation-dependent Probe Amplification Assay

In order to evaluate CNVs and methylation status, MS-MLPA was performed in all samples, as well as respective controls. This technique was conducted following the *General MS-MLPA protocol for the detection and quantification of nucleic acid sequences and methylation profiling. - MS-MLPA protocol version MSP-v005* (MRC-Holland, Amsterdam, The Netherlands) and the manufacture's recommendations (MRC-Holland 2015). The reagents required for this experiment were provided by MRC-Holland (Amsterdam, The Netherlands), except the restriction enzyme *HhaI* (Promega, Madison, USA).

The probe panel used was the MRC-Holland SALSA MS-MLPA probemix ME002-C1 Tumour Suppressor-2. This probemix contains 41 probes, of which 27 have recognition site for the *HhaI* methylation-sensitive restriction enzyme and allows to ensure the promotor methylation status of 25 different genes. The other 14 probes are not digested by *HhaI* and therefore are used as a reference to digestion. All 41 probes are able to detect CNVs of 38 genes (Figure 8).

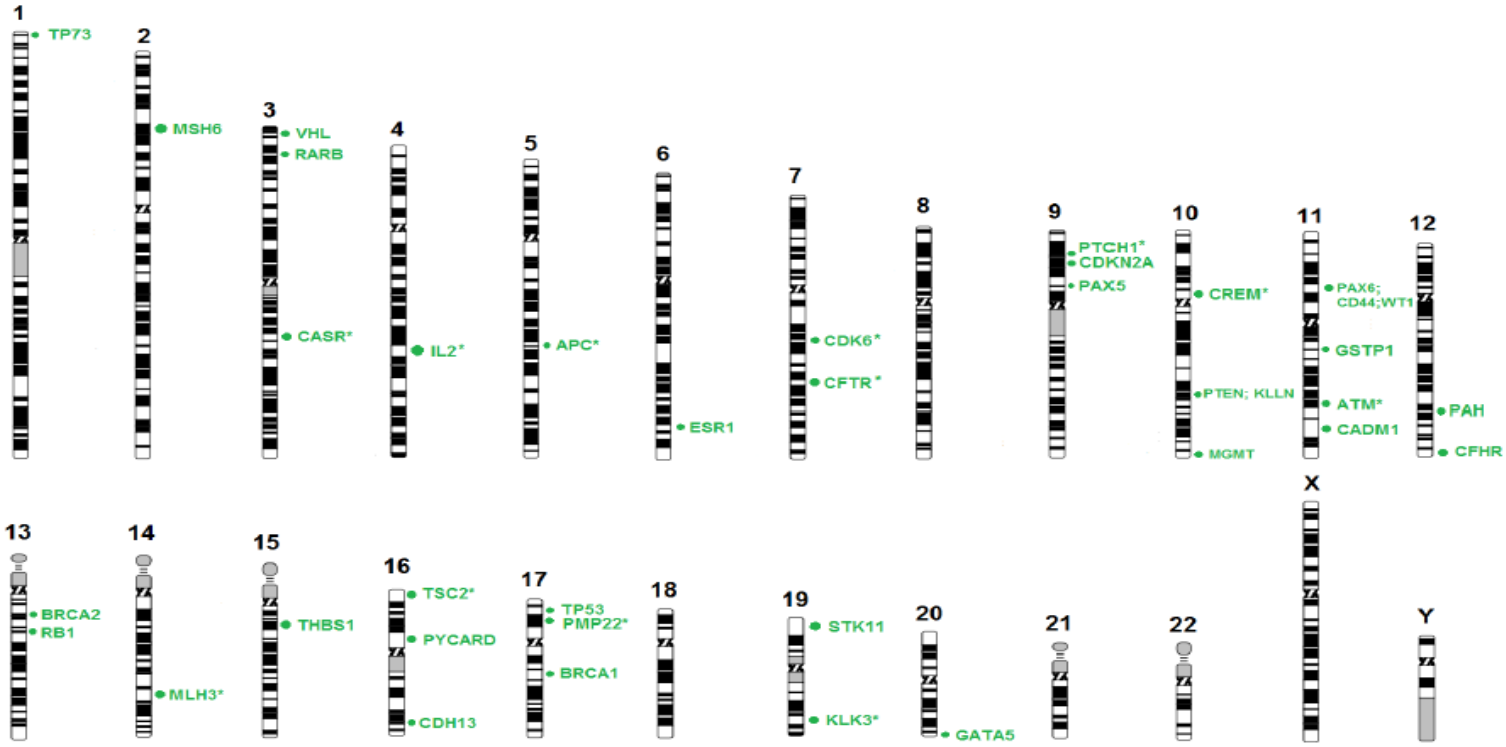

Figure 8- Chromosomal distribution of the genes studied using SALSA MS-MLPA ME002-C1 tumour suppressor-2 probemix. Reference probes are represented by *. *APC* - Adenomatous polyposis coli; *ATM* - ATM serine/threonine kinase; *BRCA1* - Breast cancer 1; *BRCA2* - Breast cancer 2; *CADM1* - Cell Adhesion Molecule 1; *CASR* - Calcium-sensing receptor; *CD44* - CD44 molecule; *CDH13* - Cadherin 13; *CDK6* - Cyclin-dependent kinase 6; *CDKN2A* - Cyclin-dependent kinase inhibitor 2A; *CFTR* - Chloride channels, ATP-gated CFTR; *CHFR* - Checkpoint with forkhead and ring finger domains; *CREM* - cAMP responsive element modulator; *ESR1* - Estrogen receptor 1; *GATA5* - GATA binding protein 5; GSTP1 - Glutathione S-transferase pi 1; IL2 - Interleukin 2; *KLK3* - Kallikrein-related peptidase 3; *KLLN* - Killin, p53-regulated DNA replication inhibitor; *MGMT* - O(6)-methylguanine-DNA methyltransferase; *MLH3* – mutL homolog 3; *MSH6* - mutS homolog 6; PAH - Phenylalanine Hydroxylase; PAX5 - Paired box 5; PAX6 - Paired box 6; PMP22 - Peripheral Myelin Protein 22; *PTCH1* - Patched 1; *PTEN* - Phosphatase and tensin homolog; *PYCARD* - PYD and CARD domain containing; *RARB* - Retinoic acid receptor beta; *RB1* – *RB* transcriptional corepressor *1*; *STK11* - Serine/threonine kinase 11; *THBS1* - Thrombospondin 1; *TP53* – Tumour protein 53; *TP73* - Tumour protein p73; *TSC2* - Tuberous Sclerosis 2; *VHL* - von Hippel-Lindau tumour suppressor; *WT1* - Wilms tumour 1.

For each MS-MLPA reaction 100 ng of DNA in a 5 µl volume were used. The DNA was denatured for 10 minutes at 98°C and then cooled to 25°C. Afterwards, the probe mix was added and in order to allow the hybridization and the samples were heated at 95°C for one minute and maintained at 60°C for 15-16 hours. After hybridization, the samples were divided in two reactions: ligase enzyme was added to the first tube; ligase and restriction enzymes were added to the second tube. The enzymes addition was performed at 48°C and the ligation reaction occurred also at 48°C for 30 minutes. After the enzymes inactivation, for five minutes at 98°C, PCR was carried out using FAM-labelled primers, dNTPs and SALSA polymerase. PCR consisted on 35 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 60°C, extension for 60 seconds at 72ºC, a final extension for 20 minutes at 72ºC and a hold at 15ºC.

Denaturation, hybridization, ligation and PCR reactions were performed by using the thermocycler ABI 2720 (Applied Biosystems, Foster City, CA, USA).

For the next step, fragment separation by capillary electrophoresis (CE), 0,70µL of the PCR product were combined with 9,4µL of a solution with *RoxTM* (Applied Biosystems, Foster City, USA) and highly deionized formamide (Applied Biosystems, Foster City, USA). The capillary electrophoresis was performed using the GeneScan ABI PRISM 3130 capillary electrophoresis system (Applied Biosystems, California, USA). The obtained electropherograms were analysed by the GeneMapper v4.1 (Applied Biosystems, Foster City, USA) software. For each MS-MLPA reaction one negative control (without DNA) and three different references from healthy patients were used. The reference samples were previously examined by MS-MLPA and no imbalance was detected for the 41 probes analysed. Automated fragment and data analysis were obtained using Coffalyser.Net (MRC-Holland, Amsterdam, The Netherlands) program. For copy number detection, this program displayed the ratio between tumour samples and controls. The ratio values equal or higher than 1.2 were scored as numerical gains and values lower or equal to 0.8 were scored as numerical losses (Table 6). Methylation status assessment is based on comparison the signal peaks from digested and undigested samples (digested samples do not generate peaks and undigested samples generate peaks). Positive methylation was considered when the methylation ratios were equal or higher than 0.20.

Table 6- Interpretation of Copy Number Status obtained from Coffalyser.Net.

Ratio	Copy number status
≥1.2	Numerical Gain / Amplification
0.8 - 1.2	Normal
≤0.8	Numerical Loss / Deletion

3.7. Statistical analysis

The statistical analysis was performed with the Statistical Package for Social Sciences (SPSS) v21.0.

The software SPSS Statistics was used to determine the Cohen's kappa coefficient that measures the agreement between two raters. In the present study the Kappa value was used to assess the agreement between the tumour tissue sample and exfoliated cells. When the value of Kappa is equal to 1 it means that there is total agreement between the samples evaluated. On the other hand, when this value is 0 or lower, it means that there is no agreement between the samples. The significance level implemented for statistical meaning was 5 %.

4. Results

4.1. Array Comparative Genomic Hybridization

Among all 65 biopsies samples of this study, a total of 12 were analysed by aCGH. The results showed several alterations throughout the genome. The main rearrangements were detected at chromosome 3. Besides, chromosomes 8, 18, X and Y also revealed several alterations. The great majority of genetic alterations detected correspond to gains of genetic material, mostly at chromosomes 3q, 8q and Xq. However, some losses were also detected specially at chromosomes 3p, 18q and Yp and Yq (Figure 9). Considering the chromosomes referred, the minimal common regions regarding gains of genetic material were 3q26.1 - q29 (9/12), 8q22.3 - q24.3 (7/12) and Xq13.2 - q28 (5/12). On the other hand, the minimal common regions regarding losses were found at 3p22.2 - p11.1 (5/12), 18q12.2-q23 (4/12), Yq11.21 - q11.223 (5/12) and Yp11.32 - p11.2 (4/12) (Figure 9).

All samples revealed significant alterations at chromosome 3. Half of the samples revealed total gain of the long arm whereas four samples showed complete loss of the short arm. In addition, two of these six patients presented total loss of the 3p simultaneously with complete amplification of 3q. Besides that, one patient showed loss of the entire chromosome (Figure 9).

Taking chromosome 8 into account, four of 12 samples showed amplification of the short arm, from which three of them revealed also total loss of the long arm.

The samples of three patients presented amplification of the long arm of chromosome X, while the long arm from another patient sample was completely deleted.

The chromosome Y displayed significant alterations in nine of the 12 samples analysed. Two patients revealed deletions at p11.32-p11.2 and q11.21-q11.23 regions, whereas another patient showed amplification at the same regions.

