DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Characterization of the Genetic and Epigenetic Profile of Tongue Squamous Cell Carcinoma

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Isabel Carreira (Universidade de Coimbra) e da Professora Doutora Paula Veríssimo (Universidade de Coimbra)

Sofia Matos Lisboa

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Abbreviations

Akt/PKB – Protein kinase β

AL – Allelic imbalance

APC – Adenomatous polyposis coli

ATM – ATM serine/threonine kinase

BRCA1 – Breast cancer1

BRCA2 – Breast cancer 2

CADM1 – Cell adhesion molecule 1

CASR – Calcium-sensing receptor

CCND1 – Cyclin D1

CD44 – CD44 molecule

CDDP – Cis-diamminedichloroplatinum

CDH1 – E-cadherin

CDH13 – Cadherin 13

CDK – Cyclin-dependent kinase

CDK6 – Cyclin-dependent kinase 6

CDKN1A – Cyclin-dependent kinase inhibitor 1A

CDKN1B – Cyclin-dependent kinase inhibitor 1B

CDKN2A – Cyclin-dependent kinase inhibitor 2A

CFTR – Cystic fibrosis transmembrane conductance regulator

CHFR – Checkpoint with forkhead and ring finger domains

CSS – Cause-specific survival

COBRA – Combined Bissulfite Restriction Analysis

CREM – cAMP responsive element modulator

CT – Chemotherapy

DAPK1 – Death-associated protein kinase 1

DNMT – DNA methyltransferase

ECS – Extracapsular spread

EGFR – Epidermal growth factor receptor

ERBB2 – Erb-b2 receptor tyrosine kinase 2

ERK – Extracellular-signal-regulated kinase

ESR1 – Estrogen receptor 1

FFPE – Formalin-fixed paraffin embedded

GATA5 – GATA binding protein 5

gDNA – genomic DNA

GST – Glutatione S-transpherase

GSTP1 – Glutathione S-transferase pi 1

HIF1α – Hypoxia-inducible factor 1α

HNSCC – Head and neck squamous cell carcinoma

HPV – Human papilloma virus

HRAS – Harvey rat sarcoma viral oncogene homolog

H&E – Hematoxylin and eosin

IARC – International Agency for Research on Cancer

IL2 – Interleukin 2

IL6 – Interleukin 6

Jack/STAT – Janus kinase – signal transducer and activator of transcription

KLK3 – Kallikren-related peptidase 3

KLLN – Killin

LOH – Loss of heterozygoty

MAPK – Mtitogen-activated protein kinase

MDM2 – MDM2 proto-oncogene, E3 ubiquitin protein ligase

MGMT – O-6-methylguanine-DNA methyltransferase

MLH1 – MutL homolog 1

MLH3 – mutL homolog 3

MLPA – Multiplex Ligand-dependent Probe Amplification

MS-MLPA – Methylation-specific Multiplex Ligand-dependent Probe Amplification

MSH6 – mutS homolg 6

MSP – Methylation-specific PCR

MYC – V-myc avian myelocytomatosis viral oncogene homolog

NRAS – Neuroblastoma RAS viral (v-ras) oncogene homolog

OCC – Oral cavity cancer

OCSCC – Oral cavity squamous cell carcinoma

OS – Overall survival

OSCC – Oral squamous cell carcinoma

OPSCC – Oropharyngeal squamous cell carcinoma

PAH – Phenylalanine hydroxylase

PAX5 – Paired box 5

PAX6 – Paired box 6

PBS – Phosphate-buffered saline

PMP22 – Peripheral myelin protein 22

PTCH1 – Patched 1

PTEN – Phosphate and tensin homolog

PYCARD – PYD and CARD domain containing

RARB – Retinoic acid receptor, beta

RASSF1 – Ras association (RalGDS/AF-6) domain family member 1

RB1 – Retinoblastoma 1

RT – Radiation therapy

RUNX3 – Runt-related transcriptional factor 3

SCC – Squamous cell carcinoma

SEER – Surveillance, Epidemiology and End Results

STAT3 – Signal transducer and activator of transcription 3 (acute-phase response factor)

STK11 – Serine/threonine kinase 11

THBS1 – Thrombospondin 1

TNM – Tumor, Nodes and Metastasis

TP53 – Tumor protein p53

TP73 – Tumor protein p73

TSC2 – Tuberous sclerosis 2

TSCC – Tongue squamous cell carcinoma

TSG – Tumor suppressor gene

UICC – Union Internationale Contre le Cancer

VEGF – Vascular endothelial growth factor

VHL – von-Hippel Lindau

WIF1 – WNT inhibitory factor 1

WT1 – Wilms tumor 1

Wtn – Wingless type

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CHAPTER 1 – LITERATURE REVIEW and OBJECTIVES

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CHAPTER 3 – RESULTS

Caracterização do Perfil Genético e Epigenético do Carcinoma Epidermóide da Língua

Resumo

O carcinoma epidermóide da língua é uma neoplasia invasiva, caracterizada por metástases precoces e extensivas dos gânglios linfáticos, o que contribui para a agressividade destes tumores. A sua incidência tem vindo a aumentar mundialmente, sendo o tumor maligno mais comum da cavidade oral. O risco de doentes diagnosticados com cancro da língua virem a desenvolver uma recidiva é superior ao dos diagnosticados com qualquer outro subtipo de cancro da cabeça e pescoço. De acordo com o sistema de classificação TNM (*Tumor-Nodes-Metastasis*), doentes em estadios mais iniciais da doença teoricamente deveriam apresentar um prognóstico mais favorável. Porém, estes doentes, contrariamente ao antecipado, apresentam um prognóstico menos favorável quando comparados com doentes em estadios mais avançados de outros subtipos de carcinomas da cabeça e pescoço. Isto revela que o presente sistema de classificação não é o mais adequado para um prognóstico correcto dos doentes com cancro da língua. Diferenças na evolução clínica dos doentes que varia de acordo com a localização anatómica do tumor, sugere a possibilidade de os mecanismos moleculares envolvidos no processo carcinogéneo dos diferentes tipos de cancro da cabeça e pescoço serem também eles distintos. Por este motivo é importante estudar a língua como uma localização anatómica isolada, de forma a identificar as principais alterações moleculares específicas envolvidas no desenvolvimento e progressão do carcinoma epidermóide da língua. O principal objectivo do presente estudo foi a caracterização do perfil genético e epigenético de 31 carcinomas primários da língua, obtidos aquando remoção cirúrgica, através da técnica de MS-MLPA (*Methylation-specific Multiplex Ligation-dependent Probe Amplification*). As alterações mais frequentes foram detectadas nos genes *WT1*, *PAX5*, *GATA5*, *MSH6*, *PYCARD*, *STK11*, *CDKN2A*, *CHFR*, *BRCA1*, *GSTP1*, *TP53*, *RARB* e *CADM1*, sugerindo a importância destes genes no processo carcinogéneo. A metilação do gene *MSH6* poderá estar associada a estadios mais avançados da doença e ao desenvolvimento de metástases. O presente estudo revelou diferentes alterações genéticas e epigenéticas que poderão estar envolvidas no desenvolvimento e progressão do carcinoma da língua, estabelecendo-se em paralelo uma associação com as características clínicas e patológicas dos doentes. Os genes identificados servem de base para estudos futuros com uma maior amostragem, de forma a estabelecer uma associação estatisticamente

significativa entre genótipo e fenótipo. A identificação de biomarcadores irá permitir um diagnóstico cada vez mais precoce, uma avaliação assertiva do prognóstico e subdividir os doentes de acordo com o seu perfil genómico e clínico, de forma a prever mais eficazmente a sua evolução clínica e qual o melhor tratamento a seguir. O desenvolvimento e optimização de metodologias que permitem a extracção de DNA genómico pouco fragmentado e em elevada quantidade e pureza a partir de amostras de tecido parafinizado é essencial para um aumento da amostragem utilizada em estudos genéticos. O presente trabalho permitiu também optimizar e estabelecer um método eficiente e reprodutível de extracção de DNA a partir de amostras de tecido incluído em parafina de carcinomas da língua, obtendo-se concentrações relativamente elevadas de DNA, com elevada pureza.

Palavras-chave: Carcinoma epidermóide da língua; *Methylation-specific Multiplex Ligation-dependent Probe Amplification*; extracção de DNA; tecido incluído em parafina.

Characterization of the Genetic and Epigenetic Profile of Tongue Squamous Cell Carcinoma

Abstract

Tongue squamous cell carcinoma (TSCC) is the most common malignancy in the oral cavity, characterized by high recurrence rates, reduced overall survival and increasing incidence worldwide. A higher risk of locoregional failure is observed in these carcinomas as compared with other head and neck subsites, contributing to the invasiveness and aggressiveness of these tumors. Applying the Tumor, Nodes and Metastasis (TNM) classification system, patients with early stage TSCC would theoretically represent those with better prognosis. However, this group of patients has the worse prognosis as compared with other head and neck subsites with more advanced stage disease, revealing that the current clinicopathological criteria does not comprehensively differentiate patient prognosis. The discrepancy in patients' outcome according to the anatomical subsites of head and neck squamous cell carcinoma (HNSCC) highlights the presence of different molecular mechanisms underlying tumorigenesis. For this reason, further investigation specifically at the tongue subsite would be of benefit to determine subsite-specific molecular drivers of carcinogenesis in a single and relatively homogeneous site. The main objective of the present thesis was the characterization of the genetic and epigenetic profile by Methylation-specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) technique of 31 primary tongue tumors, collected from patients with TSCC, upon resection surgery. The most frequently altered genes in the present cohort were *WT1*, *PAX5*, *GATA5*, *MSH6*, *PYCARD*, *STK11*, *CDKN2A*, *CHFR*, *BRCA1*, *GSTP1*, *TP53*, *RARB* and *CADM1*, suggesting the important role of the present genes in the development and progression of TSCC. Methylation of *MSH6* gene seemed to be associated with more advanced stages of the disease and metastasis. The present study revealed several genetic and epigenetic alterations that may play a role in TSCC development in association with patient's clinicopathological features. The highlighted genes provide a basis for further research in larger cohorts that may lead to the identification of candidate biomarkers allowing for a better diagnosis, prognosis and accurate risk-stratification of patients, as well as choose the most adequate treatment and predict treatment response in TSCC. The development of methods that allow the recovery of optimal quality DNA from formalin-fixed paraffin embedded (FFPE) tissues is essential to increase the cohorts for cancer research. In the present study an efficient and reproducible method of DNA

extraction from FFPE tongue tumor samples, yielding relatively high concentrations and high purity DNA was established.

Key-words: Tongue squamous cell carcinoma; Methylation-specific Multiplex Ligation-dependent Probe Amplification; DNA extraction; formalin-fixed paraffin embedded tissues.

CHAPTER 1
LITERATURE REVIEW and OBJECTIVES

Chapter 1 – Literature review and objectives

1 – Anatomical considerations, epidemiology and risk factors

1.1 – Anatomical considerations

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignancy worldwide and consists of a heterogeneous group of tumors of which squamous cell carcinoma constitutes the largest histological group (90-95%). HNSCC develops from the mucosa of the upper aerodigestive tract, affecting the nasal cavity, paranasal sinuses, nasopharynx, hypopharynx, larynx, trachea, oral cavity, oropharynx and salivary glands. Commonly, HNSCC are grouped in three major groups: oral cavity, pharynx and larynx (Figure 1). (Leemans, Braakhuis, & Brakenhoff, 2011; Rousseau & Badoual, 2012; Takes et al., 2010)

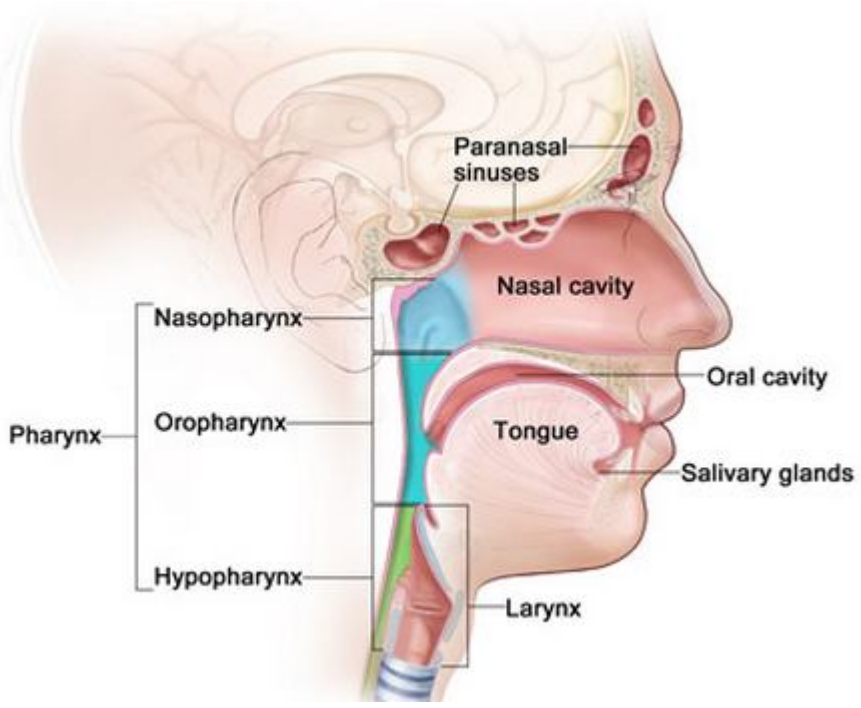

Figure 1 – Anatomical sites and subsites of head and neck squamous cell carcinoma. Adapted from National Cancer Institute, 2012b.

Oral squamous cell carcinoma (OSCC), a subsite of HNSCC, is an invasive epithelial neoplasm with predisposition to early and extensive lymph node metastases.

This carcinoma arises from different anatomic sites within the oral cavity: oral soft tissues, such as gingival and alveolar mucosa, floor of the mouth, tongue, soft and hard palates and tonsils (Figure 2). The most predominant anatomic location of oral tumors is the tongue, followed by floor of the mouth, as demonstrated in Figure 3. (Cancela et al., 2010; Jin & Jin, 2011)

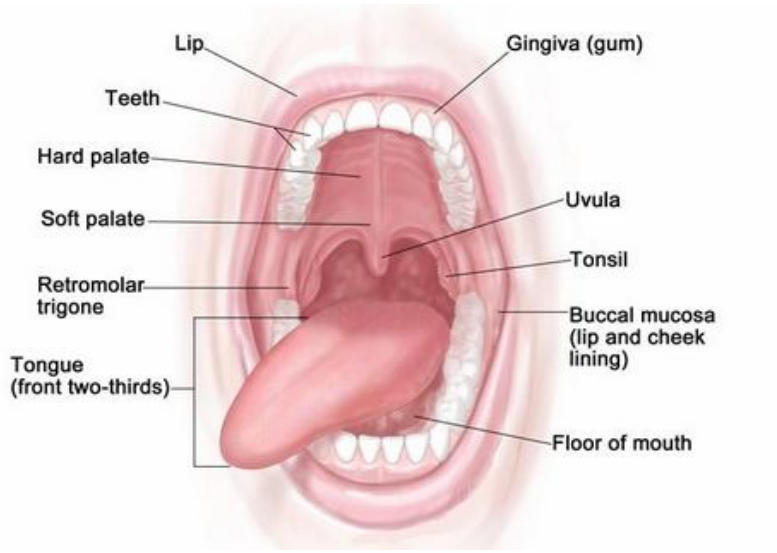

Figure 2 – Anatomy of the oral cavity. Adapted from National Cancer Institute, 2012a.

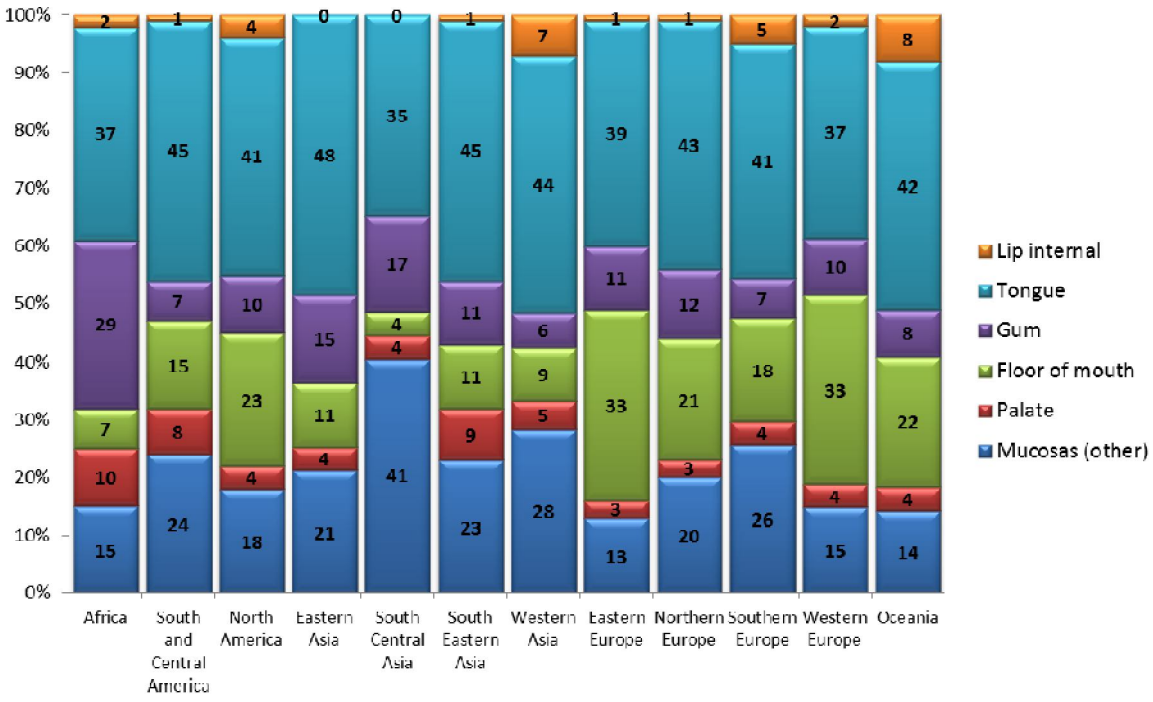

Figure 3 – Subsite distribution of OSCC among different world regions (1998-2002). Adapted from Cancela et al., 2010.

Tongue squamous cell carcinoma (TSCC) arises from the squamous epithelium of the oral/lingual tongue, which consist of the anterior two thirds of the tongue. Circumvallate papillae separate the anterior tongue of the oral cavity from the posterior/base of tongue which forms part of the oropharynx. This anatomical distinction is essential, due to differences in pathogenesis, behavior and identity of tumors arising from these two sites. The most marked difference is that a large number of carcinomas of the posterior tongue, oropharyngeal squamous cell carcinomas (OPSCCs), develop after Human Papillomavirus (HPV) infection, with a good prognosis when compared to not HPV-related (HPV-negative) HNSCCs.

1.2 – Epidemiology

HNSCC normally arises after the fifth decade of life and of the total patients diagnosed with the disease only 40-60% survive for 5 years. (Leemans, Braakhuis, & Brakenhoff, 2011) From the head and neck cancers, OSCCs are the most common; however incidence is not well documented, as these carcinomas are usually grouped with other subsites. Still oral cavity cancer (OCC) is thought to be the 8th most frequent cancer among males and 14th among females worldwide. (Cancela et al., 2010) Together, lip and OCC account for 2,1% of all cancers, with two thirds occurring in men. (Ferlay et al., 2015) Incidence of this cancer varies widely reflecting geographic differences in exposure to risk factors, as will be discussed in the next section. (Cancela et al., 2010; Ferlay et al., 2015) The highest incidence rates are found in Melanesia region, South Central Asia and Eastern Europe. Furthermore, a higher burden of lip and oral cancer is observed in less developed regions, registering 77% of the 145,000 deaths from these diseases in the year 2012. (Ferlay et al., 2015) In Portugal, Registos Oncológicos Regionais data from 2001 reported a higher incidence rate of OCC in men (11.4 per 100,000 habitants) than in women (2.8). The tongue subsite had the highest incidence rate (2.8), followed by lip (1.5). (Bento, 2009; Santos, & Teixeira, 2011)

Early diagnosis may occur due to the accessibility of oral cavity for visual screening, but most oral cavity tumors are diagnosed at an advanced stage, which contributes to the poor overall 5-year survival rate. (Silva et al., 2011) In 2008, Instituto Português de Oncologia do Porto showed that diagnosis at advanced stages occurred in 76.9% of the tongue cancer cases and 61.4% for other oral cavity subsites. (Instituto

Português de Oncologia do Porto, 2008) The overall 5-year survival rate of OCC was 36.4%, between 2000 and 2006. (Bento, 2009; Santos, & Teixeira, 2011) Advanced stage at initial diagnosis, locoregional recurrence, further primary tumors and lymph node metastasis contribute to the limited survival and high mortality rates in OSCC. (Silva et al., 2011)

Historically, OSCC has been associated with significant tobacco and alcohol exposure, arising more commonly in older men. However, more recently, incidence of OSCC is decreasing, accompanied by opposite increasing incidence of TSCC, particularly in younger patients with limited exposures to risk factors. According to Surveillance, Epidemiology and End Results (SEER) published analysis, TSCC was stable during the years 1975 to 2007, but was paradoxically increasing in women, specifically on a subgroup of young, white women. (Goepfert, Kezirian, & Wang, 2014; Patel et al., 2011) This was also verified in a later study, in which Li et al. (2014) analyzed 89 patients diagnosed with TSCC, where non-smokers were younger than smokers and were more likely to be female. During the same period of time and also using the SEER database, a study conducted by Mehta & Schantz (2010) reported a two-fold increase in moderately and poorly differentiated tongue tumors, with a concomitant decrease in incidence of well-differentiated tumors. Thus, overall ratio of grade III to grade I increased from 2.27:1 to 12.5:1.

Despite the advances in cancer diagnosis, management and treatment, patients' outcome has not significantly improved in the last decades. Patients diagnosed with TSCC have a poorer prognosis and survival when compared with patients diagnosed with other oral cavity and head and neck cancer subsites. (Goldstein et al., 2012; Rusthoven et al., 2008) Rusthoven et al. (2008) analysis of 6791 patients with stage I and stage II OSCCs found 5-year overall survival (OS) and cause-specific survival (CSS) rates of 60,9% and 83,5%, respectively, for patients diagnosed with TSCC in opposite to 64,7% and 94,1%, respectively, for patients with other subsites of OCSCC. Additionally, patients with stage I and II TSCC had an unfavorable SCC when compared with staged-matched patients with other HNSCC subsites.

1.3 – Risk factors

The main risk factors in HNSCC development are tobacco exposure and alcohol consumption. These two substances have a synergistic effect between them and avoiding consumption could prevent up to 90% of HNSCCs, particularly larynx and hypopharynx tumors. Smokers and alcohol drinkers are at higher risk of developing second primary oral cancer and experiencing more severe outcomes than patients who abstain from these substances. (Massano et al., 2006; Morse et al., 2007; Poveda-Roda et al., 2010)

The risk of developing head and neck cancer is increased by smoking at a young age and during a long period of time. Furthermore, a high number of cigarettes smoked per day and deep smoke inhalation also contribute to boost the risk. (Hashibe et al., 2007; Morse et al., 2007; Rousseau & Badoual, 2012) The evident association between smoking and OSCC should lead to total smoking cessation among patients diagnosed with this disease. However, even after medical advice, many patients continue to smoke, despite the fact that it affects patient survival, increases the risk of tumor relapse and adds limitations to treatment efficacy. (Poveda-Roda et al., 2010)

Other than smoking, betel quid (*Piper betle*) chewing is also a common habit in some regions of Asia and has been specifically correlated with poor prognosis in OSCC. High rates of oral and oropharyngeal SCC are documented in developing countries, mainly due to tobacco chewing habits, especially when consumed in betel quid containing areca nut (*Areca catechu*). In India, chewing tobacco habits account for 50% of men and over 90% of women with this type of tumors. (Rousseau & Badoual, 2012) This allied to the fact that 80% of the smokers worldwide live in developing countries reflects the higher burden of OSCC in these areas. In contrast, cigarette smoking in most developed countries is decreasing and its consumption is becoming equal in both genders, namely in European eastern countries where male consumption is decreasing whereas increasing in females. (Cancela et al., 2010; Lo et al., 2003; Rousseau & Badoual, 2012)

Difficulties in access screening by a dental or medical professional and access to treatment when the disease has already progressed increase the burden of OSCC in developing countries. Although, in developed countries treatment is easily available, early diagnosis of oral lesions is still problematic, mainly due to the lack of general population and professional´s awareness. Health programs targeting populations at

higher risk must be developed in order to improve prevention and minimize consequences of the disease. (Cancela et al., 2010)

Other etiology factors of OSCC have been established, such as nutrition, occupational exposure to carcinogens, socioeconomic conditions and, particularly, HPV infection. (Gillison et al., 2000; Massano et al., 2006; Rousseau & Badoual, 2012; Syrjänen, 2005) Occupational exposure to carcinogens substances, particularly in air suspension, such as polycyclic aromatic hydrocarbons, has been associated to a higher risk of developing HNSCC. (Rousseau & Badoual, 2012) Lower socioeconomic status and education is associated with worse patient outcome, as a result of poorer oral hygiene and difficult access to medical care. (Massano et al., 2006; Silva et al., 2011)

Despite the decrease of smoking habits in developed countries, OSCC incidence in some of these countries is increasing, especially in Western European countries such as Belgium, Denmark, Greece, Portugal and Scotland. In Eastern Europe, OSCC constitutes a real public health issue, with increasing mortality rates observed for over the last two decades. (La Vecchia et al., 2004) This may be explained by the increasing prevalence of HPV infections and its association with OSCC in this continent, especially high rates of SCC of the oropharynx caused by HPV. (Marur et al., 2010; Smith et al., 2004)

1.4 – Role of HPV in HNSCC

HNSCC incidence of specific subsites has been decreasing; however, tongue and OPSCC are becoming more prevalent, which has been related to an increase in oral and oropharyngeal HPV infection, particularly HPV16, 18 and 33. (Kreimer et al., 2005; Leemans, Braakhuis, & Brakenhoff, 2011; Smith et al., 2004) HPV16 is the most prevalent HPV type in cervical SCC and is also the most common type present in HPV-positive HNSCCs. (Kreimer et al., 2005)

HPV-positive and HPV-negative head and neck tumors represent different clinicopathological and molecular entities (Table I). HPV-positive HNSCC patients are, on average, 5 years younger than their HPV-negative counterparts and often have lower alcohol and/or tobacco exposure. Moreover these are usually younger adults that engage risk sexual practices, namely oral sex with multiple partners. HNSCC associated with HPV infection differ in aetiological factors, molecular identity and prognosis. HPV-

positive HNSCC has been associated with a more favorable clinical outcome, with an increased survival of up to 60-80%. (Gillison et al., 2000; Leemans, Braakhuis, & Brakenhoff, 2011; Mehta, Yu, & Schantz, 2010; Smith et al., 2004)

Table I – Differences between HPV-positive and HPV-negative HNSCCs. Adapted from Leemans, Braakhuis, & Brakenhoff, 2011; Marur et al., 2010.

Feature	**HPV-positive HNSCC**	**HPV-negative HNSCC**
Incidence	Increasing	Decreasing
Aetiology	Oral sex	Smoking, excessive alcohol use
Age	Under 60 years	Above 60 year
TP53 mutations	Infrequent	Frequent
Predilection site	Oropharynx	None
Prognosis	Favorable	Poor
Survival	Improved	Unchanging

Despite initial reports on the low percentage of HPV-associated HNSCCs, the link between HPV and HNSCC development is becoming more firmly establish, as a result of newer PCR-based methods that have improved detection accuracy and reliability. In fact HPV infection has recently been recognized as a primary cause of OPSCC, including the tonsils and base of tongue. Recent studies have demonstrated the presence of HPV genome in 47% to 63% SCC of the oropharynx. (Fakhry et al., 2008; Kreimer et al., 2005; Mehta, Yu, & Schantz, 2010; Smith et al., 2004)

The major geographic areas affect by oropharynx HPV-positive SCC are North America and Asia, where HPV prevalence is significantly higher than compared to Europe (Table II). (Kreimer et al., 2005) In United States the percentage of oropharynx SCC caused by HPV is 40-80%, whereas in Europe it varies from 20% in areas with high rates of tobacco use to 90% registered in Sweden. (Marur et al., 2010)

Table II – Prevalence of HPV. Adapted from Kreimer et al., 2005.

