Universidade de Aveiro Secção Autónoma das Ciências da Saúde

2015

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GENETIC AND EPIGENETIC CHARACTERIZATION OF LARYNGEAL CARCINOMA

CARACTERIZAÇÃO GENÉTICA E EPIGENÉTICA DO CARCINOMA DA LARINGE

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Isabel Marques Carreira, Professora Associada com Agregação da Faculdade de Medicina da Universidade de Coimbra, e co-orientação da Professora Doutora Sandra Rebelo, Professora Auxiliar Convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro.

“We should not be afraid of confrontations. Even the planets collide and chaos stars are born”

Charles Chaplin

o júri

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Agradecimentos

À Professora Doutora Isabel Carreira, pela oportunidade que me concedeu para realizar este projeto, pela orientação e todo o apoio no desenvolvimento deste trabalho

À Professora Doutora Sandra Rebelo cuja co-orientação foi essencial para a entrega deste trabalho. Agradeço também o acompanhamento e os ensinamentos dados não só durante o mestrado como também na licenciatura.

À Ilda Ribeiro, um especial agradecimento por toda a paciência que tiveste e por estares sempre pronta a ajudar-me durante a realização deste projeto. Obrigado por todas as dicas e conselhos que deste, tanto na componente prática como na análise e discussão dos resultados. Obrigado pela simpatia com que me recebeste e pelo convívio.

A toda a equipa do Laboratório de Citogenética e Genómica da Faculdade de Medicina da Universidade de Coimbra, pela simpatia com que me receberam e pelo convívio. Um agradecimento especial ao Nuno Lavoura e ao Miguel Pires por todo apoio prestado na componente prática.

Às meninas do LCG, Joana Rodrigues, Mariana Val, Sofia Lisboa, Inês Tavares e Marília Santiago pelo convívio e por todo o apoio.

Ao Centro Hospitalar da Universidade de Coimbra, em particular ao Dr. Jorge Migueis do Serviço de Otorrinolaringologia e à Dra. Maria José Julião do Serviço de Anatomia Patológica por toda a cooperação e disponibilidade prestada na elaboração deste projeto

À minha segunda família de Aveiro, um agradecimentos especial à Carla, Catarina, Helena, Jéssica e à Joana Seara pela amizade e pelos bons momentos que passamos juntas ao longo destes cinco anos. Sem vocês nunca me teria tornado na pessoa que sou hoje.

Às meninas de Coimbra, Joana e Laetitia por amavelmente me terem "deixado" fazer bolos para elas. Obrigado pela amizade, convívio, ajuda, pelos bons e maus momentos que passamos juntas e sobretudo pela paciência ao longo deste ano.

Aos meus pais, porque sem eles nunca chegaria onde cheguei. Obrigado por me disponibilizarem tudo o que necessitei, pelo apoio incondicional, encorajamento e carinho. A toda a minha família e em particular à minha irmã que, ainda que impossível de aturar, me ajudou a ultrapassar os dias menos bons.

Palavras-chave

Carcinoma das células escamosas da laringe (CCEL), variação do número de cópias (CNVs), metilação de DNA, MLPA, MS-MLPA, Array CGH.

Resumo

O carcinoma da laringe pertence a uma grande família de tumores conhecida como Cancro da Cabeça e do Pescoço que é considerado o sexto tipo de tumor mais maligno em todo o mundo. Dentro desta família, os tumores podem ter origem em diversos locais anatómicos, sendo a laringe o segundo órgão mais comummente afetado. O cancro da laringe apresenta uma incidência mundial de 1,9% e uma taxa de mortalidade elevada de 1,6%. Apesar dos avanços tecnológicos na área do diagnóstico e da terapêutica, a taxa de sobrevivência ao fim de 5 anos não apresentou melhorias significativas. As baixas taxas de sobrevivências são explicadas essencialmente pelo diagnóstico tardio, pela agressividade do tumor e pela sua propensão a desenvolver metástases. Desta forma, torna-se essencial a identificação de biomarcadores com valor de diagnóstico e prognóstico a fim de detetar a presença do tumor numa fase mais precoce. Este estudo surge com o objetivo principal de caracterizar o perfil genético e epigenéticos do carcinoma das células escamosas da laringe com recurso às técnicas de MLPA, MS-MLPA e *array* CGH, usando oito amostras tumorais e sete amostras não-tumorais contra laterais ao tumor, ambas coletadas após cirurgia A análise genética revelou uma maior taxa de ganho de material genético nos cromossomas 3q, 8q, 11q, 14q13.1, Xp22.31, Xq21.1 e perda de material genético nos cromossomas 3p, 9p23.1 e Y. O ganho dos genes *MYC* e *TNFRSF1A* revelou ser o evento mais comum entre as amostras analisadas. Relativamente ao perfil epigenético, observou-se que os genes *CDKN2A*, *CHFR*, *RARβ* e *RASSF1* se encontravam metilados nas amostras em estudo. Em suma, este trabalho permitiu identificar algumas alterações genéticas e epigenéticas descritas na literatura como estando associadas ao CCEL, assim como alterações associadas ao desenvolvimento tumoral. Foram ainda identificadas alterações que ainda não foram reportadas como estando associadas ao cancro. Desta forma, este estudo piloto permitiu dar início ao estudo de potenciais biomarcadores associados ao CCEL. Porém, novos estudos devem ser realizados, com um número de amostras superior, de forma a identificar alterações genéticas significativas no desenvolvimento e progressão do CCEL e associa-las às características clinico patológicas dos doentes.

Keywords

Laryngeal Squamous Cell Carcinoma (LSCC), copy number variations (CNVs), DNA methylation, MLPA, MS-MLPA, Array CGH.

Abstract

Laryngeal carcinomas belong to a bigger family of tumours known as Head and Neck Cancer (HNC). HNC is the sixth most malignant type of cancer in the world and it can arise from several anatomical sites. Among them, the larynx is the second most common affect organ. The incidence of laryngeal carcinoma is 1,9% worldwide and it presents a high mortality rate (1,6%). Despite technological advances in diagnosis and treatment fields, the 5 year-survival rate did not improved significantly. The low survival rates are mainly explained by a late diagnosis, tumour aggressiveness and the fact that laryngeal carcinoma metastasize easily. Taking this into consideration, it is essential to identify biomarkers with significant diagnostic and prognostic value in order to anticipate the detection of laryngeal carcinoma in an early stage. This study arises mainly for characterize the genetic and epigenetic profile of laryngeal squamous cell carcinoma (LSCC). Eight LSCC samples and seven non-tumour samples contralateral to the primary tumour were collected upon resection surgery and characterized by MLPA, MS-MLPA and array CGH. The results showed that gain of genetic material was mainly present in chromosomes 3q, 8q, 11q, 14q13.1, Xp22.31 and Xq21.1 while genetic loss occurred mainly in chromosomes 3p, 9p23.1 and Y. Gain of *MYC* and *TNFRSF1A* was the most common event among the tumour samples included in this study. Regarding the methylation profile, the genes *CDKN2A*, *CHFR*, *RARβ* e *RASSF1* were the only ones which were methylated in this samples. In conclusion, this study allowed to identify genetic alterations associated with LSCC that have already been reported in scientific papers as well as alterations that have been associated with tumour development and progression. In addition, a few genetic alterations which have never been reported as being associated with human cancer before were identified. Nevertheless, new studies must be carried out, with a higher number of samples. Ultimately, the main goal would be to identify genetic alterations significantly associated with LSCC progression and establish a correlation with clinicopathological data.

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Abbreviations

ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
aCGH	Array Comparative Genomic Hybridization
AKT	Protein kinase B
ALS2CL	ALS2 C-terminal like
APC	Adenomatous Polyposis Coli
ASAP1	ArfGAP with SH3 domain, ankyrin repeat and PH domain 1
ATM	ATM serine/threonine kinase
BFB	Breakage–Fusion–Bridge
BRCA1	Breast Cancer 1, early onset
BRCA2	Breast Cancer 2, early onset
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
BTNL3	Butyrophilin-like 3
CADM1	Cell adhesion molecule 1
CASP8	Caspase 8, apoptosis-related cysteine peptidase
CASR	Calcium-sensing receptor
CCND1	Cyclin D1
CCNL1	Cyclin L1
CD27	CD27 molecule
CD38	CD 38 molecule
CD44	CD44 molecule (Indian blood group)
CD99	CD99 molecule
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
CDH13	Caherin 13
CDK2	Cyclin Dependent Kinase 2
CDK4	Cyclin Dependent Kinase 4
CDK6	Cyclin Dependent Kinase 6
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CDKN1B	Cyclin-Dependent Kinase Inhibitor 1B
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CDKN2B	Cyclin-Dependent Kinase Inhibitor 2B
CDKN2D	Cyclin-Dependent Kinase Inhibitor 2D
CELF2	CUGBP, Elav-like family member 2
CGH	Comparative Genomic Hybridization
CHD5	Chromodomain-Helicase-DNA Binding Protein 5
CHEK1	Checkpoint kinase 1
CHFR	Checkpoint with Forkhead and Ring finger domains, E3 ubiquitin protein ligase
CHL1	Cell adhesion molecule L1-like
CHUC	Coimbra Hospital and University Centre
CLDN10	Claudin 10
CNV	Copy Number Variation
CREM	cAMP responsive element modulator
CSMD1	CUB and Sushi multiple domains 1
CTBS	Chitobiase, di-N-acetyl-
CTNNB1	Catenin (cadherin-associated protein), beta 1, 88kDa

CTTN (*EMS1*)	Cortactin
Cy3	Cyanine 3
Cy3	Cyanine 4
DAPK	Death Associated Protein Kinase 1
DEPDC1B	DEP domain containing 1B
DISC	Death-Inducing Signalling Complex
DNMT1	DNA methyltransferase 1
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
DPYD	Dihydropyrimidine dehydrogenase
DCUN1D1	DCN1, defective in cullin neddylation 1, domain containing 1
EGFR	Epidermal Growth Factor Receptor
EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1
ELISA	Enzyme-Linked Immunosorbent Assay
ESR1	Estrogen Receptor 1
ESRG	Embryonic stem cell related (non-protein coding)
FADD	Fas associated protein with dead domain
FGF4	Fibroblast growth factor 4
FHIT	Fragile Histidine Triad
GALR1	Galanin receptor 1
GATA4	GATA binding protein 4
GBE1	Glucan (1,4-alpha-), branching enzyme 1
GPC5	Glypican 5
GPC6	Glypican 6
GSTP1	Glutathione S-Transferase Pi 1
H2AFX	H2A histone family, member X
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	*Human Pappillomavirus*
hTERC	human Telomerase RNA Component
IARC	International Agency for Research on Cancer
IGF2	Insulin-like Growth Factor 2
IGF1R	Insulin-like Growth Factor 1 Receptor
IL1A	Interleukin 1, alpha
IL18	Interleukin 18
JAK	Janus kinase
KCNRG	Potassium channel regulator
KLLN	Killin, p53-regulated DNA replication inhibitor
KLK3	Kallikrein-related peptidase 3
LAMP3	Lysosomal-associated membrane protein 3
LMNA	Lamin A/C
LSCC	Laryngeal Squamous Cell Carcinoma
LOH	Loss of heterozygosity
LRTM1	Leucine-rich repeats and transmembrane domains 1
LRRFIP1	Leucine rich repeat (in FLII) interacting protein 1
MAGT1	Magnesium transporter 1
MCCC1	Methylcrotonoyl-CoA carboxylase 1 (alpha)
MET	MET proto-oncogene, receptor tyrosine kinase

MFHAS1	Malignant fibrous histiocytoma amplified sequence 1
MGMT	O-6-methylguanine-DNA methyltransferase
MIR1204	microRNA 1204
MLH1	mutL homolog 1
MLH3	mutL homolog 3
MLPA	Multiple Ligation-dependent Probe Amplification
MS-MLPA	Methylation Specific MLPA
mTOR	Mechanistic target of rapamycin (serine/threonine kinase)
MTUS1	Microtubule associated tumor suppressor 1
MYC	v-myc avian myelocytomatosis viral oncogene homolog
MUC4	Mucin 4
MUC20	Mucin 20
N33	Tumour suppressor candidate 3
NOS1	Nitric oxide synthase 1 (neuronal)
NOTCH1	Notch 1
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
SCC	Squamous Cell Carcinoma
SMAD2	SMAD family member 2
SMAD4	SMAD family member 4
STAT	Signal transducers and activators of transcription
ORAOV1	Oral Cancer Overexpressed 1
PAH	Phenylalanine hydroxylase
PARK2	Parkin RBR E3 ubiquitin protein ligase
PCR	Polymerase chain reaction
PEX13	Peroxisomal biogenesis factor 13
PI3KCA (PI3K)	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PKHD1	Polycystic kidney and hepatic disease 1 (autosomal recessive)
POMT2	Protein-O-mannosyltransferase 2
PPFIA1	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1
PPIL2	Peptidylprolyl isomerase (cyclophilin)-like 2
PRPF31	pre-mRNA processing factor 31
PSOCFS	Portuguese Society of Otorhinolaryngology and Cervico-Facial Surgery
PTK2	Protein tyrosine kinase 2
PVT1	Pvt1 oncogene (non-protein coding)
RARB	Retinoic Acid Receptor, Beta
RASSF1	Ras Association (RalCDS/AF-6) domain Family member 1A
RB (RB1)	Retinoblastoma 1
RECQL4	RecQ protein-like 4
RPIA	Ribose 5-phosphate isomerase A
S100A4	S100 calcium binding protein A4
SNX6	sorting nexin 6
SPG11	Spastic paraplegia 11 (autosomal recessive)
STK11	Serine/threonine kinase 11
TIMP3	TIMP metallopeptidase inhibitor 3
TNFRSF1A	Tumour Necrosis Factor Receptor Superfamily, member 1A
TNK2	Tyrosine kinase, non-receptor, 2

TP53	Tumour Protein p53
TP63	Tumour Protein p63
TP73	Tumour Protein p73
TSC2	Tuberous Sclerosis 2
WDR36	WD repeat domain 36
WFS1	Wolfram syndrome 1 (wolframin)
WHSC1	Wolf-Hirschhorn syndrome candidate 1
WISP1	WNT1 inducible signaling pathway protein 1
USP25	Ubiquitin specific peptidase 25
VCX3A	Variable charge, X-linked 3A
VHL	von Hippel-Lindau tumour suppressor, E3 ubiquitin protein ligase

1 Introduction

1.1 The Larynx

The larynx is an organ located in the throat with approximately 5 centimetres wide. It is made by a rigid wall reinforced by hyaline cartilage and smaller elastic cartilages, all of which are connected by ligaments. Also, the surface of larynx is composed by stratified squamous epithelium and, at some points, this epithelium undergoes a transition to ciliated pseudostratified columnar epithelium. Underneath this epithelium there are several mucous and serous glands (1,2).

The larynx can be divided in three mains parts (Figure 1):

I. Top: Supraglottis (the area above the vocal cords; contains the epiglottis cartilage);
II. Middle: Glottis (where vocal cords are located);
III. Bottom: Subglottis (the area below the vocal cords which is connected to trachea) (1,2).