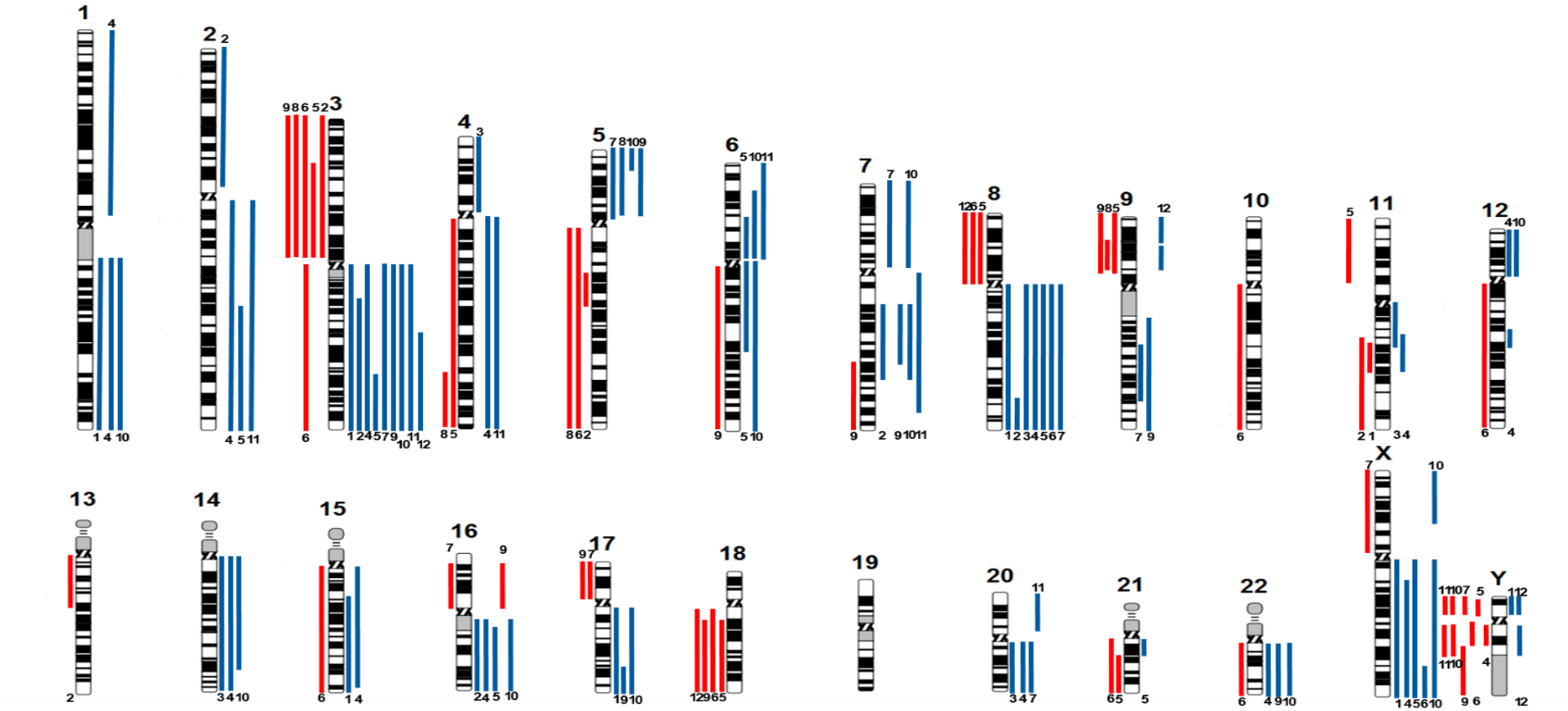

Figure 9- Summary of the more significant chromosomal gains and losses detected by aCGH in 12 OSCC samples. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material.

4.2. Methylation-Specific Multiplex Ligation-dependent Probe Amplification

4.2.1. Tumour samples characterization

In order to examine the most significant genetic alterations involved in OSCC, 65 samples from tumour tissue were analysed by MS-MLPA using *SALSA MLPA probemix ME002-C1.* The software GeneMapper v41.1 was used to obtain electropherograms with a peak pattern for each sample.

For each sample, two electropherograms were acquired: one for CNVs and other for the methylation profiles. *ME002-C1* probemix contains nine control fragments that are summarized on Table 7.

Table 7- Quality control fragments of MS-MLPA. (MRC-Holland, 2015).

Control fragments	Length (nucleotides, nt)	Interpretation
92 nt brenchmark probe	92	Normal probe, which forms a benchmark, used to compare the other quality control fragments.
Q-fragments	64, 70, 76 and 86	High when DNA amount is insufficient or the ligation reaction failed. When all Q-fragment are greater than ⅓ (33%) of the 92 nt control fragment means that DNA quantity of the samples is too low.
D-fragments	88 and 96	Low when occurred a poor DNA denaturation. When the signal is lower than 40% of the 92 nt control fragment means that problems in the DNA denaturation process have occurred.
X and Y fragments	100 and 105	Control for sample exchange.

As mentioned before, for each MS-MLPA reaction, three reference samples and one negative control were used. For each sample, the peak pattern obtained for the references should

not present genetic abnormalities (Figure 10). On the order hand, in the negative control (no DNA) the Q- fragments should be greater than 1/3 of the 92nt control fragment.

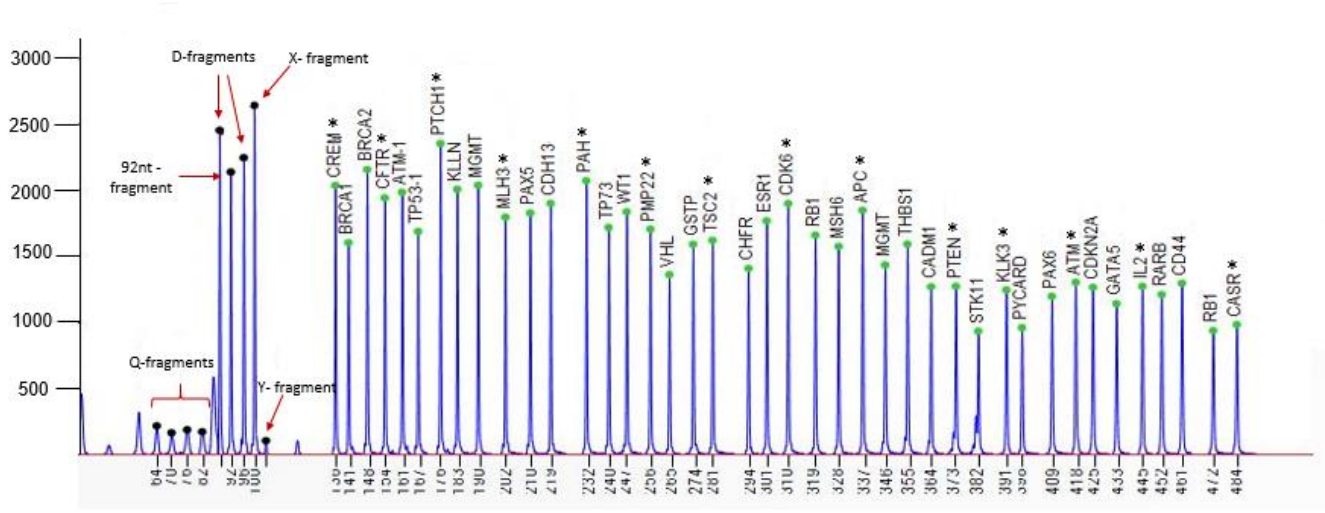

Figure 10- CNVs electropherogram of a male control sample analysed by MS-MLPA using SALSA MLPA probemix ME002. * - reference probe.

For the analysis of the methylation electropherograms, it is important to note that only the probes without site for *Hhal* or the genes that are methylated, and therefore amplified in PCR reaction, are able to create a peak (Figure 11).

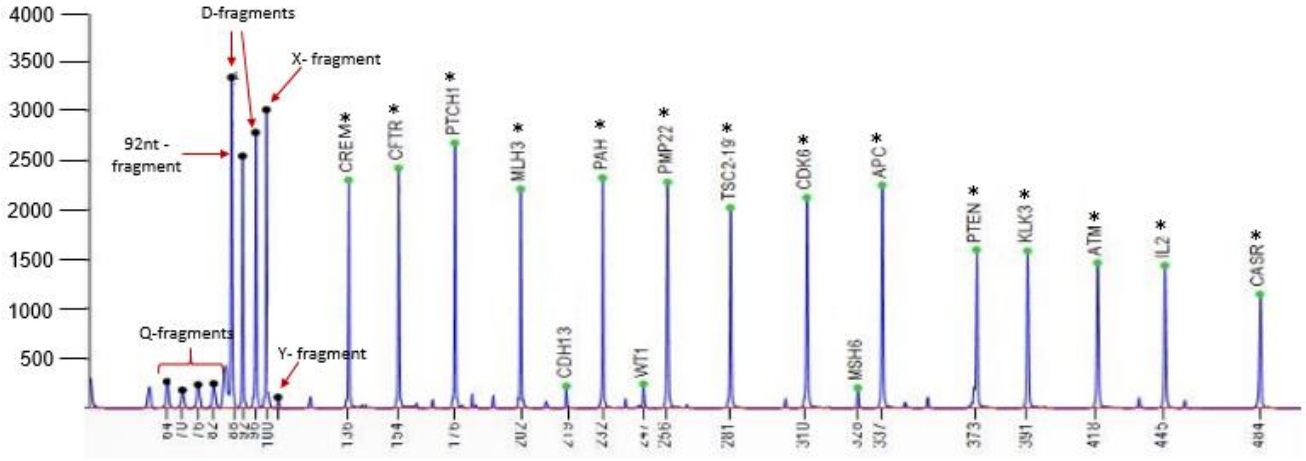

Figure 11- Methylation electropherogram of a male control sample analysed by MS-MLPA using SALSA MLPA probemix ME002. * - reference probe.

4.2.1.1.Copy number variations

From the 65 tumour samples analysed by MS-MLPA, only five (7.7%) did not display any alteration in copy number of the 41 genes probes evaluated (Table 8). All genes analysed showed at least one alteration, including the reference genes.

Table 8- Genetic imbalances detected by MS- MLPA in tumour tissue samples. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. * - Reference probes.

Region	1p	2p	3p		3q	4q	5q	6q	7q		9p			10p	10q				11p			11q				12q		13q			14q	15q	16p		16q	17p		17q	19p	19q	20q
Patient \ Gene	*TP73*	*MSH6*	*VHL*	*RARB*	*CASR**	*IL2**	*APC**	*ESR1*	*CDK6**	*CFTR**	*CDKN2A*	*PAX5*	*PTCH1**	*CREM**	*KLLN*	*PTEN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM**	*ATM**	*CADM1*	*PAH*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*MLH3**	*THBS1*	*TSC2**	*PYCARD*	*CDH13*	*TP53*	*PMP22**	*BRCA1*	*STK11*	*KLK3**	*GATA5*
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Table 8 (continuation) - Genetic imbalances detected by MS- MLPA in tumour tissue samples. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. * - Reference probes

Region	1p	2p	3p		3q	4q	5q	6q	7q		9p			10p	10q				11p			11q				12q		13q			14q	15q	16p		16q	17p		17q	19p	19q	20q
Patient \ Gene	*TP73*	*MSH6*	*VHL*	*RARB*	*CASR**	*IL2**	*APC**	*ESR1*	*CDK6**	*CFTR**	*CDKN2A*	*PAX5*	*PTCH1**	*CREM**	*KLLN*	*PTEN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM**	*ATM**	*CADM1*	*PAH*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*MLH3**	*THBS1*	*TSC2**	*PYCARD*	*CDH13*	*TP53*	*PMP22**	*BRCA1*	*STK11*	*KLK3**	*GATA5*
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Table 8 (continuation) - Genetic imbalances detected by MS- MLPA in tumour tissue samples. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. * - Reference probes.