	No. studies	No. cases	Overall HPV prevalence (95% CI)	HPV16 prevalence (95% CI)
Oral cavity				
Europe	15	744	16,0 (13,4-18,8)	10,8 (8,6-13,2)
North America	8	577	16,1 (13,2-19,4)	10,1 (7,7-12,8)
Asia	13	1,133	33,0 (30,3-35,8)	22,3 (20,3-25,2)
Other	2	188	18,1 (12,9-24,3)	14,9 (10,1-20,8)
Oropharynx				
Europe	17	529	28,2 (24,4-32,2)	23,8 (20,2-27,7)
North America	7	285	47,0 (41,1-53,0)	42,1 (36,3-48,1)
Asia	4	54	46,3 (32,6-60,4)	35,2 (22,7-49,4)
Other	2	101	36,6 (27,3-46,8)	33,7 (24,6-43,8)
Larynx				
Europe	19	799	21,3 (18,5-24,3)	13,8 (11,5-16,4)
North America	7	297	13,8 (10,1-18,3)	10,1 (7,0-14,1)
Asia	8	306	38,2 (32,8-43,9)	26,5 (21,6-31,8)
Other	1	33	48,5 (30,8-66,5)	45,5 (28,1-63,6)

Although association between HPV and OPSCC has been well established, with strong and consistent epidemiological relation with sexual behavior, the role of the HPV in OSCC pathogenesis is still controversial. (Lingen et al., 2013; Mehta, Yu, & Schantz, 2010) A large multicenter case-control study conducted by International Agency for Research on Cancer (IARC) found that the odds of detecting antibodies against HPV16 major capsid protein (L1) and/or E6 or E7 were significantly higher among cases with OCC than among controls (OR 1,5; 95% CI and OR 2,9; 95% CI, respectively). (Herrero et al., 2003) Miller & Johnstone (2001), in a large meta-analysis of 94 reports with a total of 4680 samples analyzed, also revealed an increased probability of detecting HPV in tissue with precancerous and cancerous features compared with normal mucosa. These investigators found the probability of detecting HPV to be 10,0% (95% CI 6,1-14,6) for normal oral mucosa, 22,2% (95% CI 15,7-29,9) for benign leukoplakia, 26,2% (95% CI 19,6-33,6) for intraepithelial neoplasia, 29,5% (95% CI 23,0-36,8) for verrucous carcinoma and 46,5% (95% CI 37,6-55,5) for OSCC. Additionally, other case-control studies reviewed by Mehta, Yu, & Schantz (2010) show a sixfold increase in the risk of oral cancer associated with oral HPV infections. However, overall prevalence of HPV-positive oral cavity tumors of the IARC study was 3,9% and 12% in a study conducted by Gillison et al. (2000) Moreover, Lingen et al. (2013) reported low etiological fraction for high-risk HPV in OSCC, after evaluation of 409 consecutive cases of OSCC, diagnosed in North America from 2005 to 2011, for high-risk HPV E6/E7 oncogene expression. From the total cases, 5.9% were expression

positive for high-risk HPV E6/E7 (95% CI 0.8-3.6), 3,7% for HPV16 (95% CI 1.8-5.5) and 2.2% for other high-risk HPV types (95% CI 0.8-3.6). Within the oral cavity the numbers of HPV-positive tumors in different subsites were the follow: 9 present in floor of the mouth, 6 in anterior tongue, 4 in alveolar process, 3 in hard palate, 1 in gingive and 1 in lip. These investigators found significant association of HPV-positive OSCCs with male gender, small tumor stage, poor tumor differentiation and basaloid histopathology, analogous to oropharynx cancers.

The rising incidence of TSCC, particularly among young patients with no history of tobacco use, has raised the question of whether or not HPV might have a role in tongue carcinogenesis. Surprisingly, this increase in incidence has not been significantly associated with infection of this virus. Li et al. (2014) studied a group of 89 patients, in which non-smokers were younger than smokers and were more likely females. The young age and fewer *TP53* mutations of non-smokers raised the possibility of a viral role in development of this disease; however identity of such virus was not determined. Although it is now clear that HPV-positive OPSCC constitutes a unique epidemiological and clinical entity, HPV role in pathogenesis of OSCCs, particularly TSCCs, has not yet been established. Further investigation is required in order to identify HPV relationship with development of these tumors or other undiscovered environmental or biological risk factors beyond traditional ones, such as tobacco exposure.

2 – Current clinicopathological staging criteria

The current most widely adopt staging criteria for management of HNSCC is the Tumor, Nodes and Metastasis (TNM) classification system of the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). The T-category describes the extent of the primary tumor; the N-category indicates the presence and extent of regional lymph node metastasis and the M-category reveals the presence or absence of distant metastasis. Combination of different T, N and M categories are grouped in I, II, III and IV stages, where increasing stage is associated with decreasing prognosis. The most common tumors of the head and neck have their own TNM classification; these include lip and oral cavity, pharynx (oropharynx, nasopharynx and hypopharynx), larynx, maxillary sinus, nasal cavity and ethmoid sinus,

mucosal malignant melanoma, major salivary glands and thyroid gland. (Table III and IV) (Sobin, Gospodarowics, & Witteking, 2009)

Table III – TNM classification system to define lip and oral cavity cancer, of which the oral tongue is a subsite. Adapted from Sobin, Gospodarowics, & Witteking, 2009.

Primary Tumor (T)	
TX	Primary tumor cannot be assessed.
T0	No evidence of primary tumor.
Tis	Carcinoma *in situ*.
T1	Tumor ≤2 cm in greatest dimension.
T2	Tumor >2 cm but ≤4 cm in greatest dimension.
T3	Tumor >4 cm in greatest dimension.
T4a	Moderately advanced local disease.
	(Lip) Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face, that is, chin or nose.
	(Oral cavity) Tumor invades adjacent structures only (e.g., through cortical bone [mandible or maxilla] into deep [extrinsic] muscle of tongue [genioglossus, hyoglossus, palatoglossus and styloglossus], maxillary sinus, or skin of face).
T4b	Very advanced local disease.
	Tumor invades masticator space, pterygoid plates, or skull base and/or encases internal carotid artery.
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed.
N0	No regional lymph node metastasis.
N1	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension.
N2	Metastasis in a single ipsilateral lymph node, >3 cm but ≤6cm in greatest dimension.
	Metastasis in multiple ipsilateral lymph nodes, none >6cm in greatest dimension.
	Metastasis in bilateral or contralateral lymph nodes, none >6cm in greatest dimension.
N2a	Metastasis in single ipsilateral lymph node, >3cm but ≤6cm in greatest dimension.
N2b	Metastasis in multiple ipsilateral lymph nodes, none >6cm in greatest dimension.
N2c	Metastasis in bilateral and contralateral lymph nodes, none >6cm in greatest dimension.
N3	Metastasis in a lymph node >6cm in greatest dimension.
Distant Metastasis (M)	
M0	No distant metastasis.
M1	Distant metastasis.

Table IV – TNM classification system according to anatomic stage / prognostic groups for lip and oral cavity cancer, of which oral tongue is a subsite (T – primary tumor; N – regional lymph nodes; M – distant metastasis). Adapted from Sobin, Gospodarowics, & Witteking, 2009.

Stage	T	N	M
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N1	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	Any T	N3	M0
	T4b	Any N	M0
IVC	Any T	Any N	M1

TNM classification and other staging systems have been essential for prediction of clinical tumor evolution and patient prognosis, selection of the best treatment modality, comparison of treatment results in groups of patients with similar prognosis and exchange of information between clinicians and researches. However, these classification systems are based almost exclusively on anatomic information and the recent shift towards nonsurgical treatments, such as radiotherapy and chemotherapy, has increased the discussion of whether or not this information alone is enough for management and treatment of HNSCC. Although, prognosis tends to be worse in patients belonging to higher-stage groups, it is also evident that within these same stage groups significant differences in prognosis may be observed, due to tumor biology differences, as well as environmental-factors related with risk factor exposure, that are not included in the TNM classification system. (Takes et al., 2010)

Another category used within the TNM system is histopathological grading, designated with the G prefix (Table V). Tumor grade description is based on microscopic appearance of abnormal tumor cells and tumor tissue, indicating how quickly the tumor is likely to grow and spread. Tumors are classified as well differentiated when the cells of the tumor and the organization of the tumor's tissue are close to those of normal cells and tissue. However, when tumors present abnormal cells

and lack normal tissue structures, these are classified as undifferentiated or poorly differentiated tumors. Well differentiated tumors tend to grow and spread at a slower rate and are normally associated with better prognosis, whereas higher histopathological grades, by growing and spreading more quickly, require more aggressive and immediate treatment. Although, histopathologic grading helps the prediction of tumor progression and prognosis, it entails observation subjectivity of the pathologist. For this reason, histopathological grading would benefit from further immunohistochemical and molecular analysis, particularly in establishing the true identity of undifferentiated tumors (G4). (Takes et al., 2010) Genetic studies would be of special interest, as the correct diagnosis allows for an adequate choice of therapy. Therefore, the possibility of including these studies in routine diagnosis should be considered and further investigated, as difficulties arise when classifying tumors of the head and neck.

Table V – Definition of the G-category apply to all head and neck sites, except thyroid and mucosal malignant melanoma. Adapted from Sobin, Gospodarowics, & Witteking, 2009.

Histopathological Grading (G)	
GX	Grade of differentiation cannot be assessed.
G1	Well differentiated.
G2	Moderately differentiated.
G3	Poorly differentiated.
G4	Undifferentiated.

HNSCC progression has been presumed to occur in a stepwise fashion, starting from the local primary site to invasion of regional lymph nodes and then to distant sites. Although, accordingly with this assumption metastasis is considered a late event in head and neck tumors progression, it is now recognized that metastasis may occur in early stages. Thus some stage group defining criteria is inconsistent. For instance, a T1 tumor that has spread to regional nodes is classified as T1N2c and is completely different from a tumor classified as T4N0, yet both tumors are stage IV tumors. Another example, that involves nodal status having a greater impact on prognosis than the status of the primary tumor, is that T1N1 and T3N0 tumors are both considered stage III tumors; however the first may have a worse clinical prognosis than the latter. The stages I-IV are considered to group together T, N and M combinations with similar survival, however patients within the same group may have very different tumor biology and treatment needs.

Again, staging might be helpful in defining prognosis but less applicable in decision-making regarding treatment. (Takes et al., 2010)

Regarding TSCC, TNM staging also does not systematically reflects patient prognosis, due to its limited ability to identify high risk disease. Liao et al. (2007) conducted a retrospective review of 513 consecutive patients with stage III-IVA OSCC, which included 170 TSCCs, who underwent radical surgery. The authors did not find significant differences in overall survival between pathologically (p-prefix) staged pT4N0, pT3N0 and pT1-3N1 tumors (p=1.0, p=0.6 and p=0.6, respectively). Furthermore, although pT4N0 oral cavity tumors qualify for stage IVA status that is associated with poor prognosis, in patients with stage IVA OSCC, the survival rates for pT4N0 are better than those for no pT4N0 (pT4N1 and pTanyN2) and similar to those of patients with pathological stage III. These results suggest that patients with pT4N0 tumors could be managed as pathological staged III patients. Another study conducted by Ebrahimi et al. (2011) challenges the current TNM staging system, which classifies all patients with bone invasion throughout the cortex as T4, a single high-risk category that assigns patients to stage IV disease with resultant prognostic and management implications. On a multivariate analysis of T4 OSCCs predominantly defined by the presence of medullary bone invasion, these investigators found that patient survival differed significantly according to tumor size (p=0.03), suggesting that tumor size is a more important predictor of survival than medullary invasion. These two studies indicate that TNM classification system does not effectively differentiate patient prognosis.

The shortcomings of the TNM staging system accentuate the need to identify molecular biomarkers, as well as determine their clinical relevance, in order to improve diagnosis, prognosis and treatment of HNSCC, particularly TSCC. Further research of genetic and epigenetic markers will help establish a more specific stratification of patients, based on molecular assessment of tumor features. Nevertheless, changes and revisions to the current classification system need to be validated and supported by large cohorts. Although different putative molecular markers have been identified in HNSCC, when referring to the tongue subsite this is hampered by common use of heterogeneous cohorts, combining different head and neck subsites.

3 – Molecular changes

Evolution and progression of HNSCC are thought to result from a multistep process involving alterations of cellular and molecular pathways in the squamous epithelium. These pathways include growth factor receptors, signal transducer, and transcription factors, which regulate DNA damage response, cell cycle arrest and programmed cell death. Increasing evidence suggests that molecular progression from potentially malignant lesions to invasive disease comprises a series of genetic and epigenetic alterations that are present at different stages of progression (Figure 4). (Crowe et al., 2002; Haddad & Shin, 2008; Leemans, Braakhuis, & Brakenhoff, 2011).

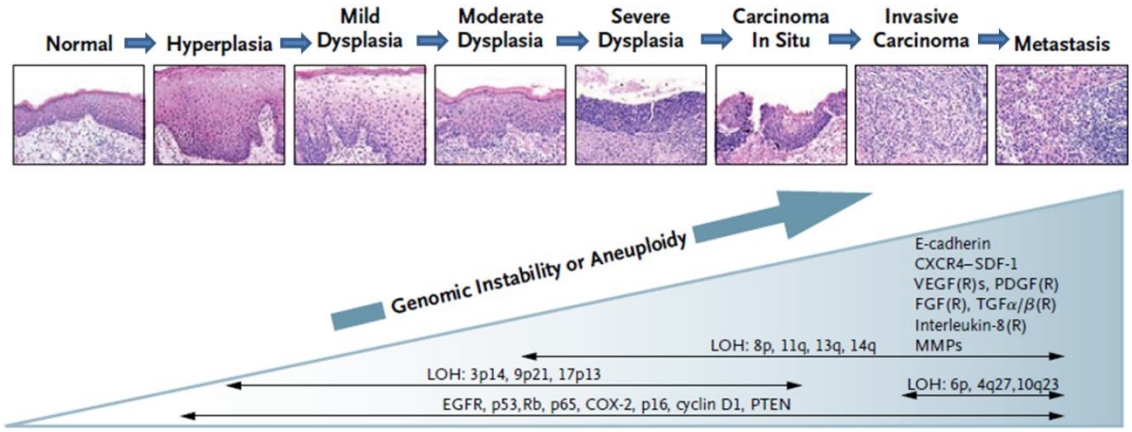

Figure 4 – Models of progression and genetic instability in HNSCC. Adapted from Haddad & Shin, 2008.

Although, a great deal of studies on the identification of candidate genes in HNSCC has been published over the years, only a minority has focused on the oral tongue subsite alone. In order to gain a comprehensive view of the genetic and epigenetic alterations underlying HNSCC, especially the ones associated with development and progression of TSCC, the most relevant and most widely studied alterations will be discussed in this section.

3.1 – Conventional and molecular cytogenetic alterations

HNSCC malignancy hallmarks include chromosomal aberrations such as deletions, amplifications and structural rearrangements. During progression of the disease a pattern of DNA allelic loss and gain is observed, particularly losses at chromosomal arms 3p, 9p and 17p and gains at chromosomal arms 3q, 5p, 7p, 8q and 11q. (Gollin, 2014; Scully, Field, & Tanzawa, 2000a; Silva et al., 2011)

Loss of heterozygosity (LOH) has been identified at different chromosomal loci in association with different stages of HNSCC progression. (Haddad & Shin, 2008) LOH in 9p21 in oral dysplasia and OSCC has been reported at a frequency of 30% and 70-80%, respectively, revealing the potential value in early diagnosis and tumor surveillance of this early and common event in oral malignancy. (Silva et al., 2011)

In OSCC, cell disassociation from the primary tumor usually results in metastasis within regional lymph nodes. The identification of molecular markers associated with regional metastatic behavior that may represent prognostic indicators, as well as increased knowledge of their role in metastatic behavior may be useful in clinical decision regarding treatment modality. The most frequently reported cytogenetic changes correlated with dysplasia, invasion, nodal and distant metastasis in HNSCC include losses at 9p21, 3p14, 3p21 and 17p13 and gains at 11q13. Additionally, LOH due to losses reported in chromosomal arms 7q, 10q, 11p, 15q and 20p and gains at 19q have been associated with metastatic phenotypes. (Scully, Field, & Tanzawa, 2000a; Silva et al., 2011) In order to exclude possible bias from other head and neck sites, Hannen et al. (2004) analyzed a group of tumors uniformly composed of TSCCs, by comparative genomic hybridization, and found that metastatic behavior of metastasize and non-metastasize primary tongue tumors is different, as metastasized tumors had significantly more chromosome copy number variations than non-metastasized. The most frequent aberration in the two groups were gains on chromosomal arms 8q and 3q, in concordance with aberrations already described in HNSCC.

Accumulation of numerous genetic alterations in tumorigenesis and concomitant genomic instability underlying amplification or deletion or up-regulation or down-regulation of different oncogenes and tumor-suppressor genes has been widely studied in these tumors. Thus, several candidate genes and biomarkers involved in tumor

development and progression have been identified over the years (Table VI). (Haddad & Shin, 2008; Scully, Field, & Tanzawa, 2000b; Silva et al., 2011)

Table VI – Candidate biomarkers and common gene alterations in oral carcinoma. Adapted from Scully, Field, & Tanzawa, 2000b; Silva et al., 2011.

Gene / Biomarker	Chromosomal Position	Function	Clinical Significance
NRAS	1p13.2	Signaling, growth	Poor prognosis
RARB	3p24	Cell growth and differentiation	Decreased overall survival
VEGF	6p12	Angiogenesis	Consideration for targeted therapy
CDKN1A	6p21.1	Cell-cycle regulation	Tumorigenesis
EGFR	7p12	Cell proliferation, growth	Nodal metastases; more rapid clinical course, consideration for targeted therapy
MYC	8q24	Cell growth, apoptosis	Tumor progression
CDKN2A	9p21	Senescence, cell-cycle progression	Decreased overall survival
MGMT	10q26	Promoter methylation	Decreased overall survival
CCND1	11q13	Cell-cycle regulation	Nodal metastases; more rapid clinical course
HRAS	11p15.5	Signaling, growth	Poor prognosis
CDKN1B	12p13.1-p12	Cell-cycle progression	Poor prognosis
MDM2	12q13-q14	Cell-cycle regulation	Tumorigenesis
TP53	17p13.1	Cell-cycle regulation	Decreased overall survival
ERBB2	17q11.2-q12	Cell proliferation, growth	More rapid clinical course
STAT3	17q21	Cytokine signaling, cell proliferation	Decreased survival

Cancer cells overcome senescence and obtain limitless replicative potential through changes in cell cycle regulation pathways. In HNSCC altered genes involved in cell cycle regulation are the ones encoding proteins in the p53 and RB pathways. (Hanahan & Weinberg, 2011; Kastan & Bartek, 2004)

The p53 signaling pathway responds to intrinsic and extrinsic stress signals that can disrupt DNA replication and cell division. Stress signals are transmitted to p53 protein by posttranslational modifications, resulting in activation of p53 as a

transcriptional factor that initiates cell cycle arrest, cellular senescence or apoptosis. Another important role of this protein is stimulation of DNA repair after DNA damage. (Harris & Levine, 2005) Alterations in tumor suppressor gene *TP53*, mapped at 17p13, through LOH, point mutations, deletions and insertions are early events in HNSCC. Mutations in this gene are the most common reported mutations in these tumors, being somatic mutations found in 60-80% of the cases. (Agrawal et al., 2011; Leemans, Braakhuis, & Brakenhoff, 2011) Early *TP53* mutations commonly occur in guanine nucleotide, probably due to exposure to carcinogens in tobacco smoke, and are maintained through metastasis. (Agrawal et al., 2011; Denaro et al., 2011; Govindaraja, Chandramouli, & Chandramouli, 2010; Haddad & Shin, 2008) In OSCC, mutations in *TP53* exons 5-9 were found in 60% of the cases in association with overexpression of p53. The prevalence of positive immunohistochemical detection of p53 has been reported between 30-50%, occurring frequently in adjacent non-tumoral mucosa, which indicates that these alterations are early events in squamous cell carcinoma development. (Bettendorf, Piffkò, & Bànkfalvi, 2004) Furthermore, 17p13 LOH in poorly differentiated tumors suggests that loss of p53 function may be associated with the transition from preinvasive to invasive head and neck tumors. (Denaro et al., 2011)

The role of p53 as a prognostic marker of head and neck tumors is controversial. However, a large study of 420 heterogeneous HNSCC, including 180 tumors of the oral cavity, found association of TP53 mutations with significantly worse patient survival, particularly disruptive mutations ($p=0.003$). (Poeta et al., 2007)

The aberrant p53 protein activity may also be caused by aberrant production of other proteins that regulate its activity, such as mouse double minute 2 (MDM2) and viral proteins. (Leemans, Braakhuis, & Brakenhoff, 2011) MDM2, a negative regulator of *TP53*, is a cellular proto-oncogene amplified in 25-40% of all human cancers. In HNSCC, MDM2 expression or upregulation varies from 40-80%. This protein is a p53 target and in turns it limits the amount of p53, by targeting the latter and promoting its degradation. The relationship between these two molecules is vital for regulation of proliferation and apoptosis. In this type of cancer, inactivation of p53 is frequently due to MDM2 binding and at the same time MDM2 low expression is associated with mutations in p53 that prevents upreguation of MDM2. (Denaro et al., 2011) Additionally, interactions between p53 and viral protein E6 encoded by oncogenic HPV types, mainly HPV16 and -18, results in increased ubiquitin-dependent proteolysis of p53. A high frequency of *TP53* mutations in HPV-negative cases contrasts with

expression of wild-type p53 in HPV-positive tumors and this seems to be related with better prognosis in patients with HPV-positive OSCC. (Gillison et al., 2000; Massano et al., 2006)

Mapped at 13q14, retinoblastoma 1 (*RB1*) tumor suppressor gene is involved in cell cycle control. Mutations of *RB1* and loss of Rb protein activity result in uncontrolled cell proliferation. In HNSCC, decreased Rb expression has been observed in 6-74% of the tumors and LOH in 14-37%. (Bettendorf, Piffko, & Bankfalvi, 2004)

CCND1, located on chromosome 11q13, is a proto-oncogene that encodes cyclin D1 protein. Cyclins function as regulators of cyclin-dependent kinases (CDKs) and exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. Cyclin-D1-CDK4/6 complex formation and subsequent Rb phosphorylation result in cell cycle G1/S transition. This cyclin activity is inhibited by several tumor suppressor genes, including *CDKN2A*, *CDKN1A* and *CDKN1B*. Amplification and overexpression of *CCND1* are independent prognostic factors in HNSCC. Amplification of this gene has been reported in over 80% of these tumors (Smeets et al., 2006) and increased cyclin D1 expression has been associated with the presence of regional nodal metastases and advanced tumor stage. (Bettendorf, Piffko, & Bankfalvi, 2004; Massano et al., 2006; Scully et al., 2000a, 2000b) In oral cancer, cyclin D1 expression and amplification was observed in 20-68% cases and, even though, association with recurrence, nodal metastasis and survival has been verified in HNSCC, further investigation is needed to find specific clinical associations with cyclin D1 alterations in OSCC alone. (Bettendorf, Piffko, & Bankfalvi, 2004; Massano et al., 2006; Scully et al., 2000a, 2000b)

CDKN2A, located on chromosome 9p21, encodes $p16^{INK4A}$ that inhibits cell cycle progression via inhibiting phosphorylation of Rb. $p16^{INK4A}$ inhibition of cyclin D1 interaction with CDK4/6 maintains the hypophosphorilated form of Rb, sustaining the Rb-mediated sequestration of E2F transcriptional factor, thereby preventing cell cycling progression from G1 to S phase. *CDKN2A* is frequently inactivated in HNSCC by mutation or methylation in combination with chromosome loss or, more commonly, by homozygous deletion. Decreased $p16^{INK4A}$ expression is associated with reduced survival, increased recurrence rates and nodal metastasis. (Bettendorf, Piffko, & Bankfalvi, 2004; Leemans, Braakhuis, & Brakenhoff, 2011) Negative or low $p16^{INK4A}$ expression has been reported in up to 83% of OSCCs and up to 60% of potentially malignant lesions. (Silva et al., 2011) Despite the large number of studies investigating

the relevance of CDKN2A alteration in HNSCC, its role in TSCC and impact on patient prognosis is still unclear.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase and a member of the ErbB family of cell-surface receptors. When phosphorylated, EGFR can signal through mitogen-activated protein kinase (MAPK), protein kinase β (Akt/PKB), extracellular-signal-regulated kinase (ERK) and Janus kinase – signal transducer and activator of transcription (Jack/STAT) pathways. (Hynes & Lane, 2005) EGFR and its ligands are essential for cellular proliferation, migration, adhesion, invasion, metastasis and angiogenesis. Activation of *EGFR* oncogene is either by mutation or amplification. In HNSCC, mutations are rarely found and amplifications are documented in 30% of these tumors. Although overexpression data alone is unreliable, EGFR is overexpressed in over 80% of HNSCC and is associated with poor prognosis, more aggressive phenotypes and resistance to chemotherapeutic agents. For this reason the efficacy of agents that target EGFR has attracted interest in anticancer drug development. (Hama et al., 2009; Leemans, Braakhuis, & Brakenhoff, 2011; Temam et al., 2007) For instance, development of anti-EGFR monoclonal antibodies, such as cetuximab, in combination with high-dose radiation has shown to lead to a better patient prognosis. (Hama et al., 2009; Silva et al., 2011) In OSCC, *EGFR* gene amplification was found in 30% of tumors and has also been observed in potentially malignant lesions. Overexpression of EGFR has been frequently associated with advanced T stage of the primary tumor, diffuse tumor invasiveness and high incidence of cervical node metastases. Expression of EGFR has also been correlated with lower histologic tumor differentiation and shorter patient survival. (Bettendorf, Piffko, & Bankfalvi, 2004; Massano et al., 2006; Ulanovski et al., 2004)

The *vascular endothelial growth factor* (*VEGF*) plays a decisive role in the development of blood vessels by stimulation of vasculogenesis and angiogenesis. Four subtypes have been described – A, B, C, and D. Expression of this growth factor has been described in OSCC, correlating VEGF-A and VEGF-B with angiogenesis and VEGF-C and VEGF-D with the risk of nodal metastases. Up-regulation of the latter two in the invasive front of the tumor revealed their possible role in tumor invasion and metastases development. (Shintani et al., 2004) High expression of VEGF has also been correlated with worse prognosis in OSCC. (Massano et al., 2006; Uehara et al., 2004)

The *von-Hippel Lindau* (*VHL*) gene is a tumor suppressor gene, mapped at 3p26-p25, that codes VHL protein, contributing to the ubiquitination and proteasomal

degradation of hypoxia-inducible factor 1α (HIF1α), a transcriptional factor for the *VEGF*, underlying tumor growth and angiogenesis. In TSCC, LOH of *VHL* gene was found at high frequency in 45.5% of tumors and has been found less likely to occur in younger patients, which indicates that development and progression of these tumors may differ between patients within different age groups. (Asakawa et al., 2008)

Over expression of *MYC* and *RAS* gene families is an important event in head and neck tumor progression and has been correlated with poor prognosis. Member of the *RAS* gene family, *NRAS* oncogene encodes N-ras protein, which increased expression in dysplasia has been associated with early steps of oral carcinoma development. Another member of the Ras family is *HRAS;* mutations in this gene have been reported in 35% of oral tumors in Asian populations, particularly linked with betel nut chewing. *c-Myc* oncogene encodes the transcriptional factor c-Myc involved in gene expression regulation. Over expression of this transcriptional factor results of gene amplification and is a common event in OSCC, associated with loss of differentiation. (Bettendorf, Piffko, & Bankfalvi, 2004; Silva et al., 2011)

Besides, few studies have been focusing specifically on the tongue subsite, this number has been increasing, with evidence that tumors of different subsite of the head and neck behave differently and have their own identity. Previous reports on candidate biomarkers involved in oral cavity tumorigenesis (Table VI) represent a great starting point for identification of putative TSCC markers. However, special attention must be pay to the relative frequency of the oral cavity subsites investigated in different cohorts, as anatomical proximity does not necessarily means identical tumor features.

3.2 – Hypermethylated loci

Promoter methylation profiling of primary head and neck tumors found methylation of tumor suppressor genes *CHFR, RARB, DAPK1, RASSF1A* and *APC* to be the most frequently reported epigenetic event in HNSCC. (Chen et al., 2007; Demokan & Dalay, 2011; Yalniz et al., 2011) Although a vast number of hypermethylated loci have been reported in HNSCC (Table VII), few were the studies focusing specifically on the tongue subsite. Aberrant epigenetic changes have been described during the development and progression of TSCC in genes responsible for cell signaling, growth, motility, angiogenesis and cell cycle control.

Phosphatase and tensin homolog (*PTEN*) is a tumor suppressor gene is located at 10q23.3 and plays an important role in tumorigenesis, thus somatic mutation or deletion of this gene has been described in a variety of tumor types. Loss of PTEN expression was demonstrated in 29% of TSCCs, associated poor patient outcome with reduced overall survival and event-free survival. Also, when compared with tumor grade and nodal status, PTEN expression is an independent predictor of poor outcome. (Lee et al., 2001) Although genetic alterations of *PTEN* are rare in HNSC (5-10%), in TSCC these alterations are not uncommon and play an important role in tumorigenesis and progression. Since other mechanism may be responsible for *PTEN* gene loss of function, such as promoter hypermethylation, the frequency of *PTEN* alterations may be underestimated in HNSCC. Further research is needed in order to establish the possible epigenetic role in *PTEN* inactivation in these tumors. (Lee et al., 2001; Molinolo et al., 2009)

Loss of *CDKN2A* expression by promoter hypermethylation has been described as an early event in oral cancer. In a study conducted by Cao et al. (2009), promoter methylation of this gene occurred in 41% of the 78 patients analyzed with histologically confirmed mild or moderate oral epithelial dysplasia and was significantly associated with a higher rate of progression to oral cancer (P=0.013). Particularly, in TSCC, this epigenetic event may serve as a useful molecular marker for local recurrence prediction. A prospective study performed by Sinha et al. (2009) identified *CDKN2A* promoter hypermethylation in 86.8% of the 38 tongue carcinomas evaluated. Promoter methylation was present in 43,3% of the 30 patients with histologically free margins, which predicted a 6.3-fold increased risk of having local recurrence as compared to patients whose margins were negative for *CDKN2A* promoter methylation.