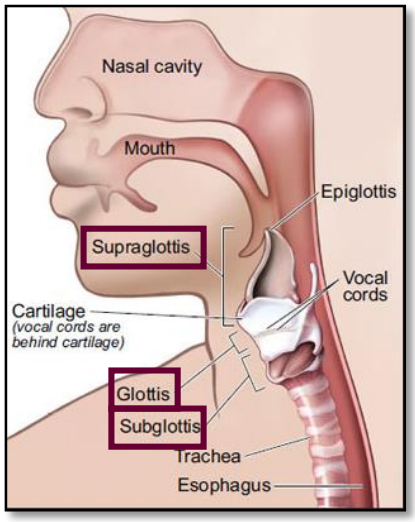

Figure 1 - Anatomy of Larynx. Adapted from: National Cancer Institute - U.S. Department of Health and Human Services 2014 (2)

The larynx is responsible for three physiological functions:

I. Breathing: the larynx allow the passage of air, from pharynx to trachea;
II. Speaking: through vibration of vocal cords;
III. Swallowing: epiglottis prevents the entrance of food and liquid into lungs (1,2).

1.2 Cancer

A neoplasm can be defined as an abnormal mass in a specific tissue made by transformed cells which continue to replicate uncoordinated and excessively, even after the cessation of the initial stimuli that promoted the change. Genetic changes are associated to the origin of all neoplasms, causing unregulated proliferation independent of physiologic growth-regulatory stimuli (3). Furthermore, the cells undergo a set of changes involving not

only uncontrolled proliferation but also misregulated differentiation and loss of checkpoint control and accumulation of chromosomal aberrations and aneuploidies (4).

Neoplasm can also be referred as a tumour and it can be divided into benign and malignant, based on its potential clinical behaviour (3):

- Benign tumours usually remain localized and do not spread to other sites. Also, they are easily removed by local surgery and the patient generally survives
- Malignant tumours, also known as cancers, are characterized by invasion and destruction of adjacent structures, metastasis, high risk of recurrence and death.

Nevertheless, all tumours are composed by two basic components: (1) parenchyma, which is made by transformed cells (neoplastic cells) and determinates the biologic behaviour of tumours; (2) stroma, made by connective tissue, blood vessels and host-derived inflammatory cells, which is fundamental to support the growth of cells (3).

1.2.1 Hallmarks of Cancer

The hallmarks of cancer are a set of characteristics which are developed during the multistep development of tumours. They are involved in tumour growth and its spread to other sites (metastization), allowing us to understand the diversity of neoplastic diseases (5,6).

According to Hanahan and Weinberg (2000), there are six hallmarks of cancer: sustaining proliferative signalling, evading growth suppressors, resisting cell death, inducing angiogenesis, activating invasion and metastasis and enabling replicative immortality. Genomic instability and inflammation underlie these hallmarks. In the last years, more hallmarks have been suggested: reprogramming of energy metabolism, evading immune destruction, tumour-

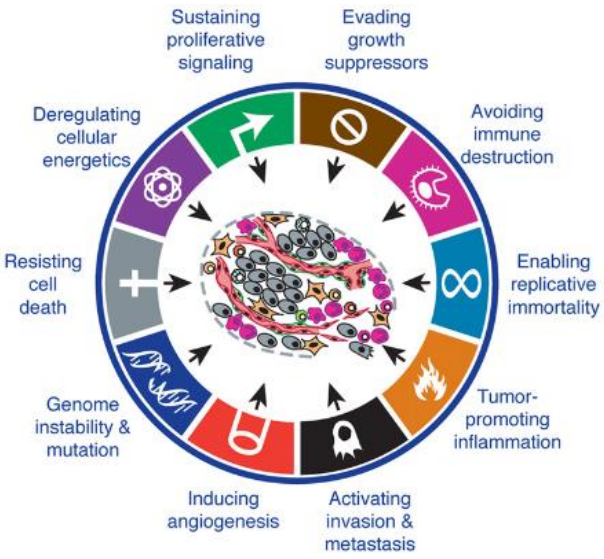

Figure 2 - The Hallmarks of Cancer. Adapted from Hanahan D and Weinberg RA 2011 (6)

promoting inflammation and genome instability and mutation (Figure 2) (5,6).

1.3 Head and Neck Cancer and Laryngeal Carcinoma

Laryngeal carcinoma is part of a bigger family of tumours known as Head and Neck Cancers. Besides larynx, this family of tumours can arise from nine more anatomic sites: nasal cavity/paranasal, oral cavity, salivary gland, trachea, thyroid, nasopharynx, oropharynx and hypopharynx. At least 90% of all histological head and neck cancers are represented by squamous cell carcinoma (SCC) and they generally arise from the oral cavity, oropharynx, larynx and hypopharynx. Therefore, the term *head and neck squamous cell carcinoma* is frequently used to refer this anatomical subsites (7–9). According to WHO (World Health Organization), SCC represents 95% of the malignancies that affect the larynx. In most cases, laryngeal cancer has origin on the vocal cords in the glottic region (50%-60%) or in supraglottic region (30%-40%) (10,11).

1.4 Laryngeal Carcinoma

1.4.1 Clinical features

The signs and symptoms depend on the localization and the size of the tumour. The most common ones are voice changes/hoarseness which is considered to be an early symptom in glottis cancer. Symptoms of supraglottic and hypopharyngeal tumours may include not only voice changes but also dysphagia (trouble swallowing), lump/mass in the neck, haemoptysis (coughing of blood) and odynophagia (painful swallowing). Finally, in subglottic tumours symptoms such as dyspnoea (breathing discomfort/impaired breathing) and stridor (abnormal high-pitched sound produced by turbulent airflow through a partially obstructed airway) are common (2,10).

1.4.2 TNM classification

The TNM classification (Table 1 and 2) is an anatomically based system used worldwide for cancer staging. TNM classification is divided in three categories: (1) T: primary tumour site; (2) N: regional lymph node involvement; (3) M: presence/absence of metastases. Therefore it records the primary and regional nodal extent of the tumour as well as presence or absence of metastastic spread.

Table 1 - TNM Clinical Classification. Adapted from: Sobin et al. (2011) (12)

T – Primary Tumour
Tx: Primary tumour cannot be assessed
T0: No evidence of primary tumour
T1-T4: Carcinoma *in situ*.
N – Regional Lymph Nodes
Nx: Regional lymph nodes cannot be assessed
N0: No regional lymph node metastasis
N1: Metastasis in a single ipsilateral lymph node ≤ 3cm in dimension
N2a: Metastasis in a single ipsilateral lymph node whose dimension ranges from 3 cm to 6 cm
N2b: Metastasis in multiple ipsilateral lymph nodes ≤ 6 cm in dimension
N2c: Metastasis in bilateral or contralateral lymph nodes ≤ 6 cm in dimension
N3: Metastasis in a lymph node > 6 cm in dimension
M – Distant Metastasis
M0: No distant metastasis
M1: Distant metastasis.

Table 2 - Stage Grouping was developed to condensate the three categories of TNM clinical classification. In the TNM system, carcinoma in situ is considered Stage 0, stages I and II are tumours localized in the organ of origin, tumours that spread to lymph nodes are in stage III while stage IV corresponding to tumour with distant metastasis. Adapted from: Sobin et al. (2011) (12)

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

1.4.3 Epidemiology and Risk Factors

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumour in the world and the laryngeal carcinoma is the second one within HNSCC (13). More specifically, according to GLOBOCAN 2012, the incidence of laryngeal carcinoma worldwide was 1.9% (138 102 new cases), more than 73 000 people died (1,6%) and the 5-year prevalence was about 2.5% (14,15).

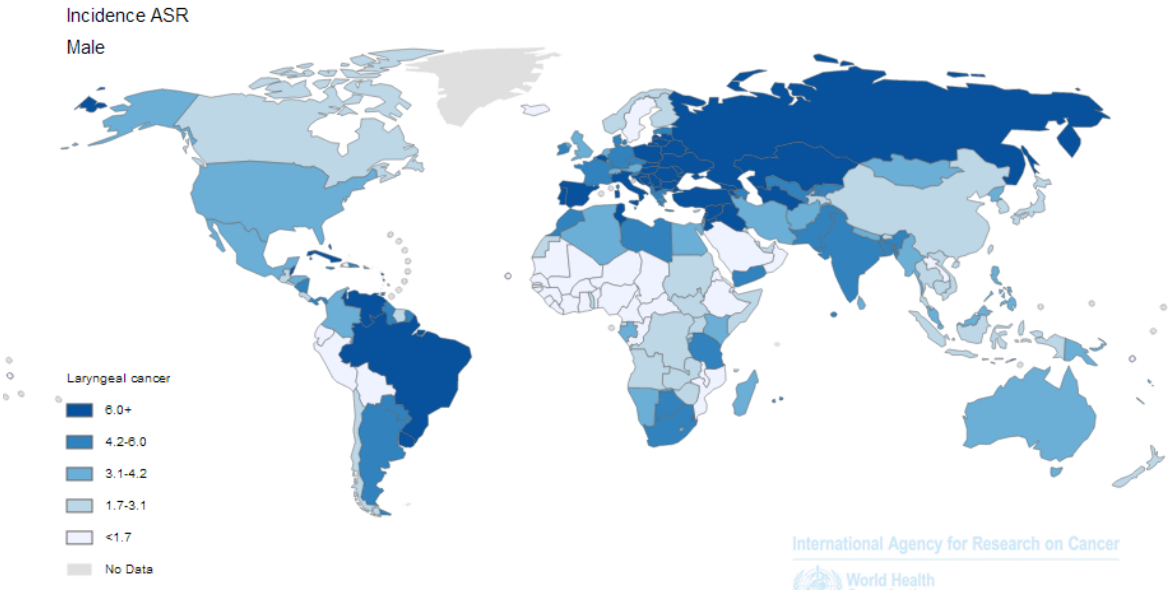

Figure 3 - Laryngeal Carcinoma Incidence Worldwide in 2012. Source: GLOBOCAN 2012 (14)

According to Portuguese Society of Otorhinolaryngology and Cervico-Facial Surgery (SPORL), Portugal is the third European country with the highest number of laryngeal carcinoma cases (16).

It is known that laryngeal cancer risk is strongly linked to age and sex. Overall, the incidence of this carcinoma is higher in adult males, between 60 and 70 years old (10,17).

Alcohol and tobacco are the two main risk factors of larynx cancer in developed countries, in the western world, and they account for the majority of cases. Through epidemiological studies it was possible to identify an association between alcohol drinking with laryngeal cancer risk, as well as, a synergistic effect with tobacco smoking (10,17,18). Moreover, it is known that tobacco on its own is responsible for 85% - 90% of laryngeal squamous cell carcinoma (LCSS) (11).

Other risk factors are the exposure to other compounds, such as metal dust, cement dust, varnish and lacquer, as well as ionizing radiation, diesel exhausts, sulphuric acid mists and mustard gas. Also, through epidemiological studies, it was possible to identify an

association between cancer of the larynx and *Human Papillomavirus* (HPV) (10,17). Among all HPV subtypes detected in patients with cancer of the larynx, the prevalence of infection by HPV-16 and -18 seems to be much higher than the infection by other types (13). X. Li et al. (2013) showed a strong association between HPV-16 with laryngeal carcinoma (19). However, the findings regarding the frequency of HPV infection among laryngeal invasive lesions or carcinomas are quite heterogeneous (19,20). According to Torrente et al. (2011), the method of detection of HPV-16 may explain this heterogeneity. It appeared that those studies that used ELISA (enzyme-linked immunosorbent assay) were consistent with HPV being an important risk factor only for tonsillar carcinoma while those that used tumour-based DNA amplification suggested that HPV were also a risk factor for oral, laryngeal and oropharyngeal cancer. Other factors that may influence are methodological differences, quality of the sample and sample size (21). Both techniques have different sensitivities and specificities that may explain the inconsistence between results. Also, some smaller studies might be under the influence of a selection bias explained either by the preferential inclusion of specific samples or by submission and publication of studies with high HPV prevalence.

1.4.4 HPV-Induced Carcinogenesis

HPV is an 8 kb circular double-stranded DNA non-enveloped virus which belongs to the family *Papillomaviridae*. However, there is only one strand of the genome which is transcribed into two different classes of proteins expressed by alternative splicing: early non-structural regulatory proteins (E1-E7) and late structural proteins (L1 and L2) (22). HPV is an obligatory intranuclear virus which means that it must infect mitotically active cells in order to establish itself in epithelia. More than 200 different HPV genotypes have already been described and they can be classified as high-, intermediate or low-oncogenic risk according to their association with cervical cancer. By 1995, IARC (International Agency for Research on Cancer) had already recognized that high-risk HPV-16 and -18 were carcinogenic in humans. In HNSCC, HPV-16 is, by far, the most common genotype (90-95%) (21).

The infection by HPV can achieve basal and parabasal cells of epithelium through the site of mucosal injury, metaplastic epithelium or through squamocolumnar junction. In the larynx, HPV-induced proliferation results in a metaplastic alteration and formation of multi-layered squamous cell epithelium. In HPV-induced carcinogenesis (Figure 4),

integration of HPV DNA into the host cell genome is crucial. After integration, E1 or E2 open reading frames are disrupted or deleted, leading to loss of gene expression and upregulation of viral oncoproteins E6 and E7 and consequently to DNA instability. More specifically, E6 binds and induces the degradation of p53 suppressor protein while E7 protein binds to pRb (retinoblastoma protein) which leads to facilitation of DNA replication proteins expression. The molecular consequence of such expression is cell cycle entry and inhibition of p53-mediated apoptosis which will lead to virus replication and accumulation of DNA aberrations. Also, the oncogenic infection associated with E6 and E7 expression in basal layer, where stem cells are located, leads to disruption of cell cycle checkpoints. Additionally, E6 interferes with DNA repair enzymes and E7 induce chromosome abnormalities by the disruption of centrosome synthesis. Overall, the genetic instability induced by HPV may lead to emergence of tumorigenic cells. Alongside with molecular events there are morphologic alterations of epithelium which are represented by an increased mitotic rate, aneuploidies and increased rate of mutation in the host cell (21,23).