Region	1p	2p	3p		3q	4q	5q	6q	7q		9p			10p	10q				11p			11q				12q		13q			14q	15q	16p		16q	17p		17q	19p	19q	20q
Patient \ Gene	*TP73*	*MSH6*	*VHL*	*RARB*	*CASR**	*IL2**	*APC**	*ESR1*	*CDK6**	*CFTR**	*CDKN2A*	*PAX5*	*PTCH1**	*CREM**	*KLLN*	*PTEN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM**	*ATM**	*CADM1*	*PAH*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*MLH3**	*THBS1*	*TSC2**	*PYCARD*	*CDH13*	*TP53*	*PMP22**	*BRCA1*	*STK11*	*KLK3**	*GATA5*
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Table 8 (continuation) - Genetic imbalances detected by MS- MLPA in tumour tissue samples (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. * - Reference probes.

Region	1p	2p	3p		3q	4q	5q	6q	7q		9p			10p	10q				11p			11q				12q		13q			14q	15q	16p		16q	17p		17q	19p	19q	20q
Patient \ Gene	*TP73*	*MSH6*	*VHL*	*RARB*	*CASR**	*IL2**	*APC**	*ESR1*	*CDK6**	*CFTR**	*CDKN2A*	*PAX5*	*PTCH1**	*CREM**	*KLLN*	*PTEN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM**	*ATM**	*CADM1*	*PAH*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*MLH3**	*THBS1*	*TSC2**	*PYCARD*	*CDH13*	*TP53*	*PMP22**	*BRCA1*	*STK11*	*KLK3**	*GATA5*
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The alterations more commonly detected were gains of genetic material (Table 8). The most common alteration detected was gain of *STK11* (19p13.3), present in 24 (36.9%) of the patients. The gain of *PYCARD* (16p11.2) was the second most common genetic imbalance detected, present in 19 (29.2%) samples. The genes *BRCA1* (17q21.31), *CHFR* (12q24.33) were amplified in 15 (23.1%) and 14 patients (21.5%), respectively. Moreover, gains of the genes *MSH6* (2p16), *GATA5* (20q13.33) and *VHL* (3p25.3) were detected in 12 patients (18.5%) (Figure 12). No patient showed gain of the gene *CADM1* (11q23.2) (Table 8).

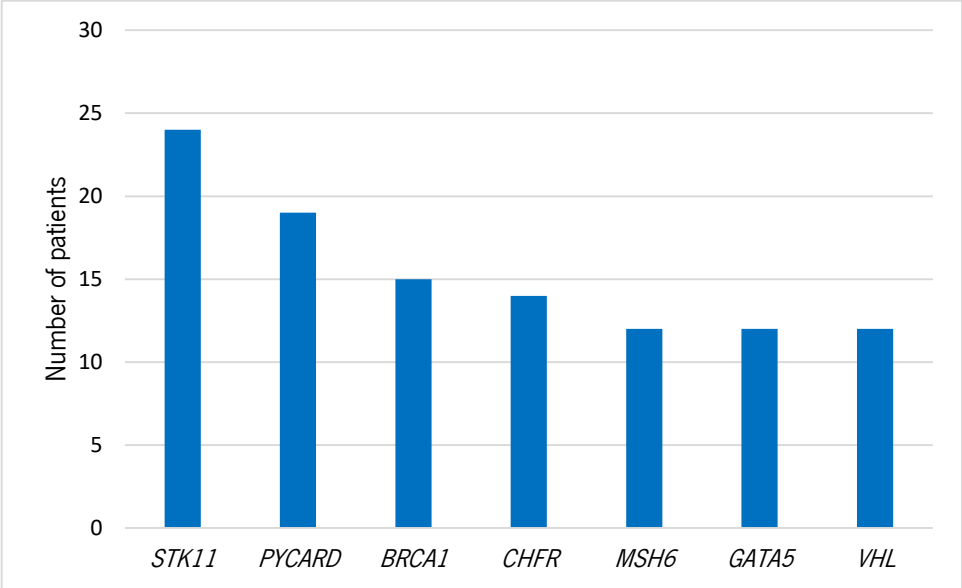

Figure 12- The most frequent gains detected in 65 OSCC samples using SALSA MLPA probemix ME002.

The most common alteration regarding losses of genetic material was the deletion of *CDKN2A* (9p21), detected in 15 (23.1%) of the 65 patients. Although the deletion of *VHL* (3p25.3) is present in 10 (15.4%) patients, its amplification was detected in a higher number of samples. The probemix used in this study contains two probes for the *ATM* (11q22-q23) gene: one specific for the exon 1 and other specific for the exon 26. The results showed deletion of this gene in 10 (15.4%) and nine (13.8%) patients of exon 26 and exon 1, respectively. The genes *RARB* (3p24) and *CADM1* (11q23.2) were deleted in eight (12.3%) patients (Figure 13). The genes *TP73* (1p36.3), *CDK6* (7q21-q22), *PTCH1* (9q22.1-q31), *GSTP1* (11q13.2), *PAH* (12q22-q24.2), *CHFR* (12q24.33), *CDH13* (16q23.3) and *STK11* (19p13.3) did not display deletions in the cohort (Table 8).

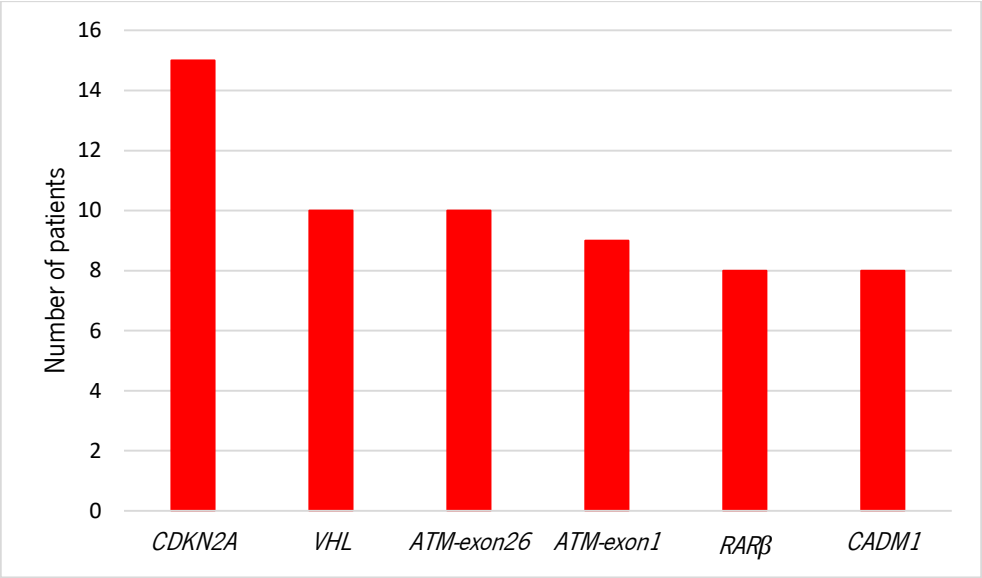

Figure 13- The most frequent deletions detected in 65 OSCC samples sing SALSA MLPA probemix ME002.

Overall, the genetic profile of the tumour samples analysed in this study was mostly characterized by gains of genetic material, essentially on chromosomes 16p and 19p. Losses of genetic material were mostly frequent at chromosomes 3p, 9p and 11q.

4.2.1.2. Methylation profile

To analyse the methylation status of the tumour samples, a cut-off was established. Accordingly, a gene was considered methylated when the percentage of methylation was equal or higher than 20%.

Seven out of 65 samples (10.7 %) did not show any methylated gene (Table 9). However, all of these seven patients showed at least one alteration regarding CNVs.

Nine (36%) of the 25 genes analysed did not display aberrant methylation in the samples analysed, namely, *VHL* (3p25.3), *GSTP1* (11q13.2), *ATM* (11q22-q23), *BRCA2* (13q13.1), *RB1* (13q14.2), *THBS1* (15q15), *PYCARD* (16p11.2), *BRCA1* (17q21.31), *STK11* (19p13.3) (Table 9).

Taking into account the 16 genes that were methylated at least in one sample, *WT1* (11p13) was the gene most frequently methylated in the tumour samples analysed, being altered in 50 patients (76.9%). The genes *PAX5* (9p13.2), *GATA5* (20q13.33), *MSH6* (2p16), *RARβ* (3p24), revealed aberrant methylation in 23 (35.4%), 21 (32.3%), 19 (29.2%) and 14 (21.5%) patients, respectively (Figure 14). The remaining genes revealed aberrant methylation in 20% or less patients (Table 9).

Table 9- Methylation profile of tumour tissue samples analysed by MS-MLPA samples. (■) – Methylation.

Region	1p	2p	3p		6q	9p		10q			11p			11q			12q	13q			15q	16p	16q	17p	17q	19p	20q
Gene / Patient	*TP73*	*MSH6*	*VHL*	*RARB*	*ESR1*	*CDKN2A*	*PAX5*	*KLLN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM*	*CADM1*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*THBS1*	*PYCARD*	*CDH13*	*TP53*	*BRCA1*	*STK11*	*GATA5*
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Table 9 (continuation) - Methylation profile of tumour tissue samples analysed by MS-MLPA samples. (■) – Methylation.

Region	1p	2p	3p		6q	9p		10q			11p			11q			12q	13q			15q	16p	16q	17p	17q	19p	20q
Gene / Patient	*TP73*	*MSH6*	*VHL*	*RARB*	*ESR1*	*CDKN2A*	*PAX5*	*KLLN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM*	*CADM1*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*THBS1*	*PYCARD*	*CDH13*	*TP53*	*BRCA1*	*STK11*	*GATA5*
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Table 9 (continuation) - Methylation profile of tumour tissue samples analysed by MS-MLPA samples. (■) – Methylation.

Region	1p	2p	3p		6q	9p		10q			11p			11q			12q	13q			15q	16p	16q	17p	17q	19p	20q
Gene / Patient	*TP73*	*MSH6*	*VHL*	*RARB*	*ESR1*	*CDKN2A*	*PAX5*	*KLLN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM*	*CADM1*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*THBS1*	*PYCARD*	*CDH13*	*TP53*	*BRCA1*	*STK11*	*GATA5*
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Table 9 (continuation) - Methylation profile of tumour tissue samples analysed by MS-MLPA samples. (■) – Methylation.