The *O^6-methylguanine-DNA methyltransferase* (*MGMT*) gene codes the MGMT protein essential for genome stability. This detoxifying agent of DNA adducts prevents mismatch and errors during DNA replication and transcription and is also involved in the repair of naturally occurring mutagenic DNA lesion O^6-methylguanine back to guanine. Promoter hypermethylation of *MGMT* has been reported as an early event in HNSCC, revealing its potential role as a molecular marker for early detection in this type of cancer. (Chen et al., 2007; Demokan & Dalay, 2011) In OSCC, *MGMT* methylation is a common event and is present in 25-52% of primary tumors. (Ha & Califano, 2006)

Wingless type (*Wnt*) signaling has been increasingly implicated in cancer initiation and malignant transformation, particularly in OSCC. Wnt proteins bind to specific transmenbrane receptors and activate β-catenin that accumulates in the cytoplasm, being then translocated to the nucleus, which results in activation of growth promoting oncogenes and regulation of cell polarity, invasion, metastasis and angiogenesis. (Lo Muzio, 2001) Wnt antagonists, *Runt-related transcription factor 3* (*RUNX3*) and *WNT inhibitory factor 1* (*WIF1*), are negative modulators of Wnt, preventing β-catenin function as a transcriptional factor. Recent studies have revealed expression of RUNX3 downregulation by hypermethylation and methylation silencing of *WIF1* in several types of cancer including OCC. In TSCC, a study conducted by Supic *et al.* revealed promoter hypermethylation of *WIF1* and *RUNX3* in 35% and 25% of the 76 tongue carcinomas analyzed, respectively. *RUNX3* promoter hypermethylation was significantly associated with lymph node involvement (P=0.013) and tumor stage (P=0.006). Additionally, comethylation of both genes was associated with tumor stage (P=0.055) and nodal status (P=0.058). (Supic et al., 2011)

Table VII – Candidate genes frequently methylated in HNSCC. Adapted from Ha & Califano, 2006; Yalniz et al., 2011.

Gene	Chromosomal Position	Function
RASSF1	3p21.3	Tumor suppression
MLH1	3p22.3	DNA repair
RARB	3p24	Tumor suppression
APC	5q21-q22	Tumor suppression
CDKN2A	9p21	Tumor suppression
DAPK1	9q34.1	Apoptosis
MGMT	10q26	DNA repair
WIF1	12q14.2	Inhibition of Wnt signaling
CHFR	12q24.33	Early G2/M checkpoint
CDH1	16q22.1	Cell adhesion

DNA methylation assessment is an appealing and developing area in cancer research. Aberrant methylation of cytosine occurs at CpG dinucleotide (CpG islands) rich promoter regions of tumor suppressor genes and is catalyzed by DNA methyltransferases (DNMTs). Promoter hypermethylation of these genes is the best characterized epigenetic event in carcinogenesis. Tumor suppressor genes transcriptional silencing is achieved by this process, likely due to the inhibition of

transcriptional factor binding, as closed chromatin configuration leads to blockage of the assess of transcription factors to the promoter region. (Baylin & Jones, 2011)

Epigenetic alterations are susceptible to change and represent excellent candidates to disclose how environmental factors are implicated in increased risk of cancer. Normal cells gene expression patterns are maintained through organization of methylation and chromatin status, however in cancer cells expression patterns homeostasis is lost and becomes unrecognizable, as transformed cells undergo concomitant genomic hypomethylation and dense hypermethylation of CpG islands within gene regulatory regions. This results in genomic instability and transcriptional silencing of associated genes. (Esteller, 2002; Feinberg & Tycko, 2004)

Aberrant promoter hypermehylation of CpG islands is an early event in tumorigenesis, representing a potencial target for early tumor detection, as well as a biomarker of malignant transformation and predictor of tumor behavior. Also, methylation patterns are tissue-specific and have tumor-type specificity, being possibly useful in subclassifying specific tumor types and determining tissue origin in metastasis. (Baylin, 2005; Costello et al., 2000; Esteller, 2002; Feinberg & Tycko, 2004) Furthermore, as opposed to genetic events, DNA methylation is reversible, representing an attractive target for new therapeutic strategies involving inhibitors of the DNMT for reactivation of methylation-silenced genes. (Tsai & Baylin, 2011)

Promoter hypermethylation profiles of HNSCC have been widely explored. However, most promoter hypermethylation studies only evaluate a limited number of genes and usually combine different HNSCC subsites, with different tumor stages. Also, HPV-status of these tumors is not always defined, which as mentioned before is an important prognostic factor especially in oral cavity and oropharingeal cancers. Differences in composition of patient cohorts as well as methylation assessment methodologies and sample procession result in a wide range of reported hypermethylation frequencies. Thus, assessment of methylation profile of each head and neck subsites individually, particularly TSCC, as means to classify molecularly distinct groups is highly important for risk cancer evaluation, early detection, prognosis stratification, treatment modality selection and treatment response prediction.

3.3 – The relevance of studying genetic and epigenetic changes

The prognostication, management and treatment of HNSCC have been traditionally established by grouping all the different head and neck subsites together, assuming homogeneity of the disease; however, this is not supported by the distinct tumor behavior and patient outcome observed according to the different subsites from which the tumors arise. (Haddad & Shin, 2008; Rusthoven et al., 2008) In the numerous studies and cohorts published over the years, attempting to understand the pathogenesis of HNSCC, combination of different anatomical subsites has resulted in a wide range of information on genetic and epigenetic events involved in tumourgenesis, however with little regard for the individual identity of these tumors. (Bernier & Cooper, 2005; Gillison et al., 2000; Leemans, Braakhuis, & Brakenhoff, 2011; Stransky et al., 2011) Beyond any doubt, this has resulted in inadequacy to stratify patients according to risk of disease, as well as choosing the most appropriate treatment, particularly in the tongue subsite. For this reason the primary ambition of molecular studies is the identification and characterization of biomarkers in order to improve prognostic prediction and risk-stratification of patients, as well as be able to predict treatment response.

4 – Formalin-fixed paraffin embedded specimens

Formalin-fixed paraffin embedded (FFPE) tissues have been used in diagnostic pathology for decades, resulting in a vast amount of samples readily accessible for molecular research. Most importantly, almost all FFPE samples have associated pathological and clinical information, allowing for its application on association and classification studies.

Identifying molecular targets as diagnostic and prognostic markers requires optimal preservation of proteins, RNA and DNA, in human specimens. However, routine fixation methods fail to conserve the structure of these molecules, thus ability to extract adequate material from fixed tissues is limited. Several factors involved in the fixation process are listed in Table VIII. (Srinivasan, Sedmak, & Jewell, 2002)

Table VIII – Prefixation, intrafixation and postfixation parameters involved in the maintenance of the *in vivo* status of the human tissue *ex vivo*. Adapted from Srinivasan, Sedmak, & Jewell, 2002.

Prefixation	Intrafixation	Postfixation
Constant factors	**Properties of the fixatives**	**Storage parameters**
Nature of the anesthetic Duration of anesthesia Anoxic injury *in situ*	Chemistry and mechanism of action Tissue penetration	Duration Temperature Condition (vacuum/nonvacuum packed)
Variable factors	**Condition of fixation**	**Nature of the biological factor to be analyzed**
Prefixation time	Temperature Duration pH Osmolarity Concentration Size of the specimen Volume of the fixative	Proteins Enzymes Lipids Nucleic acids Mucopolysacharides Biogenic amines Glycogen

Numerous chemical fixatives have been used in the last few decades, with formaldehyde and glutaraldehyde being the most popular. (Magdeldin & Yamamoto, 2012) Since glutaraldehyde is a larger molecule, its rate of diffusion through membranes is slower, for this reason formaldehyde as a 10% neutral buffered solution, formalin, is the most widely used fixative.

Formalin fixation and paraffin wax embedding process preserves cytoskeletal structure and proteins, essential for pathological purposes, due to irreversible formalin-protein cross-links. However, formalin and other cross-linking fixatives degrade nucleic acids, being up to 30% of nucleic acids lost during fixation. (Srinivasan, Sedmak, & Jewell, 2002) DNA isolation from FFPE tissues is impaired by formalin cross-links formation and paraffin wax, resulting in low concentration yields and fragmented DNA, generally 500 bp or less.

DNA fragment size is mostly influenced by fixation process and how long it is fixed for. It can also be affected by the time since surgical resection until fixation, as tissues experience anoxia and environmental changes. The time the tissue has been stored for has also been suggested but it is found to have minor effect.

During the fixation process, DNA formalin-induced modifications depend on concentration, temperature and pH of the fixative. Temperature is directly related to extracted DNA size, lower temperatures upon fixation, particularly at 4°C, yields less fragmented DNA. Low pH environment should be avoided since acidic pH results in nucleic acid degradation. (Srinivasan, Sedmak, & Jewell, 2002)

Regarding fixation time, at least one hour is required per mm of tissue thickness, but routinely the tissues are fixed for 24 to 48 hours. This represents a major problem since size of DNA extracted from tissues fixed in formalin decreases with increasing fixation time. In order to obtain greater amounts of high-molecular DNA, tissues should be fixed for 3 to 6 hours in buffered formalin. (Srinivasan, Sedmak, & Jewell, 2002)

Ultimately, using formaldehyde as a fixative in order to preserve tissue nucleic acids should be performed under specific criteria: minimal prefixation time, preferable less than 2 hours; use of cold (4°C) 10% neutral formalin; duration of fixation from 3 to 6 hours. (Srinivasan, Sedmak, & Jewell, 2002) However, FFPE tissues samples are not obtained for molecular research purposes. Routine sample preparation consists of previous established methods for routine pathological diagnosis, with little regard for the nucleic acid degradation, thus knowledge about the effects of fixatives on the integrity and utility of the preserved DNA and RNA is increasingly important.

Overcome these major obstacles and isolate great high quality DNA from FFPE tissue from tumor samples is crucial, since these large archives frequently have historical records of patient progression and outcome, allowing retrospective studies to be performed exploring DNA changes that influence cancer development and progression.

Still caution must be taken in adapting this information because it is possible that alterations in gene expression profiles can occur either during and or after the resection of the tissue. Thus comparing the genetic profile of fresh frozen tissues and FFPE tissues is imperative to validate the latter application in future studies.

5 – Methylation-specific Multiplex Ligation-dependent Probe Amplification

The number of genes known to be hypermethylated in cancer has been largely increasing, revealing the necessity of sensitive and robust multiplex methods able to detect aberrant methylation patterns of promoter regions. This is particularly important when analyzing DNA extracted from FFPE tissue specimens that have been stored over the years, resulting in poor quality DNA. (Esteller, 2002; Nygren et al., 2005)

Over the years innumerous approaches have been used for methylation detection based on conversion of cytosine residues into uracil after bisulphite treatment. However, during subsequent PCR, these residues are converted to thymidine, resulting in different DNA sequence of the alleles that were originally methylated and the corresponding unmethylated alleles. Although techniques exist that are able to exploit this aspect, such as Methylation-specific PCR (MSP) and Combined Bissulfite Restriction Analysis (COBRA), the majority of them are laborious and/or only allow for the analysis of one gene at a time, thus not being suitable to study large numbers of FFPE tissue samples. The relatively recent developed Methylation-specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) technique represents a desirable solution, as it is able to detect changes in methylation status as well as copy number changes of up to 40 selected sequences in a reaction using only 20ng of DNA. (Nygren et al., 2005)

MS-MLPA consists of a semi-quantitive method for methylation status assessment. This variant of the MLPA technique allows for further analysis of methylation profile in addition to the copy number detection already inherent to the MLPA method, by making use of methylation-sensitive restriction enzyme. The usability of MS-MLPA for detection of epigenetic alterations has been widely recognized for tumor analysis, particularly for identification of tumor suppressor genes methylation patterns involved in tumorigenesis. (Nygren et al., 2005)

The MS-MLPA assay protocol consist of different steps: DNA denaturation and hybridization of MLPA probes, ligation and ligation plus digestion reactions, PCR amplification and subsequent capillary electrophoresis separation of amplification products, as demonstrated in Figure 5. This procedure is very similar to the conventional MLPA protocol, except that MS-MLPA assay generates two types of samples, one undigested sample for copy number detection that is processed as a standard MLPA reaction and a digested one that was additionally incubated with HhaI

endonuclease. Probes for methylation detection resemble the MLPA probes, but their target sequence contains the restriction site for methylation-sensitive enzyme. After ligation and ligation plus digestion reactions, digested probes cannot be amplified exponentially during PCR and do not produce a signal during capillary electrophoresis. The opposite occur when sample DNA is methylated, as DNA-probe hybrids are protected against HhaI digestion and ligated probes will generate a peak. (MCR-Holland, 2015a)

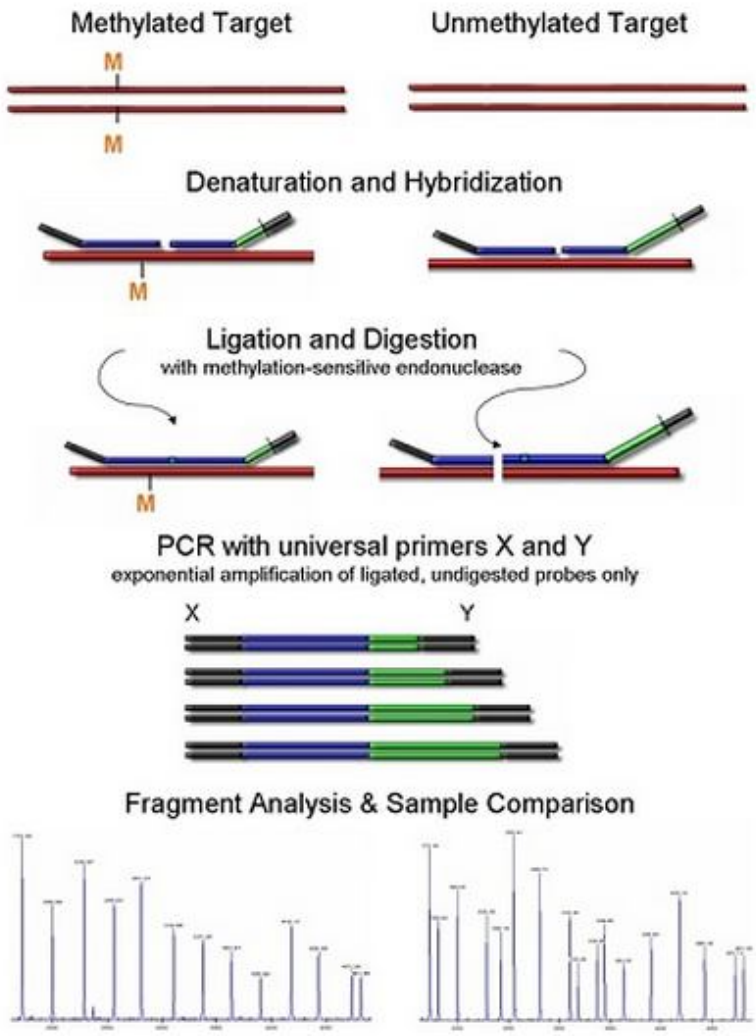

Figure 5 - Outline of the MS-MLPA procedure. Adapted from MRC-Holland, 2015a.

6 - Objectives

The main objective of the present thesis is the characterization of the genetic and epigenetic profile of 31 primary tongue tumors, collected from patients with TSCC, upon resection surgery, by MS-MLPA technique. Unraveling the genetic and epigenetic alteration patterns present in TSCC will allow for the establishment of candidate genes, as well as determine their clinical significance, providing a great opportunity to identify the molecular pathways underlying the development and progression of the disease. The association between the tongue-specific genotype with patients' clinicopathological features will contribute for the improvement of diagnosis, prognosis and accurate risk-stratification of patients, as well as choose the most adequate treatment and predict treatment response in TSCC.

Clinical assessment and histopathological evaluation of tumors often show limitations regarding prognosis and clinical decision-making due to significant diversity in patients' clinical course and treatment response. This diversity can be explained by alterations at the genetic and epigenetic level that result in unique/individual phenotypes. Identification of the genetic and epigenetic profile of TSCC will contribute to the establishment of specific molecular diagnosis for individual patients, improving prognostic prediction, treatment decision-making and ultimately to further improve the survival rate of these patients.

Limitations of the current clinicopathological staging system capability of identifying patients with high risk disease, highlights the need for prognosis biomarkers to be identified. Furthermore, aberrant DNA methylation patterns have been reported as early events in the carcinogenic process, revealing their potential role as diagnostic biomarkers. Establishing correlations between molecular alterations and clinicopathological features will warrant the future application of the MS-MLPA technique in clinical practice for early detection and patient's follow-up.

Towards the need of analyzing a great amount of tumor samples for the establishment of molecular biomarkers involved in malignant transformation and progression of TSCC, FFPE tissue samples represent a valuable resource. FFPE tissues have been used in diagnostic pathology for decades, resulting in a vast amount of samples readily accessible for molecular research. These large archives of FFPE specimens frequently have associated pathological and clinical information, allowing for its application on retrospective studies. However, DNA isolation from FFPE tissues

is impaired by formalin cross-links formation and paraffin wax, resulting in low concentration yields and fragmented DNA. Thus the present thesis also aimed the development of an optimized protocol for DNA extraction from FFPE specimens of TSCC.

CHAPTER 2

MATERIALS and METHODS

Chapter 2 – Materials and Methods

1 – Patients, tumors and control samples

The present study was approved by Medicine Faculty of University of Coimbra Ethics Committee. Thirty one fresh-frozen tumor samples and 16 matched-paired FFPE specimens were obtained from the Anatomical Pathology Department of the Coimbra Hospital and University Center, CHUC, EPE, between 2010 and 2014. Informed consent was obtained from all patients. Pathological features and clinical annotations of each patient were obtained and these included patient's sex, age at the diagnosis, tumor staging, histological differentiation, presence or absence of metastasis, treatment modality and tobacco and alcohol history. Clinicopathological features of all cases are summarized in Table IX.

All tumor tissue samples were collected upon surgical resection, followed by histopathological evaluation of the mirror sections performed by an experienced pathologist. Hematoxylin and eosin (H&E) staining was used to confirm diagnosis and select tumoral representative areas. Fresh tumor specimens were snap frozen by immersion in liquid nitrogen and stored at -80ºC until analysis. FFPE blocks preparation consisted of tissue fixation in neutral buffered formalin, followed by paraffin embedding, according to routine pathologic laboratory protocols. Five histological cuts of 20 µm thick paraffin sections were obtained from each FFPE resection block for analysis. The first and last FFPE tissue cut slides were stained with H&E and evaluation of tumor content was performed by an experienced pathologist. Areas of interest were circled on the H&E slides and manually microdissected. When only small tumoral tissue areas were identified, more cuts were performed to obtain optimal tissue quantity. Gingival samples from 16 healthy donors subjected to third molar removal were used as fresh-frozen controls and obtained from Maxillofacial Surgery and Stomatology Unit of the Coimbra Hospital and University Centre, CHUC, EPE. One non-tumoral FFPE sample was also included that consisted of 5 histological cuts of 20 µm thick paraffin section of a surgically removed prosthesis-fibroma from a healthy donor and was obtained from the Anatomical Pathology Department of the Coimbra Hospital and University Center, CHUC, EPE.

Table IX – Patient and tumor clinicopathological features.

Characteristic	No. of Patients (n=31)
Mean age, yrs (range)	63.87 (44-94)
Gender	
Male	25
Female	6
Stage	
I and II	12
III and IV	17
Not recorded	2
Treatment	
Surgery only	12
Surgery+RT	12
CT+Surgery	1
Surgery+RT+CT	5
Not recorded	1
Clinical outcome	
Death from the disease	9
Alive	22
Smoking (cigarettes per day)	
≥20	13
<20	3
None	8
Not recorded	7

RT radiation therapy, CT chemotherapy

2 – DNA extraction

2.1 – Fresh-frozen tissue

DNA was extracted from fresh-frozen tissue samples using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. (Roche Diagnostics GmbH, 2008)

2.2 – FFPE tissue

DNA was extracted from FFPE tissue samples using three different methods (A, B and C). Method A consisted of simultaneous paraffin removal and proteinase K digestion, whereas method B and C consisted of heat paraffin removal and xylene deparaffinization, respectively, followed by proteinase K digestion. For all three

methods, genomic DNA (gDNA) extraction was performed using the QIAmp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), with protocol variations.

2.2.1 – Method A

Day 1 (1st tissue digestion)

Paraffin flakes were placed in 1.5 ml microcentrifuge tubes, 400µl of *Buffer ATL* was added, vortexed and incubated at 70ºC for 1 hour at 20 rpm in a hybridization oven (Shellab, Illinois, USA), until all paraffin was liquefied. For tissue digestion 20µl of Proteinase K (20 mg/ml) was added and samples were incubated in a water bath at 54ºC overnight.

Day 2 (Paraffin removal, 2nd tissue digestion and gDNA extraction)

Paraffin was removed by immediate centrifuge 10,000 g x 5 min., as paraffin solidifies at the solution top, forming a wax disk. After paraffin removal, 20µl of Proteinase K (20mg/ml) was added and samples were incubated in a water bath at 54ºC until full tissue digestion. Genomic DNA was isolated using the QIAmp DNA FFPE Tissue kit, according to Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) (Agilent Technologies, California, USA) protocol for gDNA extraction from FFPE samples. (Agilent Technologies, 2008)

2.2.2 – Method B

Day 1 (Paraffin removal)

Paraffin flakes were placed in 1.5 ml microcentrifuge tubes, 480 µl PBS and 20 µl of 20% Tween were added, vortexed and incubated at 90ºC for 3 hours in a water bath to melt paraffin wax. Samples were immediately centrifuged at 10,000 g x 15 min. and placed on ice for 2 min. to remove solid paraffin disk. Pellet was ressuspended with 1,000 µl of 100% ethanol, vortexed and centrifuged at 10,000 g x 5 min. Ethanol was removed after wash, 400 µl of 1M NaSCN (Sigma-Aldrich, Missouri, USA) was added to the dried pellet, vortexed and incubated at 37ºC overnight at 20 rpm.

Day 2 (1st tissue digestion)

Samples were removed from hybridization oven and centrifuged at 10,000 g x 20 min. The tissue pellet was washed with 400 µl PBS, vortexed and centrifuged at

10,000 g x 20 min. Pellet was ressuspended with 360 µl of *Buffer ATL*, 40 µl of proteinase K was added, vortexed and incubated at 55ºC overnight at 20 rpm.

Day 3 (2nd tissue digestion)

Samples were centrifuged at 6,000 g x 30 sec. to drive the contents of the wall and lid, 40 µl of proteinase K was added, vortexed and incubated at 55ºC for 6 hours at 20 rpm. Samples were then centrifuged at 6,000 g x 30 sec., 40 µl of proteinase K was added, vortexed and incubated at 55ºC overnight at 20 rpm.

Day 4 (gDNA extraction)

Genomic DNA was isolated using the QIAmp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), according to Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE) (Agilent Technologies, California, USA) protocol for gDNA extraction from FFPE samples, with minor changes. (Agilent Technologies, 2008) Samples were cooled down to room temperature, centrifuged at 6,000 g x 30 sec. to drive contents off the walls and lid, 8 µl of RNase A (100 mg/mL) was added, vortexed and incubated at room temperature for 2 min. The mixture was centrifuged at 6,000 g x 30 sec., 400 µl of *Buffer AL* was added, vortexed and incubated at 70ºC for 10 min. in a water bath. Samples were centrifuged at 6,000 g x 30 sec., 440 µl of 100% ethanol was added, vortexed and centrifuged at 6,000 g x 30 sec. Each sample was transferred to two QIAamp MinElute columns, centrifuged at 6,000 g x 1 min. and flow-through was discarded. 500 µl of *Buffer AW1* was added, centrifuged at 6,000g x 1 min. and flow-through was discarded. 500 µl of 80% ethanol was added, centrifuged at 13,000 g x 3 min. and flow-through was discarded. DNA was eluted from each column with 50 µl nuclease free water (Promega, Wisconsin, USA) at 70ºC, for a total elution volume of 100 µl of each sample, incubated at room temperature for 5 min. and centrifuged at 6,000 g x 1 min. Eluted DNA solution was added again to the column, incubated at room temperature for 5 min. and centrifuged at 13,000 g x 1 min.

2.2.3 – Method C

Day 1 (Deparaffinization)

Paraffin flakes were placed in 1.5 ml microcentrifuge tubes, deparaffinized 3 times with 1000 µl of xylene, vortexed, incubated at 45ºC for 10 min. at 20 rpm and

centrifuged at 13,000 g x 5 min. The tissue pellet was washed with 1,000 µl methanol (Merck KGaA, Darmstadt, Germany), vortexed and centrifuged at 13,000 g x 5 min., washed with 1,000 µl phosphate-buffered saline (PBS), vortexed and centrifuged at 13,000 g x 5 min. Pellet was ressuspended with 1,000 µl 1M NaSCN (Sigma-Aldrich, Missouri, USA) vortexed and incubated at 37°C overnight at 20 rpm.

Day 2 (1st tissue digestion)

Samples were removed from hybridization oven, centrifuged at 13,000g x 5 min. and washed for 2 times with 1,000 µl PBS, vortexed and centrifuged at 13,000 g x 10 min. The deparaffinized tissue was ressuspended in 200 µl of *Buffer ATL* (Qiagen, Hilden, Germany), followed by 20µl of Proteinase K (20 mg/ml) digestion at 55°C for 4 hours at 20 rpm. Another 20µl of Proteinase K (20 mg/ml) were added and lysate was incubated at 55°C overnight at 20 rpm.

Day 3 and 4 (2nd tissue digestion)

Samples were removed from hybridization oven, 20 µl of Proteinase K (20 mg/ml) was added, followed by digestion at 55°C for 4 hours at 20 rpm. Another 20 µl of Proteinase K (20 mg/ml) was added and lysate was incubated at 55°C overnight at 20 rpm.

Day 5 (DNA isolation)

Genomic DNA was isolated using the QIAmp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions with some modifications. Briefly, 200 µl of *Buffer AL* was added, vortexed and incubated for 70°C for 10 min. at 20 rpm. 200 µl of 100% ethanol was added and immediately vortexed for 15 sec. The mixture was briefly centrifuged, the entire lysate was transferred to two QIAamp MinElute columns, centrifuged at 6,000g x 1 min. and flow-through was discarded. 500 µl of *Buffer AW1* was added, centrifuged at 6,000g x 1 min. and flow-through was discarded. 500 µl of *Buffer AW2* was added, centrifuged at 13,000 g x 1 min. and flow-through was discarded. In order to completely dry the membrane, the column was centrifuged at 13,000 g x 2 min. and flow-through discarded. DNA was eluted from each column with 50 µl nuclease free water (Promega, Wisconsin, USA) (100 µl total elution volume for each sample), incubated at room temperature for 5 min.

and centrifuged at 6,000 g x 1 min. Eluted DNA solution was added again to the column, incubated at room temperature for 5 min. and centrifuged at 13,000 g x 1 min.

3 – Sample analysis – concentration, purity and integrity assessment

Accurate assessment of extracted DNA quantity and quality is essential for successful molecular downstream applications. High quality DNA is defined by being free of contaminants and with minimal degradation. Measure of these parameters when extracting DNA from FFPE tissues is particularly important, as the extraction process results in various degrees of DNA degradation and contamination.

DNA purity and concentration of all samples were assessed using NanoDrop 1000 Spetrophotometer (Thermo Fisher Scientific, Wilmington, USA), according to manufacturer's instructions. A260/A280 and A260/A230 ratios were recorded, allowing for sample purity determination, as the first indicates the presence or absence of contaminating proteins and the second of organic compounds such as guanidinum isothiocyanate, alchohol and phenol, as well as cellular carbohydrates. DNA samples with A260/A280 ratio values of 1.8 to 2.0 and A260/A230 ratio values >2 were considered high-quality/pure. (Thermo Fisher Scientific, 2008) Re-quantification of DNA concentration was performed in all FFPE samples using Qubit Fluorometer 3.0 (Life Technologies, Wilmington, USA), according to manufacturer's instructions. (Thermo Fisher Scientific, 2014)

An additional concentration step was performed in 4 samples extracted with method C that presented the lowest yields of isolated DNA, using Concentrator plus (Eppendorf, Hamburg, Germany). Samples were concentrated by speed vacuum of 80 µl for 20 min. at 60°C and resuspended with 40 µl of nuclease free water. Resulting concentrated extracts concentration and purity were evaluated.

Since determination of FFPE derived DNA fragment sizes is crucial to ensure downstream processing suitability, an agarose gel electrophoresis was performed in order to assess DNA integrity and the average molecular weight of DNA samples extracted from FFPE tissue specimens. Selected samples for analysis consisted of 9 FFPE DNA samples from 3 patients (23, 24 and 26) extracted with the three different methods, 1 FFPE control sample extracted with method C, 4 concentrated DNA

samples extracted with method C (control, patient 23, 24 and 26) and 3 fresh-frozen DNA samples. DNA concentration of all analyzed samples was 23 ng/µl. Briefly, a 2% agarose gel was stained with Midori Green (Nzytech, Lisboa, Portugal). 4 µl of molecular weight 50 pb DNA Step Ladder (340 µl/ml) (Promega, Wisconsin, USA) was used. The gel was run at 100V for 50 minutes and resulting gel image obtained with Bio-Rad ChemiDoc XRS, using Quantity One v4.2 software (Bio-Rad, California, USA).