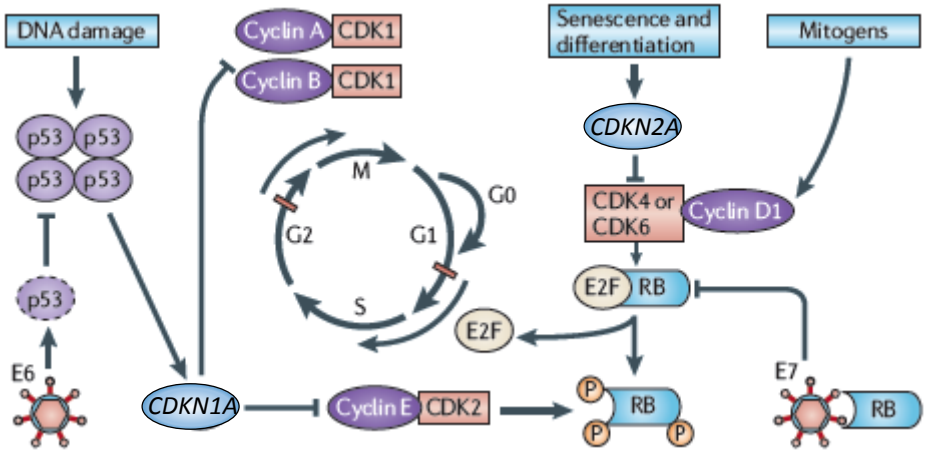

Figure 4 - Deregulation of cell cycle by HPV. Adapted from Leemans et al. (2011) (23)

1.4.5 Non HPV-Carcinogenesis

It has already been accepted that solid tumours, such as LSCC, result from a multistep process where genetic alteration are accumulated (24). Renan et al. (1993) suggested that LSCC develops by the accumulation of 6 to 10 independent genetic events (25,26). Through experimental studies, it was possible to demonstrate that the progression of the histopathological phenotype of LCSS correlates with genetic progression of HNSCC (24). Taking this into consideration, we now know that the evolution and progression of LSCC result from a multiple stepwise alterations that include not only genetic alterations but also

abnormalities in cellular and molecular pathways in the squamous epithelium. In HNSCC, it has been suggested a model of molecular progression from premalignant lesions to invasive carcinoma (27). However, since genetic alterations in laryngeal carcinomas are usually studied as part of head and neck cancers, there is not much information about molecular progression that allow a clear differentiation between laryngeal malignancies and the other cancers included in head and neck family. Also, it is not clear which genes participate in the different histopathological phases of LSCC. In Figure 5 is represented a few molecular alterations and its probable times of onset in cancer of the larynx (24,28–30).

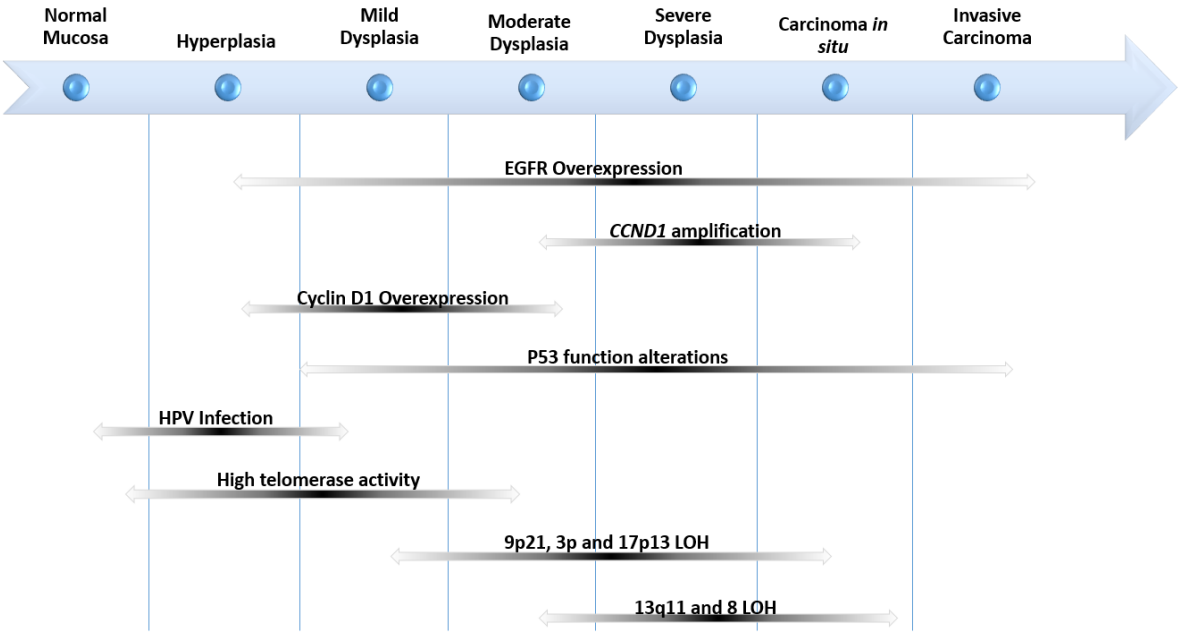

Figure 5 - A grey scale in the corresponding bars represents the most probable times of onset of some molecular alterations in laryngeal carcinogenesis. (LOH - loss of heterozygosity). Adapted from: Almadori et al. (2004) (24)

Furthermore since larynx plays a fundamental role in human speech and communication as well as breathing and swallowing, the optimal management of patients with laryngeal cancers is critical. Taking this into consideration, the study of molecular biology and tumourigenesis of laryngeal cancers seems to be mandatory in order to enhance our understanding of the evolution of this disease. The main challenge is to identify specific tumour biomarkers that will help to improve survival and preserve the function of larynx (30).

1.4.6 Multistep Laryngeal Carcinogenesis: Genetic Model of Transformation and Neoplastic Progression.

Although the overall genetic and molecular mechanisms of LSCC do not remain clear enough, Marcos et al. (2011), through the study of multigenic gains and losses by Multiple Ligation-dependent Probe Amplification (MLPA), proposed a genetic model of multistep laryngeal carcinogenesis (31). The results allowed the construction of an expanded model of transformation and neoplastic progression which include not only the classic sequence of steps (normal mucosa, precursor lesions, invasive tumour) but also normal mucosa tobacco and alcohol exposed, negative and positive lymph node primary tumour and lymph node metastases (Figure 6).

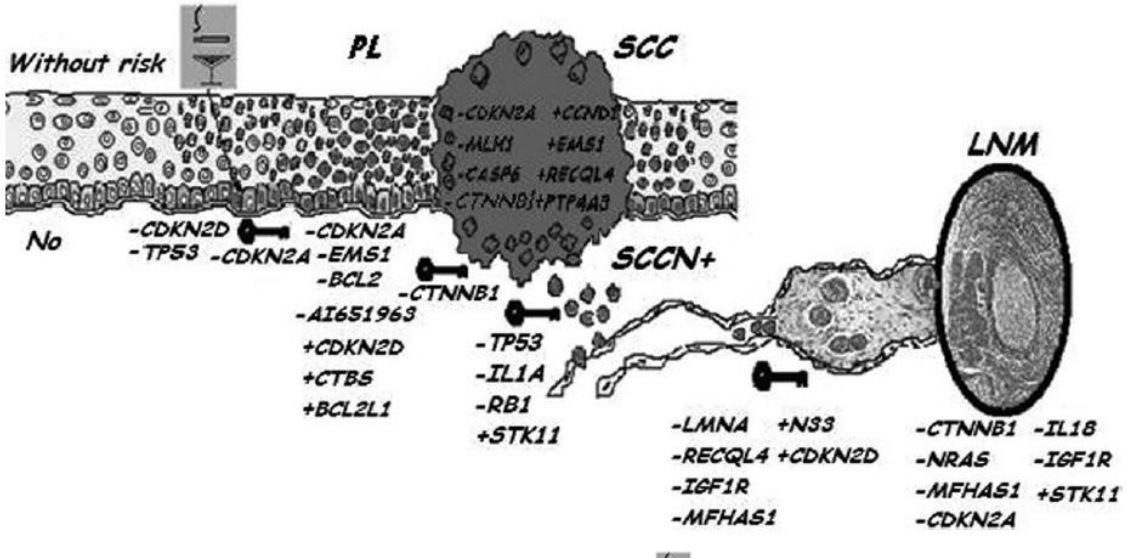

Figure 6 - Genetic progression model proposed by Marcos et al. (2011). [( ) smoker patients and also exposed to alcohol (-): genetic loss; (+): genetic gain; PL: precursor lesion; SCC: squamous cell carcinoma; SCCN+: SCC with positive lymph node; LNM: lymph node metastases; : key genes between 2 steps] (31)

1.4.7 Cytogenetic Alterations in Laryngeal Carcinoma

Since laryngeal carcinoma develops through the accumulation of multiple genetic changes, its karyotypes are generally complex and have a non-random pattern of chromosomal alterations, including deletions and amplifications. In the cancer of larynx, the structural rearrangements are frequently located in the chromosomes 1 to 5, 7, 8, 11, 12 and 15. 43% of the total breakpoints are located in pericentromeric regions, including centromeric bands p10 and q10 and juxtacentromeric bands p11 and q11. Also, the most common imbalances are partial or total loss of chromosome arms 3p, 5q, 8p, 9p, 13q, and 17p and gain of chromosomal regions 3q, 5p, 7p, 8q, 11q13,17q and 18p (32,33). By 2007, 17 recurrent structural alterations (translocations (t), isochromosomes (i) and deletions (del))

have already been identified, being i(8q), i(3q), i(5p) del(3)(p11) and gene amplification in 11q13 the most common among them (33,34). Taking this into consideration, some of these chromosomal regions that are better characterized in LSCC and/or in HNSCC will be discussed individually in the following sections.

1.4.7.1 Region 3p

Partial/total loss of 3p is one of the most common genetic alteration in LCSS. In this region are located many tumour suppressor genes such as *FHIT* (fragile histidine triad), encoded on 3p14.2. This gene was detected in LCSS and precursor lesions. The decreased expression of *FHIT*, which may be caused by deletion or promoter methylation, was detected in about 42% of LCSS and 23% of dysplasia lesions (33). In a normal physiological state, *FHIT* [Uniprot: P49789] contributes to the regulation of the expression of genes which are important for cell proliferation and survival, such as *CCND1* (cyclin D1). Also, *FHIT* has a role in the induction of apoptosis and functions as a tumour suppressor gene. Loss of FHIT protein may lead to abnormal cell proliferation probably by breakdown of G_0/G_1 arrest in the larynx and gain of apoptosis resistance during carcinogenesis (35).

Other genes mapped on this region are *RARβ* (retinoic acid receptor, beta), *CTNNβ1* (catenin (cadherin-associated protein), beta 1, 88kDa) and *MLH1* (mutL homolog 1).

The *RARβ* gene (chromosome 3p24) encodes for a retinoic acid receptor beta which participates in the mediation of cellular signalling in embryonic morphogenesis, cell growth and differentiation through binding retinoic acid. In HNSCC, loss of *RARβ* has been associated with cell immortalization and, in some patients, with resistance to growth inhibitory effects of retinoids. In laryngeal cancer specifically more studies have to be done in order to understand better the role of such loss in carcinogenesis (36,37).

β-catenin, encoded on 3p21, is part of wingless-Wnt signalling cascade which is involved in cellular proliferation and differentiation. Abnormal Wnt signalling has been associated with several human cancers. On the other hand, cytoplasmic β-catenin binds to the intracellular domain of E-cadherin in order to maintain cell adhesion. This protein is also downregulated in many human cancers, including in LCSS. Cadherin and catenin form a complex which is important not only in cell adhesion and differentiation but also in cell migration and tumour suppression. It seems that the loss of expression of catenin may be implicated in tumour invasion and metastasis in patients with supraglottic tumours.

However, more research is necessary to clear up the involvement of this protein in malignant transformation of laryngeal cells (38,39).

MLH1 [Uniprot: P40692], mapped on chromosome 3p22.3, is a DNA mismatch repair (MMR) gene. As a briefly explanation, the MMR pathway targets DNA replication errors, such as base substitution mismatches as well as insertion-deletion mismatches, that escape the proofreading function of DNA polymerase. *MLH1* has a role in DNA damage signalling and in meiosis (40). In LCSS, *MLH1* is also downregulated (26,41). Moreover, Sasiadek et al. (2006) suggested the existence of an interaction between amplification of *CCND1* and downregulation of *MLH1* in LCSS (41).

1.4.7.2 Region 3q

Genetic gain in 3q region is frequent in SCC. Furthermore, it has been reported an overlapping area of gain at 3q26 in different anatomic sites, including the larynx. In this region is located the *hTERC* gene (human telomerase RNA component) which encodes the RNA component of human telomerase. This enzyme plays a role in cellular senescence by adding telomere repeat TTAGGG. Telomerase comprises a protein component with reverse transcriptase activity as well as an RNA component that acts as template for the telomere repeat (42). When this enzyme is overexpressed, cells with critically short telomeres avoid apoptosis and amplification of *hTERC* has been seen in many tumour sample and immortalized cell lines which suggest that its transcription may be upregulated during tumourigenesis. Liu et al. (2012) found this alteration in LCSS patients with moderate dysplasia, severe dysplasia, carcinoma *in situ* and invasive carcinoma. They saw that amplification of *hTERC* was present at low frequency in normal epithelium and mild dysplasia when compared with the stages mentioned above, which may suggest that the amplification may be implied in the progression to invasive LCSS (42,43).

Gains in 3q region are commonly associated with isochromosome formation (an abnormal chromosome with two identical arms, either two short or two long arms, normally resulting from a transversal division through the centromere at meiosis II) and is usually accompanied by the loss of 3p mention above (33,44).

1.4.7.3 Region 7p

One of the major alterations on this region is the overexpression of *EFGR* (epidermal growth factor receptor) gene, which is mapped in 7p12. *EGFR* codes for a transmembrane

receptor tyrosine kinase which has a major role in the regulation of cancer cells proliferation as well as cell cycle progression (33,45). EGFR [Uniprot: P00533] is able to activate several downstream signalling cascades, including the RAS-RAF-MEK-ERK, PI3K-AKT-mTOR, PLCγ-PKC and NF-kappa-B, that regulate numerous cellular processes. LCSS patients with a high expression of EGFR tend to have a poorer prognosis (45). Braut et al. (2009) suggested an association between the increase of EGFR expression and gene amplification with the increase of biological aggressiveness of glottic lesions. They also conclude that gene amplification is an early event in glottic cancer (46).

1.4.7.4 Region 8q

In LSCC, partial or entire gain of 8q by the formation of isochromosome or unbalanced structural rearrangements is common. Within the genes mapped in this region, *MYC* (v-myc avian myelocytomatosis viral oncogene homolog) is probably the alteration more studied (33). *MYC* is mapped on 8q24.21 and codes for a transcription factor which has a role in activation and repression of transcription. Moreover, *MYC* is implicated in control of cell proliferation by upregulating cyclins and downregulating *CDKN1A*, differentiation and programmed cell death (47,48). In LSCC, amplification and overexpression of *MYC* happen frequently (30-68%) (33). *MYC* may be activated by gain or amplification in laryngeal carcinogenesis. Liu et al. (2013) found a strong association between *MYC* amplification and histopathological stages which suggests that this alteration may be implied in the development of LSCC (47). Furthermore, amplification of *MYC* gene is considered an early event during the progression of laryngeal dysplasia (47). On the other hand, Coskunpinar et al. (2014) observed a decreased expression of *MYC* in metastatic tumours. *MYC* suppresses transcription of integrins, which have an important role in metastasis of cancer of the larynx and their silencing appears to result in inhibition of metastasis. Taking this in consideration, it is easy to understand the loss of expression of *MYC* in a metastatic phenotype (48).