Region	1p	2p	3p		6q	9p		10q			11p			11q			12q	13q			15q	16p	16q	17p	17q	19p	20q
Gene / Patient	*TP73*	*MSH6*	*VHL*	*RARB*	*ESR1*	*CDKN2A*	*PAX5*	*KLLN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM*	*CADM1*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*THBS1*	*PYCARD*	*CDH13*	*TP53*	*BRCA1*	*STK11*	*GATA5*
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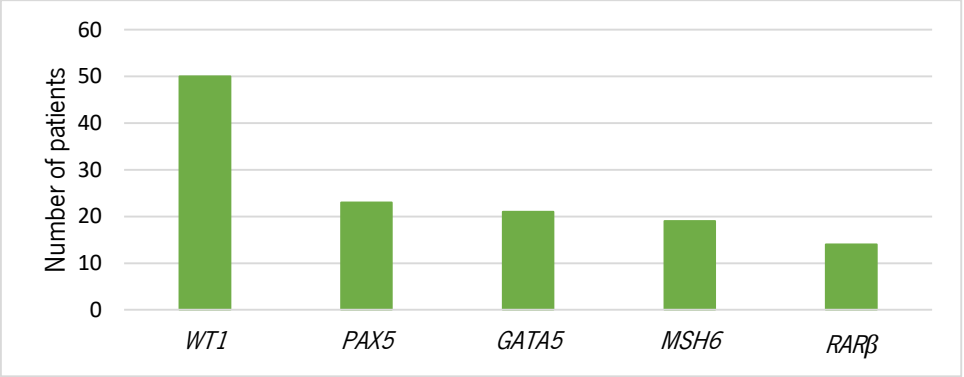

Figure 14- The most frequent methylated genes detected in 65 OSCC samples using SALSA MLPA probemix ME002

4.2.2. Non-tumour samples characterization

A total of 48 samples from the surgery resection margin (macroscopically tumour free tissue) were also analysed by MS-MLPA using *SALSA MLPA probemix ME002*. These samples belong to 48 out of the 65 patients that were involved in this study.

4.2.2.1. Copy number variations

23 (47.9%) out of the 48 non-tumour samples did not display any alteration regarding CNV (Table 10).

The copy number of the *TP73* (1p36.3), *CASR* (3q21.1), *ESR1* (6q24-q27), *CDK6* (7q21-q22), *CREM* (10p12.1-p11.1), *KLLN* (10q23), *PTEN* (10q23), *MGMT* (10q26), *PAX6* (11p13), *WT1* (11p13), *CD44* (11p13), *ATM* (11q22-q23), *CADM1* (11q23.2), *BCRA2* (3q12-q13), *THBS* (115q15) and *CDH13* (16q23.3) genes were normal in all patients. It means that 1/3 (33.3%) of the genes studied did not present alterations in CNV (Table 10).

The most common alteration detected in non-tumour samples was gain of *STK11* (19p13.3). This alteration was found in 15 patients (31.2%). Gains of *PYCARD* (16p11.2) and *VHL* (3p25.3) were found in nine patients (20%), being the second most common alterations detected. Regarding deletions, loss of *CDKN2A* (9p21) was presented in six patients (12.5%) (Figure 15). Alterations in other genes were also detected but at lower frequencies (Table 10).

Table 10- Genetic imbalances detected by MS- MLPA in non-tumour samples (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. * - Reference probes.

Region	1p	2p	3p		3q	4q	5q	6q	7q	7q	9p	9p	9q	10p	10q				11p			11q				12q		13q			14q	15q	16p		16q	17p		17q	19p	19q	20q
Patient \ Gene	*TP73*	*MSH6*	*VHL*	*RARB*	*CASR**	*IL2**	*APC**	*ESR1*	*CDK6**	*CFTR**	*CDKN2A*	*PAX5*	*PTCH1**	*CREM**	*KLLN*	*PTEN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM**	*ATM**	*CADM1*	*PAH*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*MLH3**	*THBS1*	*TSC2**	*PYCARD*	*CDH13*	*TP53*	*PMP22**	*BRCA1*	*STK11*	*KLK3**	*GATA5*
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Table 10 (continuation) - Genetic imbalances detected by MS- MLPA in non-tumour samples (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. * - Reference probes.

Region	1p	2p	3p		3q	4q	5q	6q	7q		9p			10p	10q				11p			11q				12q		13q			14q	15q	16p		16q	17p		17q	19p	19q	20q
Patient \ Gene	*TP73*	*MSH6*	*VHL*	*RARB*	*CASR**	*IL2**	*APC**	*ESR1*	*CDK6**	*CFTR**	*CDKN2A*	*PAX5*	*PTCH1**	*CREM**	*KLLN*	*PTEN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM**	*ATM**	*CADM1*	*PAH*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*MLH3**	*THBS1*	*TSC2**	*PYCARD*	*CDH13*	*TP53*	*PMP22**	*BRCA1*	*STK11*	*KLK3**	*GATA5*
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Table 10 (continuation) - Genetic imbalances detected by MS- MLPA in non-tumour samples (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. * - Reference probes.

Region	1p	2p	3p		3q	4q	5q	6q	7q		9p			10p	10q				11p			11q				12q		13q			14q	15q	16p		16q	17p		17q	19p	19q	20q
Patient \ Gene	*TP73*	*MSH6*	*VHL*	*RARB*	*CASR**	*IL2**	*APC**	*ESR1*	*CDK6**	*CFTR**	*CDKN2A*	*PAX5*	*PTCH1**	*CREM**	*KLLN*	*PTEN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM**	*ATM**	*CADM1*	*PAH*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*MLH3**	*THBS1*	*TSC2**	*PYCARD*	*CDH13*	*TP53*	*PMP22**	*BRCA1*	*STK11*	*KLK3**	*GATA5*
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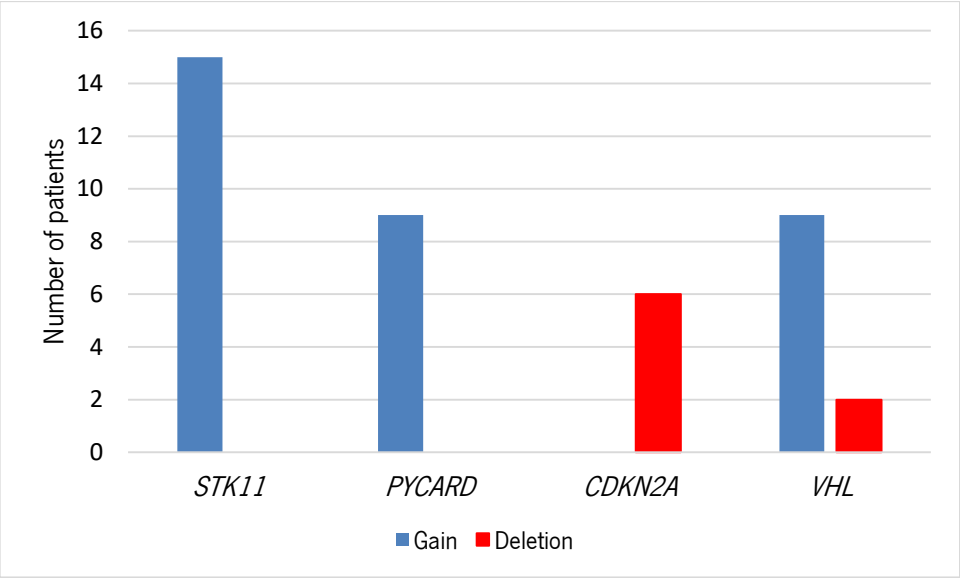

Figure 15- The most frequent CNVs detected in 48 non tumour samples using SALSA MLPA probemix ME002.

It is important to consider that in some patients, the same alteration appears in both tumour and non-tumour samples. For example, six patients (15, 23, 31, 45, 50 and 53) exhibited gain of *STK11* in both types of samples. Moreover, loss of *CDKN2A* were found in both type of samples in three patients (29, 55 and 59) (Tables 8 and 10).

Overall, the genetic profile of the non-tumour samples studied was mainly characterized by gains of genetic material, essentially on chromosomes 19p and 16p. Losses of genetic material were most frequent at chromosome 9p.

4.2.2.2. Methylations profile

17 (35.4%) out of the 48 non-tumour samples analysed did not show methylation of any gene.

Regarding methylation, 11 (44%) out of the 25 genes studied did not display alterations, namely *TP73* (1p36.3), *RARB* (3p24), *VHL* (3p25.3), *CDKN2A* (9p21), *PAX6* (11p13), *GSTP1* (11q13.2), *CADM1* (11q23.2), *RB1* (13q14.2), *THBS1* (15q15), *PYCARD* (16p11.2), and *STK11* (19p13.3) (Table 11).

As observed for tumour samples, the *WT1* (11p13) was the gene found to be the most methylated, specifically in 1/3 of the non-tumour samples (16/48). The genes *MSH6* (2p16) and *TP53* (17p13.1) were methylated in eight patients (16.6%). Furthermore, both *KLLN* (10q23) and *BRCA2* (13q13.1) revealed aberrant methylation in six patients (Figure 16). The nine remaining genes showed methylation in four or less patients (Table 11).

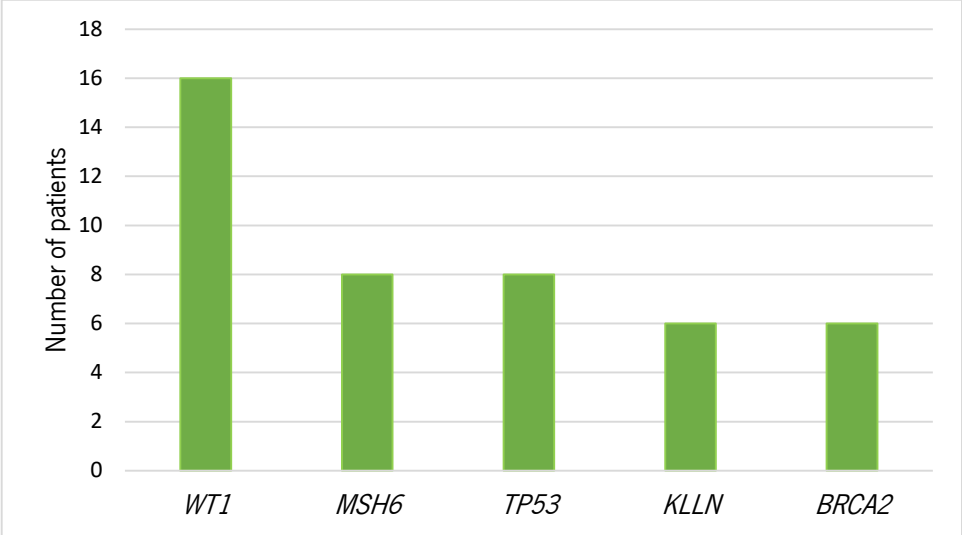

Figure 16- The most frequent methylated genes detected in 48 non-tumour samples using SALSA MLPA probemix ME002.

A couple of patients showed the same methylation status for some genes in both tumour and non-tumour samples. For example, the gene *WT1* was methylated in 12 patients (3, 6, 11,14,19, 33, 39, 40, 52, 55, 57 and 59) in both tumour and non-tumour samples (Tables 9 and 11).

Table 11- Methylation profile of non-tumour tissue samples analysed by MS-MLPA samples. (■) – Methylation.

Region	1p	2p	3p		6q	9p		10q	10q		11p			11q			12q	13q			15q	16p	16q	17p	17q	19p	20q
Gene / Patient	*TP73*	*MSH6*	*VHL*	*RARB*	*ESR1*	*CDKN2A*	*PAX5*	*KLLN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM*	*CADM1*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*THBS1*	*PYCARD*	*CDH13*	*TP53*	*BRCA1*	*STK11*	*GATA5*
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Table 11 (continuation) - Methylation profile of non-tumour tissue samples analysed by MS-MLPA samples. (■) – Methylation.