4 – MS-MLPA

In order to evaluate copy number changes and methylation status, MS-MLPA was performed in all fresh-frozen tissue samples, as well as respective controls, using the tumor specific ME002-C1 MS-MLPA probemix (MRC-Holland, Amsterdam, Netherlands), as previously described by Nygren et al. (2005). ME002-C1 MS-MLPA probemix contains 27 probe sequences, with the recognition site for the *Hha*I methylation-sensitive restriction enzyme and allows the detection of methylation status of a total of 25 different tumor suppressor genes promoter regions. The present panel also includes 14 reference probes which are not digested by HhaI. All 41 probes provide information on copy number alterations of 38 cancer related genes. *MGMT*, *ATM* and *RB1* genes are targeted by two probe sequences each. (Figure 6) (MRC-Holland, 2015b) Briefly, 120 ng DNA samples were heat-denatured at 98ºC for 10 min. and cooled down to 25ºC. After addition of target-specific MLPA probes, samples were heated at 95ºC for 1 min. and incubated at 60ºC for 15 hours. The reaction was then split into two tubes. Standard MLPA reaction was performed in one tube (copy number test), whereas the other was incubated with *Hha*I restriction enzyme (methylation test). Ligation of annealed probes was performed at 49ºC for 30 min. in buffer containing Ligase-65 for the first tube and ligation-digestion reaction in buffer containing Ligase-65 and *Hha*I for the second one. After heat inactivation of the enzymes at 98º for 5 min., PCR was carried out using FAM-labeled primers, dNTPs and SALSA polymerase. PCR consisted of 35 cycles of denaturation for 30 sec. at 95ºC, annealing for 30 sec. at 60ºC, an extension step for 60 sec. at 72ºC, a final extension for 20 min. at 72ºC and a hold at 15ºC. All reactions were performed on a thermal cycle equipped with a heat lid (ABI 2720, Applied Biosystems, California, USA). After heat-denatured, PCR products were

analyzed on a GeneScan ABI PRISM 3130 capillary electrophoresis system (Applied Biosystems, California, USA). For each set of MLPA reaction, a negative control without DNA and 3 previously analyzed DNA samples from healthy controls were included. The DNA control samples were previously analyzed and had no significant genomic imbalances for the loci being studied. In terms of copy number detection, results were displayed as a ratio between obtained signals for reference and tumor samples. Ratio values higher than 1.2 were scored as numerical gains and lower than 0.8 were scored as numerical losses. For methylation status assessment, no signal was generated when probes suffered digestion, as digested probes are not amplified during PCR. In contrast, when target DNA is methylated, probe-sample DNA hybrids are not digested by *Hha*I, the target region is amplified and a signal is generate. Quantification of the methylation status was performed by comparison of the signal peaks from digested and undigested samples. Positive methylation was scored for methylation values ≥20% (20-49% methylated; ≥50% hypermethylated) and negative scored for values <20%.

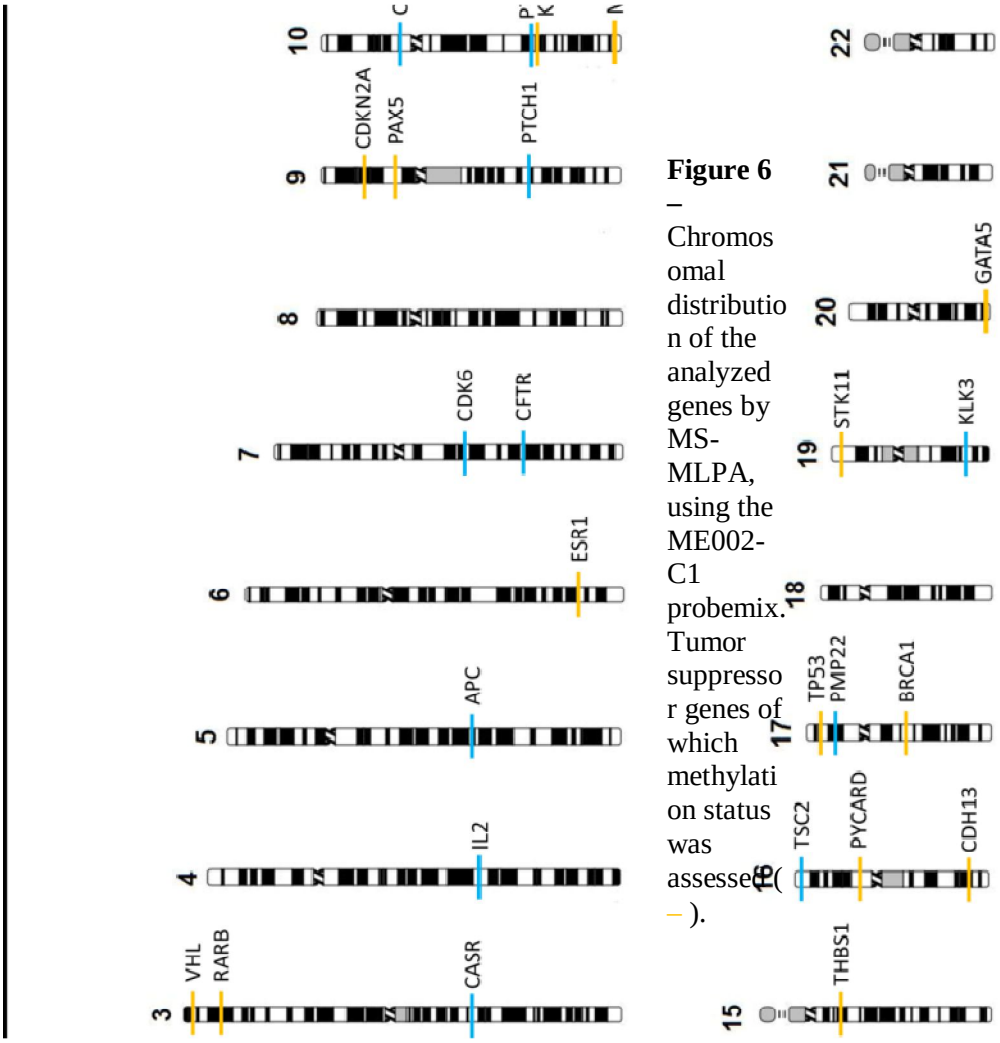

Figure 6 – Chromosomal distribution of the analyzed genes by MS-MLPA, using the ME002-C1 probemix. Tumor suppressor genes of which methylation status was assessed (–).

Table X – Genes analyzed by MS-MLPA with ME002-C1 probemix.

Gene	Chromosomal Position	Function
TP73	1p36.3	Tumor protein p73
MSH6	2p16	mutS homolg 6
VHL	3p25.3	von Hippel-Lindau tumor supressor
RARB	3p24	Retinoic acid receptor, beta
CASR	3q21.1	Calcium-sensing receptor
IL2	4q26-q27	Interleukin 2
APC	5q21-q22	Adenomatous polyposis coli

ESR1	6q24-q27	Estrogen receptor 1
CDK6	7q21-q22	Cyclin-dependent kinase 6
CFTR	7q31.2	Cystic fibrosis transmembrane conductance regulator
CDKN2A	9p21	Cyclin-dependent kinase inhibitor A
PAX5	9p13.2	Paired box 5
PTCH1	9q22.1-q31	Patched 1
CREM	10p12.1-p11.1	cAMP responsive element modulator
KLLN	10q23	Killin
PTEN	10q23	Phosphatase and tensin homolog
MGMT	10q26	O-6-methylguanine-DNA methyltranferase
PAX6	11p13	Paired box 6
WT1	11p13	Wilms tumor 1
CD44	11p13	CD44 molecule
GSTP1	11q13.2	Glutathione S-transferase pi 1
ATM	11q22-q23	ATM serine/threonine kinase
CADM1	11q23.2	Cell adhesion molecule 1
PAH	12q22-q24.2	Phenylalanine hydroxylase
CHFR	12q24.33	Checkpoint with forkhead and ring finger domains
BRCA2	13q12-q13	Breast cancer 2
RB1	13q14.2	Retinoblastoma 1
MLH3	14q24.3	mutL homolog 3
THBS1	15q15	Thrombospondin 1
TSC2	16p13.3	Tuberous sclerosis 2
PYCARD	16p11.2	PYD and CARD domain containing

CDH13	16q23.3	Cadherin 13
TP53	17p13.1	Tumor protein 53
PMP22	17p12	Peripheral myelin protein 22
BRCA1	17q21.31	Breast cancer1
STK11	19p13.3	Serine/threonine kinase 11
KLK3	19q13.41	Kallikren-related peptidase 3
GATA5	20q13.33	GATA binding protein 5

CHAPTER 3

RESULTS

Chapter 3 - Results

1 – Patients, tumors and control samples

In the present study, a cohort of 31 patients with TSCC was retrospectively identified. Patients clinicopathological features, treatment modalities, outcome and risk factor exposure are summarized in Table IX. The majority of patients was male, 80.65% (n=25/31), and ranged in age from 44 to 94 years, with an average of 63.87 years (±12.65). All patients received appropriate treatment, consisting of surgery (n=12/31, 38.71%), surgery with adjuvant radiotherapy (RT) (n=12/31, 38.71%), surgery with adjuvant chemotherapy (CT) (n=1/31, 3.23%) or surgery with adjuvant radiotherapy and chemotherapy (n=5/31, 16.13%). Sixteen patients had a smoking

history (51.61%) at the time of diagnosis or had been smokers for several years and 25.81% (n=8/31) of patients had a history of alcohol consumption.

Diagnosis and clinicopathological assessment of tumors was based on the American Joint Committee on Cancer TNM classification system. (Sobin, Gospodarowics, & Witteking, 2009) Five tumors were classified as stage I, 7 as stage II, 11 as stage III and 6 as stage IV. At the time of diagnosis, 48.39% (n=15/31) of the patients presented metastasized TSCC. Of these patients, 8 presented stage III tumors, 5 stage IV tumors and 1 a stage II tumor.

2 – DNA extraction and samples analysis

2.1 – DNA extraction from fresh-frozen tissue samples

Fresh-frozen DNA samples quantity and quality assessment with NanoDrop 1000 showed that the 31 samples had relatively high concentration values, hence MS-MLPA technic only requires as little as 20 ng of DNA. In terms of quality, registered A260/A280 and A260/A230 ratios were in general within accepted values, showing the absence of contaminants. Overall DNA samples were considered high quality and suitable for molecular downstream processing.

2.2 – DNA extraction from FFPE tissue samples – Protocol optimization

FFPE DNA samples analysis with NanoDrop 1000 revealed an overestimation of the DNA amount present. In general, DNA concentration values for FFPE DNA samples were lower than for DNA samples extracted from fresh-frozen tissues, however there were samples for which DNA concentrations were even higher than fresh-frozen DNA samples. Concentration of all FFPE DNA samples was then measured by Qubit Fluorometer 3.0, in a fluorescent-based assay that is DNA specific, revealing an overestimation of 3 to 4 times of the concentration measured by NanoDrop 1000. This is mainly due to the fact that UV absorbance measurements are not selective, resulting in higher concentration values that are influenced by the presence of contaminants such

as proteins, salts and organic compounds. Moreover, spectrophotometer measure is often inaccurate for the quantification of DNA at low concentrations.

DNA extraction from FFPE specimens was performed using three different methods (A, B and C). As shown in Figure 7, concentration values of DNA samples extracted from FFPE tissues were highly dependent on the method used. The medium amounts of extracted DNA from each method were 24.55 (±12.12) ng/µl, 25.76 (±9.27) ng/µl and 37.14 (±7.24) ng/µl for methods A, B and C, respectively (Figure 8). These results contemplate only DNA concentration values of samples extracted with the three different methods (patients 7, 8, 9,21, 23, 24, 26 and 30), enabling comparison.

Although spectrophotometer measurement is not proper for DNA quantification, it is very accurate for evaluation of the FFPE extracts purity. DNA quality assessment revealed differences between methods, being the best absorption ratios observed when using the C extraction method. For 4 FFPE DNA samples extracted using this method that had the lowest concentration yields, a concentration step was needed which resulted in a 1.5-fold increase in DNA concentration. After concentration, the 4 FFPE DNA samples extracted using this method continued to have high purity DNA.

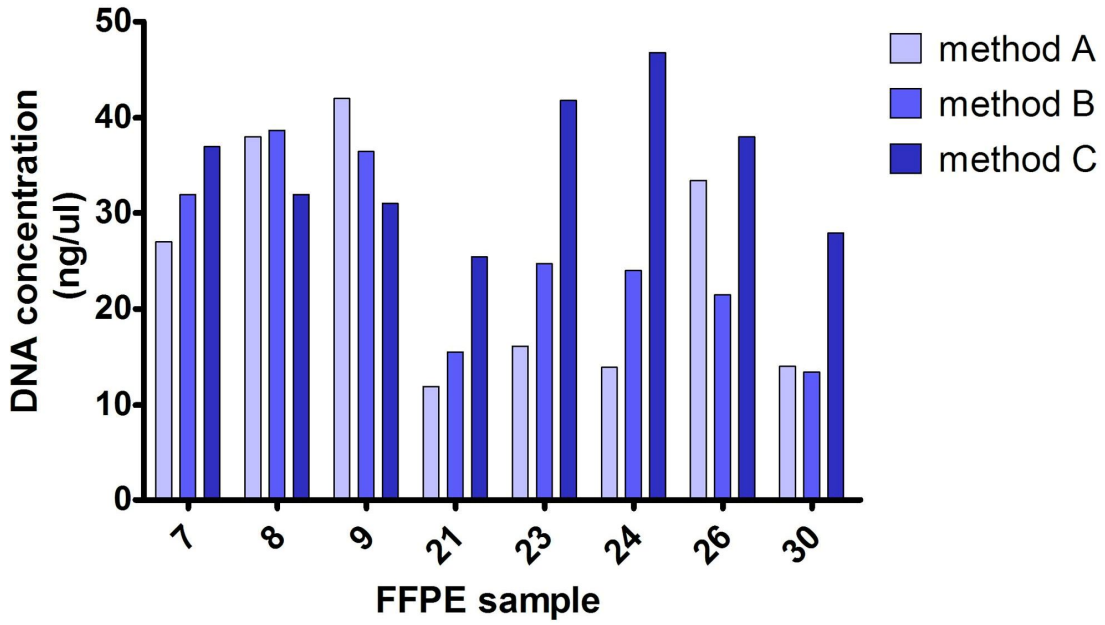

Figure 7 – Concentration values of FFPE DNA samples extracted with methods A, B and C, from patients 7, 8, 9, 21, 23, 24, 26 and 30.

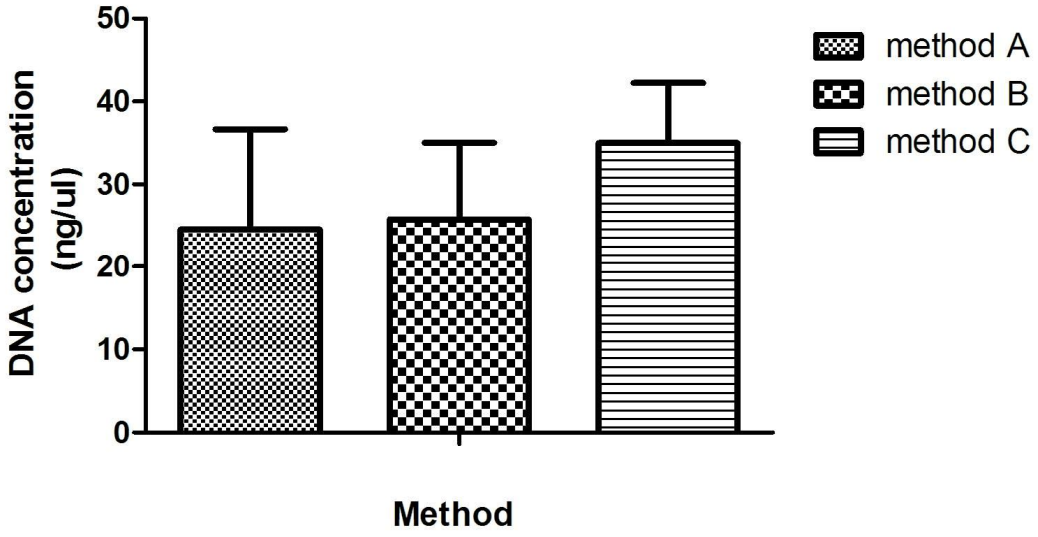

Figure 8 – Medium DNA concentration obtained using extraction methods A, B and C.

2.3 – DNA fragmentation

DNA fragmentation was evaluated by an agarose gel electrophoresis, analyzing 14 FFPE DNA samples and 3 fresh-frozen DNA samples, as shown in Figure 9. DNA extracts from fresh-frozen tissues (15, 16 and 17) did not present fragmentation and were present in enough concentration for posterior analysis by MS-MLPA technique. In contrast, FFPE DNA samples (1 to 14) showed significant fragmentation and lower concentration. Extracted FFPE DNA samples using method C (5, 9 and 13) had relatively higher DNA amounts when compared with samples extracted using method A (3, 7 and 11) and B (4, 8 and 12) and control sample extracted using method C (1). After concentration, FFPE DNA samples showed increasing DNA amounts (2, 6, 10 and 14).

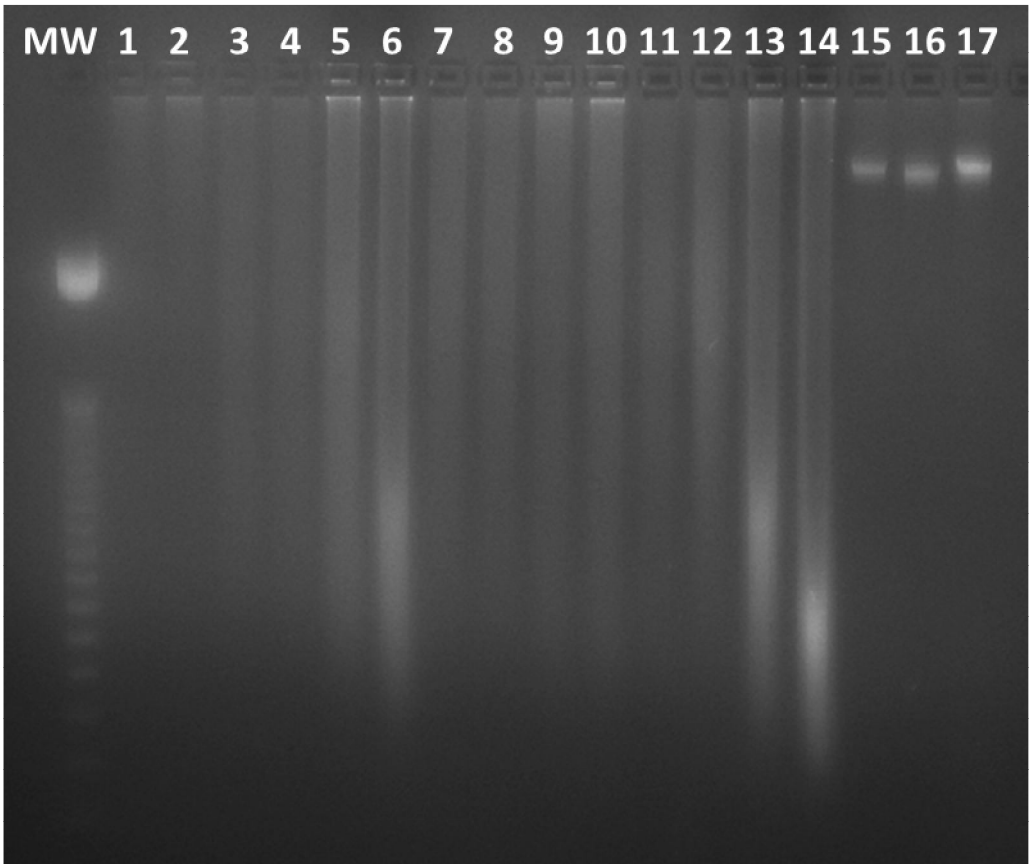

Figure 9 – Gel electrophoresis of representative DNA extracts from FFPE specimens and fresh-frozen tissue samples, assessing DNA fragmentation and molecular weight. MW – molecular weight 50 pb DNA Step Ladder; 1 – control FFPE DNA sample (method C); 2 – concentrated control FFPE DNA sample (method C); 3, 4 and 5 – FFPE DNA sample of patient 23 (method A, B and C, respectively); 6 – concentrated FFPE DNA sample of patient 23 (method C); 7, 8 and 9 – FFPE DNA samples of patient 24 (method A, B and C, respectively); 10 – concentrated FFPE DNA sample of patient 24 (method C); 11, 12 and 13 – FFPE DNA samples of patient 26 (method A, B and C, respectively); 14 – concentrated FFPE DNA sample of patient 26 (method C); 15, 16 and 17 – fresh-frozen DNA samples.

3 – MS-MLPA analysis of fresh-frozen tumor samples

Copy number abnormalities and methylation status of all 31 fresh-frozen TSCC tumor samples and 16 fresh-frozen control samples were assessed by MS-MLPA. The obtained data was analyzed with Coffyanalyzer software (MRC-Holland, Amsterdam, Netherlands), which allows DNA fragments analysis and comparative analysis of samples.

For each sample two electropherograms were generated, one for the MS-MLPA undigested product for copy number detection and one for the digested product for methylation detection. (Figure 10 and 11) In all obtained electropherograms, sample quality and quantity were assessed by registered initial peaks. MS-MLPA ME002-C1 probemix includes 9 control fragments: Q-fragments for DNA quantity control that are only visible with less than 100 ng sample DNA, D-fragments which are used to highlight incomplete denaturation, X chromosome specific fragment at 100 nt for the identification of female samples and Y chromosome specific fragment at 105 nt for the identification of male samples.

For quantitative assessment of copy number alteration, namely loss or gain of genetic material, data normalization is required. ME002-C1 panel includes 14 reference probes which are not affected by *HhaI* digestion. The peak area of each single probe amplification product is divided by the cumulative peak area of all reference probes within individual samples, in order to compensate differences in PCR efficiency. MS-MLPA is a relative technique that detects relative differences by comparison of samples peak patterns. Copy number changes detection is obtained by comparing electrophoresis peak patterns of the undigested MS-MLPA reactions of test sample and three reference samples, included in each run. Determining methylation status is achieved by comparison of electrophoresis peak pattern of test sample with digested counterpart.

MS-MLPA electropherograms of a healthy control female proband shows no copy number alterations (Figure 10-A) and no aberrantly methylated promoter regions were detected (Figure 10-B). The digested reaction of this reference sample only shows peaks from reference probes, since the target sequences of probes containing *HhaI* restriction site are unmethylated and were therefore digested.

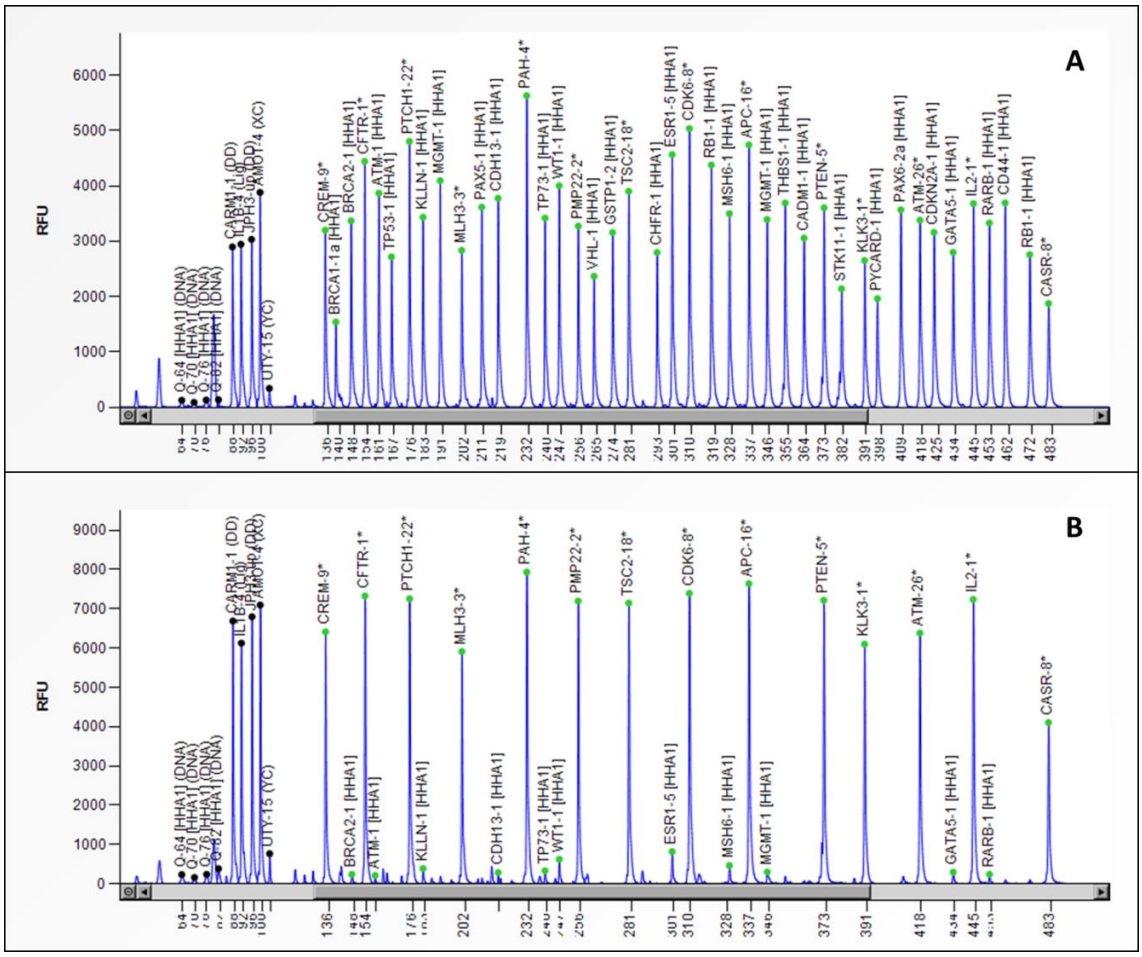

Figure 10 – Electropherograms obtained by Coffyanalyzer software. MS-MLPA results obtained for female control sample for copy number (A) and methylation status (B) detection.

MS-MLPA electropherograms obtained for patient 29 (Figure 11) show copy number changes and aberrant methylation patterns. Two probes had a reduced peak signal as compared to reference samples, revealing gene copy number loss of *PAX5* and *CADM1*. In contrast, four probes had increased peak signal, revealing numerical gains of *CDK6*, *CFTR*, *MLH3* and *PYCARD*. In addition to signal peaks of reference probes, the electropherogram of the digested reaction shows two extra peaks, resulting from the methylation of *RARB*, *PAX5* and *WT1* promoter region.

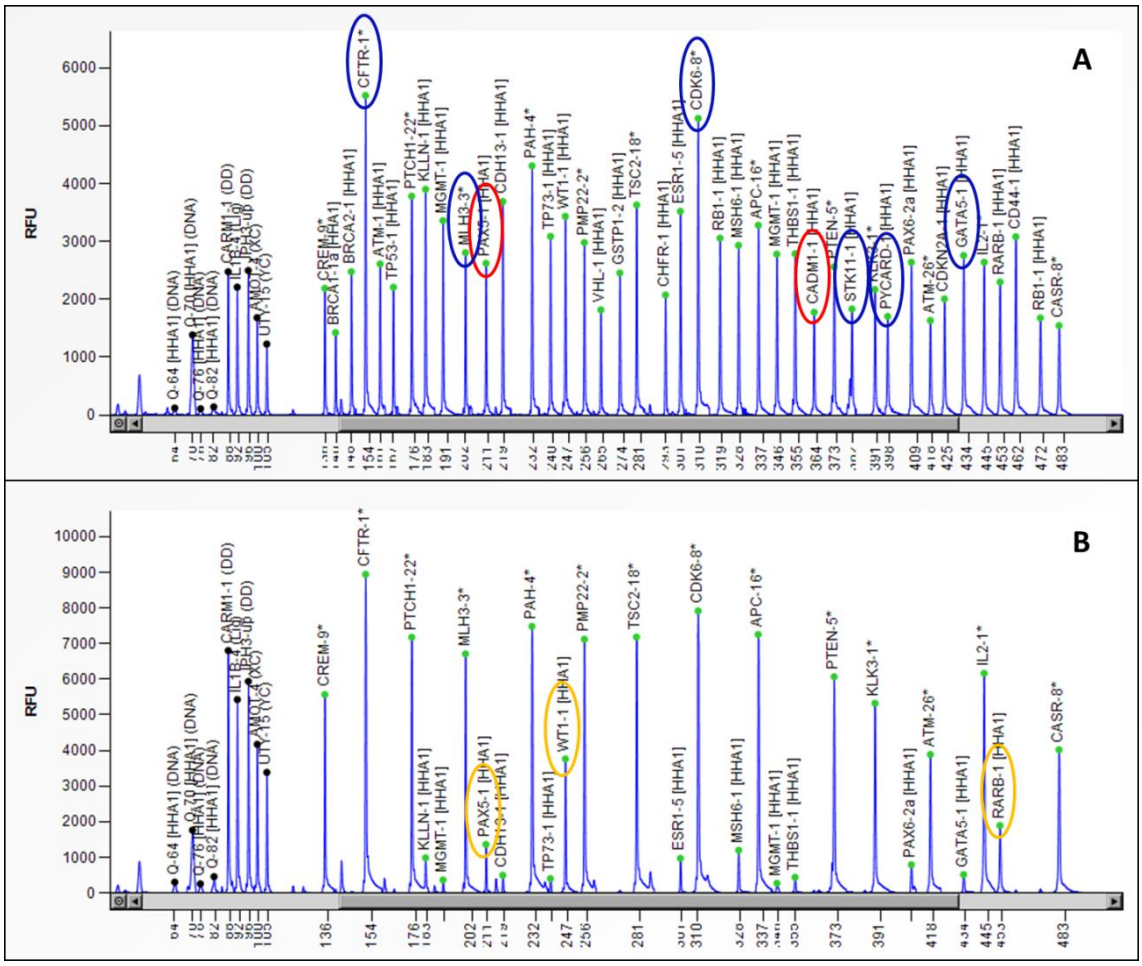

Figure 11 – Electropherograms obtained by Coffyanalyzer software. MS-MLPA results obtained for male patient 29 for copy number (A) and methylation status (B) detection.