1.4.7.5 Region 9p

In this regions is located *CDKN2A* (cyclin-dependent kinase inhibitor 2A) which is encoded on chromosomal region 9p21 (33). *CDKN2A* inhibits catalytic activity of CDK/cyclin D1 complexes through the bind to CDK4 (cyclin dependent kinase 4) and CDK6 thus. This complex is needed for RB1 protein phosphorylation as well as cell cycle

progression through G1/S restriction point (29,49). The loss of expression of *CDKN2A* has been detected in 52-82% of HNSCC, including LSCC. There three known mechanism that lead to gene inactivation in human cancers: homozygous deletions, point mutations and promoter hypermethylation. Additionally, LOH is also found in these tumours. The deregulation of cell cycle leads to genomic instability, resulting in cancer development. Also, downregulation of *CDKN2A* is associated with poor survival in patients with LCSS (29,33,49).

1.4.7.6 *Chromosomal band 11q13*

11q13 rearrangements have been extensively studied in cancer and its pathogenic importance is supported by several studies. Amplification of this region is commonly found in human cancer and, within these tumours, HNSCC seems to be the one that has a higher rate of amplification (36%) (50). Also, 11q13 amplification is associated with lymph node metastasis and decreased disease-free survival as well as overall survival (50).

The numerical and structural anomalies in 11q13 region may result by the formation of intra–chromosomal repeats that are cytogenetically visible as homogenously stained region (hsr) or by the presence of extra copies of chromosome 11 (51). Shuster et al. (2000) suggested that 11q13 amplification is most probable arised through breakage–fusion–bridge (BFB) cycles (51,52) in which the breakage of a chromosome leads to loss of telomeric end. After that, the broken end of this chromosome fuse with the newly synthesized strand, resulting in the formation of dicentric chromosome that forms a bridge during anaphase. Next, the mechanical tension applied in the centromeres by the mitotic spindles will lead to asymmetrical chromosomal break. Finally, the resulting daughter cells will contain a chromatid with an inverted repeat at the fragmented end or a chromatid with loss of the repeated segment. Unless the broken end is capped, the next BFB cycles will result in augment of the repeated segment (Figure 7) (53).

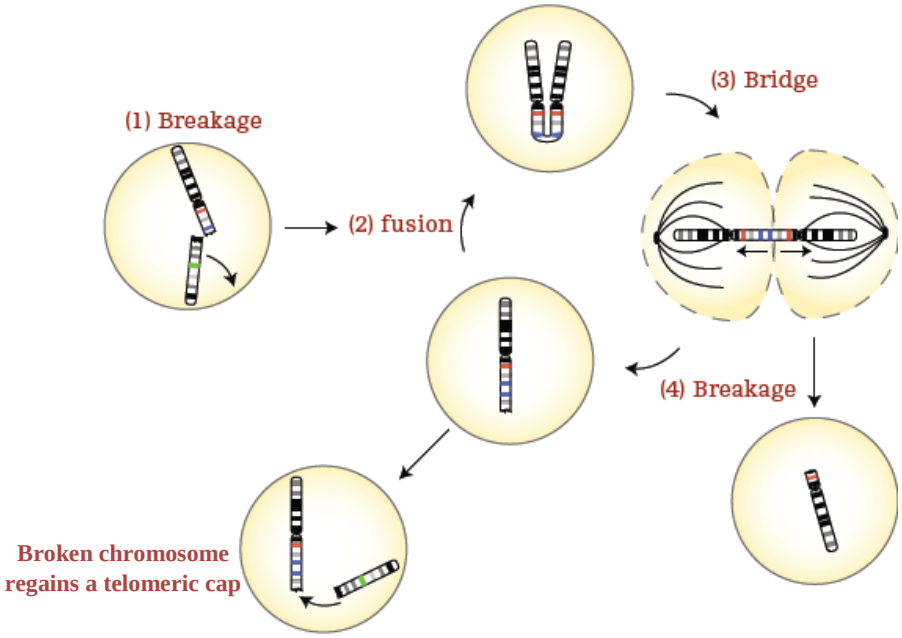

Figure 7 - The mechanism of breakage–fusion–bridge (BFB) cycles. Adapted from: Ciullo M et al. 2002 (53)

In 11q13 region are located several oncogenes, including *CCND1* and *CTTN* (cortactin). The amplification and upregulation of the oncogene *CCND1* is one of most studied alterations and it has been associated with laryngeal cancer due to alterations of cell cycle regulation. Other genes in this region, such as *FADD* (Fas associated protein with dead domain) and *ORAOV1* (oral cancer overexpressed 1) have been studied in the last years (33,51).

CCND1: Most studies focused on *CCND1* and its involvement in laryngeal cancer progression. It has been proved that *CCND1* is associated with carcinogenesis (Jin and Jin, 2007). Cyclin D1 [Uniprot: P24385] has an important role in cell cycle progression from G1 to S-phase through directly binding to CDK4 and CDK6. Overexpression of this gene allow cell growth in the absence of growth signals which is essential for cancer development (54,55). Since overexpression of cyclin D1 has been found in pre-malignant lesions, it is thought that this is an early event in tumourigenesis which is associated with a poor prognosis(33,55). According to Almadori et al. (2004) cyclin D1 overexpression always anticipates gene amplification which is thought to be a more stable and non-reversible alteration in tumour cells (24).

CTTN: this gene [Uniprot: Q14247] is involved in the organization of actin cytoskeletal and cell structure. In addition, cortactin has a role in the regulation of cell migration. Its aberrant regulation contributes to tumour cell invasion and metastasis.

Cortactin is involved in cancer cells growth as well as in tumour progression, possibly by impairing of EGFR downregulation (56,57). It has been seen that overexpression of *CTTN* results in a bigger invasive potential (58). Amplification of *CTTN* is also associated with a poor prognosis in HNSCC because expression of cortactin has been shown to correlate with lymph node metastasis (59).

FADD: *FADD* [Uniprot: Q13158] mediates FAS-induced apoptosis. FADD is an apoptotic adaptor molecule that is able to recruit caspase-8 or 10 to activate receptors (Fas or TNFRSF1A) in order to form an aggregate known as death-inducing signalling complex (DISC) which will induce caspase-8 activation. This event will lead to initiation of subsquent cascade of caspases mediating apoptosis. Its overexpression in laryngeal carcinoma seems to affect cell cycle regulation (50,60).

ORAOV1: this gene has probably a role in the development and/or progression of human cancers and it is associated with a bad prognosis and low survival rates. Although *ORAOV1* is amplified in laryngeal carcinoma, its role on laryngeal carcinogenesis is still unknown and more studies need to be carried out (51).

1.4.7.7 Region 13q

Stembalska et al. (2006) identified two critical chromosomal regions as probable targets of deletion by CGH analysis: 13(q21-q32) and 13q34. Within this regions, LOH analysis revealed three different regions of deletions: 13(q21.1-q22.1), 13(q31.1-q32.3) and 13q34. Additionally, they observed a high frequency of LOH in 13q14 which comprises *RB1* (retinoblastoma 1) gene [Uniprot: P06400], a key regulator of entry into cell division and a tumour suppressor gene (61). Although allelic loss of *RB1* gene is frequent in LSCC, the role of its inactivation is still unclear in this type of cancer (33). A few tumour suppressor genes and some genes implied in carcinogenesis are mapped in these regions. For example, *BRCA2* (breast cancer 2, early onset), mapped on 13q21 has already been associated with several types of human cancers and it predisposes to various types of cancer, including laryngeal carcinoma (61,62). Also, mutations in *ING1* (inhibitor of growth family, member 1), mapped on 13q34, have already been found in HNSCC. This particular gene is involved in control of cell cycle and apoptosis. In LCSS, Stembalska et al. (2006) identified three possible important genes within 13q31.1-q32.3 region: *GPC5* (glypican 5), *GPC6* (glypican 6) and *CLDN10* (claudin 10). The first two are involved in control of cell proliferation and

the last one codes for an integral membrane protein and a component of tight junction strands (61).

1.4.7.8 Region 17p

Alterations in the tumour suppressor gene *TP53* (17p13) are also extensively study in LSCC, suggesting that mutations may be an early event in the neoplastic transformation. *TP53* is often inactivated at the time of transition from pre-invasive to invasive carcinoma. Expression and accumulation of p53 is favoured in response to many stressful stimulus, resulting mainly in activation of genes that are responsible for DNA repair, cell cycle arrest and apoptosis. Patients with mutation in *TP53* have poor prognosis whit shorter survival or a poor response to treatment (29,33).

Todorova et al. (2014) detected this mutation in 43.5% of LSCC patients. However, they demonstrated that alterations in *TP53* gene increased with the advancing of tumour, suggesting that this event probably occurs in later stages of LSCC (29).

1.4.8 Epigenetic Alterations

Epigenetic alterations can be defined as changes in gene function that cause a stably heritable phenotype without modifying the DNA sequence which means that these alterations are not encoded in the genome (4,63). The three main systems that are involved are DNA methylation, histone modification and RNA-associated silencing. Epigenetic changes are essential for physiological processes such as differentiation, silencing of chromosomal domains, stem cell plasticity, aging and genomic imprinting (4). However they are also associated with pathophysiological conditions, including the carcinogenesis process. The disruption of any system may result in an abnormal activation or silencing genes which may lead to the development of cancer (4,13,63).

1.4.8.1 DNA methylation

Within the epigenetic modifications, DNA methylation is still the one that is best-studied in mammals. DNA methylation is defined as the addition of methyl groups (CH_3) to the cytosine at carbon 5 position in DNA, resulting in the formation of 5- methylcytosine. It contributes to normal cell development, silencing of elements that are repeated through the genome, regulation of tissue-specific gene expression and imprinted alleles. DNA methylation has extensive effects on cellular growth and genomic stability. In mammalian cells, the most studied modification occurs at CpG nucleotides, where the cytosine is

methylated within this dinucleotide. There are about 28 million CpG sites in the genome, but in spite of being distributed across the human genome, CpG nucleotides are located mainly in regions with large repetitive sequences, such as centromeric repeats and in gene regulatory regions in short CpG-rich DNA stretches known as CpG islands. This CpG islands are mainly located at 5'end that occupy approximately 60% of the gene promoters in human cells (4,64).

DNA methylation is catalysed by mammalian enzymes - DNA methyltransferases – that can be classified in: (1) *de novo* DNA methylatransferases (DNMT3A and DNMT3B) and (2) maintaining DNA methylatransferases (DNMT1). DNMT3A and DNMT3B target unmethylated CpGs and its activity is independent of replication while DNMT1 is activated during DNA replication and finishes the methylation process of a partially methylated DNA present in the cell after its division. Regardless the type of enzyme, the result of its activity will be the generation and maintenance of DNA methylation patterns that are hereditary. In tumours, although these enzymes appear to be overexpressed, so far only DNMT3B and DNMT1 were implicated in cancer development (4,65).

The methylation of CpG nucleotides contribute to oncogenesis (Figure 8) mainly through hypomethylation of cancer genome, hypermethylation of the promoters of tumour suppressor genes and through direct mutagenesis. Additionally, DNA methylation promotes the binding of chemical carcinogens to DNA and increases the rate of Ultraviolet-induced mutations.

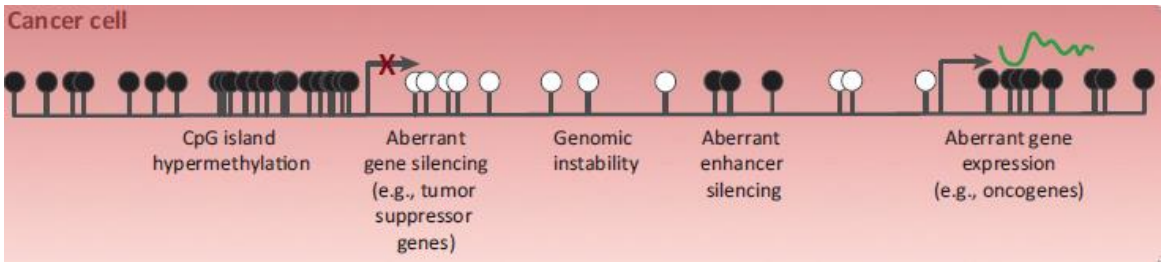

Figure 8 - DNA methylation in cancer cells. (*Black circle - methylated CpG; White circle - unmethylated CpG.)*. CpG islands hypermethylated, resulting in gene silencing and concomitant hypomethylation of CpG-poor oncogene promoters leads to genomic instability and abnormal gene expression. Adapted from: Stirzaker C et al 2014 (64)

1.4.8.1.1 DNA hypomethylation

DNA hypomethylation or, in other words, loss of DNA methylation, was the first epigenetic alteration identified in cancer cells. CpG-poor regions undergo hypomethylation during cell transformation, following by a global decrease in total genomic 5-methylcytosine in cancer cells. DNA hypomethylation occurs in many genomic sequences,

such as repetitive elements, retrotransposons (reverse transcriptase genes that can move in the genome by reverse transcription of an RNA transposition intermediate), CpG poor promoters, introns and gene deserts (regions without protein-coding sequences neither obvious biological function) (4,64,66).

According to Iliopoulos et al. (2011), three mechanisms have been proposed to understand the role of DNA hypomethylation in tumourigenesis (4):

- Undermethylated DNA can lead to genomic instability, favouring mitotic recombination, deletions and translocations as well as chromosomal rearrangements. Also, hypomethylation of retrotransposons may result in their activation and translocation to other regions, leading to further disruption of the genome;
- DNA hypomethylation may lead to activation and expression of proto-oncogenes which otherwise would be inactivated by methylation of CpG islands in the promoter regions. Some of the genes that are affected are oncogenes such as RAS and S100A4.
- Loss of methylation may lead to disruption of genomic imprinting too. This happens in colorectal neoplasia in which loss of imprinting of *IGF2* (insulin-like growth factor 2) leads to an increased risk of develop this malignancy.

1.4.8.1.2 Hypermethylation of the gene promoters

In normal human cells, CpG islands in tumour suppressor gene promoters are usually non-methylated. Rather, tumour cells are characterized by hypermethylation of promoters where CpG islands are found. This alteration is responsible for the silencing of tumour suppressor genes. Furthermore, hypermethylation of CpG islands can also inactivate other genes through the silence of transcription factors and DNA repair genes, leading to transformation of cells into a neoplastic phenotype. Hypermethylation of gene promoters leads ultimately to the formation of silent chromatin structure and aberrant silencing. The mechanism by which gene promoters are targeted for CpG hypermethylation is still unclear (4).

1.4.8.2 DNA methylation in Larynx Carcinoma

There is little information about epigenetic alterations involved in LSCC carcinogenesis. The epigenetic characteristic more studied is hypermethylation of some genes promoters. However this information and the conclusions drawn from the studies are sometimes conflicting. For example, some studies may state that these particular alterations

are connected with a set of clinic-pathological features while others do not find the same correlation. And this may be explained by the different methodologies, the stage of tumour samples as well as the site where the sample was removed. Also, intra and inter-assay variability has been considered relevant in methylation analysis studies (67,68). Some genes which have been described as having changes of the pattern of methylation during LSCC carcinogenesis are represented in Table 3.