Region	1p	2p	3p		6q	9p		10q			11p			11q			12q	13q			15q	16p	16q	17p	17q	19p	20q
Gene / Patient	*TP73*	*MSH6*	*VHL*	*RARB*	*ESR1*	*CDKN2A*	*PAX5*	*KLLN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM*	*CADM1*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*THBS1*	*PYCARD*	*CDH13*	*TP53*	*BRCA1*	*STK11*	*GATA5*
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Table 11 (continuation) - Methylation profile of non-tumour tissue samples analysed by MS-MLPA samples. (■) – Methylation.

Region	1p	2p	3p		6q	9p		10q			11p			11q			12q	13q			15q	16p	16q	17p	17q	19p	20q
Gene / Patient	*TP73*	*MSH6*	*VHL*	*RARB*	*ESR1*	*CDKN2A*	*PAX5*	*KLLN*	*MGMT*	*MGMT*	*PAX6*	*WT1*	*CD44*	*GSTP1*	*ATM*	*CADM1*	*CHFR*	*BRCA2*	*RB1*	*RB1*	*THBS1*	*PYCARD*	*CDH13*	*TP53*	*BRCA1*	*STK11*	*GATA5*
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4.2.3. Comparison between biopsies samples and exfoliated cells

A total of 59 samples of exfoliated cells (acquired by scraping the tumour surface of patients diagnosed with OSCC) were also analysed by MS-MLPA using *SALSA MLPA probemix ME002*. These samples belong to 59 out of the 65 patients that were involved in this study.

The MS-MLPA results acquired from exfoliated cells were compared with the tumour tissue results in order to evaluate the agreement between both types of samples. The software SPSS was used to determine the Cohen's kappa coefficient that measure the agreement between both, exfoliated cells and tumour tissue.

Regarding CNVs, the results showed agreement in 22 (57.9%) out of the 38 genes studied, specifically: *TP73, MSH6, RARB, CASR, APC, ESR1, CFTR, CDKN2A, PAX5, CREM, KLLN, PTEN, MGMT, PAX6, WT1, CADM1, RB1, PYCARD, TP53, PMP22, KLK3, GATA5* (Table 12). Some of the referred genes are presented in Figure 17 with the respective results for both samples.

Table 12- Genes that showed agreement between tumour tissue and exfoliated cells samples, regarding CNVs. The table presents the p-value and the Kappa value, which ranges from 0 to 1. A Kappa value of 1 specifies a total agreement, whereas the Kappa value of 0 indicates no agreement between the two types of samples. The significance level implemented for statistical meaning was 5 %.

Gene	Kappa value	p-value
TP73	0.314	0.001
MSH6	0.317	0.011
RARB	0.325	0.005
CASR	0.200	0.028
APC	0.259	0.004
ESR1	0.310	0.001
CFTR	0.162	0.011
CDKN2A	0.594	<0.0001
PAX5	0.278	0.006
CREM	0.277	0.002
KLLN	0.216	0.001
PTEN	0.204	0.001
MGMT	0.251	0.018
MGMT	0.659	<0.0001
PAX6	0.200	0.011
WT1	0.195	0.006
CADM1	0.155	0.019
RB1	0.200	0.025
PYCARD	0.282	0.017
TP53	0.272	0.008
PMP22	0.300	0.010
KLK3	0.322	0.001
GATA5	0.322	0.001

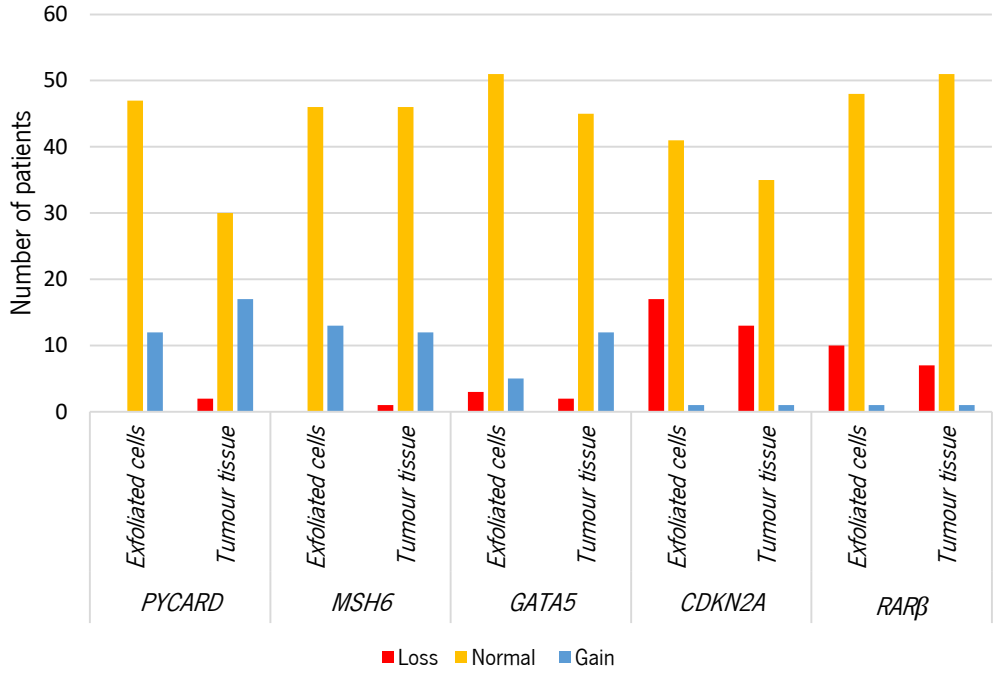

Figure 17- Five of the 22 genes that showed agreement between the tumour tissue and exfoliated cells in 59 samples analysed by MS-MLPA, regarding CNV.

Additionally, four genes with values closed to statistical significance were also found, namely, *TSC2, GSTP1*, *ATM* and *STK11.*

Concerning methylation status, the results showed agreement in 18 (72.0%) out of the 25 genes analysed, specifically: *TP73, VHL, RARβ, ESR1, CDKN2A, PAX5, MGMT, PAX6, WT1, GSTP1, CADM1, CHFR, RB1, THBS, PYCARD, CDH13, STK11, GATA5* (Table 13). Some of the genes mentioned are represented in Figure 18 with the respective results for both samples. Moreover, in six out of the 18 genes, the kappa value was equal to 1, i.e., the methylation status for these genes was the same in both types of samples. These six genes were: *VHL, CDKN2A, GSTP1, RB1, THBS* and *STK11* (Table 13).

Table 13- Genes that showed agreement between tumour tissue and exfoliated cells samples, regarding methylation profile. The table presents the p-value and the Kappa value, which ranges from 0 to 1. A Kappa value of 1 specifies a total agreement, whereas the Kappa value of 0 indicates no agreement between the two types of samples. The significance level implemented for statistical meaning was 5 %.

Gene	Kappa value	p-value
TP73	0.699	<0.0001
VHL	1.000	<0.0001
RARB	0.667	<0.0001
ESR1	0.395	0.002
CDKN2A	1.000	<0.0001
PAX5	0.474	<0.0001
MGMT	0.434	<0.0001
MGMT	0.499	<0.0001
PAX6	0.688	<0.0001
WT1	0.700	<0.0001
CD44	0.482	<0.0001
GSTP1	1.000	<0.0001
CADM1	0.687	<0.0001
CHFR	0.639	<0.0001
RB1	1.000	<0.0001
RB1	1.000	<0.0001
THBS	1.000	<0.0001
CDH13	0.487	<0.0001
STK11	1.000	<0.0001
GATA5	0.744	<0.0001

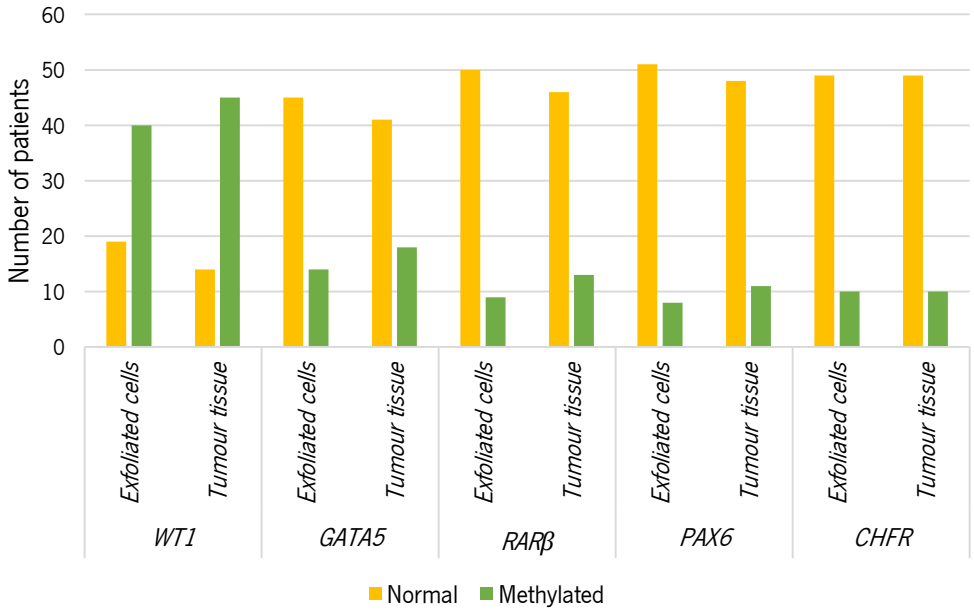

Figure 18- Five of the 18 genes that showed agreement between the tumour tissue and exfoliated cells in 59 samples analysed by MS-MLPA, regarding methylation.

5. Discussion

5.1. Genetic and epigenetic characterization of oral cancer tissue samples

The samples included in this study were genetically characterized by aCGH and MS-MLPA to evaluate CNVs and epigenetically by MS-MLPA to assess the methylation profile. The aCGH allow screening the entire genome, whereas MS-MLPA is a target technique.

5.1.1. Copy number variation

As described, the main rearrangements detected through aCGH were gains at chromosomes 3q, 8q and Xq, and losses at 3p,18q Yp and Yq. On the order hand, the most frequent CNVs detected through MS-MLPA were gains at chromosomes 16p and 19p, and losses at 3p, 9p and 11q. Therefore, these alterations will be discussed with more emphasis.

5.1.1.1. Chromosome 3

Alterations at chromosome 3 have been highly described in HNC, specially losses of 3p and gains of 3q (Gollin, 2014; Ribeiro *et al.*, 2014b).

Region 3p

Loss of 3p arm has been hugely studied and described as one of the most frequent changes in OSCC. This genetic imbalance is associated with early events in OSCC carcinogenesis, since it is present not only in tumour but also in potentially malignant lesions (Gollin, 2014; Wistuba and Meyerson, 2008). In this study five of the 12 patients analysed by aCGH presented loss of the short arm of chromosome 3.

The probemix ME002 contains two probes that analysed the CNV of two genes coded at this region, namely *VHL* (3p25.3) and *RARB* (3p24).