3.1 – Copy number alterations

MS-MLPA analysis of fresh-frozen gingival samples from 16 healthy donors, which were subjected to third molar removal, showed no significant genomic imbalances for the loci being studied. After control samples analysis, specific cut-off values for copy number gain and loss were determined and results were displayed as a ratio between obtained signals for reference and tumor samples. Ratio values higher than 1.2 were scored as numerical gains and lower than 0.8 were scored as numerical losses.

As shown in Table XI, genetic imbalances were observed in 28 (90.32%) of the 31 tumor samples analyzed. No copy number changes were detected in tumor samples from patients 9, 12 and 24 for the analyzed 38 cancer-related genes. Genetic imbalances were verified for 36 genes, with the exception of *PTEN* and *RB1* which did not exhibit

any copy number changes in the tumor samples. Overall, 150 copy number alterations were identified of which 42 were losses and 108 were gains. The number of tumor samples exhibiting copy number gains was higher than the ones showing losses. Thirteen samples (41.94%) showed both gains and losses of genetic material, whereas 14 samples (45.16%) exhibit only copy number gains and 1 sample (patient 1) showed only copy number losses.

Table XI – Copy number alterations detected by MS-MLPA using the ME002-C1 probemix in 31 fresh-frozen tumor samples from patients with TSCC. Gains – blue; Losses – red.

Chromosomal Position	Gene	Patients																													Copy Number Alterations n (%)		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Gains	Losses	Normal
1p36.3	*TP73*																																1 (3.23)	2 (6.45)	28 (90.32)
2p16	*MSH6*																																4 (12.90)	0 (0.00)	27 (87.10)
3p25.3	*VHL*																																5 (16.13)	1 (3.23)	25 (80.65)
3p24	*RARB*																																0 (0.00)	2 (6.45)	29 (93.55)
3q21.1	*CASR*																																4 (12.90)	1 (3.23)	26 (83.87)
4q26 q27	*IL2*																																0 (0.00)	4 (12.90)	27 (87.10)
5q21 q22	*APC*																																1 (3.23)	0 (0.00)	30 (96.77)
6q24-q27	*ESR1*																																0 (0.00)	2 (6.45)	29 (93.55)
7q21-q22	*CDK6*																																4 (12.90)	0 (0.00)	27 (87.10)
7q31.2	*CFTR*																																4 (12.90)	0 (0.00)	27 (87.10)
9p21	*CDKN2A*																																2 (6.45)	7 (22.58)	22 (70.97)
9p13.2	*PAX5*																																4 (12.90)	2 (6.45)	25 (80.65)
9q22.1-q31	*PTCH1*																																2 (6.45)	0 (0.00)	29 (93.55)
10p12.1-p11.1	*CREM*																																1 (3.23)	0 (0.00)	30 (96.77)
10q23	*KLLN*																																1 (3.23)	0 (0.00)	30 (96.77)
10q23	*PTEN*																																0 (0.00)	0 (0.00)	31 (100)
10q26	*MGMT*																																0 (0.00)	1 (3.23)	30 (96.77)
11p13	*PAX6*																																0 (0.00)	3 (9.68)	28 (90.32)
11p13	*WT1*																																1 (3.23)	3 (9.68)	27 (87.10)
11p13	*CD44*																																1 (3.23)	2 (6.45)	28 (90.32)
11q13.2	*GSTP1*																																8 (25.81)	0 (0.00)	23 (74.19)
11q22-q23	*ATM*																																1 (3.23)	2 (6.45)	28 (90.32)
11q23.2	*CADM1*																																0 (0.00)	4 (12.90)	27 (87.10)
12q22-q24.2	*PAH*																																0 (0.00)	1 (3.23)	30 (96.77)
12q24.33	*CHFR*																																6 (19.35)	0 (0.00)	25 (80.65)
13q12-q13	*BRCA2*																																0 (0.00)	1 (3.23)	30 (96.77)
13q14.2	*RB1*																																0 (0.00)	0 (0.00)	31 (100)
14q24.3	*MLH3*																																6 (19.35)	0 (0.00)	25 (80.65)
15q15	*THBS1*																																0 (0.00)	1 (3.23)	30 (96.77)
16p13.3	*TSC2*																																1 (3.23)	0 (0.00)	30 (96.77)
16p11.2	*PYCARD*																																13 (41.94)	0 (0.00)	18 (58.06)
16q23.3	*CDH13*																																1 (3.23)	1 (3.23)	29 (93.55)
17p13.1	*TP53*																																7 (22.58)	0 (0.00)	24 (77.42)
17p12	*PMP22*																																0 (0.00)	1 (3.23)	30 (96.77)
17q21.31	*BRCA1*																																9 (29.03)	0 (0.00)	22 (70.97)
19p13.3	*STK11*																																12 (38.71)	0 (0.00)	19 (61.29)
19q13.41	*KLK3*																																1 (3.23)	0 (0.00)	30 (96.77)
20q13.33	*GATA5*																																8 (25.81)	1 (3.23)	22 (70.97)

In the tumor tissue samples of the analyzed 31 patients, a higher frequency of copy number gains were present at chromosomal arms 16p, 19p, 11q, 17q, 7q and 20q, whereas losses were more frequent at 9p, 11p and 11q, as shown in Figure 12 and Table XI.

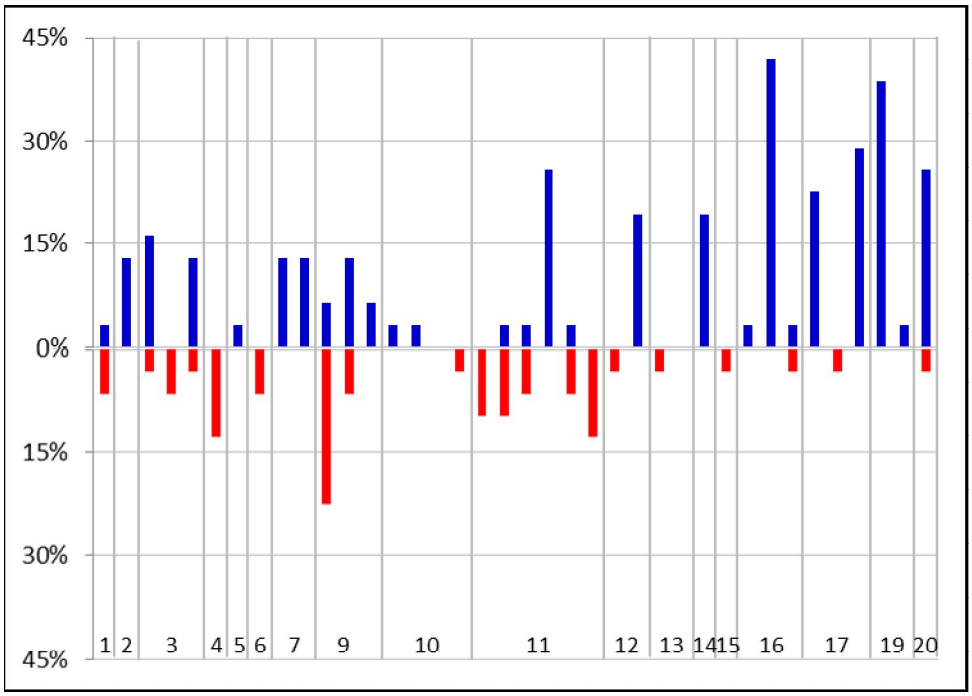

Figure 12 – Genetic imbalances detected by MS-MLPA in 31 fresh-frozen tumor samples from patients with TSCC. Each line represents one gene analyzed. Gains – blue; Losses – red.

Genes exhibiting a higher frequency of copy number gains were *PYCARD*, *STK11*, *BRCA1*, *GSTP1* and *GATA5*, whereas losses were more frequent for *CDKN2A*, *IL2*, *CADM1*, *PAX6* and *WT1*, as shown in Table XII.

Table XII – Genes presenting a higher frequency of copy number gains or losses in the 31 patients with TSCC.

Gains			**Losses**		
Gene	Chromosomal Position	Number of Patients	Gene	Chromosomal Position	Number of Patients
PYCARD	16p11.2	13	*CDKN2A*	9p21	7
STK11	19p13.3	12	*IL2*	4q26-q27	4
BRCA1	17q21.31	9	*CADM1*	11q23.2	4
GSTP1	11q13.2	8	*PAX6*	11p13	3
GATA5	20q13.33	8	*WT1*	11p13	3

The analyzed genes with a higher frequency of copy number changes in the patients samples were *PYCARD*, *STK11*, *CDKN2A*, *BRCA1*, *GATA5*, *GSTP1* and *TP53*, as shown in Figure 13. Copy number gains of *PYCARD* (16p11.2) gene were present in

41.94% (n=13/31) of the analyzed tumor samples. The second most frequent copy number change was genetic gains of *STK11* (19p13.3) that was present in 38.71% (n=12/13%) of all patient samples. Copy number losses of *CDKN2A* (9p21) were present in 22.58% (n=7/31) of tumor samples, whereas gains of this gene were present in 6.45% (n=2/31) of the samples. In 29.03% (n=9/31) of all tumor samples, copy number gains of *BRCA1* (17q21.31) were registered. Copy number changes of *GATA5* (20q13.33) were mostly gains, present in 25.81% (n=8/31) of patients. Copy number loss of *GATA5* was only verified in the tumor sample from patient 1. *GSTP1* (11q13.2) and *TP53* (17p13.1) genes only exhibit genetic material gains in 25.81% (n=8/31) and 22.58% (n=7/31) of tumor samples, respectively.

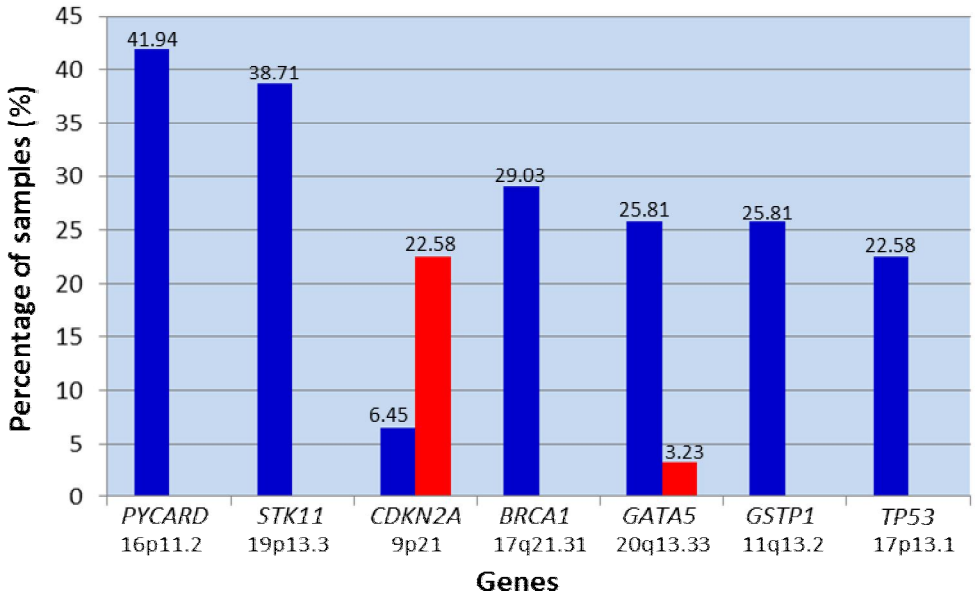

Figure 13 – Genes with a higher frequency of copy number changes in the 31 patients with TSCC. Percentage of tumor samples exhibiting gains (blue) or losses (red) in the analyzed genes.

Less frequent copy number changes were observed of *VHL*, *PAX5*, *CHFR*, *MLH3*, *CASR*, *MSH6*, *CDK6*, *CFTR*, *IL2*, *CADM1*, *WT1*, *TP73*, *CD44*, *ATM*, *PAX6*, *RARB*, *ESR1*, *PTCH1*, *CDH13*, *APC*, *CREM*, *KLLN*, *TSC2*, *KLK3*, *MGMT*, *PAH*, *BRCA2*, *THBS1* and *PMP22* genes. Gain of genetic material of *VHL* (3p25.3) was present in 16.13% (n=5/31) of tumors, whereas loss of this gene was only detected in one tumor sample. *PAX5* (9p13.2) copy number changes consisted of gains, present in 12.90% (n=4/31) of tumor samples, and losses identified in 6.45% (n=2/31) of samples.

CHFR (12q24.33) and *MLH3* (14q24.3) only exhibited gains of genetic material that were present in 19.35% (n=6/31) of tumors. Copy number gains of *CASR* (3q21.1) were present in 12.90% (n=4/31) of tumor samples, whereas loss of this gene was only present in one sample. *MSH6* (2p16), *CDK6* (7q21-q22) and *CFTR* (7q31.2) only exhibited copy number gains that were present in 12.90% (n=4/31) of the tumors. Copy number losses of *IL2* (4q26-q27) and *CADM1* (11q23.2) were identified in 12.90% (n=4/31) of tumor samples. Loss of genetic material of *WT1* (11p13) was present in 9.68% (n=3/31) of tumors, whereas gain of this gene was only detected in one of the analyzed tumors. *TP73* (1p36.3), *CD44* (11p13) and *ATM* (11q22-q23) showed copy number gains in only one tumor sample (patient 14, 19 and 21, respectively) and losses of genetic material of these genes were present in 6.45% (n=2/31) of tumors. Copy number losses of *PAX6* (11p13) were present in 9.68% (n=3/31) of tumor samples. *RARB* (3p24) and *ESR1* (6q24-q27) only exhibited copy number losses that were present in 6.45% (n=2/31) of tumor samples. Two tumor samples (6.45%) showed genetic material gains of *PTCH1* (9q22.1-q31). Copy number changes of *CDH13* (16q23.3) were present in two tumor samples (6.45%), one exhibiting gain and one loss of this gene. The genes with the lowest frequency of copy number changes were *APC* (5q21-q22), *CREM* (10p12.1-p11.1), *KLLN* (10q23), *TSC2* (16p13.3) and *KLK3* (19q13.41) which only exhibited copy number gains in 3.23% (n=1/31) of the tumors and *MGMT* (10q26), *PAH* (12q22-q24.2), *BRCA2* (13q12-q13), *THBS1* (15q15) and *PMP22* (17p12) that only showed loss of genetic material in the same percentage of samples.

3.2 – Methylation status

Methylation profile analysis by MS-MLPA of the 16 control gingival samples extracted from healthy donors, upon third molar removal, allowed determination of specific methylation cut-off values. Positive methylation was scored for methylation values ≥20% (20-49% methylated; ≥50% hypermethylated) and negative scored for values <20%. Quantification of samples methylation status was performed by comparison of the signal peak patterns from digested and undigested counterpart samples. All control samples showed methylation values <20% for all 25 tumor supreessor genes, with the exception of one that showed 43% of methylation of *CD44*.

As shown in Table XIII, aberrant methylation was observed in 96.77% (n=30/31) of the 31 tumor samples analyzed. Eight tumor samples (25.81%) showed promoter methylation in one gene, in 12.90% (n=4/31) of tumors methylation was present in two genes, in 35.48% (n=11/31) of tumors three genes were aberrantly methylated and in 22.58% (n=7/31) of tumors at least four genes showed methylation. Methylation of promoter regions was present in 14 of the total 25 cancer-related genes analyzed. Seven of these genes also showed promoter hypermehylation (*CDKN2A*, *PAX5*, *PAX6*, *WT1*, *CADM1*, *CHFR* and *GATA5*). In contrast, *TP73*, *VHL*, *CD44*, *GSTP1*, *ATM*, *BRCA2*, *RB1*, *THBS1*, *PYCARD*, *BRACA1* and *STK11* showed no aberrant promoter methylation (data not shown).

The most frequently methylated gene was *WT1,* in 77.42% (n=24/31) of tumor samples, as shown in Figure 14. Methylation of this tumor suppressor gene was verified in 41.94% (n=13/31) of patients and promoter hypermethylation was detected in 35.48% (n=11/31) of tumors. *PAX5* showed promoter methylation in 48.39% (n=15/31) of tumors, being hypermethylated in 3 (9.68%) of them. Promoter methylation of *MSH6* was present in 35.48% (n=11/31) of cases. *GATA5* promoter region showed aberrant methylation in 25.81% (n=8/31) of tumor samples. This tumor suppressor gene was methylated in 19.35% (n=6/31) of patients samples and hypermethylated in 6.45% (n=2/31). Promoter regions of tumor suppressor genes *RARB*, *ESR1* and *MGMT* did not show hypermethylation. Methylation of this three genes was detected in 16.13% (n=5/31), 12.90% (n=4/31) and 9.68% (n=3/31) of the analyzed tumors, respectively. Aberrant methylation of *CADM1* and *CHFR* promoter regions was identified in 9.68% (n=3/31) of tumors, being promoter methylation of these genes detected in 6.45% (n=2/31) of tumor samples and hypermethylation registered in 3.23% (n=1/31) of samples. *KLLN* methylation was verified in 2 (6.45%) tumor samples. Promoter methylation and hypermethylation of *PAX6* was present in 6.45% (n=2/31) of patients samples. *CDKN2A* showed only promoter hypermethylation in one of the tumor samples. *CDH13* and *TP53* promoter regions were methylated in 3.23% (n=1/31) of tumors.

Table XIII – Methylation profile of 25 tumor suppressor genes promoter regions detected by MS-MLPA using ME002-C1 probemix of 31 fresh-frozen tumor samples from patients with TSCC. Dark grey – hypermethylation (≥50%); light grey – methylation (20-49%); white – absence of methylation (<20%)

	MSH6	RARB	ESR1	CDKN2A	PAX5	KLLN	MGMT	PAX6	WT1	CADM1	CHFR	CDH13	TP53	GATA5
1														
2														
3														
4														
5														
6														
7														
8														
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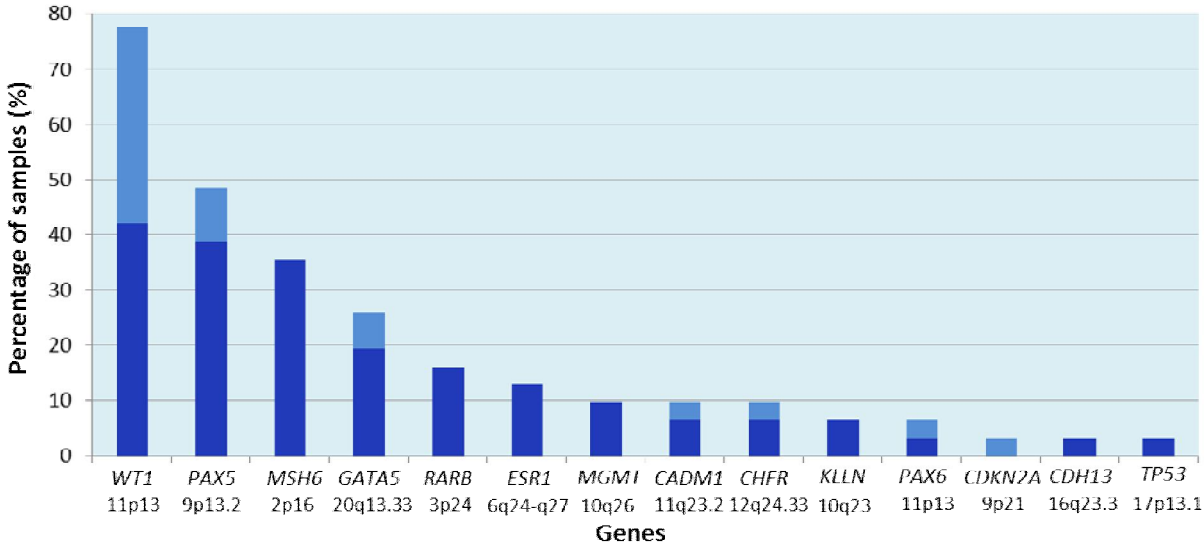

Figure 14 – Methylation of the tumor supressor genes analysed by MS-MLPA in 31 TSCC. Percentage of tumor samples presenting gene methylation (dark blue) and hypermethylation (light blue). Cut-offs: 20-49% - methylation; ≥50% - hypermethylation.

CHAPTER 4
DISCUSSION

Chapter 4 – Discussion

1 – Genetic and epigenetic profiling of TSCC by MS-MLPA

In recent years, advances in molecular research and genomics have allowed for a deeper understanding of the molecular processes involved in the initiation and progression of HNSCC. Studies and cohorts published over the years have resulted in a wide range of information on genetic and epigenetic events involved in tumourigenesis. However, the combination of different anatomical subsites within the head and neck has impaired the identification of molecular markers specific for the different primary sites, as well as their clinical significance. Beyond any doubt, this has resulted in inadequacy to stratify patients according to risk of disease, as well as choosing the most appropriate treatment, particularly in the tongue subsite. Unraveling the genetic and epigenetic alteration patterns present in TSCC will allow for the identification of candidate genes, and determine their clinical significance, which is imperative to improve diagnosis, prognosis, accurate risk-stratification of patients and choose the most adequate treatment and predict treatment response. For this reason, the present study sought to determine the genetic and epigenetic signatures of 31 primary tongue tumors collected from patients with TSCC upon resection surgery and establish a correlation between the resulting tumor-specific genetic and epigenetic changes with patient's key clinicopathological features, providing a great opportunity to identify the molecular pathways underlying the development and progression of the disease.

Analysis of genomic DNA samples extracted from fresh-frozen tumor samples and control gingival samples was performed by MS-MLPA technique using the ME002 probemix. The most remarkable advantage of using this technique is the fact that in addition to the detection of copy number alterations, MS-MLPA assay allows for the detection of aberrant methylation patterns of CpG islands on a large number of genes using one single and simple reaction. MS-MLPA requires only a minimum amount of 20ng DNA in each sample and is simple to preform, allowing for a large number of samples to be simultaneously analyzed. Thus, this simple to perform, sensitive and reproducible technique is suitable for multiplex analysis. (Nygren et al., 2005) ME002 probemix includes 27 probes for methylation status detection of 25 different tumor suppressor genes that contain a restriction site for the methylationsensitive restriction enzyme HhaI and 14 reference probes that are not affected by HhaI digestion, the total 41 probes allow for the detection of copy number alterations. (MCR-Holland, 2015b)

The present probemix panel was chosen due to the fact that copy number loss, copy number gain or methylation of the genes target by the probes included in this particular tumor suppressor kit had been reported in the literature in association with different types of tumors, including head and neck cancers.

For MS-MLPA data analysis, Coffalyser software was used as it has been recommended and designed by MRC-Holland as the optimal analysis program for this technique including accurate analysis algorithm and quality checks. (MRC-Holland, 2015b) (Homig-Holzel & Savola, 2012) Specific cut-off values were established for detection of copy number alterations and methylation status assessment. Copy number changes were detected by comparing the relative signal strength of the probes between patients DNA samples and normal reference samples. The theoretically expected ratios between obtained signals for reference samples and tumor samples of 0.0, 0.5, 1.0 and 1.5 indicate absence of the target region (homozygous deletion), loss of one of the two alleles (heterozygous deletion), presence of the two alleles (wild-type) and allele gain (duplication), respectively. The Coffalyser software uses stringent cut-off values of 0.7 and 1.3 for heterozygous deletion or duplication, respectively. However, when analyzing tumor samples, these sharp cut-off values are not applicable as obtained ratios will depend on the percentage of different cell types present in the samples. The differences in tumor content resulting from contaminating normal cells present in tumor samples and tumor heterogeneity impair the detection of aberrations, as these will be only present in a subpopulation of cells from which genomic DNA was extracted for analysis. (Jeuken et al., 2006) The signals generated for the genomic DNA present in diploid normal cells will cause dilution of signals representing the copy number changes in the genome of tumor cells. For this reason, in the present study, less stringent specific cut-off values for copy number changes were established: ratio values higher than 1.2 were scored as numerical gains and lower than 0.8 were scored as numerical losses. In a study attempting to establish the applicability of the MLPA technique for the reliable identification of different region-specific genetic aberrations of gliomas, Jeuken et al. (2006) verified that this technique enables the correct identification of copy number changes when the percentage of tumor cell represents at least 50% of the tumor sample, setting the threshold to detect losses and gains at 0.8 and 1.2, respectively. Quantification of samples methylation status was performed by comparison of the signal peak patterns from digested and undigested counterpart samples. Methylation specific cut-off values were determined after methylation profile analysis by MS-MLPA

of the 16 control gingival samples extracted from healthy donors, upon third molar removal. Positive methylation was scored for methylation values ≥20% (20-49% methylated; ≥50% hypermethylated) and negatively scored for values <20%. The choice of cut-off values by control samples analysis is crucial for an accurate assessment, since methylation levels and the subsequent effect on gene expression differ according to tissue histological type. (Lim et al., 2014a; Liu, Ji, & Qiu, 2013) Low levels of methylation are usually unlikely to represent a driving alteration in tumorigenesis, however the specific level of methylation that results in altered gene expression has not been yet identified. Thus, analysis of methylation patterns of equivalent normal tissue types and tumoral subsite allows for the establishment of the significant levels of methylation. Similar to the effects on copy number detection, normal tissue contamination of the tumor samples resulting from infiltrating stroma and tumor heterogeneity may lead to the detection of low levels of methylation that may be discarded. (Lim et al., 2014a)

Analysis by MS-MLPA of all fresh-frozen control samples from healthy individuals with no history of neoplasia diagnosis, at the time of gingival sample collection, showed no significant genomic imbalances in terms of copy number changes for the 41 loci being studied. Thus, these samples were suitable for inclusion in MS-MLPA analysis of tumors samples as controls for copy number aberration detection. In terms of methylation status, all control samples had no significant percentage of promoter region methylation for the 25 tumor suppressor genes analyzed, with the exception of one which showed 43% of methylation in *CD44* gene. Aberrant methylation patterns may be detected in individuals with normal phenotype. The significance of this level of methylation and its impact on gene expression may be further analyzed by enlarging the number of normal control samples from healthy individuals in order to determine if this epigenetic alteration is a relatively common event in healthy individuals. Interestingly, methylation of *CD44* was not detected for any of the analyzed tumor samples. Thus, methylation of this gene may not play a significant role in TSCC development and progression.

Another important aspect to be considered when performing the MS-MLPA technique for tumor samples analysis is the choice of adequate reference probes. Tumor samples analysis is a complex process as a variety of loci of different chromosomes can be affected. Different reference probes are included in the probemix for data normalization in order to allow the comparison of probe signals between different

samples. For this reason, the reference probes should only target chromosomal regions in which copy number changes are not expected. It is important to avoid probemix panels in which reference probes target oncogenes or tumor suppressor genes that have been associated to initiation or progression of the type of cancer in analysis. (Homig-Holzel & Savola, 2012) Reference probes included in the ME002 probemix target *CASR*, *IL2*, *APC*, *CDK6*, *CFTR*, *PTCH1*, *CREM*, *PTEN*, *ATM*, *PAH*, *MLH3*, *TSC2*, *PMP22* and *KLK3* genes. (MRC-Holland, 2015b) In the present analysis, copy number alterations of these genes were present in only a few samples. *MLH3* and *CASR* specifically targeted by reference probes were the most altered, being alterations of these genes present in less than 6 tumor samples ($n \leq 6/31$, 19.35%). *PTEN* and *ATM* showed no alteration in none of the samples. The remaining genes were altered in less than 4 samples ($n \leq 4/31$, 12.90%). The reference probes included in the ME002 probemix used in the present study were adequate for a reliable analysis of TSCC, as a low number of genetic imbalances were detected for the genes specifically targeted by these probes and these were distributed on several chromosomes, lowering the risk of affecting the normalization process and increasing the robustness of the results. (Homig-Holzel & Savola, 2012) (Jeuken et al., 2006)

In all 31 tongue tumor samples included in this study, a total of 233 genetic and epigenetic alterations were observed, of which 42 consisted of copy number losses, 108 were gains of genetic material and 83 comprised aberrant methylation. As expected, a significantly higher number of genetic imbalances and methylation aberration patterns were detected in tumor tissue as compared to normal controls, revealing the increasing number of genetic and epigenetic alterations and chromosomal instability characteristic of the carcinogenic process. From all 38 analyzed cancer-related genes, only *PTEN* and *RB1* showed no alteration in any tumor sample, suggesting that these two genes may not play an important role in TSCC development and progression. However, due to the relatively small size of the present cohort and the fact that only one and two probes were target-specific for the *PTEN* and *RB1* genes, respectively, further validation of the present results is needed. Alterations of *TP73*, *VHL*, *CASR*, *IL2*, *APC*, *ESR1*, *CDK6*, *CFTR*, *PTCH1*, *CREM*, *KLLN*, *MGMT*, *PAX6*, *CD44*, *ATM*, *PAH*, *BRCA2*, *MLH3*, *THBS1*, *TSC2*, *CDH13*, *PMP22* and *KLK3* genes were only present in less than 22.58% ($n<7/31$) of tumor samples, suggesting that the present genes could play a small role in the onset and progression of TSCC and do not represent suitable molecular markers of tongue tumorigenesis. Since the scope of the present work is to identify genetic and

epigenetic alterations that are representative of the biological processes underlying TSCC, the most frequent alterations present in the analyzed tumor samples will be discussed in detail in association with their clinical relevance.