Table 3 - Genes with altered methylation pattern in laryngeal cancer

Gene	Gene name	Location	Function	Level of methylation*	References
APC	Adenomatous Polyposis Coli	5q21-q22	Tumour Suppression	(+)	(30,67)
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	16p22.1	Cell adhesion	(+)	(13,38,69)
CDH13	Cadherin 13	16q23.3	Cell adhesion	(+)	(30)
CDKN2A (p16)	Cyclin-Dependent Kinase Inhibitor 2A	9p21.3	Tumour Suppression	(+)	(13,68,70,71)
CDKN2B	Cyclin-Dependent Kinase Inhibitor 2B (p15, inhibits CDK4)	9p21	Tumour Suppression	(+)	(30,67)
CHD5	Chromodomain-Helicase-DNA Binding Protein 5	1p36.3	Tumour Suppressor	(+)	(13,72)
CHFR	Checkpoint with Forkhead and Ring finger domains, E3 ubiquitin protein ligase	12q24.33	Mitotic Checkpoint	(+)	(30,67)
DAPK	Death-Associated Protein Kinase 1	9q34.1	Apoptosis	(+)	(13,67,68,73–75)

ESR1	Estrogen Receptor 1	6q24-q27	Hormone and DNA binding, activation of transcription; Metastasis-suppressor properties	(+)	(30)
FHIT	Fragile Histidine Triad	3p14.2	Tumour Suppressor	(+)	(73)
GSTP1	Glutathione S-Transferase Pi 1	11q13.2	Protection against DNA damage caused by glutathione	(+)	(30,73)
MGMT	O-6-methylguanine-DNA methyltransferase	10q26	DNA Repair	(+)	(68,71,73,75)
MLH1	mutL homolog 1	3p21.3	DNA repair	(+)	(26,68)
RARβ	Retinoic Acid Receptor, beta	3p24	Tumour Suppression	(+)	(30,67,73)
RASSF1	Ras association (RalGDS/AF-6) domain family member 1A	3p21.3	Tumour Suppression	(+)	(73,75)
S100A4	S100 calcium binding protein A4	1q21.3	Oncogene	(-)	(13,76)
TP73	Tumour Protein p73	1p36.3	Pro-apoptotic and anti-apoptotic properties	(+)	(30)

* (+) – Hypermethylated ; (-) – Hypomethylated

Chromodomain-Helicase-DNA Binding Protein 5 (CHD5): Loss of *CHD5* has been observed in laryngeal carcinoma. *CHD5* [Uniprot: Q8TDI0] is a tumour suppressor gene characterized by a chromodomain, a helicase ATP-binding domain and a functional domain. It is located in the nucleus associated with heterochromatin. *CHD5* is involved in cell proliferation and differentiation through modification of chromatin. *CDKN2A* is a potential

downstream activated gene that regulates the p53/TP53 pathway which prevents cell proliferation (13). Loss of heterozygosity and instability of 1p36 was detected in laryngeal carcinomas. A study made by Wang et al. (2011) shown a decreased *CHD5* mRNA and protein expression levels in LSCC when compared to clear surgical margin tissues. They also observed that downregulation of *CHD5* was significantly associated with advanced stages of LCSS. This study allow them to conclude that decreased *CHD5* expression may contribute to invasion and aggression of LCSS through the decreased apoptosis, increased proliferation and invasiveness. Furthermore, hypermethylation of the gene promoter was identified in Hep-2 cell line as well as in 60% of the LSCC samples (13,72).

***E-cadherin (CDH1)*:** E-cadherin is a calcium dependent cell adhesion glycoprotein encoded by *CDH1* gene located in the chromosome 16q22.1. This transmembrane glycoprotein has a highly conserved cytoplasmic tail which interacts with the cytoskeleton. It is involved in adhesion of epithelial cells as well as cell attachment, cell polarity and tissue architecture. E-cadherin is a key component of adherens junctions between epithelial cells. It is thought that its loss of function may contribute to progression of cancer through the increase of cell proliferation, invasion and/or metastasis (13,77). Rodrigo et al. (2002) demonstrated that E-cadherin is abnormally expressed in supraglottic squamous cell carcinomas and suggested that a decreased *CHD1* expression may be a predictor of lymph node metastases (77,78). Also, in LSCC it has already been seen a correlation between reduced E-cadherin expression and decreased survival rates as well as vascular invasion. Some of the reasons that may explain these observations are *CDH1* mutation, loss of heterozygosity or promoter hypermethylation. On the other hand, loss of E-cadherin was also associated with activation of epidermal growth factor receptor (EGFR) pathways which will promote cell proliferation. Moreover, in head and neck cancers, loss of E-cadherin is frequently found associated with promoter hypermethylation. Although epigenetic silencing is considered to be one of the main mechanisms of E-cadherin loss, more research is necessary to be done in order to identify more specific epigenetic changes in laryngeal SCC patients (13,38).

Starska et al. (2013) accessed the *CDH1* expression levels as well as the DNA methylation *status* of this gene and the results showed a high positive expression of mRNA and protein nuclear fraction of E-cadherin in laryngeal carcinoma tissues with lower

aggressive behaviour (tumour front grading[1]: 6-13) On the other hand, more invasive and aggressive laryngeal tumours (pTNM[2] classification pT3 – pT4) demonstrated a higher frequency of *CDH1* methylation status in the tumour samples. This findings suggest that e-cadherin promoter is under epigenetic control and that *CDH1* promotor methylation is connected with an aggressive, invasive and metastatic phenotype (69).

Cyclin-dependent kinase inhibitor 2A (CDKN2A): *CDKN2A* is a key regulator of cell cycle that is methylated in HNSCC (68). Pierini et al. (2014) accessed methylation status of *CDKN2A* in 100 laryngeal carcinoma samples and obtained a frequency of 47.4%. They also establish an association between *CDKN2A* promoter methylation and increased tobacco carcinogen exposure which leads to gene inactivation, accumulation of genetic abnormalities and finally to cancer development. Also, a significant correlation was attributed to promoter hypermethylation and invasion of regional lymph nodes by cancer cells, suggesting that this event play a role in tumour cell migration (68).

Death-Associated Protein Kinase 1 (DAPK1): *DAPK1* codes for a calcium/calmodulin dependent serine/threonine kinase which is mapped in chromosome 9q21.33. It is a tumour suppressor gene involved in many cellular signalling pathways that control cell survival, apoptosis and autophagy (13). In previous studies, the methylation of *DAPK1* was proven to be a frequent event in laryngeal carcinoma (13,73,74). The loss of *DAPK1* expression may be implied in loss of growth control and acquirement of apoptosis resistance (73). In LCSS, López et al. (2014) observed an association between the absence of methylated *DAPK1* and advanced-stage tumours. It is known that the methylation of promoter region plays a major part in controlling *DAPK1* transcription in LCSS (13,67).

O-6-methylguanine-DNA methyltransferase (MGMT): *MGMT* [Uniprot: P16455], mapped in 10q26.3, encodes the enzyme O-6-methylguanine-DNA methyltransferase, which is involved in DNA repair through defence against the effects of O6-methylguanine . The enzyme is irreversibly inactivated after repairing alkylated guanine in DNA by transferring the alky group at the O-6 position to cysteine residue in the enzyme.

[1] Sum of 6 parameters (cytoplasmic differentiation, nuclear polymorphism, number of mitosis, mode of infiltration, depth of invasion and plasmalymphocytic infiltration) which were graded from 1 to 4. The maximum score was 24 points and the group studied was divided into 3 subgroups (6-13, 14-21 and ≥ 22 points).

[2] TNM classification of 2003 for head and neck carcinomas applied for classification of primary tumours (pT).

Hypermethylation of *MGMT* has been associated with cases where there are lymph node involvement. Pierini et al. (2014), through the methylation analysis of tumour tissues from 100 patients with LSCC, obtained a percentage of *MGMT* methylation of 60,8% while Paluszczak et al. (2011) obtained a frequency of 54% after analyse 41 cases of LSCC (68,73). The loss of expression may be implied in increased mutation rate due to impairment of DNA repair mechanism induced by cigarette smoke nitrosamines. Once the DNA is damaged, the acquirement of a bigger migration potential as well as enhanced invasiveness is easier (68,71,73).

Retinoic Acid Receptor, beta (RARβ): In HNSCC, loss of *RARβ* expression has been associated with increased keratinizing squamous differentiation in abnormal cells (67). Recently, Paluszczak et al. (2011) showed that, in laryngeal cancer, that lack of hypermethylation of *RARβ* is linked to a late-stage disease where lymph nodes are already involved. Also, methylated *RARβ* seems to facilitate the acquirement of uncontrolled proliferation and apoptosis inhibition (73). On the other hand Fernando López et al. (2014) observed that hypermethylation of *RARβ* was more frequent in LSCC samples which were poorly differentiated with little evidence of keratinization, suggesting that hypermethylation of the gene promoter is an early event of LSCC (67).

Checkpoint with Forkhead and Ring finger domains, E3 ubiquitin protein ligase (CHFR): *CHFR* [Uniprot: Q96EP1] is mapped on chromosome 12q24.33 and codes for an E3 ubiquitin protein ligase. This enzyme is involved in mitotic checkpoints by delaying chromosome condensation in response to mitotic stress caused by microtubule poisons. In HNSCC, promoter hypermethylation of *CHFR* has been found in up to 30% of the cases. López et al. (2014) accessed methylation status of *CHFR* in 53 LSCC samples and 11% of them were aberrantly methylated. Moreover, the samples that had the *CHFR* methylated belonged mainly to a stage IV group of samples, suggesting that aberrant methylation of *CHFR* could emerge as a predictor of late stage LCSS (67). Stephen et al. (2010) also found *CHFR* methylation as one of the most frequently methylated genes (12 of 79 LSCC samples). However this event did not come up as a an independent predictor of late stage LCSS (30).

mutL homolog 1 (MLH1): *MLH1* is a DNA mismatch repair gene which is frequently found methylated in dysplasic lesions of HNSCC (30). Sasiadek et al. (2004) concluded that LOH and methylation were the most important silencing mechanisms of

MLH1 in laryngeal cancer (26). Furthermore, Pierini et al. (2014) found *MLH1* hypermethylation frequency of 46.4% (45 of 100 LSCC samples) and a positive association with lymph node metastases (68).

S100 calcium binding protein A4 (S100A4): *S100A4* encodes a protein that belong to the S100 family of proteins. They are localized in the cytoplasm and/or nucleus and they have been associated with several physiological functions such as regulation of cell cycle progression and differentiation, tubulin polymerization, motility and invasion. *S100* genes comprise at least 13 members which are encoded on chromosome 1q21. S100A4 protein is able to promote angiogenesis, to induce degradation of extracellular matrix and to interact with cytoskeletal proteins which are involved in cell motility (79).

In LSCC, Liu et al. (2010) reported a higher expression in metastatic lymph nodes when compared to LSCC tissue and adjacent normal mucosa, suggesting that *S100A4* may has a role in metastasis of LSCC. The mechanism responsible for regulation of *S100A4* expression is not clear yet. However, this study also suggested that *S100A4* is regulated by DNA methylation. Therefore, hypomethylation seems to lead to high levels of *S100A4* expression which are associated with metastatic progression in LSCC (79). In tumour development and progression, S100A protein seems to be involved in not only in cell motility, invasion and migration but also in cell apoptosis, cell growth and differentiation. (76).

1.4.9 Changes in Signalling Pathways

In the past few years there have been many studies focused on tumour biology, including several subcellular pathways that have potential roles in tumour progression. Although there are evidences that demonstrate the essential role of signalling pathways in development and progression of head and neck cancers, the studies concerning alterations in these pathways on LSCC are little and further studies are needed to understand better the biology of LSCC individually (80,81). Some of these studies are going to be mentioned in the next sections.

1.4.9.1 *NOTCH signalling pathway*

Notch signalling pathway initiates when occurs an interaction receptor-ligand between two neighbouring cells, leading to proteolytic cleavages that release the cytoplasmic portion of Notch (IC) from de membrane. After Notch-IC enters into the nucleus, it binds to

a transcription factor known as CSL. Next, co-activators are recruited as well as histone acetyltransferases, leading to activation of CSL. In the absence of Notch signalling, CSL acts as transcriptional repressor by its binding to the promoters of its target genes and also the recruitment of co-repressors and histone deacetylases (82).

The Notch signalling pathway is implied in many cellular processes, including the maintenance of stem cells, cell fate, proliferation and differentiation of epithelial cells and apoptosis (82).

In 2009, Jiao et al. suggested for the first time a potential association between the role of *NOTCH1* signalling pathway and LSCC progression by using laryngeal squamous cell carcinoma cell line Hep-2. Notch1 is one of the multifunctional transmembrane Notch receptors that have important roles in cellular differentiation and carcinogenesis. After activation of this receptor, it will be translocated to the nucleus and it will transactivates many target genes. Jiao et al. (2009) demonstrated that activation of Notch1 signalling pathway inhibits cell proliferation and is capable of cell cycle arresting in G_0 to G_1 phases as well as inducing apoptosis and reducing migration ability. These functions were coupled with alterations of numerous genes such as: (1) downregulation of cyclin D1 and *CDK2* and upregulation of *p53* important for cell cycle arrest; (2) overexpression of caspase-9 and caspase-3 which have a role in apoptosis and (3) downregulation of matrix metalloproteinases 2 and 9 which are important for loss of the ability of cell migration (80).

1.4.9.2 EGFR-mTor signalling pathway

One of the most studied alterations in carcinogenesis is the overexpression of the transmembrane tyrosine kinase *EGFR* which seems to be implied in cell transformation. The AKT/mTOR axis is one of the downstream pathways regulated by *EGFR* and belongs also to the 3-kinase (PI3K)-AKT pathway which has been implied in multiple cell functions such as proliferation, metabolism, autophagy, migration and apoptosis, being aberrantly activated in transformed cells (81,83). Lui et al. (2013) observed that, within HNSCC group, the prevalence of PI3K pathway mutations was higher in laryngeal tumours (84). AKT and mTOR proteins modulate cell cycle. Additionally, the latest represents a point where other cellular signalling pathways converge and so it is involved in apoptosis, cell survival, cell transformations, invasion, metastasis and angiogenesis in many human cancers (81).

LSCC patients in which mTOR is highly expressed seem to have a significant shorter disease free survival. Lionello et al (2014) found a direct and strong association between

EGFR and mTOR expression in LSCC cells. Their results also suggest that mTOR pathway might be involved in neo-angiogenesis in LSCC (81). Dionysopoulos et al. (2013) found also a strong interdependent relationship between mTOR and *CCND1* expression and observed that patients with both molecules expressed had higher risk for shorter disease free survival than patients with high expression of only one (45).