VHL is acknowledged as a tumour suppressor gene that codes for VHL protein. This protein is responsible for targeting hypoxia-inducible factors (HIF) for ubiquitylation and proteasomal degradation. Loss of *VHL* leads to accumulation of HIF-1α, which is responsible for the transcription activation of several genes involved in angiogenesis and glycolysis (Gossage *et al.*, 2015; Zhang *et al.*, 2014). In fact, *VHL* is deleted in 10 out of the 65 samples studied, being the second most common deletion detected. However, this gene was amplified in more samples of the studied cohort (12/65). One of the patients that displayed deletion of this gene was also analysed by aCGH and the result was concordant. However, another patient that showed gain of *VHL* through MS-

MLPA, did not display this alteration in aCGH results. Therefore, the tumour suppressor role of *VHL* in OSCC cannot be confirmed in this study. Consequently, further studies are required in order to understand the role of *VHL* in OSCC.

RARB is considered to be the most powerful tumour suppressor among retinoic acid receptors. Lack of this gene can be associated with genetic rearrangements or epigenetic changes (Albino-Sanchez *et al.*, 2016). In the present study, deletion of *RARB* was found in only eight patients. Three out of the eight samples that showed deletion of this gene were also evaluated through aCGH and the results were concordant. The methylation profile of this gene will be further discussed.

Region 3q

Gains of the long arm of chromosome 3 have been reported as one of the most frequent imbalance, not only in OSCC but also in other cancers such as cervical, esophageal, and lung carcinomas (Oga *et al.*, 2001; Rao *et al.*, 2004; Qin *et al.*, 2005; Mendez and Ramirez, 2013). 3q gain is associated with advanced stages of OSCC since it is rarely present in oral potentially malignant lesions (Oga *et al.*, 2001). Bockmuhl *et. al* (2002) suggested that this alteration is a possible marker for invasion and metastasis and it is associated with poor prognosis (Bockmuhl *et al.*, 2002).

Our results corroborate the literature since 3q gain was the most frequent rearrangement detected thought aCGH analysis. Nine of the 12 patients revealed gain of 3q26.1-q29. However, it is not possible to infer the association between the stage of disease and this specific alteration since one patient that showed this alteration was diagnosed at stage I and one of the patients that did not display this alteration was diagnosed at stage IV and had metastasis. Probably, this association would be possible with a higher cohort.

The MS-MLPA probemix used in this study evaluate only the CNV of one gene at this region: *CASR* (3q21.19). This gene showed amplification in eight patients and deletion in four. Therefore, it seems that this gene is not helpful to understand the genetic profile of OSCC.

5.1.1.2. Chromosome 8

Genetic imbalances at chromosome 8 were also reported in OSCC, specifically loss of the short arm and gain of the long arm. The combination of these two events suggest the formation of isochromosomes (Gollin, 2001). However, aCGH studies revealed that gain of 8q is more frequent

than loss of the short arm of chromosome 8 (Silva Veiga *et al.*, 2003). Our results are in agreement with the literature since in this study the presence of gains of 8q was much higher than loss of 8p.

Region 8q

Gain of the long arm of chromosome 8 were identified in numerous cancers such as breast, prostate and also HNSCC (Kuijper *et al.*, 2009; El Gammal *et al.*, 2010; Silva Veiga *et al.*, 2003). Studies in HNSCC showed gains at this region, specifically at 8q21-q23, 8q21.1-q21.3, 8q22-q24.2 and 8q24.3 (Silva Veiga *et al.*, 2003). Moreover, Hermsen *et al.* detected gains at 8q23-q24 in laryngeal and pharyngeal squamous cell carcinomas (Hermsen *et al.*, 2001). Gains at the long arm of chromosome 8 have been associated with lower long-term disease-free survival and a short overall survival (Silva Veiga *et al.*, 2003). In the present study, the aCGH results showed gains of this region in seven out of the 12 patients, specifically gains at 8q22.3-q24. Three of the patients that showed 8q amplification died from the disease and one had metastasis.

In OSCC, according with the literature, smaller gains at 8q, specifically at 8q22-q23 were typically detected in early stages of the disease whereas gains involving the entire arm were usually detected at more advanced stages (Salahshourifar *et al.*, 2014). In fact, in the present study, the smallest gain detected at 8q corresponds to a patient that was diagnosed at stage I of the disease. In contrast, the other six patients were diagnosed at more advances stages and the aCGH results detected gain of the entire arm.

The amplification/gain of some genes coded at this region has been associated with oral cancer tumorigenesis, such as *MYC* and *PTK2* (Salahshourifar *et al.*, 2014). However, the role of 8q amplification in OSCC is not yet clarified (Gollin, 2014). Therefore, further studies are required in order to understand the role of this alteration and make it possible to improve the therapy applied to OSCC patients that present specific gains at this region. In this study, no gene coded at this region was assessed for CNVs through MS- MLPA methodology.

5.1.1.3. Chromosome 9

Region 9p

Losses at the short arm of chromosome 9 have been highly described in OSCC, specially losses at 9q21-q22 (Gollin, 2001; Gollin, 2014; Xiao *et al.*, 2001; Miyahara, 2000; Nakanishi *et al.*, 1999). Within this region, deletion of *CDKN2A* is frequently detected in OSCC. *CDKN2A* is coded at 9p21.3 and acknowledge as a tumour suppressor gene. This gene is a negative regulator

of cell proliferation. Therefore, loss of *CDKN2A* leads to uncontrolled cell proliferation. This alteration has been associated with early stages of the disease and related with poor prognosis (Park *et al.*, 2007; Dong *et al.*, 2012).

Our results corroborate the literature hence loss of *CDKN2A* was the most frequent deletion detected, being present in 15 of the tumour samples analysed by MS-MLPA. Moreover, five of the samples that displayed this alteration were also evaluated through aCGH and the results were concordant for three patients. Although these results do not appear significant, it was already described that tumour samples have always contamination with normal cells that contain two copies of this gene (such as stromal cells and lymphocytes). Therefore, these normal cells can "mask" the alterations of the tumours cells (Akervall *et al.*, 2003).

Besides *CDKN2A*, the MS-MLPA probemix used in the present study assess also the CNV of one more gene coded at 9p: *PAX5*. However, few alterations were found suggesting that alterations on the copy number of this genes are not relevant in OSCC characterization.

5.1.1.4. Chromosome 11

Region 11q

Regarding the long arm of chromosome 11, the most frequent alterations detected in OSCC are amplification of 11q13 and deletions of distal 11q, specifically 11q23-25 (Gollin, 2014). The aCGH results of this study showed deletion of the distal region of 11q in three patients (25%). Ambatipudi *et al.* established that deletions of distal region of 11q are associated with poor clinical outcomes (Ambatipudi *et al.*, 2011). In fact, in this study, two out of the three patients who showed this deletion died from the disease and another one, diagnosed at stage I, presented metastasis.

11q distal codes for several DDR genes, being *ATM* the most relevant in OSCC pathogenesis (Sankunny *et al.*, 2014). Parikh *et al.* described that loss of *ATM* leads to compromised DNA damage response and reduced sensitivity to ionizing radiation, triggering genomic instability and, consequentially, tumour progression (Parikh *et al.*, 2007). As already described, the probemix *ME002* contains two different probes for assessment of *ATM* gene, one for exon 1 and another for exon 26. Loss of this gene was found in 10 patients, for exon 26, and 9 patients, for exon 1, suggesting that deletion of this gene is relevant for OSCC carcinogenesis

CADM1 is another gene mapped at 11q23-q25, (precisely at 11q23.4) and has been associated with carcinogenesis. Silencing of this gene can be a result of promotor methylation or allelic loss (Allinen *et al.*, 2002). Alterations of *CADM1* were detected in several types of cancers

such breast, cervical, lung and also OSCC (Allinen *et al.*, 2002; Mazumder Indra *et al.*, 2011; van den Berg *et al.*, 2011; Hayama, 2009). *CADM1* encodes a transmembrane glycoprotein involved in cell interaction of epithelial cells and mediates cell-to-cell adhesion (Masuda, 2002). Therefore, losses of this gene may be involved in metastasis. MS-MLPA results revealed deletion of this gene in eight out of the 65 patients. However, up to our current knowledge, only two of them developed a metastasis.

5.1.1.5. Chromosome 16

Region 16p

Regarding CNV, the second most frequent alteration identified by MS-MLPA was gain of *PYCARD*, being detected in 19 (29.2%) patients. Five of these 19 samples were also analysed by aCGH and in three of them the gain of *PYCARD* was also detected.

PYCARD is mapped at 16p11.2 and codes an adaptor protein that is formed by two protein-protein interaction domains, a N-terminal pyrin domain (PYD) and a C- terminal caspase-recruitment domain (CARD). This gene is involved in apoptotic pathways via caspase activation (NCBI, gene ID: 66824). It is recognized as a TSG since it is silenced by promotor methylation in several cancers such as glioblastoma, prostate, colorectal and breast cancer (Stone *et al.*, 2004; Leclerc *et al.*, 2013; Collard *et al.*, 2006; Conway *et al.*, 2000). However, our results showed that this gene appears to have an oncogenic role in OSCC pathogenesis. Nevertheless, further studies should be developed for a better understanding of the role of *PYCARD* in OSCC.

5.1.1.6. Chromosome 18

Region 18q

The aCGH results revealed that loss of the long arm of chromosome 18 were frequently detected. Losses at 18q12.2-q23 were found in 25% of the patients. Alterations at this region were previously identified in HNSCC, including OSCC (Jin and Mertens, 1993; Pearlstein *et al.*, 1998; Takebayashi *et al.*, 2004; Martin *et al.*, 2008; Ribeiro *et al.*, 2014b).

This alteration is associated with tumour progression since loss of 18q is more frequent at advanced stages comparatively to early stages of the disease (Takebayashi *et al.*, 2004). Moreover, it is hypothesized that LOH of 18q is related to poor survival and leads to aggressive tumour

behaviour (Pearlstein *et al.*, 1998; Takebayashi *et al.*, 2004). In fact, one of the four patients that presented this alteration already died from the disease.

No gene mapped at chromosome 18p was evaluated by MS-MLPA. However, some TSGs mapped at this region were already identified as being lost in OSCC, such as *CDH2* (Cadherin 2) (18q11.2), *BCL2* (BCL2, apoptosis regulator) (18q21.3), *DCC* (DCC netrin 1 receptor) (18q21.3), *GALR1* (18q23) and *PARD6G* (18q23) (Ribeiro *et al.*, 2014b; Gollin, 2014). Therefore, it would be interesting to evaluate the CNV of these genes.

5.1.1.7. Chromosome 19

Regarding MS-MLPA results, the most frequent genetic alteration detected was the gain of *STK11*.

STK11 is mapped at 19p13.3 and encodes a serine/threonine protein kinase. This gene is considered a TSG and its loss has been found in several types of tumours such as breast cancer, lung adenocarcinoma, endometrial adenocarcinoma, larynx and pharynx carcinomas.(Zhuang *et al.*, 2006; Sanchez-Cespedes *et al.*, 2002; Contreras *et al.*, 2008; Guervos *et al.*, 2007).