The most frequently altered gene in the present cohort was the *WT1*, which was consistently altered in 90.32% (n=28/31) of tumor samples. Aberrant methylation of this gene was present in 24 tumor samples, whereas copy number changes were less frequent, being losses and gains of genetic material verified in 3 and 1 samples, respectively. Mapped at 11p13, *WT1* gene was first isolated as a tumor suppressor gene that was inactivated in a subset of patients with Wilms' tumor (Call et al., 1990) and encodes a transcriptional factor that plays an important role in cell growth and differentiation (Sugiyama, 2010). This transcriptional factor has four zinc fingers and has been associated with the transcriptional regulation of genes such as insulin-like growth factor (IGF)-II (Drummond et al., 1992) and retinoic acid receptor (RAR)-α (Goodyer et al., 1995). During development, WT1 is manly expressed in the developing kidney, gonad and mesothelium. (Davies et al., 1999) Methylation of *WT1* in tumors suggests a decrease in the expression of this gene in concordance with a tumor suppressor role that has been previously described (Jomgeow et al., 2006). However, overexpression of *WT1* has been verified in a variety of human cancers, such as leukemia (Inoue et al., 1994), astrocytic tumors (Yusuke Oji et al., 2004), colorectal adenocarcinoma (Oji et al., 2003b), esophageal cancer (Oji et al., 2004b), prostate cancer (Gregg et al., 2010) and HNSCC (Oji et al., 2003), indicating that *WT1* plays an oncogenic role rather than acting as a tumor suppressor in the tumorigenesis of these cancers. Also, reinforcing this idea is the fact that growth of cancer cells showing high levels of *WT1* expression was consistently inhibited by treatment with *WT1* antisense oligomers. (Algar et al., 1996; Oji et al., 1999; Yamagami et al., 1996) Oji et al. (2003) analyzed *WT1* gene expression using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in 56 HNSCCs, of which the majority was from the tongue subsite (25). Overexpression of this gene was present in 68% of the tongue tumors, suggesting an important role of *WT1* in TSCC tumorigenesis. These investigators also found a significant correlation between poor histological tumor differentiation and more advanced stages of HNSCC with the high levels of *WT1* expression. Brett, Pandey, & Fraizer (2013) showed an inverse relationship between WT1 and E-cadherin expression levels in prostate cancer cells. High levels of WT1 expression were able to dampen E-cadherin levels and enhanced the cells migratory

ability, thus contribute to the development of metastatic phenotype. *WT1* gene may serve as a potential predictive marker of cell migration and metastasis in prostate cancer. (Brett, Pandey, & Fraizer, 2013) In a study conducted by Hylander et al. (2006), the correlation of overexpression of *WT1* and higher tumor grade and stage was demonstrated in a large set (100) of epithelial ovarian cancers. Overexpression of *WT1* also represents an attractive target for immunotherapy. After immunization with WT1-specific cytotoxic T cells, an *in vivo* murine leukemia model showed tumor regression. (Oka et al., 2002) Additionally, WT1 peptide vaccination in patients with WT1 expressing breast or lung cancer, myelodysplastic syndrome, or acute myeloid leukemia was able to induce WT1-specific cytotoxic T lymphocytes and showed positive clinical responses such as reduction in tumor size without damage to normal tissues. (Oka et al., 2004) These results are inconsistent with the high levels of methylation observed in the present cohort that suggest a tumor suppressor role of the *WT1* gene. Other studies have reported promoter methylation of *WT1* in several human neoplasms, such as colorectal cancer (Hiltunen et al., 1997), breast cancer (Laux et al., 1999), ovarian cancer (Kaneuchi et al., 2005), lung cancer (Nikolaidis et al., 2012) and OSCC (Viet & Schmidt, 2008) (Gasche et al., 2011). Promoter methylation of *WT1* gene has been indicated as a significant clinical biomarker in cervical cancer (Lai et al., 2008) and lung cancer screening (Nikolaidis et al., 2012). DNA methylation analysis of 44 OSCCs conducted by Jithesh *et al.*, showed that *WT1* was differentially methylated between extracapsular spread (ECS) and non-ECS tumor samples, revealing the possible role of *WT1* methylation as a diagnostic and prognostic predictor in oral carcinogenesis. (Jithesh et al., 2013) Hypermethylation of *WT1* gene has been previously described in tissue and saliva samples of patients with OSCC (Viet & Schmidt, 2008), in accordance to the results of the present TSCC cohort that showed high levels of methylation of this gene, being *WT1* hypermethylated in 35.48% (n=11/31) of tumor samples. Viet *et al*. methylation analysis of saliva samples before and after treatment showed that *WT1* methylation was completely reversed after successful treatment of OSCC patients, suggesting the role of this gene in oral carcinogenesis and the potential utility of methylation analysis in saliva samples for early detection and evaluation of treatment response. Gasche *et al*. demonstrated alterations in *WT1* methylation patterns by interleukin-6 (IL-6) mediated chronic inflammation in oral cancer cells. IL-6 was able to alter *WT1* methylation patterns from a hemimethylated to a fully methylated state, suggesting that IL-6 promotes tumorigenesis by altering DNA methylation in OSCC.

(Gasche et al., 2011) Methylation of *WT1* was present in a significantly high percentage of the tongue tumors, suggesting the important role of this gene as a tumor suppressor in TSCC carcinogenesis. Also, given the fact that overexpression of *WT1* has been associated with poor histological differentiation and increased tumor stage, the present gene represents a potential prognostic marker in TSCC. (Oji et al., 2003) Although the high levels of methylation of the present gene indicate low levels of WT1 gene expression, further validation of the present results may be performed by proteomic or gene expression studies.

PAX5 was the second most frequently altered gene in the tumor samples analyzed (n=21/31, 67.74%). *PAX5* showed promoter methylation in 48.39% (n=15/31) of tumors and copy number losses and gains of this gene were present in 2 and 4 tumor samples, respectively. *PAX5* gene is located on chromosome region 9p13 and belongs to the PAX family of genes that includes nine developmentally regulated genes, which encode transcription factors that have key roles in organ development and differentiation of tissue during embryogenesis. PAX5 protein is expressed during B-cell development, with the exception of plasma cells. (Adams et al., 1992) Additionally to the normal physiological process carried out by PAX5 during development, this transcriptional factor also plays an important role in tumorigenesis. Frequent methylation of *PAX5* gene has been reported in ductal carcinoma *in situ*, invasive breast cancer, neuroendocrine carcinomas and HNSCC. (Guerrero-Preston et al., 2014; Moelans, Verschuur-Maes, & Van Diest, 2011) Guerrero-Preston et al. (2014) have identified promoter methylation of *PAX5* gene as a frequent event in HNSCC, associated with low *PAX5* expression levels, suggesting a tumor suppressor role of this gene in head and neck carcinogenesis. Methylation of *PAX5* gene was higher in HPV-negative tumors (83%) when compared to HPV-positive tumors (25%). Given that the HPV-positive HNSCC has been associated with a more favorable clinical outcome, with an increased survival of up to 60-80% (Gillison et al., 2000; Leemans, Braakhuis, & Brakenhoff, 2011; Marur et al., 2010), *PAX5* gene represents a potential molecular marker for prognosis and patient outcome prediction. These investigators also found an interesting relationship between *TP53* and *PAX5*, as 79% of the samples presenting mutations of the *TP53* gene also showed promoter methylation of *PAX5*, contributing to support the idea that promoter methylation and somatic mutations represent the main cause of gene inactivation and pathway disruption in HNSCC. Patients with combined *PAX5* methylation and *TP53* mutations had a worse overall survival than patients

presenting only the latter. (Guerrero-Preston et al., 2014) In superficial bladder carcinoma, concomitant high expression levels of PAX5 and TP53 have been correlated with higher recurrence and progression rates. (Babjuk et al., 2002) Norhany et al. (2006) reported high levels of *PAX5* expression in primary tumor samples from patients with OSCC, OSCC-derived cell lines and leukoplakia lesions, suggesting that PAX5 plays an important role during oral carcinogenesis. Overexpression of PAX5 in premalignant oral lesions and early stages reveals the potential role of the present gene as a biomarker for diagnosis and early detection of these tumors. (Norhany et al., 2006) In the present study, the high percentage of the tongue tumors with methylation of *PAX5* gene suggests the important role of this gene as a tumor suppressor in TSCC carcinogenesis. Of note is also the potential role of this gene as a molecular marker in early detection and prognostic prediction in TSCC.

The third gene that showed a higher number of genetic and epigenetic alterations in the population of tongue tumor samples was *GATA5* (n=17/31, 54.84%). Genetic imbalances of *GATA5* were present in 9 tumors analyzed tumors, being mainly gains of genetic material detected (n=8/31, 25.81%). Promoter methylation of *GATA5* was verified in 8 patients with TSCC (25.81%). Mapped at 20q13.33, *GATA5* gene encodes a transcription factor that contains two GATA-type zinc fingers. *GATA5* guides differentiation and development of endoderm-derived organs. (Laverriere et al., 1994) A tumor suppressor role for *GATA5* has been described, resulting from methylation and loss of expression of this gene in glioblastoma (Rankeillor et al., 2014), lung (Guo et al., 2004), gastric (Akiyama et al., 2003), esophageal (Guo et al., 2006), colorectal (Hellebrekers et al., 2009) and ovarian (Wakana et al., 2006) cancers. In glioblastoma, promoter methylation of *GATA5* has been associated with poor outcome, revealing the predictive/prognostic significance of this gene. (Rankeillor et al., 2014) In lung cancer, concomitant promoter methylation of *GATA4* and *GATA5* genes was significantly associated with increased methylation frequency and increasing patient age. (Guo et al., 2004) In a study conducted by Hellebrekers et al. (2009), frequent promoter methylation of *GATA5* was verified in colorectal carcinoma, suggesting an important role of this tumor suppressor gene in colorectal carcinogenesis. Induced *GATA5* overexpression in human colorectal cancer cell lines resulted in suppressed colony formation, proliferation, migration, invasion and anchorage-independent growth, revealing the tumor suppressive response of this gene. This study also suggested *GATA5* promoter hypermethylation as a potential biomarker for noninvasive colorectal cancer detection.

Gasche et al. (2011) demonstrated alterations in *GATA5* methylation patterns by interleukin-6 (IL-6) mediated chronic inflammation in oral cancer cells, promoter region hypermethylation of this gene and concomitant downregulation of its expression were observed following IL-6 exposure, suggesting that epigenetic silencing may results from chronic inflammation in OSCC. Promoter methylation of *GATA5* has been reported in a variety of other tumor types, but not in TSCC. The present cohort shows a novel observation that *GATA5* methylation is present in 25.81% of patients, revealing the importance of the present gene in tongue tumorigenesis.

Genetic and epigenetic alterations of *MSH6* were also frequent in the population of tumors of the present study (n=15/31, 48.39%). Promoter methylation of MSH6 was more frequent than genetic imbalances, being observed in 35.48% (n=11/31) of tumor samples. *MSH6* gains of genetic material were verified in 4 of the patients, of which one showed also methylation of this gene. *MSH6* gene is located at chromosomal region 2p16 and encodes a mismatch repair protein involved in the DNA mismatch repair system, which main function is to repair mispaired bases in DNA sequence, resulting from replication errors in order to prevent mutation accumulation. MSH6 forms a heterodimeric complex with MSH2 (MutSα) that recognizes mispaired DNA bases and repairs base-base and small insertion/deletion mispairs. (Acharya et al., 1996; Gazzoli & Kolodner, 2003; Genschel et al., 1998) Elevated percentage of promoter methylation of *MSH6* has been reported in ductal carcinoma *in situ* and invasive breast cancer. (Moelans et al., 2011) In glioblastomas, significant low levels of MSH6 protein expression have been associated with recurrence after adequate treatment. (Felsberg et al., 2011) Particularly in HNSCC, low levels of expression of the *MSH6* gene have been associated with an increased risk of developing this type of cancer. (Wei et al., 1998) In the present cohort, the frequent promoter methylation of *MSH6*, suggest the important role of this mismatch repair gene in tongue carcinogenesis. Additionally, *MSH6* methylation was present in 16.67% (n=2/12) of the 12 staged as I and II tumors and 47.06% (n=8/17) of the staged III and IV tongue tumors. Promoter methylation of this gene was also found to be a more common event in patients with metastasis at the time of diagnosis, as *MSH6* methylation was present in 8 of the 15 patients with metastasized TSCC (53.33%) and only 2 of the 12 patients without metastasis (16.67%) had methylation of the mismatch repair gene. The present results, suggest that *MSH6* promoter region methylation may be associated with more advanced stages of the

disease and metastatic phenotype in TSCC, additional studies with a larger number of subjects are still warranted to confirm these findings.

PYCARD and *STK11* genes only presented genetic imbalances, namely copy number gains in the tumor samples analyzed. *PYCARD* and *STK11* showed frequent gains of genetic material in 41.94% (n=13/31) and 38.71% of tumors, respectively. Mapped at 16p11.2, *PYCARD* gene codes a bipartite signaling protein, containing two protein-protein interaction domains, a pyrin domain (PYD) and caspase recruitment domain (CARD), that is involved in apoptosis and inflammation. Overexpression or oligomerization of PYCARD induces apoptosis via a caspase-8 dependent mechanism in epithelial cells. (Masumoto et al., 2003; McConnell & Vertino, 2004) Epigenetic silencing of *PYCARD* gene by promoter methylation has been reported in various tumor types, such as glioblastoma (Stone et al., 2004), breast (Conway et al., 2000), prostate (Collard et al., 2006) and colorectal (Yokoyama et al., 2003) cancers. However, in the present cohort no tumor samples exhibited methylation of this gene, suggesting that this mechanism of epigenetic silencing is not involved in tongue tumorigenesis. On the other hand, copy number gains of *PYCARD* were rather a frequent event in the analyzed samples, suggesting an oncogenic role of *PYCARD* in TSCC development and progression. Drexler *et al*. demonstrated that PYCARD may influence tumor growth in completely different directions, as this protein limits keratinocyte proliferation by interactions with p53, possibly through p53 activation, but it also presents a proinflammatory role in infiltrating cells that benefits tumor development. (Drexler et al., 2012) *STK11* is located at chromosomal region 19p13.3 and encodes a serine/threonine protein kinase. During development, high levels of *STK11* expression occur in heart esophagus, pancreas, kidney, colon lung, small intestine and stomach, whereas in adult tissues expression of this protein is more common in epithelium, ovaries, testis, myocytes in skeletal muscle and glia cells. (Sanchez-Cespedes, 2007) *STK11* has a tumor suppressor role in breast cancer, being low levels of expression of this gene associated with decreased survival, which indicates expression of *STK11* as potential prognostic marker in these tumors. (Shen et al., 2002) Zhi-Gang *et al.* demonstrated overexpression of STK11, *in vitro,* in breast cancer cells in association with significant inhibition of migration and invasion. Additionally, *in vivo* studies demonstrated that high levels of expression of this gene resulted in low tumor growth and decreasing of lung metastasis. (Zhuang et al., 2006) Qiu et al. (2006) reported A205T point mutation of the *STK11* gene results in inactivation of protein function and

is involvement in HNSCC carcinogenesis in association with loss of cell growth inhibition by this tumor suppressor gene. (Qiu et al., 2006) *STK11* gene shows frequent loss in several types of cancer , such as lung adenocarcinoma (Sanchez-Cespedes et al., 2002), endometrial adenocarcinoma (Contreras et al., 2008) and intestinal polyposis (Sanchez-Cespedes, 2007), which is not in concordance with the present results that show only gains of the present gene, revealing the potential role as a oncogene of *STK11* in TSCC.

Alterations of *CDKN2A* gene were mainly genetic imbalances. Copy number losses and gains of this gene were present in 22.58% (n=7/31) and 6.45% (n=2/31) tumor samples, respectively. Promoter methylation of *CDKN2A* was only observed in one tumor. Mapped at 9p21, *CDKN2A* gene encodes $p16^{INK4A}$ protein involved in the inhibition of cell cycle progression from G1 to S phase. Disruption of CDKN2A is considered to be an early event in HNSCC tumorigenesis. (Reed et al., 1996) *CDKN2A* is frequently inactivated in HNSCC by mutation or methylation in combination with chromosome loss or, more commonly, by homozygous deletion. Decreased $p16^{INK4A}$ expression is associated with reduced survival, increased recurrence rates and nodal metastasis. (Bettendorf et al., 2004; Leemans, Braakhuis, & Brakenhoff, 2011) Negative or low $p16^{INK4A}$ expression has been reported in up to 83% of OSCCs and up to 60% of potentially malignant lesions. (Silva et al., 2011) Cyclin D1 and $p16^{INK4A}$ play an important role in cell cycle control, being overexpression of the first and loss of expression of the latter common events in HNSCC. Bova et al. (1999) were the first who sought to determine the relationship between expression of this two molecules and disease outcome in TSCC. Overexpression of cyclin D1 was observed in 68% of the tumors, associated with increased lymph node stage (P= 0.014), increased tumor stage (P= 0.003) and reduced disease-free (P= 0.006) and overall (P= 0.01) survival. Loss of $p16^{INK4A}$ expression in 55% of tumors was correlated to reduced disease-free (P=0.007) and overall (P= 0.014) survival. Cyclin D1 overexpression and loss of $p16^{INK4A}$ expression are established independent predictors of death from TSCC. Additionally, simultaneous assessment of these proteins expression defines subgroups of patients at increased risk of relapse. (Bova et al., 1999) Loss of *CDKN2A* expression by promoter hypermethylation has been described as an early event in oral cancer. In a study conducted by Cao et al. (2009), promoter methylation of this gene occurred in 41% of the 78 patients analyzed with histologically confirmed mild or moderate oral epithelial dysplasia and was significantly associated with a higher rate of progression to oral

cancer (P=0.013). Particularly, in TSCC, this epigenetic event may serve as a useful molecular marker for local recurrence prediction. A prospective study performed by Sinha *et al.* identified *CDKN2A* promoter hypermethylation in 86.8% of the 38 tongue carcinomas evaluated. Promoter methylation was present in 43,3% of the 30 patients with histologically free margins, which predicted a 6.3-fold increased risk of having local recurrence as compared to patients whose margins were negative for *CDKN2A* promoter methylation. (Sinha et al., 2009) A large cohort of 131 patients with TSCC conducted by Lim et al. revealed the frequency of *CDKN2A* mutations, homozygous loss, hemizygous loss and promoter methylation of 20%, 7%, 31% and 18%, respectively, demonstrating that *CDKN2A* alteration is a frequent event in TSCC tumourigenesis. (Lim et al., 2014b) Although, conducted in a relative small group of oral tongue carcinomas, studies have shown that both *TP53* mutations and *CDKN2A* alteration are speculated to be relevant to patients developing TSCC at a young age, who develop disease without risk factor exposure and may also be associated with high risk disease. (Heaton et al., 2014; Li et al., 2014)

Genetic and epigenetic changes observed in *CHFR*, *BRCA1*, *GSTP1*, *TP53*, *RARB* and *CADM1* genes were present in 22.58-29.03% of the analyzed tongue tumors. Although the frequency of alterations of these genes in the present cohort in study was lower, these represent important markers of TSCC development and progression. However, in order to establish a significant correlation between the present genes and tongue carcinogenesis, additional studies comprising a larger number of samples are needed.

CHFR exhibited promoter methylation in 3.23% (n=3/31) of the tumors and gains of genetic material in 19.35% (n=6/31) of samples. Mapped at chromosomal region 12q24.33, *CHFR* codes a checkpoint protein in the G2S to M phase transition, playing an important role in cell cycle regulation. (Gao et al., 2009) Frequent promoter methylation of the present gene has been reported in HNSCC (Yalniz et al., 2011), however in lower frequency than for gastric cancer (Gao, Xin, Zhang, & Zhou, 2008; Oki et al., 2009). *CHFR* methylation has also been reported in cervical (Banno et al., 2007), ovarian (Gao et al., 2008) and endometrial (Yanokura et al., 2007) cancers. Syed *et al.* reported *CHFR* promoter methylation in association with higher overall survival and progression-free survival, suggesting the role of the present gene as a potential biomarker in outcome prediction. Additionally, RNAi-dependent silencing of the present gene in HNSCC cancer cells increased sensitivity to cisplatin and irradiation,

revealing the possible role of *CHFR* in treatment adequate choice and prediction of treatment response in HNSCC. (Syed et al., 2005)

BRCA1 and *GSTP1* genes showed only copy number gains in 29.03% (n=9/31) and 25.81% (n=8/31) of the tumors, respectively. *BRCA1* is located at chromosomal region 17q21.31 and is involved in cell cycle progression, DNA repair pathways and apoptotic processes. Mutations of this gene are associated with 40-45% of cases of hereditary breast cancers. (Mitrovic et al., 2013) In the present cohort, *BRCA1* gain of genetic material may be indicative of increased expression of this gene, but in order to confirm that further expression and proteomic analysis is required. Overexpression of the present gene has been previously reported in breast cancer. (Mitrovic et al., 2013; Rosen, Fan, & Isaacs, 2005) Saiki *et al.* demonstrated *BRCA1* increased levels of expression in cis-diamminedichloroplatinum(CDDP)-resistant cell lines as a predictive marker of taxane sensitive HNSCC. These two types of antitumor agents are the most active against HNSCC, thus the possible role of *BRCA1* in establishing the inverse relationship between resistances of these chemotherapeutic agents is crucial for clinical management when patients develop CDDP resistance. (Saiki et al., 2011) Mapped at 11q13.2, *GSTP1* encodes the GSTP1 protein, belonging Glutatione S-transpherase (GST) family that plays an essential role in carcinogens and cytotoxic drugs detoxification by conjugation with glutathione. In oral carcinogenesis, *GSTP1* is the most common expressed GST isoform and is responsible for the detoxification of carcinogens present in tobacco. (Geisler & Olshan, 2001) GSTP1 reduced activity has been previously described in association with enhanced chemotherapy response in HNSCC, suggesting an important role of the present gene in prediction of treatment response. (Ruwali et al., 2009)

Genetic imbalances of *TP53* were present in 22.58% (n=7/31) of the tumor samples and were all numerical gains. Promoter methylation of *TP53* was only present in one patient. Mapped at 17p13, *TP53* encodes p53 protein involved in cell cycle arrest, cellular senescence and apoptosis. (Harris & Levine, 2005) Alterations in in this gene through LOH, point mutations, deletions and insertions are early events in HNSCC. (Agrawal et al., 2011; Leemans, Braakhuis, & Brakenhoff, 2011) In terms of clinical outcome, TP53 mutations represent one of the worst molecular alterations in patients with HNSCC, as patients with disruptive mutation of this gene have a reduced survival and are more likely to relapse after complete resection and radiation therapy. (Poeta et al., 2007) In the present cohort, the increase frequency of copy number gains

observed for *TP53*, suggest an increase expression of this gene that may be further validated by expression and proteomic studies. In literature, the prevalence of positive immunohistochemical detection of p53 has been reported between 30-50%, occurring frequently in adjacent non-tumoral mucosa, which indicates that these alterations are early events in squamous cell carcinoma development. (Bettendorf et al., 2004) Thus, these results indicate *TP53* gene as a potential molecular marker for the early detection and the assessment of histopathologically free margins in TSCC. Furthermore, 17p13 LOH in poorly differentiated tumors suggests that loss of p53 function may be associated with the transition from preinvasive to invasive head and neck tumors. (Denaro et al., 2011)

RARB and *CADM1* showed genetic and epigenetic alterations in 22.58% (n=7/31) of the analyzed tongue tumors. Promoter methylation of *RARB* and *CADM1* was identified in 16.13% (n=5/31) and 9.68% (n=3/31) of patients, respectively. These two genes exhibited only genetic material loss in 6.45% (n=2/31) and 12.90% (n=4/31) of tumor samples, respectively. *RARB* is mapped at chromosomal region 3p24 and is involved in cellular signaling in embryonic morphogenesis, cell growth and differentiation. Loss of *RARB* gene is frequently caused by promoter methylation rather than deletion (Shaw et al., 2008) (Yalniz et al., 2011), which is in concordance with the present results for TSCC. Methylation of *RARB* has been reported in leukemia (Galm et al., 2004), HNSCC (Chen et al., 2007; Yalniz et al., 2011), breast (Moelans et al., 2011; Zhu et al., 2010) and prostate (Tang et al., 2013) cancer. *RARB* promoter methylation has been suggested as an early and frequent event in HNSCC. Methylation of this gene has been reported at a higher frequency in premalignant and invasive tumors of the head and neck, indicating that epigenetic silencing of *RARB* by the present mechanism may be used in early detection and prognostication of these tumors. (Chen et al., 2007; Maruya et al., 2004) Mapped at 11q23.2, *CADM1* encodes a transmembrane glycoprotein involved in cell interaction of epithelial cells and mediates cell-to-cell adhesion (Masuda et al., 2002). Silencing of *CADM1* occurs mostly by promoter methylation, but could also occur in combination with allelic loss. (Allinen et al., 2002; Fukami et al., 2003; Van Den Berg et al., 2011) In the present cohort, inactivation of *CADM1* was associated with methylation and copy number loss of this gene, but none of the patients showed concomitant silencing of *CADM1* by these two mechanisms. CADM1 promoter methylation has been reported in different tumor types, such as cervical (Overmeer et al., 2008), breast (Allinen et al., 2002), lung (Van Den Berg et al.,

2011) and oral (Hayama et al., 2009) cancers. Frequent *CADM1* alterations have been reported in HNSCC (Sanchez-Cespedes et al., 2000; Worsham et al., 2006), particularly in OSCC (Hayama et al., 2009), suggesting that the present gene is involved in oral carcinogenesis.

In the present cohort, the genetic and epigenetic characterization of tongue tumors highlighted several genes and pathways that play an important role in TSCC. Thus the identified genes represent putative biomarkers for diagnosis, prognosis, treatment choice and prediction of treatment response in carcinoma of tongue. Although no significant association between genotype and phenotype was establish, methylation of *MSH6* gene showed elevated association with advanced tumor stage and metastasis. In order to more firmly establish this and other associations between genetic and epigenetic changes and clinicopathological features of patients with TSCC, a larger number of samples is warranted and FFPE specimens represent the answer.

2 – Protocol optimization for genomic DNA extraction from FFPE samples

Molecular studies for identification of genes and pathways associated with cancer development and progression have been mainly focused on surgically removed snap-frozen tumor samples, in order to grant the highest possible amount of high quality DNA for analysis. However, the limited number of available fresh-frozen tumor samples represents a disadvantage. The solution resides in the FFPE specimens that have been routinely used in diagnostic pathology for decades, resulting in a vast amount of samples and representing the most readily available source of tumor tissue. Most importantly, almost all FFPE samples have associated pathological and clinical information, allowing for its application on association and classification studies.

Formalin is the most widely used fixative in routine diagnostic pathology laboratories and tissue fixation with this 10% neutral buffered formaldehyde solution results in DNA-protein cross-links formation and DNA fragmentation due to low pH upon fixation. Since genetic studies are based in DNA analysis throughout PCR-dependent techniques, recovering DNA from FFPE tissue samples has been proven a challenge. Fixation-induced DNA modifications include intra- and interstrand cross-linking (Srinivasan et al., 2002), extensive strand cleavage and base modification (Rait

et al., 2006). Translation research would benefit from the optimization of the DNA extraction protocols from these specimens, by allowing the isolation of great high quality DNA for downstream molecular biology applications.

Innumerous studies have focused on the optimal recovery of DNA from FFPE specimens by developing optimized methods for this purpose. However, the reproducibility of the different methodologies has been proven difficult, as different DNA quality assessment measures, FFPE inter-sample variability, DNA fragment size and different performed methods impair comparison of the different results. (Gilbert et al., 2007; Hostetter et al., 2009) For this reason, the second aim of the present work was to compare three different methods of DNA extraction from FFPE specimens obtained from the same pathological laboratory that were fixed using a routine and well establish method and obtained from the same tissue source in order to minimize sample to sample variation for the development of an efficient and reproducible method of DNA extraction from FFPE tongue tumor samples.

For DNA extraction optimization, 5 histological cuts of 20 μm thick paraffin sections were obtained from each FFPE resection block, for a total of 37 FFPE samples obtained from 16 TSCC patients of the 31 previously analysed and 1 healthy individual. DNA extraction was performed by three different methods, A, B and C. Method A consisted of simultaneous paraffin removal and proteinase K digestion, whereas method B and C consisted of heat paraffin removal and xylol deparaffinization, respectively, followed by proteinase K digestion. Genomic DNA isolation was then performed by column-based DNA extraction commercial kit. As accurate assessment of extracted DNA quantity and quality is essential for successful molecular downstream applications and particular important for appraisal of FFPE DNA extracts as these frequently exhibit high degrees of fragmentation and contamination, spectroscopy and flourimetric analysis was performed by NanoDrop and Qubit assay, respectively.