1.4.9.3 JAK-STAT pathway

STAT (Signal transducers and activators of transcription) proteins are cytoplasmic transcription factors that transduce signals from cytokines and growth factors to the nucleus. Also they regulate the expression of several target genes (85). The JAK-STAT pathway is widely implicated in normal cellular functions such as proliferation and differentiation, angiogenesis, innate and adaptive immunity and apopotisis. The abnormal activation of this pathway contributes to formation and progression of human cancers. Overall, constitutively activated JAK-STAT signalling pathway have been implicated not only in the progression but also in prognosis of cancer and its resistance to conventional treatment (86).

One of the members of STAT family, *STAT3*, has been considered to be an oncogene which have a key role in promoting proliferation, differentiation and cell cycle progression. Some studies had shown that inhibition of *STAT3* activity in human cancer cells induces apoptosis and/or cell cycle arrest, abrogation of transforming growth factor and suppression of oncogenic cell growth (87). Gao et al. (2005), by inhibiting *STAT3* expression with a siRNA, demonstrated that downregulation of *STAT3* leaded to growth suppression and to induction of apoptosis in Hep2 LCSS cell line (87). Also, through the usage of a selective JAK-2 inhibitor, Zhang et al. (2010) suggested that down-regulation of *STAT3* is associated with decreased proliferation and increased apoptosis in the same cell line (85).

1.5 Cytogenetic and Cytogenomic Lab Techniques and Cancer Genetics

1.5.1 Array CGH (comparative genomic hybridization)

The array CGH is a technique that allows the screening of the complete genome with the purpose of detecting genomic imbalances, namely copy number variations (CNVs). This technique is based on CGH, a competitive hybridization reaction in which the genome of a test sample or control compete for binding to a preparation of normal metaphase chromosomes (88). The array CGH (aCGH) combines the CGH and microarray techniques. The difference lies in the fact that it is used a solid support, usually a glass microscope slide,

with small cloned or synthesized DNA fragments (oligonucleotides with about 200kb in size) instead of using metaphase chromosomes. These oligonucleotides represent target areas of the genome and that alone increases the resolution of traditional CGH technique that allowed only the detection of chromosome aberration between 5 and 10 Mb. Each microarray chip contains up to 180 000 oligonucleotides (89–92).

The underlying mechanism of aCGH is based on the following steps: extraction of DNA sample and controls, marking them with different fluorescent probes (Cy3 and Cy5), mixing and homogenisation of the samples and application on the microarray. Subsequently, the relative fluorescent intensity of the signal is captured and quantified by an imaging software and the values are compared between patients and controls. Finally, it is obtained a representative plot of the ratio test versus control at different positions in the genome. The ratio of red fluorescence (Cy5) and green (Cy3) in each spot represents the quantity of DNA sample. In a situation where the amount of DNA in the test sample and in the control are equal the fluorescence ratio will be 1:1, showing a yellowish colour. When there are differences in the amount of DNA, namely gain or loss of genetic material, the proportion of fluorescent Cy3: Cy5 changes (89–92).

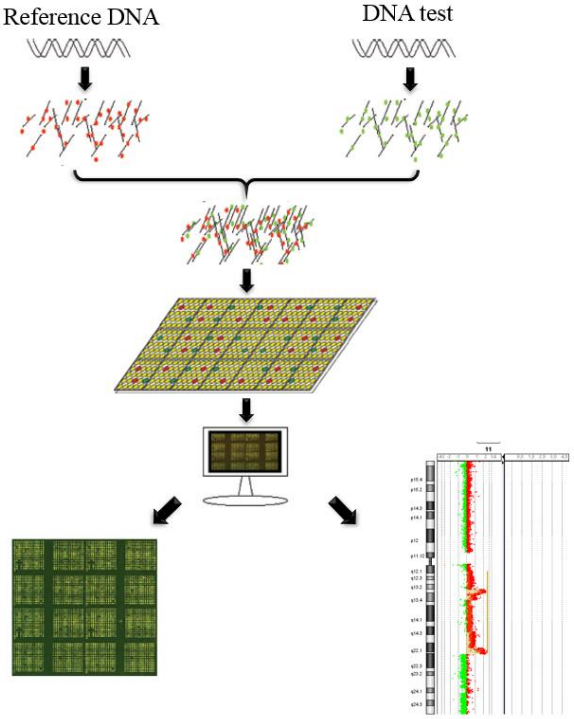

Figure 9 - Schematic representation of the principle underling CGH microarray technology. Adapted from: Bejjani BA and Shaffer LG 2006 (92)

The main advantage of aCGH lies in its simplicity, automation, high resolution and the possibility of simultaneous detection of imbalances caused by aneuploidy, deletions,

duplications and/or amplifications in any locus represented on the array. Also, aCGH does not require cell culture neither big amounts of DNA (0.2-1.0µg, depending on the microarray format). The biggest limitation of arrays lies in the fact that it do not discriminate potentially pathogenic loci of segmental duplications that are often found in the genome of normal individuals and thus can induce a wrong interpretation of the results. Moreover, aCGH is not capable of detecting balance chromosomal abnormalities, such as balanced translocations, insertions, inversions neither triploidies (89,91).

Array CGH has been considered a useful tool in research. Through this technique is possible to identify DNA copy number profiles for various cancers which are widely associated with multiple gains and losses of total/partial chromosomal regions. Since aCGH does not require cell culture and metaphase chromosomes, it is considered a highly desired technique for studding genetic patterns of solid tumours, which access directly the DNA content and link it to any possible dosage alteration to chromosome abnormalities. Furthermore, the hope lies in the discovery of associations between these profiles and prognostic markers that will be helpful to follow-up patients and to guide clinical treatments (92).

1.5.2 Multiple Ligation-dependent Probe Amplification (MLPA) assay

Currently, most of the techniques for detecting CNVs are not capable of detecting deletion or duplication of a single exon. Furthermore, these methods are time consuming, expensive, difficult to apply the analysis of multiple samples and require large amounts of DNA. The MLPA technique thus arises as an attempt to overcome some of these limitations. The underlying principle of MLPA is based on hybridization of two probes to the target sequence. Each probe is composed by one short synthetic oligonucleotide and one phage M13-derived long probe oligonucleotide. Both oligonucleotides have target-specific sequence, on 3’ end, and a universal primer sequence, on 5´end, which allows a simultaneous multiplex PCR amplification. One half-probe contains also a stuffer sequence of 19-370 nucleotides between the 5 'and 3' ends that allows the differentiation during electrophoresis of the length of the probe itself and the size of the amplification product (93–95).

The main steps of the MLPA reaction are DNA denaturation and probes hybridization, ligation reaction, PCR amplification and detection and quantification of the product (130 to 480bp) by capillary electrophoresis. In the first step, after addition of the two half-probes, they will be able to recognize target-specific sequences, and only in the presence of a match these two oligonucleotides will be ligated and amplified. Next, the PCR reaction is performed with only a pair of primers in which one of them is fluorescently labelled. Since only the ligated probes will be amplified in this step, the number of probe ligation products is a measure of the number of target sequences in the sample. Finally the products are separated by capillary electrophoresis following by measurement of the height of PCR derived fluorescence peaks in order to quantify the amount of amplification product after comparing it with control samples (93–95).

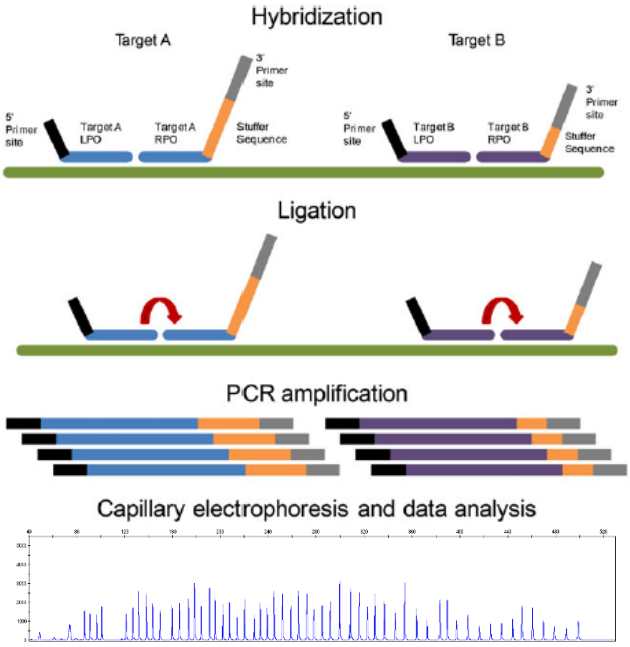

Figure 10 - Schematic representation of the principle underling MLPA assay. Adapted from: Willis AS et al 2012 (180)

The results are presented as ratio DNA sample versus DNA control and if the value lies between 0.8 and 1.2 that means that both of the alleles are present in the sample. On the other hand, if values are greater than 1.2 that means that there is a gain of genetic material while values below 0.8 means that a loss occurred (Table 5). The control present in SALSA MLPA kits comprises nine control fragments: four Q fragments (64, 70, 76 and 82 nt), a 92 nt brenchamark probe, two D fragments (88 and 96 nt) a X fragment and a Y fragment (Table 7) (93–95).

The main advantages of this assay are: (1) multiplex technique with a high throughput, (2) low cost and technically uncomplicated method and (3) detection of small rearrangements. On the other hand, the main limitations are: (1) only detect sequences recognized by the probes used, (2) more sensitive to contaminants and DNA degradation than PCR, (3) cannot be used in single cells and (4) it is not suitable for detection of unknown point mutations (93,96,97).

1.5.3 Methylation Specific Multiple Ligation-Dependent Probe Amplification (MS-MLPA) assay

In addition to be able to determinate the copy number, MS-MLPA also is useful to access the methylation status of up to 50 DNA sequences in a single reaction (98).

In MS-MLPA, the probe sets contain many methylation-specific probes. The sequence targeted mentioned above contains a restrictions site, an unmethylated GCGC sequence, which is recognized by HhaI endonuclease. The mechanism underlying the MS-MLPA is similar to the MLPA. However, after the hybridization step, the mixture is divided into two parts, being one treated as a normal MLPA reaction and in the other HhaI is added. The enzyme will digest the probes hybridized to unmethylated DNA and the ones hybridized with methylated DNA remain undigested due the presence of methyl group. An undigested ligated probe will be amplified by PCR reaction, resulting in a normal peak signal. The amplification products (64-500nt) are separated by capillary electrophoresis too. The levels of DNA methylation are obtained through the comparison of the peak height of methylation specific probes, between DNA sample and control (93,98).

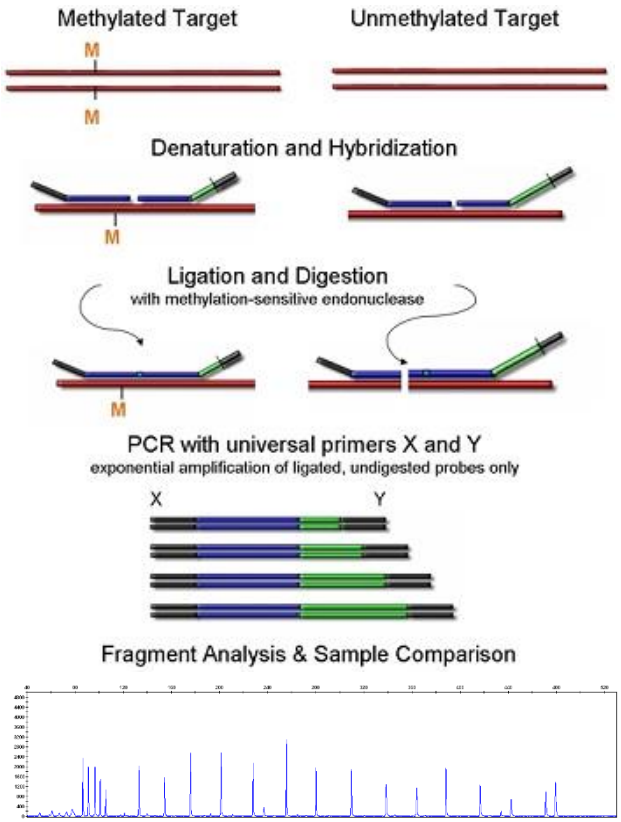

Figure 11 - Schematic representation of the principle underling MS-MLPA assay. Adapted from: MRC-Holland (181).

The main applications of MLPA and MS-MLPA in the field of molecular studies of cancer include the study of germ line deletions and/or duplications in hereditary cancers, analysis of somatic deletions/duplications in genes involved in tumour progression and response to therapy and analysis of DNA methylation, mainly as mechanism of tumour suppressor genes silencing (93).

2 Aims

As mentioned, larynx plays a major role in maintenance of basic features of human life such as communication, breathing and swallowing. Tumours in this region compromise all of these functions as well as other vital anatomical structures, leading to dramatic decrease in the patient's quality of life. On the other hand, despite the improvements observed in multimodal therapies, 5-year survival rates does not improved significantly.

The study of genetic and epigenetic alterations in the progression of laryngeal cancer is considered to be pivotal for the development of new strategies of diagnosis and treatment, thus assisting in the development of personalized medicine strategies for each patient. However, the number of studies concerned specifically genetic alterations in LSCC is limited, because these tumours are usually studied as part of HNSCC. The lack of knowledge about this particular subject arises several problems, including the lack of correlation between molecular alterations and LSCC progression as well as its histophatological phases.

Taking this into consideration, the main goal of this project is to make a genetic and epigenetic characterization, through array CGH, MLPA and MS-MLPA techniques, of fresh frozen tissue samples obtained from patients diagnosed with LSCC. Moreover, since *SALSA MLPA probemix P428* was originality developed for detection of copy number variations of genomic DNA sequences which are known to have diagnostic and prognostic importance in HNSCC, it is also the propose of this project to evaluate if this probemix is suitable to detect genetic alterations in LSCC.

3 Materials and Methods

3.1 Samples

The fresh-frozen tissue samples analysed in this study were obtained upon resection surgery of eight patients diagnosed with LSCC in the Department of Otorhinolaryngology - Head and Neck Surgery of Coimbra Hospital and University Centre (CHUC). After the surgery, the samples were collected from the tumour tissue in the Department of Pathology of CHUC. All samples were given to the Cytogenetics and Genomics Laboratory in Faculty of Medicine of University of Coimbra for genetic and epigenetic studies, between December 2013 and February 2015. For each patient, one sample of tumour and non-tumour were collected. Patient 7 was the only one who was not possible to collect a non-tumour sample. Regarding the non-tumour samples, they were contralateral to the primary tumour. Regarding the control samples, four palatine uvulas were used in order to perform genetic and epigenetic analysis. Those samples were obtained upon resection surgery of patients diagnosed with sleep apnoea and/or snoring in the Department of Otorhinolaryngology - Head and Neck Surgery of CHUC. These patients did not have clinical history of cancer.