At first sight, the MS-MLPA results seem to indicate that *STK11* plays an oncogenic role in OSCC since it was amplified in 36.9% of the patients and no deletion was found for this gene. However, it must be taken into account that aCGH results showed deletion of this gene in three of the 12 patients. So, the MS-MLPA probe used to analyse this gene cannot be the most appropriated to infer the effects of *STK11* alterations in OSCC. A study developed in HNSCC lines by Qiu *et al.* revealed that A205T point mutation of the *STK11* gene originate inactive proteins (Qiu *et al.*, 2006). Additionally, Tan *et al.* demonstrated that *STK11* is frequently mutated in tongue carcinoma (Tan *et al.*, 2014). Bearing this in mind, it can be hypothesized that even though a certain region of *STK11* is amplified, some alterations can occur on other gene regions producing non-functional transcripts.

Zhuang-Gang *et al.* revealed that overexpression of *STK11*, *in vitro*, in breast cancer cells is associated with significant inhibition of migration and invasion. Additionally, *in vivo* studies demonstrated that high levels of expression of this gene resulted in low tumour growth and decreasing of lung metastasis (Zhuang *et al.*, 2006). Therefore, another possible explanation for our results is that gains of *STK11* may be associated with lower risk of metastasis development.

Considering the disagreement between our results and the literature it is imperative to perform more accurate studies in order to determine the *STK11* alterations that can be involved in OSCC.

5.1.1.8. Chromosome Y

Chromosome Y codded genes that are involved in cell cycle control, signal transduction, cell proliferation, protein degradation and gene expression (Veiga *et al.*, 2012).

Alterations of chromosome Y have been described in several diseases including cancer. Loss of this chromosome were identified in numerous tumours such as leukaemia, bladder cancer, esophageal carcinoma, prostate cancer, gastric cancer, colorectal carcinoma and also in HNSCC (Park *et al.*, 2009; Aly and Khaled, 2002; Hunter *et al.*, 1993; Jordan *et al.*, 2001; Castedo *et al.*, 1992; Veiga *et al.*, 2012). The results of this project corroborate the literature as loss of chromosome Y was frequently found, specifically at Yq11.21-q11.223 (5/12) and Yp11.32-p11.2 (4/12). Silva Veiga *et al.* suggested that loss of chromosome Y is independent of the onset age of the disease (Veiga *et al.*, 2012). The age of the 12 patients included in the present study varies from 49 to 90. In fact, no correlation was found between age and loss of chromosome Y since both younger and older displayed this alteration. Although loss of chromosome Y has already been described in several tumours, the role of this alteration in OSCC remains poorly understood.

The MS-MLPA probemix used in this study, does not evaluate any gene mapped at chromosome Y.

5.1.2. Methylation profile

Epigenetic modifications and particularly DNA methylation are considered early events in the carcinogenesis process. Moreover, this kind of alterations are reversible. Therefore, knowledge about the epigenetic alterations of OSCC can be important to early stages of diagnosis and to develop new target therapies.

The most frequent methylated genes in this study were *WT1, PAX5, GATA5, MSH6* and *RARB*, evidencing that promotor methylation of these genes may have important roles in OSCC carcinogenesis.

5.1.2.1. *WT1*

WT1 is mapped at 11p13 and encodes a transcriptional factor that plays important roles in cell growth and differentiation (Sugiyama, 2010). *WT1* was first isolated as a TSG, being inactivated in Wilms' tumours (Call *et al.*, 1990). However, its tumour suppressor role has been

controversial. Overexpression of *WT1* was found in several types of tumours such as stomach, colorectal, lung, breast, thyroid and liver cancer, revealing its oncogenic role in these tumours (Mikami *et al.*, 2013). Moreover, Oji *et al.* showed overexpression of this gene in HNSCC. In the present study *WT1* appears to play a tumour suppressor function since it was methylated in 76.9% of the patients. These results are in agreement with previous studies that also identified methylation of this gene in OSSC patients (Viet and Schmidt, 2008; Gasche *et al.*, 2011; Ribeiro *et al.*, 2016). A study developed in OSCC patients showed that *WT1* was differentially methylated between extracapsular spread and non-extracapsular spread tumour samples, suggesting that the methylation status of *WT1* represent a prognostic predictor in oral carcinogenesis (Jithesh *et al.*, 2013). Recently, Ribeiro *et al.* found that *WT1* promotor methylation is correlated with early OSCC stages (Ribeiro *et al.*, 2016). Viet *et al.* analysed saliva samples of OSCC patients before and after treatment and the results showed that methylation of *WT1* is completely reversed after treatment. Therefore, they suggest that this gene can be a potential biomarker to early diagnosis and to evaluate the response to treatment (Viet and Schmidt, 2008).

5.1.2.2. *PAX5*

PAX5 is mapped at 9p13.2 and belongs to the PAX family of genes. This gene plays important roles in organ development and tissue differentiation during embryogenesis (Norhany *et al.*, 2006).

Although alterations of *PAX5* have been described in several tumours, the role of this gene in tumorigenesis is still controversial. Overexpression of *PAX5* was found in different tumours such as bladder carcinoma, OSCC, and small-cell lung cancer, suggesting an oncogenic role for this gene (Babjuk *et al.*, 2002; Norhany *et al.*, 2006; Kanteti *et al.*, 2009). Contrarily, several studies revealed methylation of *PAX5* in different tumours, including OSCC, proposing a tumour suppressor role for this gene. Liu *et al.* reported that *PAX5* is methylated and consequently downregulated in hepatocellular carcinoma (Liu *et al.*, 2011). A study developed in gastric cancer showed that *PAX5* acts as a TSG through regulation of the p53 pathway (Deng *et al.*, 2014). Guerrero-Preston identified promotor methylation of *PAX5* as being a frequent event in HSCC (Guerrero-Preston *et al.*, 2014). More recently, the results of Ribeiro *et al.* showed that *PAX5* promotor methylation was a frequent epigenetic event in OSCC, especially in tongue tumours (Ribeiro *et al.*, 2016). In the present study, *PAX5* was methylated in 35.4% of the samples, corroborating the studies that describe a tumour suppressor role for this gene.

5.1.2.3. *GATA5*

GATA5 is mapped at 20q13.33 and encodes a transcription factor that plays key roles in differentiation and development of endoderm-derived organs (Patient and McGhee, 2002). This gene is described as a TSG since its silencing prevents normal differentiation and contributes to tumour development (Fu *et al.*, 2007).

In this study *GATA5* was methylated in 32.3%, being the third most common methylated gene identified. Thus, this gene appears to play a tumour suppressor role in OSCC carcinogenesis. Methylation of *GATA5* has been described in several types of tumours such as lung, colorectal, renal, glioblastoma and also OSCC (Guo *et al.*, 2004; Hellebrekers *et al.*, 2009; Peters *et al.*, 2014; Rankeillor *et al.*, 2014; Ribeiro *et al.*, 2016). Guo *et al.* concluded that in lung cancer, increased methylation frequency of *GATA5* is associated with increased age of patients (Guo *et al.*, 2004). A study developed by Peters *et al.* revealed that hypermethylation of *GATA5* in renal cell carcinoma is associated with tumour progression and poor prognosis (Peters *et al.*, 2014). Moreover, in glioblastoma, promoter methylation of this gene was already correlated with poor outcome (Rankeillor *et al.*, 2014). More recently, a 93 cohort study revealed that there is an association between *GATA5* methylation status and prognosis. Ribeiro *et al.* concluded that patients that displayed promotor methylation of *GATA5* had worse prognosis (Ribeiro *et al.*, 2016). These findings reveal that *GATA5* plays an important role in oral carcinogenesis. So, this gene represents a potential target for new therapeutic approaches.

5.1.2.4. *MSH6*

MSH6 is mapped at 2p16 and encodes a mismatch repair protein involved in the DNA mismatch repair system. Promotor methylation of *MSH6* has been reported in several tumours such as breast cancer, glioblastoma, HNSCC and OSCC (Moelans *et al.*, 2011; Felsberg *et al.*, 2011; Wei *et al.*, 1998; Ribeiro *et al.*, 2016). Our results are in agreement with previous studies since *MSH6* was methylated in 29.2% of the patients.

Felsberg *et al.* showed that in glioblastoma, *MSH6* methylation are associated with tumour recurrence (Felsberg *et al.*, 2011). Ribeiro *et al.* demonstrated that promotor methylation of *MSH6* is related with development of metastasis or relapses during or after treatment. Moreover, they also reported that methylation of this gene is associated with worse prognosis (Ribeiro *et al.*, 2016). Therefore, *MSH6* methylation profile is a potential biomarker for assessment of tumour behaviour

in OSCC patients. Patients with this alteration should be more frequently followed up in order to detect possible metastasis or relapses.

5.1.2.5. *RARB*

As previously referred, loss of *RARB is* commonly detected in cancer, including in OSCC. It is suggested that *RARB* alterations are frequently caused by promoter methylation rather than deletion (Shaw *et al.*, 2008). Our results corroborate this idea since methylation of *RARB* was found in 14 patients whereas deletion was found in only 8 patients.

Methylation of *RARB* was previously reported in several tumours such as leukaemia, HNSCC, breast, prostate and also in OSCC (Galm *et al.*, 2004; Chen *et al.*, 2007; Marzese *et al.*, 2012; Tang *et al.*, 2013; Ribeiro *et al.*, 2016). Marzese *et al.* suggested that methylation of this gene is associated with poor prognosis in breast cancer (Marzese *et al.*, 2012). Moreover, methylation of *RARB* was already reported at a higher frequency in potentially malignant HNSCC lesions, indicating that epigenetic silencing of *RARB* is associated with early stages of the disease (Chen *et al.*, 2007). Accordingly, *RARB* represents a potential marker for early detection and prognostication of OSCC.

5.2. Non-tumour tissue

Through genetic analysis of oral lesions and benign mucosa, Califano *et al.* evidenced that early genetic events may be shared by both lesions and benign mucosa. Therefore, they concluded that cells of the same local anatomical area derived from the same clone (Califano *et al.*, 1996). Taking this into account, the genetic and epigenetic background of non-tumour samples (collected from the resection margin of the surgery) were evaluated in this project by MS-MLPA in order to compare the results with those obtained in tumour samples. It was aimed to investigate which alterations would be present in both samples in order to speculate possible early changes in carcinogenesis. Moreover, it was intended to discover possible alterations in non-tumour samples that can be associated with risk of tumour relapses or metastasis development.

Overall, the alterations found in non-tumour samples were also present in the corresponding tumour tissue or/and in exfoliated cells samples.

From the 48 samples analysed, only seven patients did not exhibit genetic and epigenetic alterations: 23 did not display alterations in CNV and 17 did not show methylation in any gene. In

fact, acknowledging that epigenetic alterations are considered early events in carcinogenesis, it was expected to find methylation alterations in a higher percentage than CNV.

Regarding CNVs, the alterations detected were quite similar to those obtained in tumour samples. However, as expected, the number of patients that displayed these alterations were lower in non-tumour samples. Then, the most frequent alterations detected in non-tumour samples were gains of *STK11* (19p13.3), *PYCARD* (16p11.2) and *VHL* (3p25.3) and losses of *CDKN2A* (9p21) (Figure 15). In what concerns methylation, although *WT1* (11p13) was the gene most frequently methylated in both samples, the methylation profile of non-tumour was significantly different from tumour. Besides that, *WT1*, *MSH6* (2p16) and *TP53* (17p13.1) were the genes most commonly methylated in non-tumour samples (Figure 16).