Determination of DNA concentrations was performed by Qubit assay, as this represents a more adequate methodology than NanoDrop for accurate evaluation of DNA quantity present in samples containing low amounts of DNA that are characteristic of extraction from FFPE samples. NanoDrop showed a 3 to 4-fold increase in registered concentrations values than the obtained using Qubit assay. This is explained by the fact that NanoDrop UV absorbance is not selective; resulting in higher concentration values that are influenced by the presence of contaminants such as proteins, salts, organic compounds and paraffin wax in DNA extracts. Although

spectrophotometer measurement is not proper for FFPE DNA quantification, it is very accurate for evaluation of the FFPE extracts purity, by A260/A280 and A260/A230 ratios measurement. The first indicates the presence or absence of contaminating proteins and the second whether organic compounds such as guanidinum isothiocyanate, alchohol and phenol, as well as cellular carbohydrates are present. DNA samples with A260/A280 ratio values of 1.8 to 2.0 and A260/A230 ratio values >2 were considered high-quality/pure. (Thermo Fisher Scientific, 2008)

The total amount of DNA extracted was highly dependent on the method used. The medium amounts of extracted DNA from each method were 24.55 (±12.12) ng/µl, 25.76 (±9.27) ng/µl and 37.14 (±7.24) ng/µl for methods A, B and C, respectively. Samples consisted of 5 histological cuts of 20 µm thick paraffin sections, but whenever only small tumor tissue areas were identified, more cuts were performed to obtain optimal tissue quantity. Although, similar tissue quantity was used for extraction, laser-based microdissection of present FFPE samples would allow a more precise cut of the tumor tissue, thus sustaining a consistently amount of tissue in all samples for a more rigorous evaluation of DNA yields variation using the three different methods. Laser-based microdissection of FFPE tissue samples enables a more detailed molecular analysis of tumors, allowing the retrieval of representative tumor content without the contamination of normal cells, as well as obtaining cells subpopulations of interest.

Additionally, A260/A280 and A260/A230 ratios revealed that method C was the best in terms of DNA extracts purity, followed by method A and B. The present work demonstrated that method C was the most adequate for DNA extraction from FFPE tongue tumor samples and that xylol treatment allows for efficient paraffin removal from these specimens. On the contrary, heat paraffin removal is not as efficient as xylol deparaffinization, resulting in higher contamination of DNA extracts. Tissue digestion after paraffin removal performed in method B and C demonstrated to be more efficient than simultaneous deparaffinization and digestion. Further, successful DNA extraction obtained with method C also reflects the longer time needed when performing the present protocol, as longer incubation times seems to enhance paraffin removal, tissue digestion and decrease the amount of contaminants.

Four samples extracted with method C and presenting low yields of isolated DNA were selected to be concentrated in a speed vac. After concentration, these 4 FFPE DNA samples showed a 1.5-fold increase in DNA concentration and continued to

have high purity DNA, representing suitable samples for downstream molecular biology applications.

Since determination of FFPE derived DNA fragment sizes is crucial to ensure downstream processing suitability, an agarose gel electrophoresis was performed in order to assess DNA integrity and the average molecular weight of DNA samples extracted from 14 FFPE tissue specimens and 3 fresh-frozen tissues (Figure). DNA extracts from fresh-frozen tissues did not present fragmentation. In contrast, FFPE DNA samples showed significant fragmentation and lower concentration. Extracted FFPE DNA samples using method C had relatively higher DNA amounts when compared with samples extracted using method A and B and control sample extracted using method C. FFPE DNA concentrated samples showed increasing DNA amounts. DNA extraction from FFPE tissue samples often results in high fragmentation, influenced by the fixation process and how long the tissue was fixed for. It can also be affected by the time since surgical resection until fixation, as tissues experience anoxia and environmental changes. The storage time has also been suggested to contribute to FFPE DNA fragmentation, but studies report this as a minor effect. (Srinivasan et al., 2002) The present study was unable to determine the latter, since analyzed FFPE samples had been stored for a relative similar period.

The development of methods that allow the recovery of optimal quality DNA from FFPE tissues is essential to increase the cohorts for cancer research. The present work shows an efficient and reproducible method of DNA extraction from FFPE tongue tumor samples, yielding relatively high concentrations and high purity DNA.

At the present, the developed method opens the possibility of using the existing large archives of FFPE tongue tumor samples in retrospective genetic studies, as these specimens have associated pathological and clinical information. This will allow association of the genetic and epigenetic events underlying TSCC malignant transformation and progression with patients' clinicopathological features. Establishing genotype-phenotype relations will aid in the determination of the clinical significance of putative molecular markers underlying tongue tumorigeneis. This will improve diagnosis, prognosis and accurate risk-stratification of patients, as well as choose the most adequate treatment and predict treatment response. Still, the suitability of FFPE DNA extracts obtained with the present method for application in downstream molecular biology techniques such as MS-MLPA needs to be further assessed in future studies. Caution must be taken in adapting information obtained through FFPE samples

analysis, as DNA fragmentation may lead to the identification of false-positives and alterations in gene expression profiles may also occur after the resection until tissue fixation. Thus comparing the genetic profile of fresh-frozen tissues and FFPE tissues is imperative to validate the latter application in future studies.

CHAPTER 5

CONCLUSIONS

Chapter 5 - Conclusions

The present thesis has focused on the identification of genetic and epigenetic alterations underlying TSCC as putative biomarkers involved in tongue malignant transformation and progression. Additionally, the present work aimed to optimize a protocol for genomic DNA extraction from FFPE tongue tumor samples. The following conclusions were drawn from the realization of the present thesis:

- MS-MLPA technique demonstrated to be an efficient and reliable method for the multiple parallel analyses of quantitative genetic and epigenetic alterations present in these tumors.

- MS-MLPA analysis of 31 fresh-frozen TSCC tumor samples enabled the detection of copy number alterations, namely gains and losses of genetic material, of 37 from a total of 38 cancer-related genes and promoter methylation of 14 of the 25 tumor suppressor genes analyzed.

- The most frequently altered genes in the present cohort were *WT1*, *PAX5*, *GATA5*, *MSH6*, *PYCARD*, *STK11*, *CDKN2A*, *CHFR*, *BRCA1*, *GSTP1*, *TP53*, *RARB* and *CADM1*, suggesting the important role of the present genes in the development and progression of TSCC.

- Promoter methylation of *WT1* was the most frequent alteration and was present in 77.42% of the tongue tumors analyzed, suggesting the important role of this gene as a tumor suppressor in TSCC carcinogenesis. Also, given the fact that overexpression of *WT1* has been associated with poor histological differentiation and increased tumor stage, the present gene represents a potential prognostic marker in TSCC.

- *PAX5* was the second most frequently altered gene in the tumor samples analyzed (67.74%), indicating the important role of this gene in tongue malignant transformation and progression. The present gene showed promoter methylation in 48.39% of tumors. Since this gene is associated with a more favorable clinical outcome with improved survival, methylation of *PAX5* gene

represents a potential molecular marker for prognosis and patient outcome prediction.

- Methylation of *MSH6* gene showed elevated association with advanced tumor stage and metastasis in TSCC. In order to more firmly establish the correlation between genetic and epigenetic changes and clinicopathological features of patients with TSCC, a larger number of samples are warranted.

- The development of methods that allow the recovery of optimal quality DNA from FFPE specimens is essential to increase cancer cohorts. Thus, from the three DNA extraction methods tested, method C represents an efficient and reproducible method of DNA extraction from FFPE tongue tumor samples, yielding relatively high concentrations and high purity DNA. These open new possibilities for TSCC genetic and epigenetic research.

CHAPTER 6

FUTURE PERSPECTIVES

Chapter 6 – Future Perspectives

TSCC is the most common malignancy in the oral cavity, characterized by high recurrence rates, reduced overall survival and increasing incidence worldwide. Advanced stage at initial diagnosis, locoregional recurrence, further primary tumors and lymph node metastasis contribute to the limited survival and high mortality rates in OSCC. (Silva et al., 2011) Despite the advances in cancer diagnosis, management and treatment, patients' outcome has not significantly improved in the last decades. Furthermore, patients diagnosed with TSCC have a poorer prognosis and survival when compared with patients diagnosed with other oral cavity and head and neck cancer subsites. (Goldstein et al., 2012; Rusthoven, Ballonoff, Raben, & Chen, 2008) This allied to the fact that the current clinicopathological staging system is not consistently capable of identifying patients with high risk disease, highlights the need for prognostic biomarkers to be identified. In this context, unraveling the genetic and epigenetic alteration patterns present in TSCC will allow for the establishment of candidate genes, as well as determine their clinical significance, providing a great opportunity to identify the molecular pathways underlying the development and progression of the disease.

Analysis of the genetic and epigenetic profile of 31 patients with TSCC in present study sought to determine the association between the tongue-specific genotype with patients' clinicopathological features, with the purpose of identify putative molecular markers for further improvement of diagnosis, prognosis and accurate risk-stratification of patients, as well as choose the most adequate treatment and predict treatment response in TSCC. However, limitations in establishing associations between genetic and epigenetic changes and clinicopathological features, as well as difficulty in detecting other alterations less relevant in the analyzed population of tumors, revealed the need for analyzing a greater amount of tumor samples. Since, FFPE tissues have been used in diagnostic pathology for decades, a vast amount of samples is readily accessible for molecular research. Still the most significant advantage of these archival specimens is the associated clinical and pathological information obtained over the treatment course of patients, allowing retrospective studies to be performed. DNA isolation from FFPE tissues is impaired by formalin cross-links formation and paraffin wax, resulting in low concentration yields and fragmented DNA. With the achievement of our secondary objective we optimized a protocol for DNA extraction from FFPE specimens of TSCC with relatively high concentrations and high purity DNA. Thus, the

suitability of FFPE DNA extracts obtained with this present method for application in downstream molecular biology techniques such as MS-MLPA needs to be further assessed in future studies. Caution must be taken in adapting information obtained through FFPE samples analysis, as is possible that alterations in gene expression profiles can occur either during and/or after the resection of the tissue. Thus comparing the genetic profile of fresh-frozen tissues and FFPE tissues is imperative to validate the latter application for future studies. After validation of FFPE tissues samples applicability in this type of genetic and epigenetic analysis for these particular tumors, great amounts of samples can be readily analyzed allowing for genotype-phenotype correlations. For instance, analysis of well characterized sub-set of tumors will allow the identification of phenotype-specific putative prognostic markers. In cases of tumor reoccurrence, the comparison between the genomic alterations present in the primary tumor and the secondary one will allow the establishment of bimolecular markers predictive of tumor recurrence.

The establishment of putative molecular markers involved in tongue malignant transformation and progression will allow the development of TSCC-specific probemix panels for MS-MLPA analysis of these tumors, warranting the future application of the MS-MLPA technique in clinical practice for early detection and patient's follow-up. FFPE tongue tumor samples analysis in large cohorts by grouping tumors according to well characterized clinical and pathological features may even result in the development of even more specific panels for the detection of patients histological types, tumor grade and treatment outcome prediction.

CHAPTER 7

REFERENCES

Chapter 7 - References

Acharya, S., Wilson, T., Gradia, S., Kane, M. F., Guerrette, S., Marsischky, G. T., et al. (1996). hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(November), 13629–13634. doi:10.1073/pnas.93.24.13629

Adams, B., Dorfler, P., Aguzzi, A., Kozmik, Z., Urbánek, P., Maurer-Fogy, I., et al. (1992). Pax-5 encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes and Development*, *6*, 1589–1607. doi:10.1101/gad.6.9.1589

Agilent Technologies. (2008). Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis (ULS Labeling for Blood, Cells, Tissues or FFPE). Retrieved April 15, 2015, from http://www.chem-agilent.com/pdf/G4410-90020.pdf

Agrawal, N., Frederick, M. J., Pickering, C. R., Bettegowda, C., Chang, K., Li, R. J., et al. (2011). Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science (New York, N.Y.)*, *333*(August), 1154–1157. doi:10.1126/science.1206923

Akiyama, Y., Watkins, N., Suzuki, H., Jair, K.W., van Engeland, M., Esteller, M., et al. (2003). GATA-4 and GATA-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. *Molecular and Cellular Biology*, *23*(23), 8429–8439. doi:10.1128/MCB.23.23.8429-8439.2003

Algar, E. M., Khromykh, T., Smith, S. I., Blackburn, D. M., Bryson, G. J., & Smith, P. J. (1996). A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukaemia cell lines. *Oncogene*, *12*(5), 1005–1014.

Allinen, M., Peri, L., Kujala, S., Lahti-Domenici, J., Outila, K., Karppinen, S. M., et al. (2002). Analysis of 11q21-24 loss of heterozygosity candidate target genes in breast cancer: Indications of TSLC1 promoter hypermethylation. *Genes Chromosomes and Cancer*, *34*(4), 384–389. doi:10.1002/gcc.10079

Asakawa, T., Esumi, M., Endo, S., Kida, A., & Ikeda, M. (2008). Tongue cancer patients have a high frequency of allelic loss at the von Hippel-Lindau gene and other loci on 3p. *Cancer*, *112*(3), 527–534. doi:10.1002/cncr.23200

Babjuk, M., Soukup, V., Mareš, J., Dušková, J., Sedláček, Z., Trková, M., et al. (2002). The expression of PAX5, p53 immunohistochemistry and p53 mutation analysis in superficial bladder carcinoma tissue. Correlation with pathological findings and clinical outcome. *International Urology and Nephrology*, *34*, 495–501. doi:10.1023/A:1025652203472

Banno, K., Yanokura, M., Kawaguchi, M., Kuwabara, Y., Akiyoshi, J., Kobayashi, Y., et al. (2007). Epigenetic inactivation of the CHFR gene in cervical cancer

contributes to sensitivity to taxanes. *International Journal of Oncology, 31*, 713–720.

Baylin, S. B. (2005). DNA methylation and gene silencing in cancer. *Nature Clinical Practice. Oncology, 2 Suppl 1*(December), S4–S11. doi:10.1038/ncponc0354

Baylin, S. B., & Jones, P. A. (2011). A decade of exploring the cancer epigenome — biological and translational implications. *Nature Reviews Cancer, 11*, 726–734. doi:10.1038/nrc3130

Bento, M. (2009). Comparação da incidência de cancro nos três registos oncológicos regionais, em 2011. *Roreno*.

Bernier, J., & Cooper, J. S. (2005). Chemoradiation after surgery for high-risk head and neck cancer patients: how strong is the evidence? *The Oncologist, 10*, 215–224. doi:10.1634/theoncologist.10-3-215

Bettendorf, O., Piffkò, J., & Bànkfalvi, a. (2004). Prognostic and predictive factors in oral squamous cell cancer: Important tools for planning individual therapy? *Oral Oncology, 40*, 110–119. doi:10.1016/j.oraloncology.2003.08.010

Bova, R. J., Quinn, D. I., Nankervis, J. S., Cole, I. E., Sheridan, B. F., Jensen, M. J., et al. (1999). Cyclin D1 and p16 INK4A Expression Predict Reduced Survival in Carcinoma of the Anterior Tongue. *Clin Cancer Res, 5*, 2810–2819.

Brett, A., Pandey, S., & Fraizer, G. (2013). The Wilms' tumor gene (WT1) regulates E-cadherin expression and migration of prostate cancer cells. *Molecular Cancer, 12*(1), 3. doi:10.1186/1476-4598-12-3

Call, K. M., Glaser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A, et al. (1990). Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell, 60*, 509–520.

Cancela, M. C., Voti, L., Guerra-Yi, M., Chapuis, F., Mazuir, M., & Curado, M., P. (2010). Oral cavity cancer in developed and in developing countries: population-based incidence. *Head and Neck, 32*, 357-367. doi:10.1002/hed.21193

Cao, J., Zhou, J., Gao, Y., Gu, L., Meng, H., Liu, H., & Deng, D. (2009). Methylation of p16 CpG island associated with malignant progression of oral epithelial dysplasia: a prospective cohort study. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 15*(16), 5178–5183. doi:10.1158/1078-0432.CCR-09-0580

Chen, K., Sawhney, R., Khan, M., Benninger, M. S., Hou, Z., Sethi, S., et al. (2007). Methylation of multiple genes as diagnostic and therapeutic markers in primary head and neck squamous cell carcinoma. *Archives of Otolaryngology--Head & Neck Surgery, 133*(11), 1131–1138. doi:10.1001/archotol.133.11.1131

Collard, R. L., Harya, N. S., Monzon, F. A., Maier, C. E., & O'Keefe, D. S. (2006). Methylation of the ASC gene promoter is associated with aggressive prostate cance. *Prostate*, *66*(7), 687–695.

Contreras, C. M., Gurumurthy, S., Haynie, J. M., Shirley, L. J., Akbay, E. A., Wingo, S. N., et al. (2008). Loss of Lkb1 provokes highly invasive endometrial adenocarcinomas. *Cancer Research*, *68*(3), 759–766. doi:10.1158/0008-5472.CAN-07-5014

Conway, K. E., McConnell, B. B., Bowring, C. E., Donald, C. D., Warren, S. T., & Vertino, P. M. (2000). TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. *Cancer Research*, *60*, 6236–6242.

Costello, J. F., Frühwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., et al. (2000). Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nature Genetics*, *24*(february), 132–138. doi:10.1038/72785

Crowe, D. L., Hacia, J. G., Hsieh, C. L., Sinha, U. K., & Rice, H. (2002). Molecular pathology of head and neck cancer. *Histology and Histopathology*, *17*, 909–914.

Davies, R., Moore, A., Schedl, A., Bratt, E., Miyahawa, K., Ladomery, M., et al. (1999). Multiple roles for the Wilms' tumor suppressor, WT1. Retrieved from http://cancerres.aacrjournals.org/content/59/7_Supplement/1747s.abstract

Demokan, S., & Dalay, N. (2011). Role of DNA methylation in head and neck cancer. *Clinical Epigenetics*, *2*, 123–150. doi:10.1007/s13148-011-0045-3

Denaro, N., Lo Nigro, C., Natoli, G., Russi, E. G., Adamo, V., & Merlano, M. C. (2011). The Role of p53 and MDM2 in Head and Neck Cancer. *ISRN Otolaryngology*, *2011*, 1–8. doi:10.5402/2011/931813

Drexler, S. K., Bonsignore, L., Masin, M., Tardivel, A., Jackstadt, R., & Hermeking, H. (2012). Tissue-specific opposing functions of the inflammasome adaptor ASC in the regulation of epithelial skin carcinogenesis. *Proc Natl Acad Sci USA*, *109*(45), 18384–18389. doi:10.1073/pnas.1209171109/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1209171109

Drummond, I. A, Madden, S. L., Rohwer-Nutter, P., Bell, G. I., Sukhatme, V. P., & Rauscher, F. J. (1992). Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science (New York, N.Y.)*, *257*(July), 674–678.

Ebrahimi, A., Murali, R., Gao, K., Elliott, M. S., & Clark, J. R. (2011). The prognostic and staging implications of bone invasion in oral squamous cell carcinoma. *Cancer*, *117*, 4460–4467. doi:10.1002/cncr.26032

Esteller, M. (2002). CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene*, *21*, 5427–5440. doi:10.1038/sj.onc.1205600

Fakhry, C., Westra, W. H., Li, S., Cmelak, A., Ridge, J. A., Pinto, H., et al. (2008). Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *Journal of the National Cancer Institute*, *100*, 261–269. doi:10.1093/jnci/djn011

Feinberg, A. P., & Tycko, B. (2004). The history of cancer epigenetics. *Nature Reviews. Cancer*, *4*(February), 143–153. doi:10.1038/nrc1279

Felsberg, J., Thon, N., Eigenbrod, S., Hentschel, B., Sabel, M. C., Westphal, M., et al. (2011). Promoter methylation and expression of MGMT and the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 in paired primary and recurrent glioblastomas. *International Journal of Cancer*, *129*, 659–670. doi:10.1002/ijc.26083

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M. et al. (2015). Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer*, *136*, E359-E386.

Fukami, T., Fukuhara, H., Kuramochi, M., Maruyama, T., Isogai, K., Sakamoto, M., et al. (2003). Promoter methylation of the TSLC1 gene in advanced lung tumors and various cancer cell lines. *International Journal of Cancer*, *107*(April), 53–59. doi:10.1002/ijc.11348

Galm, O., Wilop, S., Reichelt, J., Jost, E., Gehbauer, G., Herman, J. G., & Osieka, R. (2004). DNA methylation changes in multiple myeloma. *Leukemia : Official Journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, *18*, 1687–1692. doi:10.1038/sj.leu.2403434

Gao, Y. J., Xin, Y., Zhang, J. J., & Zhou, J. (2008). Mechanism and pathobiologic implications of CHFR promoter methylation in gastric carcinoma. *World Journal of Gastroenterology*, *14*(32), 5000–5007. doi:10.3748/wjg.14.5000

Gao, Y., Lou, G., Zhang, G. M., Sun, X. W., Ma, Y. Y., Yang, Y. M., & Liu, G. (2009). CHFR promoter hypermethylation and reduced CHFR mRNA expression in ovarian cancer. *Int J Biol Markers*, *24*(2), 83–89.

Gasche, J. A., Hoffmann, J., Boland, C. R., & Goel, A. (2011). Interleukin-6 Promotes Tumorigenesis by Altering DNA Methylation in Oral Cancer Cells. *Int J Cancer*, *129*(5), 1053–1063. doi:10.1002/ijc.25764

Gazzoli, I., & Kolodner, R. D. (2003). Regulation of the Human MSH6 Gene by the Sp1 Transcription Factor and Alteration of Promoter Activity and Expression by Polymorphisms. *Molecular and Cellular Biology*, *23*(22), 7992–8007. doi:10.1128/MCB.23.22.7992

Geisler, S. A., & Olshan, A. F. (2001). GSTM1, GSTT1, and the risk of squamous cell carcinoma of the head and neck: a mini-HuGe review. *American Journal of Epidemiology*, *154*(2), 95–105.

Genschel, J., Littman, S. J., Drummond, J. T., & Modrich, P. (1998). Isolation of MutSbeta from human cells and comparison of the mismatch repair specificities of MutSbeta and MutSalpha. *The Journal of Biological Chemistry*, *273*(31), 19895–19901. doi:10.1074/jbc.273.31.19895

Gilbert, M. T. P., Haselkorn, T., Bunce, M., Sanchez, J. J., Lucas, S. B., Jewell, L. D., et al. (2007). The Isolation of Nucleic Acids from Fixed, Paraffin-Embedded Tissues-Which Methods Are Useful When? *PLoS ONE*, *2*(6). doi:10.1371/journal.pone.0000537

Gillison, M. L., Koch, W. M., Capone, R. B., Spafford, M., Westra, W. H., Wu, L., et al. (2000). Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *Journal of the National Cancer Institute*, *92*(9), 709–720. doi:10.1093/jnci/92.9.709

Goepfert, R. P., Kezirian, E. J., & Wang, S. J. (2014). Oral Tongue Squamous Cell Carcinoma in Young Women: A Matched Comparison—Do Outcomes Justify Treatment Intensity? *ISRN Otolaryngology*, *2014*, 1–6. doi:10.1155/2014/529395

Goldstein, D. P., Bachar, G. Y., Lea, J., Shrime, M. G., Patel, R. S., Gullane, P. J. et al. (2012). Outcomes of squamous cell cancer of the oral tongue managed at the Princess Margaret Hospital. Head and Neck. doi:10.1002/hed.23001

Gollin, S. M. (2014). Cytogenetic Alterations and their Molecular Genetic Correlates in Head and Neck Squamous Cell Carcinoma: A Next Generation Window to the Carcinoma: A Next Generation Window to the Biology of Disease. *Genes, Chromosomes & Cancer, 53*, 972-990. doi:10.1002/gcc.22214

Goodyer, P., Dehbi, M., Torban, E., Bruening, W., & Pelletier, J. (1995). Repression of the retinoic acid receptor-alpha gene by the Wilms' tumor suppressor gene product, wt1. *Oncogene*, *10*(6), 1125–1129.

Govindaraja, C., Chandramouli, A., & Chandramouli, C. (2010). P53 mutations in head and neck squamous cell carcinoma. *Int J Pharm Biomed Res*, *1*(October 2006), 117–121. Retrieved from http://www.researchgate.net/publication/46418117_8._P53_mutations_in_head_and_neck_squamous_cell_carcinoma/file/72e7e51a92070dec0a.pdf

Gregg, J. L., Brown, K. E., Mintz, E. M., Piontkivska, H., & Fraizer, G. C. (2010). Analysis of gene expression in prostate cancer epithelial and interstitial stromal cells using laser capture microdissection. *BMC Cancer*, *10*, 165. doi:10.1186/1471-2407-10-165

Guerrero-Preston, R., Michailidi, C., Marchionni, L., Pickering, C. R., Frederick, M. J., Myers, J. N., et al. (2014). Key tumor suppressor genes inactivated by “greater promoter” methylation and somatic mutations in head and neck cancer. *Epigenetics*, *9*(May 2015), 1031–1046. doi:10.4161/epi.29025

Guo, M., Akiyama, Y., House, M. G., Hooker, C. M., Heath, E., Gabrielson, E., et al. (2004). Hypermethylation of the GATA genes in lung cancer. *Clinical Cancer Research*, *10*(410), 7917–7924. doi:10.1158/1078-0432.CCR-04-1140

Guo, M., House, M. G., Akiyama, Y., Qi, Y., Capagna, D., Harmon, J., et al. (2006). Hypermethylation of the GATA gene family in esophageal cancer. *International Journal of Cancer*, *119*(April), 2078–2083. doi:10.1002/ijc.22092

Ha, P. K., & Califano, J. a. (2006). Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. *Lancet Oncology*, *7*, 77–82. doi:10.1016/S1470-2045(05)70540-4

Haddad, R. I., & Shin, D. M. (2008). Recent Advances in Head and Neck Cancer. *The New England Journal of Medicine*, *359*, 1143–1154.

Hama, T., Yuza, Y., Saito, Y., O-uchi, J., Kondo, S., Okabe, M., et al. (2009). Prognostic significance of epidermal growth factor receptor phosphorylation and mutation in head and neck squamous cell carcinoma. *The Oncologist*, *14*, 900–908. doi:10.1634/theoncologist.2009-0058

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. doi:10.1016/j.cell.2011.02.013

Hannen, E. J. M., Macville, M. V. E., Wienk, S. M., Slootweg, P. J., Manni, J. J., Hanselaar, A. G. J. M., & De Wilde, P. C. M. (2004). Different chromosomal imbalances in metastasized and nonmetastasized tongue carcinomas identified by comparative genomic hybridization. *Oral Oncology*, *40*, 364–371. doi:10.1016/j.oraloncology.2003.09.011

Harris, S. L., & Levine, A. J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene*, *24*, 2899–2908. doi:10.1038/sj.onc.1208615

Hashibe, M., Brennan, P., Benhamou, S., Castellsague, X., Chen, C., Curado, M. P., et al. (2007). Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: Pooled analysis in the international head and neck cancer epidemiology consortium. *Journal of the National Cancer Institute*, *99*(10), 777–789. doi:10.1093/jnci/djk179

Hayama, M., Uzawa, K., Kasamatsu, A., Higo, M., Norhany, S., Kouzu, Y., et al. (2009). Downregulation of Tumour Suppressor Gene TSLC1 mRNA in Human Oral Squamous Cell Carcinoma. *Asian Journal of Oral and Maxillofacial Surgery*, *21*(3-4), 75–80. doi:10.1016/S0915-6992(09)80001-0

Heaton, C. M., Durr, M. L., Tetsu, O., Van Zante, A., & Wang, S. J. (2014). TP53 and CDKN2a mutations in never-smoker oral tongue squamous cell carcinoma. *Laryngoscope*, *124*(7), 267–273. doi:10.1002/lary.24595

Hellebrekers, D. M. E. I., Lentjes, M. H. F. M., Van Den Bosch, S. M., Melotte, V., Wouters, K. a D., Daenen, K. L. J., et al. (2009). GATA4 and GATA5 are potential

tumor suppressors and biomarkers in colorectal cancer. *Clinical Cancer Research*, *15*(16), 3990–3997. doi:10.1158/1078-0432.CCR-09-0055

Herrero, R., Castellsagué, X., Pawlita, M., Lissowska, J., Kee, F., Balaram, P., et al. (2003). Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. *Journal of the National Cancer Institute*, *95*(23), 1772–1783. doi:10.1093/jnci/djg107

Hiltunen, M. O., Koistinaho, J., Alhonen, L., Myöhänen, S., Marin, S., Kosma, V. M., et al. (1997). Hypermethylation of the WT1 and calcitonin gene promoter regions at chromosome 11p in human colorectal cancer. *British Journal of Cancer*, *76*, 1124–1130. doi:10.1038/bjc.1997.522

Homig-Holzel, C., & Savola, S. (2012). Multiplex ligation-dependent probe amplification (MLPA) in tumor diagnostics and prognostics. *Diagn Mol Pathol*, *21*(4), 189–206. doi:10.1097/PDM.0b013e3182595516

Hostetter, G., Kim, S. Y., Savage, S., Gooden, G. C., Barrett, M., Zhang, J., et al. (2009). Random DNA fragmentation allows detection of single-copy, single-exon alterations of copy number by oligonucleotide array CGH in clinical FFPE samples. *Nucleic Acids Research*, *38*(2), 1–12. doi:10.1093/nar/gkp881

Hylander, B., Repasky, E., Shrikant, P., Intengan, M., Beck, A., Driscoll, D., et al. (2006). Expression of Wilms tumor gene (WT1) in epithelial ovarian cancer. *Gynecologic Oncology*, *101*, 12–17. doi:10.1016/j.ygyno.2005.09.052

Hynes, N. E., & Lane, H. a. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. *Nature Reviews. Cancer*, *5*(5), 341–354. doi:10.1038/nrc1667

Inoue, K., Sugiyama, H., Ogawa, H., Nakagawa, M., Yamagami, T., Miwa, H., et al. (1994). WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood*, *84*, 3071–3079.