Table 4 – Clinical Characterization of patients diagnosed with LSCC that were included in the study. (♀)-Female; (♂)-Male; pTNM- TNM classification of Malignant Tumours 2009; NA – No data available

Patient	Sex	Age	Anatomical Site	pTNM		
				pT	pN	pM
1	♀	40	Larynx	T3	N2	Mx
2	♂	55	Larynx	T4	N0	Mx
3	♂	40	Palate/Left Tonsil		NA	
4	♂	71	Pharynx		NA	
5	♂	64	Larynx		NA	
6	♂	60	Supraglottic	T4	N2c	Mx
7	♂	61	Epiglottis	T3	Nx	Mx
8	♂	69	Hypopharynx	T3	N2	Mx

3.2 DNA extraction

Genomic DNA was extracted from fresh frozen tissues using *High Pure PCR Template Preparation Kit* (Roche GmbH, Manmheim, Germany), according to the manufacture's recommendations (99).

3.3 DNA quantification and assessment of DNA purity

After DNA extraction, DNA quantification (ng/µL) and its purity were assessed by spectrophotometer NanoDrop-1000 (Thermo Fisher Scientific, Wilmington, USA) using 2µL of sample. The DNA purity was evaluated using the values of the ratio between absorbance at 260 nm and 280 nm (A_{260}/A_{280}) and between absorbance at 260 nm and 230 nm (A_{260}/A_{230}). The DNA is considered to be pure when value of the ratio A_{260}/A_{280} is approximately 1,8 and the value of the ratio A_{260}/A_{230} is between 1,8 and 2,2 (100).

3.4 Array CGH (comparative genomic hybridization)

Copy number variation (CNVs) of the samples were assessed through *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis* (Agilent Technologies, Santa Clara, California, USA) according to the manufacture's recommendations (101). Array CGH was carried out using an Agilent SurePrint G3 Human Genome microarray 180K (Agilent Technologies, Santa Clara, CA, USA), an oligonucleotide microarray containing approximately 180,000 60-mer probes with a 17-kb average probe spacing. For each array CGH experiment, 1100ng of DNA were used in a total volume of 26µL. Also, for each CGH reaction, one sample and one sex-matched control were used and they were labelled with Cy5 and Cy3 respectively. The fluorescent DNA labelling was followed by purification and the degree of labelling was accessed by NanoDrop-1000 (Thermo Scientific, Wilmington, USA). The expected specific activity of Cy3 or Cy5 labelled samples with a 1µg input of gDNA is 25-40 pmol/µg and 20-35 pmol/µg respectively. The followed steps include clean-up, hybridization and microarray wash. During the clean-up, the excess of primers and nucleotides were removed using Amicon 30-kDA individual filters (Millipore, Billerica, MA, USA). After, Cy5-labelled tumour sample and Cy3-labelled control were combined with Human Cot-1 DNA (Kreatech Diagnostics, Amsterdam, Netherlands), treated with Agilent blocking agent and 2x Hi-RPM buffer and hybridized in a 4x180K oligonucleotide slide, at 65 °C for 24 h in a hybridization oven (Agilent Technologies) at a constant rotation of 20 rpm. The hybridized slides were scanned with a microarray scanner (scanner C, Agilent) and the data was processed with the Feature Extraction software v10.7. The data from the slide images was analysed using an aberration calling algorithm, ADM-2, and a threshold filter that requires at least three contiguous probes. Finally, the results were analysed using Agilent Genomic Workbench v6.5.

In this project, eight fresh frozen tissue tumour samples and two controls sex-matched were analysed.

3.5 Multiplex Ligation-dependent Probe Amplification (MLPA) Assay

CNVs were also assessed by MLPA assay, following the protocol *MLPA protocol for the detection and quantification of nucleic acid sequences - MLPA DNA Protocol version MDP-005* (MRC-Holland, Amsterdam, The Netherlands) and the manufacture's recommendations (95). The probemix used to detect copy number changes in the samples was *SALSA MLPA probemix P428* (MRC-Holland, Amsterdam, The Netherlands) which contains 41 probes for 12 different chromosomal regions that have been suggested to have clinical and/or prognostic relevance for HNSCC. This kit also contain 11 reference probes which detect 10 different autosomal chromosomal locations that have been suggested as being relatively quiet in HNSCC (Figure 12) (32,37,50,102–113).

In order to ensure the presence of sufficient DNA for analysis it was used a total volume of 5µL with approximately 100ng of DNA which were heated at 98ºC for 10 minutes. Next, the probemix was added to the samples following by its heating at 95ºC for 1 minute and its incubation for 15h at 60ºC. After hybridization, the ligation reaction was performed for 15 minutes at 54ºC following by inactivation of ligase enzyme and multiplex PCR reaction (35 cycles of 30s at 95ºC, 30s at 60ºC and 1min at 72ºC). All the reactions were carried out in the thermal cycler ABI 2720 (Applied Biosystems, Foster City, CA, USA). After PCR amplification, 1µL of sample and 9,4µL of mixture Rox™ plus HiDi formamide (Applied Biosystems, Foster City, USA) were applied in order to allow the correct fragment separation by capillary electrophoresis through ABI PRISM™ 3130 Genetic Scan (Applied Biosystems, Foster City, USA). For each MLPA experiment, three controls and one negative control were used. The obtained electropherograms were analysed using the software GeneMapper v4.1 (Applied Biosystems, Foster City, USA) and the software Coffalyser.Net (MRC-Holland, Amsterdam, The Nertherlands) displayed the ratio between tumour samples and controls. The interpretation of ratio values was done according to Table 5.

Table 5 - Interpretation of MLPA results given by Coffalyser.Net

Ratio	Copy number status
≥ 1,2	Numerical Gain/Amplification
0,8-1,2	Normal
< 0,8	Numerical Loss

In this project, eight fresh frozen tissues tumour and seven non-tumour samples and four controls were analysed by MLPA.

3.6 Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) Assay

As mentioned, MS-MLPA assay is a modification of the conventional MLPA assay. In addition to detect CNVs, it also assesses the methylation status of a sample simultaneously in a single reaction. The protocol used was *General MS-MLPA protocol for the detection and quantification of nucleic acid sequences and methylation profiling. - MS-MLPA protocol version MSP-v004* (MRC-Holland, Amsterdam, The Netherlands), and all the manufacture's recommendations were followed (98).

The procedure underlying the MS-MLPA assay is similar to the MLPA assay. However, after the hybridization step, the mixture is divided into two parts, being one treated as a normal MLPA reaction and in the other restriction HhaI endonuclease (Promega, Madison, USA) is added. The probemix used was *SALSA MS-MLPA probemix ME001-C2 Tumour suppressor-1* (MRC-Holland, Amsterdam, The Netherlands), which contains 26 probes that are able to detect the methylation status of 24 different tumour suppressor genes that are frequently silenced by methylation in tumours. Also, this probemix contains 15 reference probes which are not sensitive to Hha1 activity. Besides that, all 41 probes are able to detect copy number variations (Figure 12) (114–119).

The interpretation of the copy number variation was made as described in MLPA assay. The level of methylation percentage was evaluated using the values provided by the software Coffalyser.Net (MRC-Holland, Amsterdam, The Nertherlands). Thus, samples with genes in which the methylation percentage is greater than 20% were considered to be methylated.

In this project, eight fresh frozen tissues tumour and seven non-tumour samples and four controls were analysed by MS-MLPA.

Figure 12 - Chromosomal distribution of the genes studied using SALSA MLPA probemix P428 (●) and SALSA MS-MLPA ME001-C2 tumour suppressor probemix (●). Reference probes are represented by *. (Exception: CDK6 is a target probe in P428 while in ME001-C2 is a reference probe; different exons of BRCA2 are used as a target probes in both probemixes while another one is also used as reference probe in ME001-C2).

4 Results

4.1 Array CGH (comparative genomic hybridization)

Eight fresh-frozen samples were analysed through Array CGH and the results showed several structural rearrangements. With the data given by Feature Extraction software v10.7 and Agilent Genomic Workbench v6.5, a human ideogram showing a summary of chromosomal gains and losses was built (Figure 13). Overall, the majority of the chromosomal alterations detected were gains, especially on chromosome 3q, 6p25.3, 8q, 11q, 14q13.1 and chromosome X. However, significant losses of genetic material were also observed, namely in chromosomes 3p, 6q, 8p, Yp and Yq. The minimal common regions that were found amplified among patients were 3q26.1 (8/8), dup(3)(q26.33q27.3) (6/8), 3q29 (6/8), 6p25.3 (6/8), 8q24.21 (6/8), 11q13.3 (6/8), 14q13.1 (5/8), Xp22.31 (4/8) and Xq21.1 (5/8) while the minimal common regions that were found deleted were del(3)(p26.3p12.1) (3/8), del(6)(q24.3q27) (3/8), 8p23.1 (7/8), 8p11.22 (4/8), 9p23.1 (3/8), 12p13.31 (3/8), del(Y)(p11.32p11.2) (4/7) and del(Y)(q11.21q11.221) (7/7) (Table 6). In one patient there was total loss of chromosome 3 short arm and overall amplification of its long arm.

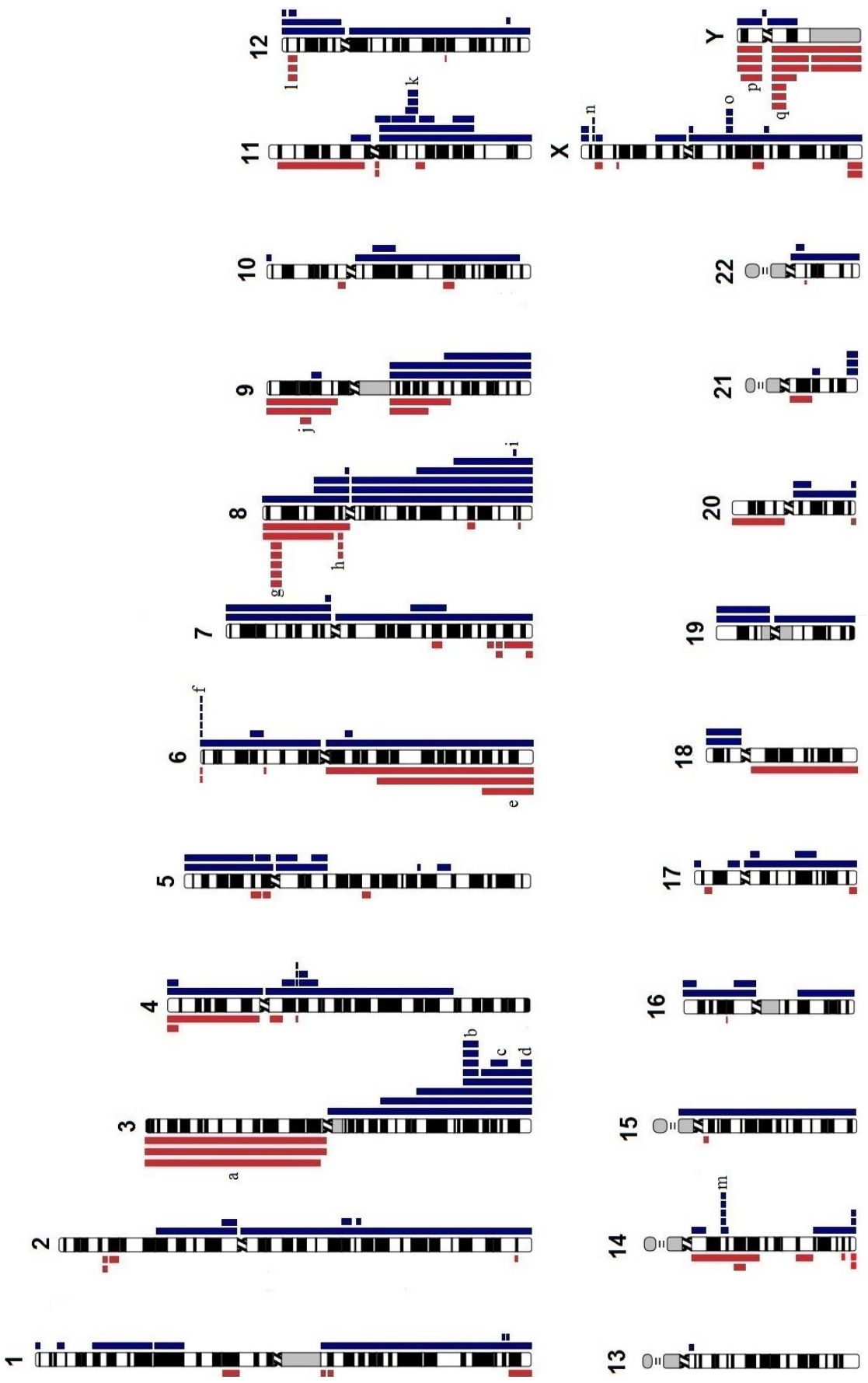

Figure 13 - Summary of chromosomal gains and losses found in eight fresh-frozen samples of LCSS detected by Array CGH. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material. a – del(3)(p26.3p12.1); b – 3p26.1; c – dup(3)(q26.33q27.3); d – 3q29; e – del(6)(q24.3q27); f – 6p25.3; g – 8p23.1; h – 8p11.22; i – 8q24.21; j – 9p21.3; k – 11q13.3; l – 12p13.31; m – 14q13.1; n – Xp22.31; o – Xq21.1; p – del(Y)(p11.32p11.2); q – del(Y)(q11.21q11.221).

Table 6 – Summary of the most common chromosomal alterations detected by Array CGH. (Chr) – Chromosome; (*) – Presence of several genes in chromosomal regions which are common variants in healthy people

Chr	Array CGH findings		Size (bp)	Genes
	Gains	Losses		
3		del(3)(p26.33 p12.1)	86,390,377	*CHL1, VHL, RARβ, RASSF1, MLH1, CTNNβ1, ALS2CL, HESRG, LRTM1, FHIT, GBE1*
	3q26.1		26,568	*No genes*
	dup(3)(q26.33 q27.3)		4,364,765	*DCUN1D1, LAMP3, MCCC1, EIF4G1, PIK3CA*
	3q29		2,717,766	*MUC20, MUC4, TNK2*
6	6p25.3		34,297	*
		del(6)(q24.3q 27)	23,442,704	*
8		8p23.1	483,767	*Defensin family*
		8p11.22	125,449	*
		8q24.21	327,790	*MYC, PVT1, MIR1204, ASAP1*
9	9p21.3		181,477	*CDKN2A, CDKN2B*
11	11q13.3		225,686	*ANO1, FADD, PPFIA1, CTTN*
12		12p13.31	505,76	*No genes*
14	14q13.1		72,265	*SNX6*
X	Xp22.31		19,254	*VCX3*
	Xq21.1		1,985	*MAGT1*
Y		del(Y)(p11.32 p11.2)	733,1100	*CD99*
		del(Y)(q11.21 q11.221)	80,064	*No genes*

4.2 Multiplex Ligation-dependent Probe Amplification (MLPA) Assay

Eight fresh-frozen tumour tissues samples and seven non-tumour samples were genetically characterized by MLPA using *SALSA MLPA probemix P428.* The obtained electropherograms, which were analysed using the software GeneMapper v4.1, showed a peak pattern for each sample.