The first genetic progression model for HNC was proposed by Califano *et al.*, in 1996. They suggested that deletion of the 9p21 was an early event, followed by 17p13 and 3p21 deletion (Califano *et al.*, 1996). Later, Braakhuis *et al.* proposed a second model suggesting that the earliest genetic alteration is loss of 17p, followed by alterations of 3p, 9p, 8p and 18q. They also emphasised that the presence of cells genetically altered in a certain anatomical area is a risk factor for cancer initiation (Braakhuis *et al.*, 2003). Thereupon, it can be hypothesized that the patients of the present cohort that displayed deletion of *CDKN2A* and/or methylation of *TP53* in non-tumour samples have more probability of develop metastasis.

In our results, the presence of promotor methylation of *WT1* in non-tumour cells suggests that this alteration is associated with early stages of the disease, which was already described in the literature.

As previously mentioned, methylation of *MSH6* is associated with metastasis and/or tumour relapses. However, to the best of our knowledge, this alteration is not already related with the stage of disease. The preliminary results of non-tumour sample revealed that *MSH6* methylation can be an early event.

As described before, the role of *STK11, PYCARD* and *VHL* in carcinogenesis is still controversial and, for that reason, it is difficult to interpret the alterations of these genes in non-tumour samples.

5.3. Exfoliated cells: a viable tool for oral squamous cell carcinoma screening?

Although oral cavity is easy to access, OSCC is often diagnosed at advanced stages of disease. Moreover, OSCC is strongly associated with tumour relapses and metastasis development. According to the literature, screening the population at risk and early detection of malignant lesion decreases not only the morbidity but also the mortality of oral cancer (Reddy *et al.*, 2014). Biopsy is the gold standard for diagnosis. However, taking numerous biopsies to screening and to follow up the patients is not practical since this technique is uncomfortable for the patients. Therefore, this work aimed to validate a non-invasive method for screening the risk population and to follow up the patients diagnosed with OSCC. In this line, MS-MLPA technique was applied in exfoliated cells acquired from 59 OSCC patients. The results were further compared with tumour tissue samples from the same patients. The agreement between both samples was evaluated through statistical analysis.

As described above, regarding CNVs, it was found agreement in 22 out of the 38 genes evaluated (Table 12). Additionally, as respects to methylation, the kappa value had statistical significance for 18 out of the 25 genes assessed (Table 13). Therefore, the results obtained revealed that the great majority of the alterations present in tumour tissue were also present in exfoliated cells. Moreover, in general, genes that showed no alterations in the tissue, also exhibited no changes in exfoliated cells. So, the results are truly promising, revealing that this non-invasive approach can be able to detect genetic and epigenetic imbalances of OSCC. Although the agreement for some genes have not yet reached, it must be taken into account that this is a pilot study. Consequently, with an increased cohort, an agreement for more genes is expected, especially for those that already presented values closed to statistically significant. Therefore, it is our belief that this non-invasive method can be accepted as a powerful tool to identify the alterations involved in the OSCC carcinogenesis. Nevertheless, further studies should be performed in order to validate this approach as a viable tool for potentially malignant and malignant lesions of the oral cavity diagnosis.

In what concerns exfoliated cells samples, although it was proved that MS-MLPA is capable of detecting genetic and epigenetic alterations of tumours, the accuracy of this technique to detect alterations involved in premalignant lesions and early stages of OSCC should be assessed. One of the drawbacks of exfoliated cells samples is the lower amount of DNA frequently obtained in the samples. Apparently, it can be supposed that an increased amount of cells should be collected. However, as tumours were located at certain anatomical regions, the scrapping process should be

also confined to restricted locals, in order to have minimal contamination with normal cells. Thus, a possible solution is to try to optimize the DNA extraction protocols.

5.4. Comparison between MS-MLPA and aCGH

Overall, the results between aCGH and MS-MLPA regarding CNV are often concordant. When different results were observed, it was frequently verified that the MS-MLPA ratios were near to the cut-offs established, with the exception of the *STK11* gene. Moreover, another reason that can explain the differences detected is the fact that the sensitivity of these techniques is highly dependent on the proportions of tumour and normal samples. Therefore, the contamination of the tumour samples with normal cells can influence the results. It must also be taken into account that some of the most common alterations detected by aCGH were not evaluated by the probemix used in MS-MLPA assay.

aCGH allows the detection of chromosomal imbalances through all the genome, making this a powerful tool to identify genetic alterations involved in tumorigenesis. However, it is an expensive technique that generates a high amount of data, which requires more care and attention in order to overcome the associated difficulties of interpretation. Therefore, aCGH is not the most appropriate technique for diagnostic routine. Contrarily, MS-MLPA is a faster, directed and cheaper technique that is able to analyse up to 50 probes in the same reaction. Accordingly, MS-MLPA is a viable technique to perform in clinical and daily analysis and it is also able to detect genetic and epigenetic alterations of tumour samples. Nonetheless, this technique only detects alterations in a set of genes, depending on the probemix used. Considering all these facts, aCGH seems to be a more robust technique to detect new genomic imbalance that can be used as biomarkers of cancer. After identification of the main genomic aberrations, a MS-MLPA probe panel can be developed and applied in diagnostic routine.

The probemix used in this study is not specific for OSCC but for cancer in general. The results obtained by aCGH in this study suggest that a new probe panel, more specific for OSCC could be developed. For instance, although gains at 8q were frequently detected by aCGH, no probe evaluating the CNV of genes mapped at this region is present in the *ME002* probemix. As mentioned before, the amplification of some genes mapped at 8q has been reported in OSCC, such as *MYC, PTK2* and *LRP12*. Thus, it would be interesting to evaluate CNV of some of this genes. Losses at 18q were also commonly detected in aCGH results and therefore it would also be pertinent to analyse some TSGs mapped at this region, as *CDH2*, *BCL2* and *DCC,* for example.

Moreover, some gene probes existent in ME002 probemix appears not have influence in OSCC carcinogenesis such as *PTEN, PAH* and *THBSS.* These genes did not display significant alterations or presented practically the same (and also low) number of deletions and amplifications for the same gene. Nevertheless, the cohort should be increased in order to confirm these hypotheses.

MS-MLPA has reference probes that are used to normalize the values obtained by the target-specific probes. As cancer is a heterogeneous and instable disease, the choice of adequate references to analyse tumour sample is extremely difficult. Consequently, in this study all reference probes presented CNV, demonstrating that these references may not be the most appropriated to analyse OSCC samples.

The probes designed for *VHL* and *STK11* appear not to be ideal to detect the CNV of these two genes. Both genes are described as been deleted in OSCC, which is also identified by aCGH in the present cohort. However, the MS-MLPA results showed gain of these genes in numerous samples, specially gains of *STK11*. As only a small length of each gene was evaluated, it can be suggested that even the region evaluated was amplified, other regions of these genes can be deleted.

6. Conclusions

Overall, the aims proposed for this project were accomplished. Tumour tissue samples, non-tumour samples and exfoliated cells from tumour were genetically and epigenetically characterized and the following conclusions were drawn:

- The two techniques (aCGH and MS-MLPA) used in this study confirmed to be reliable methods to detect genetic and epigenetic alterations present in OSCC samples;
- aCGH showed several chromosomal rearrangements through all the genome, specially gains at 3q, 8q, Xq and losses at 3p, 18q, Yp and Yq. In general, these results are in concordance with previous OSCC studies.
- The main CNVs detect by MS-MLPA were gains of *STK11* and *PYCARD* and losses of *CDKN2A, VHL* and *ATM.*
- Concerning methylation status, *WT1* was the gene most frequently methylated in the present cohort. Methylation of *WT1* was identified in nearly 80% of the tumour samples, revealing its tumour suppressor role in OSCC carcinogenesis. Besides, methylation of *PAX5*, *GATA5*, *MSH6* and *RARB* were also frequently found. Therefore, these genes revealed to be potential OSCC biomarkers.
- The analysis of non-tumour samples allowed to identify alterations that are possibly involved with early stages of OSCC, specifically, deletion of *CDKN2A*, methylation of *TP53* and methylation of *WT1*. Moreover, the alterations detected in non-tumour samples were also identified in tumour samples (from tissue or/and exfoliated cells), suggesting that the corresponding patients have a higher risk of developing tumour relapses or metastasis.
- The comparison of the MS-MLPA results of tissue samples and exfoliated cells revealed agreement in 22 of the 38 genes evaluated for CNV and in 18 of the 25 genes assessed for methylation status. The MS-MLPA results of exfoliated cells were truly promising since it was found a high agreement between this type of samples and tissue samples. This is a huge step in an attempt to validate this non-invasive methodology for screening the oral cavity and to follow up the patients diagnosed with OSCC.
- In general, the aCGH and MS-MLPA results were in agreement. However, the MS-MLPA probemix used did not contain probes to detect genetic alterations that were frequently found by aCGH. Moreover, the STK11 results were contradictory between the two methods. It is suggested that the probe used to evaluate CNV of *STK11* should be altered or additional probes to analyse this gene should be added to the ME002 probemix.

- Overall, the MS-MLPA probemix used in this study was efficient for detecting genetic and epigenetic changes of OSCC samples. However, the probemix is not specific for oral cancer but for cancer in general. Therefore, the aCGH technique can be initially used to screen the entire genome and to identify the genetic and epigenetic alterations frequently found in OSCC. Furthermore, a new and more specific MS-MLPA probemix should be developed and consequently used in diagnostic routine.

7. Future perspectives

OSCC results from accumulation of numerous genetic and epigenetic changes, followed by clonal expansion. Therefore, one of the aims of this project was characterize to OSCC molecular profiles in order to find potential biomarkers that can be used to detect OSCC in early stages and also predict the disease progression. In fact, in this study, we elucidate some genetic and epigenetic characteristics of OSCC patients. However, this study is not concluded. Since aCGH allows screening the entire genome, it is important to apply this technique in all samples in order to find additional genetic alterations that can be implicated in OSCC carcinogenesis. Then, considering that MLPA is a more reliable and easy to perform technique, it would be essential to develop a new probe panel, specific for oral cancer. Moreover, an increased cohort would also be important to establish more correlations between the genetic and epigenetic profiles and the corresponding patients' clinic-pathological features.

In spite of technological advances in cancer management and treatment, the outcome of OSCC patients remains low, mainly due to diagnosis in advanced stage and frequent development of loco-regional recurrences. Accordingly, it is also our aim to try to validate a new and non-invasive method for early detection and to follow up patients after treatment. The results of the exfoliated cells sample were promising. However, a bigger cohort is imperative in order to increase the agreement between the alterations detected through this non-invasive method and through biopsy samples. Additionally, since contamination with normal cells can mask some alterations, which are typical of tumours, it is crucial to evaluate the percentage of tumour cells present in both types of samples (biopsy and exfoliated cells) in order to ensure that the results are not influenced by normal cells.

Having into account that the five-year survival rate of OSCC remains poor, it is also important to follow-up the patients in order to evaluate the efficiency of treatment and to predict the disease progression. Therefore, it would be interesting to analyse exfoliated cells samples from OSCC patients, collected at different time points, during and after treatment. Thus, an early intervention to prevent tumour relapses or metastasis could be achieved through the analysis of genetic and epigenetic alterations.

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