Instituto Português de Oncologia do Porto. (2008). R. *Registo Oncológico Do IPO-PORTO - 2008*.

Jeuken, J., Cornelissen, S., Boots-Sprenger, S., Gijsen, S., & Wesseling, P. (2006). Multiplex ligation-dependent probe amplification: a diagnostic tool for simultaneous identification of different genetic markers in glial tumors. *The Journal of Molecular Diagnostics : JMD*, *8*(4), 433–443. doi:10.2353/jmoldx.2006.060012

Jeuken, J. W. M., Cornelissen, S. J. B., Vriezen, M., Dekkers, M. M. G., Errami, A., Sijben, A., et al. (2007). MS-MLPA: an attractive alternative laboratory assay for robust, reliable, and semiquantitative detection of MGMT promoter hypermethylation in gliomas. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, *87*(October), 1055–1065. doi:10.1038/labinvest.3700664

Jin, C., & Jin, Y. (2011). Head and neck: Oral squamous cell carcinoma. *Atlas of Genetics and Cytogenetics in Oncology and Haematology*, *11*(1), 46–49. doi:10.4267/2042/38389

Jithesh, P. V., Risk, J. M., Schache, A. G., Dhanda, J., Lane, B., Liloglou, T., & Shaw, R. J. (2013). The epigenetic landscape of oral squamous cell carcinoma. *British Journal of Cancer*, *108*(2), 370–379. doi:10.1038/bjc.2012.568

Jomgeow, T., Oji, Y., Tsuji, N., Ikeda, Y., Ito, K., Tsuda, A., et al. (2006). Wilms' tumor gene WT1 17AA(-)/KTS(-) isoform induces morphological changes and promotes cell migration and invasion in vitro. *Cancer Science*, *97*(4), 259–270. doi:10.1111/j.1349-7006.2006.00169.x

Kaneuchi, M., Sasaki, M., Tanaka, Y., Shiina, H., Yamada, H., Yamamoto, R., & Dahiya, R. (2005). WT1 and WT1-AS Genes Are Inactivated by Promoter Methylation in Ovarian Clear Cell Adenocarcinoma. *Cancer*, *104*(9), 1924–1930. doi:10.1002/cncr.21397

Kastan, M. B., & Bartek, J. (2004). Cell-cycle checkpoints and cancer. *Nature*, *432*(November), 316–323. doi:10.1038/nature03097

Kreimer, A. R., Clifford, G. M., Boyle, P., & Franceschi, S. (2005). Human Papillomavirus Types in Head and Neck Squamous Cell Carcinomas Worldwide : A Systematic Review Human Papillomavirus Types in Head and Neck Squamous Cell Carcinomas Worldwide : A Systematic Review, *14*(February), 467–475. doi:10.1158/1055-9965.EPI-04-0551

La Vecchia, C., Lucchini, F., Negri, E., & Levi, F. (2004). Trends in oral cancer mortality in Europe. *Oral Oncology*, *40*, 433–439. doi:10.1016/j.oraloncology.2003.09.013

Lai, H. C., Lin, Y. W., Huang, T. H. M., Yan, P., Huang, R.-L., Wang, H. C., et al. (2008). Identification of novel DNA methylation markers in cervical cancer. *International Journal of Cancer. Journal International Du Cancer*, *123*, 161–167. doi:10.1002/ijc.23519

Laux, D. E., Curran, E. M., Welshons, W. V, Lubahn, D. B., & Huang, T. H. (1999). Hypermethylation of the Wilms' tumor suppressor gene CpG island in human breast carcinomas. *Breast Cancer Research and Treatment*, *56*, 35–43.

Laverriere, A. C., MacNeill, C., Mueller, C., Poelmann, R. E., Burch, J. B. E., & Evans, T. (1994). GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. *Journal of Biological Chemistry*, *269*(37), 23177–23184.

Lee, J. I., Soria, J. C., Hassan, K. a, El-Naggar, a K., Tang, X., Liu, D. D., et al. (2001). Loss of PTEN expression as a prognostic marker for tongue cancer. *Archives of Otolaryngology--Head & Neck Surgery*, *127*, 1441–1445.

Leemans, C. R., Braakhuis, B. J. M., & Brakenhoff, R. H. (2011). The molecular biology of head and neck cancer. *Nature Reviews. Cancer*, *11*(1), 9–22. doi:10.1038/nrc2982

Li, R., Faden, D. L., Fakhry, C., Jiao, Y., Wang, Y., Wilkerson, M. D., et al. (2014). Clinical, Genomic and Metagenomic Characterization of Oral Tongue Squamous Cell Carcinoma in Patients Who Do Not Smoke. *Head Neck*. doi:10.1002/hed.23807

Liao, C. T., Chang, J. T. C., Wang, H. M., Ng, S. H., Hsueh, C., Lee, L. Y., et al. (2007). Survival in squamous cell carcinoma of the oral cavity: Differences between pT4 N0 and other stage IVA categories. *Cancer*, *110*(June), 564–571. doi:10.1002/cncr.22814

Lim, A. M., Candiloro, I. L., Wong, N., Collins, M., Do, H., Takano, E. A, et al. (2014a). Quantitative methodology is critical for assessing DNA methylation and impacts on correlation with patient outcome. *Clinical Epigenetics*, *6*(1), 22. doi:10.1186/1868-7083-6-22

Lim, A. M., Do, H., Young, R. J., Wong, S. Q., Angel, C., Collins, M., et al. (2014b). Differential mechanisms of CDKN2A (p16) alteration in oral tongue squamous cell carcinomas and correlation with patient outcome. *International Journal of Cancer*, *135*, 887–895. doi:10.1002/ijc.28727

Lingen, M. W., Xiao, W., Schmitt, A., Jiang, B., Pickard, R., Kreinbrink, P., et al. (2013). Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. *Oral Oncology*, *49*(1), 1–8. doi:10.1016/j.oraloncology.2012.07.002

Liu, Y., Ji, Y., & Qiu, P. (2013). Identification of thresholds for dichotomizing DNA methylation data. *Journal on Bioinformatics and Systems Biology*, *8*. doi:10.1186/1687-4153-2013-8

Lo Muzio, L. (2001). A possible role for the WNT-1 pathway in oral carcinogenesis. *Critical Reviews in Oral Biology and Medicine : An Official Publication of the American Association of Oral Biologists*, *12*(2), 152–165.

Lo, W. L., Kao, S. Y., Chi, L. Y., Wong, Y. K., & Chang, R. C. S. (2003). Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: factors affecting survival. *Journal of Oral and Maxillofacial Surgery*, *61*(201), 751–758. doi:10.1016/S0278-2391(03)00149-6

Magdeldin, S., & Yamamoto, T. (2012). Toward deciphering proteomes of formalin-fixed paraffin-embedded (FFPE) tissues. *Proteomics*, *12*, 1045–1058. doi:10.1002/pmic.201100550

Marur, S., D'Souza, G., Westra, W. H., & Forastiere, A. a. (2010). HPV-associated head and neck cancer: A virus-related cancer epidemic. *The Lancet Oncology*, *11*(8), 781–789. doi:10.1016/S1470-2045(10)70017-6

Maruya, S., Issa, J. J., Weber, R. S., Rosenthal, D. I., Haviland, J. C., Lotan, R., & El-naggar, A. K. (2004). Differential Methylation Status of Tumor-Associated Genes in Head and Neck Squamous Carcinoma : Incidence and Potential Implications Differential Methylation Status of Tumor-Associated Genes in Head and Neck Squamous Carcinoma : Incidence and Potential Im, *10*(713), 3825–3830.

Massano, J., Regateiro, F. S., Januário, G., & Ferreira, A. (2006). Oral squamous cell carcinoma: Review of prognostic and predictive factors. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*, *102*, 67–76. doi:10.1016/j.tripleo.2005.07.038

Masuda, M., Yageta, M., Fukuhara, H., Kuramochi, M., Maruyama, T., Nomoto, A., & Murakami, Y. (2002). The tumor suppressor protein TSLC1 is involved in cell-cell adhesion. *Journal of Biological Chemistry*, *277*(34), 31014–31019. doi:10.1074/jbc.M203620200

Masumoto, J., Dowds, T. A., Schaner, P., Chen, F. F., Ogura, Y., Li, M., et al. (2003). ASC is an activating adaptor for NF-κB and caspase-8-dependent apoptosis. *Biochemical and Biophysical Research Communications*, *303*, 69–73. doi:10.1016/S0006-291X(03)00309-7

McConnell, B. B., & Vertino, P. M. (2004). TMS1/ASC: the cancer connection. *Apoptosis*, *9*(1), 5–18.

Mehta, V., Yu, G. P., & Schantz, S. P. (2010). Population-based analysis of oral and oropharyngeal carcinoma: Changing trends of histopathologic differentiation, survival and patient demographics. *Laryngoscope*, *120*(November), 2203–2212. doi:10.1002/lary.21129

Miller, C. S., & Johnstone, B. M. (2001). Human papillomavirus as a risk factor for oral squamous cell carcinoma: A meta-analysis, 1982-1997. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, *91*(6), 622–635. doi:10.1067/moe.2001.115392

Mitrovic, O., Micic, M., Cokic, V., Koko, V., Djikic, D., Budec, M., et al. (2013). BRCA1 and TOP2A gene amplification and protein expression in four molecular subtypes of breast cancer. *Archives of Biological Sciences*, *65*(2), 511–518. doi:10.2298/ABS1302511M

Moelans, C. B., Verschuur-Maes, A. H. J., & Van Diest, P. J. (2011). Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma in situ and invasive breast cancer. *Journal of Pathology*, *225*(June), 222–231. doi:10.1002/path.2930

Molinolo, A. A., Amornphimoltham, P., Squarize, C. H., Castilho, R. M., Patel, V., & Gutkind, J. S. (2009). Dysregulated molecular networks in head and neck carcinogenesis. *Oral Oncology*, *45*(4-5), 324–334. doi:10.1016/j.oraloncology.2008.07.011

Morse, D. E., Psoter, W. J., Cleveland, D., Cohen, D., Mohit-Tabatabai, M., Kosis, D. L., & Eisenberg, E. (2007). Smoking and drinking in relation to oral cancer and oral epithelial dysplasia. *Cancer Causes & Control : CCC*, *18*(9), 919–929. doi:10.1007/s10552-007-9026-4

MRC-Holland. (2015a). Methylation-specific MLPA® (MS-MLPA). Retrieved April 08, 2015, from https://mlpa.com/WebForms/WebFormMain.aspx

MRC-Holland. (2015b). SALSA MLPA ME002 Tumor supressor mix 2 probemix. Retrieved April 15, 2015, from http://www.mlpa.com/WebForms/WebFormProductDetails.aspx

National Cancer Institute. (2012a). Anatomy of the Oral Cavity. Retrieved January 26, 2015, from http://www.cancer.gov/cancertopics/pdq/treatment/lip-and-oral-cavity/Patient/page1/AllPages

National Cancer Institute. (2012b). Head and Neck Cancer Regions. Retrieved January 26, 2015, from http://www.cancer.gov/cancertopics/factsheet/Sites-Types/head-and-neck

Nikolaidis, G., Raji, O. Y., Markopoulou, S., Gosney, J. R., Bryan, J., Warburton, C., et al. (2012). DNA methylation biomarkers offer improved diagnostic efficiency in lung cancer. *Cancer Research*, *72*(22), 5692–5701. doi:10.1158/0008-5472.CAN-12-2309

Norhany, S., Kouzu, Y., Uzawa, K., Hayama, M., Higo, M., Koike, H. *et al.* (2006). Overexpression of PAX5 in oral carcinogenesis. *Oncology Reports*, *16*, 1003–1008.

Nygren, A. O. H., Ameziane, N., Duarte, H. M. B., Vijzelaar, R. N. C. P., Waisfisz, Q., Hess, C. J., et al. (2005). Methylation-Specific MLPA (MS-MLPA): Simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Research*, *33*(14), 1–9. doi:10.1093/nar/gni127

Oji, Y., Inohara, H., Nakazawa, M., Nakano, Y., Akahani, S., Nakatsuka, S., et al. (2003). Overexpression of the Wilms' tumor gene WT1 in head and neck squamous cell carcinoma. *Cancer Science*, *94*(6), 523–529.

Oji, Y., Ogawa, H., Tamaki, H., Oka, Y., Tsuboi, a, Kim, E. H., et al. (1999). Expression of the Wilms' tumor gene WT1 in solid tumors and its involvement in tumor cell growth. *Japanese Journal of Cancer Research : Gann*, *90*(February), 194–204. doi:S0910505099800647 [pii]

Oji, Y., Suzuki, T., Nakano, Y., Maruno, M., Nakatsuka, S.-I., Jomgeow, T., et al. (2004). Overexpression of the Wilms' tumor gene WT1 in primary astrocytic tumors. *Cancer Science*, *95*(10), 822–827.

Oji, Y., Yamamoto, H., Nomura, M., Nakano, Y., Ikeba, A., Nakatsuka, S., et al. (2003b). Overexpression of the Wilms' tumor gene WT1 in colorectal adenocarcinoma. *Cancer Science*, *94*(8), 712–717.

Oji, Y., Yano, M., Nakano, Y., Abeno, S., Nakatsuka, S., Ikeba, A., et al. (2004b). Overexpression of the Wilms'tumor gene WT1 in esophageal cancer. *Anticancer Research*, *24*(5B), 3103-3108.

Oka, Y., Tsuboi, a, Elisseeva, O. a, Udaka, K., & Sugiyama, H. (2002). WT1 as a novel target antigen for cancer immunotherapy. *Current Cancer Drug Targets*, *2*, 45–54. doi:10.2174/1568009023334088

Oka, Y., Tsuboi, A., Taguchi, T., Osaki, T., Kyo, T., Nakajima, H., et al. (2004). Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(38), 13885–13890. doi:10.1073/pnas.0405884101

Oki, E., Zhao, Y., Yoshida, R., Masuda, T., Ando, K., Sugiyama, M., et al. (2009). Checkpoint with forkhead-associated and ring finger promoter hypermethylation correlates with microsatellite instability in gastric cancer. *World Journal of Gastroenterology*, *15*, 2520–2525. doi:10.3748/wjg.15.2520

Overmeer, R. M., Henken, F. E., Snijders, P. J., Claassen-Kramer, D., Berkhof, J., Helmerhorst, T. J., et al. (2008). Association between dense CADM1 promoter methyation and reduced protein expression in high-grade CIN and cervical SCC. *Journal of Pathology*, *215*, 388–397. doi:10.1002/path.2367

Patel, S. C., Carpenter, W. R., Tyree, S., Couch, M. E., Weissler, M., Hackman, T., et al. (2011). Increasing incidence of oral tongue squamous cell carcinoma in young white women, age 18 to 44 years. *Journal of Clinical Oncology*, *29*(11), 1488–1494. doi:10.1200/JCO.2010.31.7883

Poeta, M. L., Manola, J., Goldwasser, M. A, Forastiere, A., Benoit, N., Califano, J. A, et al. (2007). TP53 mutations and survival in squamous-cell carcinoma of the head and neck. *The New England Journal of Medicine*, *357*, 2552–2561. doi:10.1056/NEJMoa073770

Poveda-Roda, R., Bagán, J. V., Jiménez-Soriano, Y., Margaix-Muñoz, M., & Sarrión-Pérez, G. (2010). Changes in smoking habit among patients with a history of oral squamous cell carcinoma (OSCC). *Medicina Oral, Patologia Oral Y Cirugia Bucal*, *15*(5), 4–9. doi:10.4317/medoral.15.e721

Qiu, W., Schönleben, F., Thaker, H. M., Goggins, M., & Su, G. H. (2006). A novel mutation of STK11/LKB1 gene leads to the loss of cell growth inhibition in head and neck squamous cell carcinoma. *Oncogene*, *25*(20), 2937–2942. doi:10.1038/sj.onc.1209325

Rait, V. K., Zhang, Q., Fabris, D., Mason, J. T., & O'Leary, T. J. (2006) Conversions of Formaldehyde-modified 2'-Deoxyadenosine 5'-Monophosphate in Conditions Modeling Formalin-fixed Tissue Dehydration. *J Histochem Cytochem, 54*(3), 301–310. doi:10.1369/jhc.5A6725.2005

Rankeillor, K. L., Cairns, D. A., Loughrey, C., Short, S. C., Chumas, P., Ismail, A., et al. (2014). Methylation-specific multiplex ligation-dependent probe amplification identifies promoter methylation events associated with survival in glioblastoma. *Journal of Neuro-Oncology*, *117*, 243–251. doi:10.1007/s11060-014-1372-y

Reed, a L., Califano, J., Cairns, P., Westra, W. H., Jones, R. M., Koch, W., et al. (1996). High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. *Cancer Research*, *56*, 3630–3633.

Roche Diagnostics GmbH. (2008). High Pure PCR Template Preparation Kit. Retrieved April 14, 2015, from https://lifescience.roche.com/wcsstore/RASCatalogAssetStore/Articles/05204933001_03.08.pdf

Rosen, E. M., Fan, S., & Isaacs, C. (2005). BRCA1 in hormonal carcinogenesis: Basic and clinical research. *Endocrine-Related Cancer*, *12*, 533–548. doi:10.1677/erc.1.00972

Rousseau, A., & Badoual, C. (2012). Head and Neck: Squamous cell carcinoma: an overview. *Atlas of Genetics and Cytogenetics in Oncology and Hematology*.

Rusthoven, K., Ballonoff, A., Raben, D., & Chen, C. (2008). Poor prognosis in patients with stage I and II oral tongue squamous cell carcinoma. *Cancer*, *112*(November), 345–351. doi:10.1002/cncr.23183

Ruwali, M., Pant, M. C., Shah, P. P., Mishra, B. N., & Parmar, D. (2009). Polymorphism in cytochrome P450 2A6 and glutathione S-transferase P1 modifies head and neck cancer risk and treatment outcome. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *669*, 36–41. doi:10.1016/j.mrfmmm.2009.04.007

Saiki, Y., Ogawa, T., Shiga, K., Sunamura, M., Kobayashi, T., & Horii, A. (2011). A Human Head and Neck Squamous Cell Carcinoma Cell Line with Acquired cis-Diamminedichloroplatinum-Resistance Shows Remarkable Upregulation of BRCA1 and Hypersensitivity to Taxane. *International Journal of Otolaryngology*, *2011*, 1–4. doi:10.1155/2011/521852

Sanchez-Cespedes, M. (2007). A role for LKB1 gene in human cancer beyond the Peutz-Jeghers syndrome. *Oncogene*, *26*(May), 7825–7832. doi:10.1038/sj.onc.1210594

Sanchez-Cespedes, M., Esteller, M., Wu, L., Nawroz-Danish, H., Yoo, G. H., Koch, W. M., et al. (2000). Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Research*, *60*(20), 892–895.

Sanchez-Cespedes, M., Parrella, P., Esteller, M., Nomoto, S., Trink, B., Engles, J. M., et al. (2002). Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Research*, *62* (13), 3659–3662.

Santos, L. L., & Teixeira, L. M. (2011). Oncologia Oral. *LIDEL - Edições Técnicas, LDA*

Scully, C., Field, J. K., & Tanzawa, H. (2000a). Genetic aberrations in oral or head and neck squamous cell carcinoma (SCCHN): 1. Carcinogen metabolism, DNA repair and cell cycle control. *Oral Oncology*, *36*, 256–263. doi:10.1016/S1368-8375(00)00007-5

Scully, C., Field, J. K., & Tanzawa, H. (2000b). Genetic aberrations in oral or head and neck squamous cell carcinoma 3: Clinico-pathological applications. *Oral Oncology*, *36*, 404–413. doi:10.1016/S1368-8375(00)00023-3

Shaw, R. J., Hall, G. L., Lowe, D., Liloglou, T., Field, J. K., Sloan, P., & Risk, J. M. (2008). The role of pyrosequencing in head and neck cancer epigenetics: correlation of quantitative methylation data with gene expression. *Otolaryngol Head Neck Surg*, *134*(3), 251–256. doi:10.1001/archoto.2007.50

Shen, Z., Wen, X. F., Lan, F., Shen, Z. Z., & Shao, Z. M. (2002). The tumor suppressor gene LKB1 is associated with prognosis in human breast carcinoma. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *8*(July), 2085–2090.

Shintani, S., Li, C., Ishikawa, T., Mihara, M., Nakashiro, K. I., & Hamakawa, H. (2004). Expression of vascular endothelial growth factor A, B, C, and D in oral squamous cell carcinoma. *Oral Oncology*, *40*, 13–20. doi:10.1016/S1368-8375(03)00127-1

Silva, S. D. Da, Ferlito, A., Takes, R. P., Brakenhoff, R. H., Valentin, M. D., Woolgar, J. A., et al. (2011). Advances and applications of oral cancer basic research. *Oral Oncology*, *47*(9), 783–791. doi:10.1016/j.oraloncology.2011.07.004

Sinha, P., Bahadur, S., Thakar, A., Matta, A., Macha, M., Ralhan, R., & Gupta, D. (2009). Significance of promoter hypermethylation of p16 gene for margin assessment in carcinoma tongue. *Head and Neck*, *31*(November), 1423–1430. doi:10.1002/hed

Smeets, S. J., Braakhuis, B. J. M., Abbas, S., Snijders, P. J. F., Ylstra, B., van de Wiel, M. A, et al. (2006). Genome-wide DNA copy number alterations in head and neck squamous cell carcinomas with or without oncogene-expressing human papillomavirus. *Oncogene*, *25*(October 2005), 2558–2564. doi:10.1038/sj.onc.1209275

Smith, E. M., Ritchie, J. M., Summersgill, K. F., Klussmann, J. P., Lee, J. H., Wang, D., et al. (2004). Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. *International Journal of Cancer. Journal International Du Cancer*, *108*(April 2003), 766–772. doi:10.1002/ijc.11633

Sobin, L. H., Gospodarowicz, M. K., & Witteking, C. (2009). TNM Classification of Malignant Tumours, 7th Edition. *International Union Against Cancer*.

Srinivasan, M., Sedmak, D., & Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *The American Journal of Pathology*, *161*(6), 1961–1971. doi:10.1016/S0002-9440(10)64472-0

Stone, A. R., Bobo, W., Brat, D. J., Devi, N. S., Van Meir, E. G., & Vertino, P. M. (2004). Aberrant methylation and down-regulation of TMS1/ASC in human glioblastoma. *The American Journal of Pathology*, *165*(4), 1151–1161. doi:10.1016/S0002-9440(10)63376-7

Stransky, N., Egloff, A. M., Tward, A. D., Kostic, A. D., Cibulskis, K., Sivachenko, A., et al. The mutational landscape of head and neck squamous cell carcinoma. *Science (New York, N.Y.)*, *333*(August), 1157–1160. doi:10.1126/science.1208130

Sugiyama, H. (2010). WT1 (Wilms' tumor gene 1): Biology and cancer immunotherapy. *Japanese Journal of Clinical Oncology*, *40*(April), 377–387. doi:10.1093/jjco/hyp194

Supic, G., Kozomara, R., Jovic, N., Zeljic, K., & Magic, Z. (2011). Hypermethylation of RUNX3 but not WIF1 gene and its association with stage and nodal status of tongue cancers. *Oral Diseases*, *17*, 794–800. doi:10.1111/j.1601-0825.2011.01838.x

Syed, N., Smith, P., Sullivan, A., Attard, M., Farrel, P., Wong, H., et al. (2005). Transcriptional silencing of Chfr predicts clinical outcome to chemoradiotherapy in head and neck cancer. *American Association of Cancer Research*, *46*.

Syrjänen, S. (2005). Human papillomavirus (HPV) in head and neck cancer. *Journal of Clinical Virology*, *32*, 59–66. doi:10.1016/j.jcv.2004.11.017

Tang, D., Kryvenko, O. N., Mitrache, N., Do, K. C., Jankowski, M., Chitale, D. A., et al. (2013) Methylation of the RARB gene increases prostate cancer risk in black Americans. *J Urol*, *190*(1):317-324. doi:10.1016/j.juro.2013.01.083

Takes, R. P., Rinaldo, A., Silver, C. E., Piccirillo, J. F., Haigentz, M., Suárez, C. et al. (2010). Future of the TNM classification staging system in head and neck cancer. *Head and Neck*, *32*, 1693-1711. doi:10.1002/hed.21361.

Temam, S., Kawaguchi, H., El-Naggar, A. K., Jelinek, J., Tang, H., Liu, D. D., et al. (2007). Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer. *Journal of Clinical Oncology*, *25*(16), 2164–2170. doi:10.1200/JCO.2006.06.6605

Thermo Fisher Scientific. (2008). NanoDrop 1000 Spectrophotometer V3.7 User's Manual. Retrieved April 14, 2015, from http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf

Thermo Fisher Scientific. (2014). Qubit® 3.0 Fluorometer – user guide. Retrieved April 15, 2015, from http://tools.lifetechnologies.com/content/sfs/manuals/qubit_3_fluorometer_man.pdf

Tsai, H.-C., & Baylin, S. B. (2011). Cancer epigenetics: linking basic biology to clinical medicine. *Cell Research*, *21*(3), 502–517. doi:10.1038/cr.2011.24

Uehara, M., Sano, K., Ikeda, H., Sekine, J., Irie, A., Yokota, T., et al. (2004). Expression of vascular endothelial growth factor and prognosis of oral squamous cell carcinoma. *Oral Oncology*, *40*, 321–325. doi:10.1016/j.oraloncology.2003.08.020

Ulanovski, D., Stern, Y., Roizman, P., Shpitzer, T., Popovtzer, A., & Feinmesser, R. (2004). Expression of EGFR and Cerb-B2 as prognostic factors in cancer of the tongue. *Oral Oncology*, *40*, 532–537. doi:10.1016/j.oraloncology.2003.11.004

Van Den Berg, R. M., Snijders, P. J. F., Grünberg, K., Kooi, C., Spreeuwenberg, M. D., Meijer, C. J. L. M., et al. (2011). Comprehensive CADM1 promoter methylation analysis in NSCLC and normal lung specimens. *Lung Cancer*, *72*(3), 316–321. doi:10.1016/j.lungcan.2010.10.005

Viet, C. T., & Schmidt, B. L. (2008). Methylation array analysis of preoperative and postoperative saliva DNA in oral cancer patients. *Cancer Epidemiology Biomarkers and Prevention*, *17*(December), 3603–3611. doi:10.1158/1055-9965.EPI-08-0507

Wakana, K., Akiyama, Y., Aso, T., & Yuasa, Y. (2006). Involvement of GATA-4/-5 transcription factors in ovarian carcinogenesis. *Cancer Letters*, *241*, 281–288. doi:10.1016/j.canlet.2005.10.039

Wei, Q., Eicher, S. A., Guan, Y., Cheng, L., Xu, J., Young, L. N., et al. (1998). Reduced Expression of hMLH1 and hGTBP/hMSH6: A Risk Factor for Head and Neck Cancer. *Cancer Epidemiology, Biomarkers & Prevention*, *7*(April), 309–314

Worsham, M. J., Chen, K. M., Meduri, V., Nygren A. O., Errami, A., Schouten, J.P., & Benninger M. S. (2006) Epigenetic events of disease progression in head and neck squamous cell carcinoma. *Otolaryngol Head Neck Surg*, *132*(6), 668–77.

Yalniz, Z., Demokan, S., Suoglu, Y., Ulusan, M., & Dalay, N. (2011). Simultaneous methylation profiling of tumor suppressor genes in head and neck cancer. *DNA and Cell Biology*, *30*(1), 17–24. doi:10.1089/dna.2010.1090

Yamagami, T., Sugiyama, H., Inoue, K., Ogawa, H., Tatekawa, T., Hirata, M., et al. (1996). Growth inhibition of human leukemic cells by WT1 (Wilms tumor gene) antisense oligodeoxynucleotides: implications for the involvement of WT1 in leukemogenesis. *Blood*, *87*(7), 2878–2884.

Yanokura, M., Banno, K., Kawaguchi, M., Hirao, N., Hirasawa, A., Susumu, N., et al. (2007). Relationship of aberrant DNA hypermethylation of CHFR with sensitivity to taxanes in endometrial cancer. *Oncology Reports*, *17*, 41–48.

Yokoyama, T., Sagara, J., Guan, X., Masumoto, J., Takeoka, M., Komiyama, Y., et al. (2003). Methylation of ASC/TMS1, a proapoptotic gene responsible for activating

procaspase-1, in human colorectal cancer. *Cancer Letters*, *202*, 101–108. doi:10.1016/j.canlet.2003.08.027

Zhu, W., Qin, W., Hewett, J. E., & Sauter, E. R. (2010). Quantitative evaluation of DNA hypermethylation in malignant and benign breast tissue and fluids. Int J Cancer, 126 (2), 474–482. doi:10.1002/ijc.24728

Zhuang, Z. G., Di, G. H., Shen, Z. Z., Ding, J., & Shao, Z. M. (2006). Enhanced expression of LKB1 in breast cancer cells attenuates angiogenesis, invasion, and metastatic potential. *Molecular Cancer Research : MCR*, *4*(November), 843–849. doi:10.1158/1541-7786.MCR-06-0118