The quality control of MLPA reaction is ensured by the presence of nine control fragments:

Table 7 - MLPA quality control fragments (95)

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

As mentioned, for each MLPA reaction, three reference samples and one negative control are used. Regarding the control samples, they should present a MLPA peak pattern of DNA sample without any genomic abnormalities (Figure 14). Since the negative control does not have DNA, the Q-fragments are greater than 33% of the 92nt control fragment.

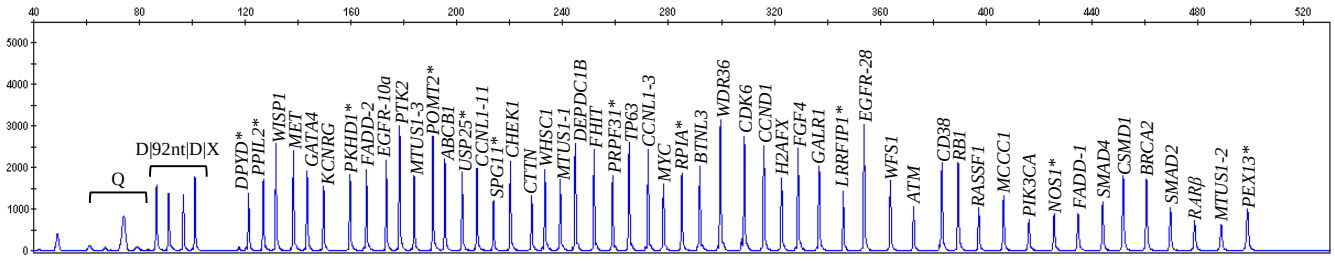

Figure 14 – Electropherogram of a female control sample analysed by MLPA using *SALSA MLPA probemix P428*. * - reference probe

In patient 7, the comparison between reference controls and the sample showed an increase of the peaks corresponding the genes *EGFR* – exon 10a (7p11.2), *EGFR* – exon 28 (7p11.2), *MYC* (8q24.21), *WISP1* (8q24.22) and *PTK2* (8q24.3) and decreased peaks corresponding to the genes *FHIT* (3p14.2), *WHSC1* (4p16.3), *ABCB1* (7q21.12), *MET* (7q31.2), *GATA4* (8p23.1-p22) and *MTUS1* – exon 3 (8p22) (Figure 15).

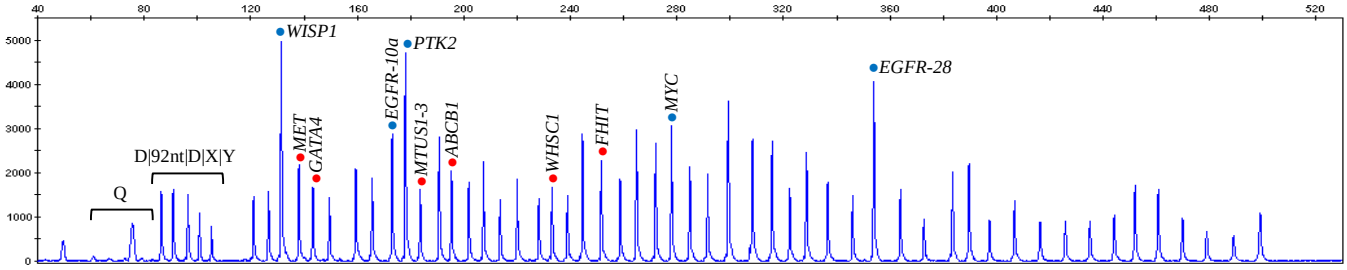

Figure 15 - Electropherogram of a LCSS sample from patient 7 analysed by MLPA using SALSA MLPA probemix P428. (●)-Gain of genetic material; (●)-Loss of genetic material.

4.2.1 Copy Number Variations

4.2.1.1 Tumour Samples

Regarding the tumour samples, 90% (7/8) of the patients had copy number variations in several genes while patient 6 did not have any alteration (Table 9). Among the 52 gene probes, 12 genes did not present any alteration (Table 8): WFS1 and *CD38* in chromosome 4p, *WDR36* and *BTNL3* in chromosome 5q, *ATM* in chromosome 11q, *BRCA2* and *RB1* in chromosome 13q and the reference probes *DPYP* (1p21.3), *RPIA* (2p11.2), *PKHD1* (6p21.2), *NOS1* (12q24.22) and *POMT2* (14q24.3).

Overall, the genetic profile of these tumour samples was mainly characterized by gains of genetic material, especially on chromosomes 3q (4/8), 8q (6/8) and 11q (5/8). The loss of genetic material was higher in chromosomes 3p (4/8) and 8p (4/8).

The gene *MYC* (8q24.21) had the highest number of genetic imbalances in which 75% (6/8) of the patients presented gain of genetic material. The second most common alteration was gain of genetic material in *FADD* – exon 1 (11q13.3) and *PRPF31* (19q13.42) in 62,5% (5/8) of patients. The genes *MCCC1* (3q26.33), *WISP1* (8q24.22), *PTK2* (8q24.3) and *FADD* – exon 2 (11q13.3) were exclusively amplified in 50% (4/8) of the patients which means that no deletions were observed in all the samples studied. *GATA4* (8p23.1-p22) was lost in 50% of the patients. Although *MTUS1* – exon 3 had the same genetic alteration rate (50%), patient 8 presented loss of genetic material while patients 1, 3 and 5 presented gain of it. The genes *PIK3CA* (3q26.33), *CCND1* (11q13.3), *FGF4* (11q13.3), *CTTN* (11q13.3)

and *PEX13* (2p16.1) were exclusively amplified in 37,5% of the patients. Regarding *FHIT*, it was the gene that have the highest rate of exclusive losses of genetic material among the patients included in this study (37,5%) (Figure 16).

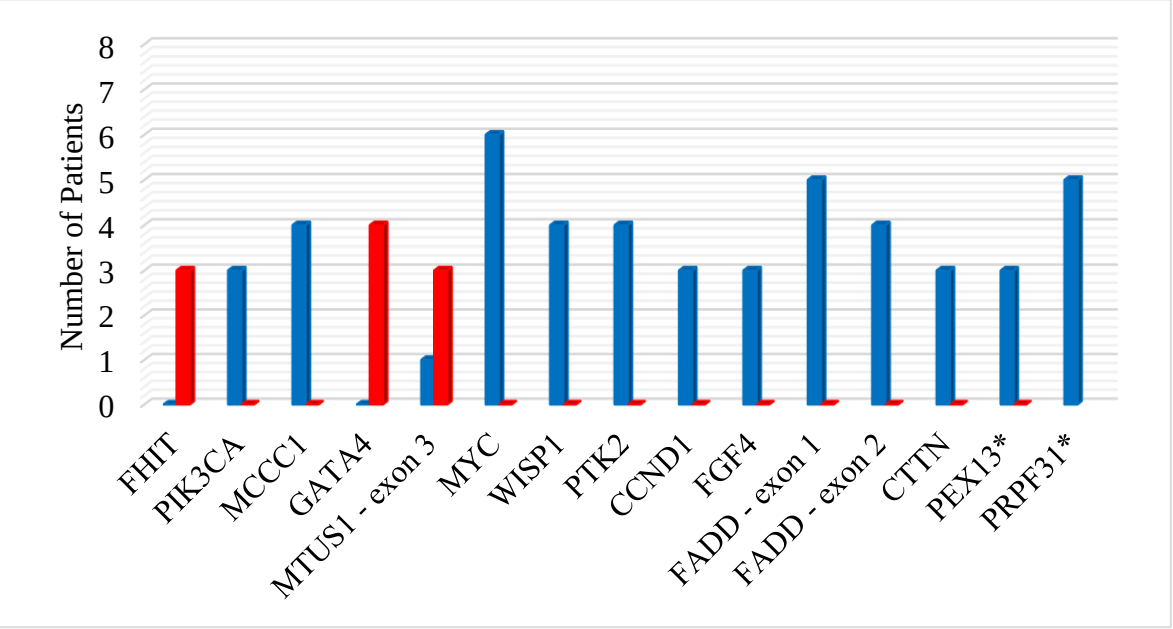

Figure 16 - The most common genetic imbalances in eight patients diagnosed with LSCC detected using SALSA MLPA probemix P428. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material; * – Reference probe

Although *CSMD1* (8p23.3), *MTUS1* – exon 1 (8p22) and *H2AFX* (11q23.3) present genetic alterations in 37,5%, it was observed both loss and gain of genetic material in these three genes: (1) *CSMD1*: lost in patients 1 and 3 and gained in patient 8; (2) *MTUS1* – exon 1: lost in patients 3 and 5 and gained in patient 8 and (3) *H2AFX*: lost in patient 5 and gained in patients 3 and 8 (Table 9).

In addition, gain of the genes *CCNL1* – exon 3 (3q25.31), *TP63* (3q28), *EGFR* – exon 10 and 28 (7p11.2), *SPG11* (15q21.1) and PPIL2 (22q11.21) was observed in 25% (2/8) of the patients while the gain of *CCNL1* – exon 11 (3q25.31), *CDK6* (7q21.2) *MTUS1 – exon* 2 (8p22), *KCNRG* (13q14.3), *SMAD2* (18q21.1) *SMAD4* (18q21.2) and *LRRFIP1* (3q37.3) was present in 12,5% (1/8). Loss of the gene *DEPDC1B* (5q12) was present in 25% (2/8) of the patients while *RARβ* (3p24), *RASSF1* (3p21.3), *CHEK1* (11q24.2), *GALR1* (18q23) and *USP25* (21q21.2) were lost in 12,5% (1/8) of the patients. Finally, *ABCB1* (7q21.12), *WHSC1* (4p16.3) and *MET* (7q31.2) presented loss in one patient and gain in another one: (1) *WHSC1:* gain in patient 3 and lost in patient 7 and (2) *ABCB1* and *MET*: both lost in patient 7 and gained in patient 8 (Table 9).

4.2.1.2 Non-Tumour Samples

Regarding all the seven non-tumour samples, 42,9% (3/7) of them were normal (patients 1, 6 and 8), without any gene or reference probe altered. Among the other non-tumour samples, it was the gain of *PRPF31* (2/7) the variation more common. Finally, alterations in the genes *PIK3CA* (3q26.33), *CDK6* (7q21.2), *SMAD2* (18q21.1), *SPG11* (15q21.1) and *USP25* (21q21.1) occurred in 14,3% (1/7) of the non-tumour samples (Table 9).

It is important to notice that, in some patients, the same alteration appears in both tumour and non-tumour samples. For example, in patient 5, *PIK3CA* is amplified in both tumour and non-tumour samples. Also, in patients 2 and 3, the gain of *PRPF31* is also present in tumour and non-tumour samples (Table 9).

Table 8 - Summary of genetic imbalances detected by SALSA MLPA probemix P428. Chr – Chromosome; (T)-Tumour Sample; (NT)-Non-tumour sample; * - Reference probe; (■) – Gene probe without any alteration in both tumour and non-tumour samples.

Chr	Gene	Gain of Genetic Material		Loss of Genetic Material	
		T	NT	T	NT
3p24.2	*RARβ*			✓	
3p21.31	*RASSF1*			✓	
3p14.2	*FHIT*			✓	
3q25.32	*CCNL1* - exon 11	✓			
	CCNL1 - exon 3	✓			
3q26.33	*PIK3CA*	✓	✓		
3q26.33	*MCCC1*	✓			
3q28	*TP63*	✓			
4p16.3	*WHSC1*	✓		✓	
4p16.1	*WFS1*				
4p15.32	*CD38*				
5q12.1	*DEPDC1B*			✓	
5q22.1	*WDR36*				
5q35.3	*BTNL3*				
7p11.2	*EGFR - exon 10a*	✓			
	EGFR - exon 28	✓			
7q21.12	*ABCB1*	✓		✓	
7q21.2	*CDK6*	✓	✓		
7q31.2	*MET*	✓		✓	
8p23.3	*CSMD1*	✓		✓	
8p23.1-p22	*GATA4*			✓	
8p22	*MTUS1 – exon 3*	✓		✓	
	MTUS1 – exon 2	✓			
	MTUS1 – exon 1	✓		✓	
8q24.21	*MYC*	✓			
8q24.22	*WISP1*	✓			
8q24.3	*PTK2*	✓			
11q13.3	*CCND1*	✓			
11q13.3	*FGF4*	✓			
11q13.3	*FADD - exon 1*	✓			
	FADD - exon 2	✓			
11q13.3	*CTTN*	✓			
11q22.3	*ATM*				
11q23.3	*H2AFX*	✓		✓	
11q24.2	*CHEK1*			✓	
13q13.1	*BRCA2*				
13q14.2	*RB1*				
13q14.3	*KCNRG*	✓			
18q21.1	*SMAD2*	✓	✓		
18q21.2	*SMAD4*	✓			
18q23	*GALR1*			✓	
1p21.3	*DPYP**				
2p11.2	*RPIA**				
2p16.1	*PEX13**	✓			
2q37.3	*LRRFIP1*	✓			
6p12.3	*PKHD1**				
12q24.22	*NOS1**				
14q24.3	*POMT2**				
15q21.1	*SPG11**	✓	✓		
19q13.42	*PRPF31**	✓	✓		
21q21.1	*USP25**		✓	✓	
22q11.21	*PPIL2**	✓			

Table 9 – Summary of genetic imbalances detected by MLPA in all the 8 patients included in this study. (Chr) - Chromosome; (T)-Tumour Sample; (NT)-Non-tumour sample; * - Reference probe; (■) – Gain of Genetic Material; (■) – Loss of Genetic Material

Chr	Gene	Patients													
		1		2		3		4		5		6		7	8	
		T	NT	T	NT	T	NT	T	NT	T	NT	T	NT	T	T	NT
3p24.2	*RARβ*					■										
3p21.31	*RASSF1*									■						
3p14.2	*FHIT*	■				■								■		
3q25.32	*CCNL1* - exon 11														■	
	CCNL1 - exon 3					■									■	
3q26.33	*PIK3CA*					■				■	■				■	
3q26.33	*MCCC1*	■				■				■					■	
3q28	*TP63*									■					■	
4p16.3	*WHSC1*					■								■		
4p16.1	*WFS1*															
4p15.32	*CD38*															
5q12.1	*DEPDC1B*									■					■	
5q22.1	*WDR36*															
5q35.3	*BTNL3*															
7p11.2	*EGFR - exon 10a*													■	■	
	EGFR - exon 28													■	■	
7q21.12	*ABCB1*													■	■	
7q21.2	*CDK6*							■							■	
7q31.2	*MET*													■	■	
8p23.3	*CSMD1*	■				■									■	
8p23.1-p22	*GATA4*	■				■				■				■		
8p22	*MTUS1 – exon 3*	■				■				■					■	
	MTUS1 – exon 2														■	
	MTUS1 – exon 1					■				■					■	
8q24.21	*MYC*	■				■		■		■				■	■	
8q24.22	*WISP1*					■				■				■	■	
8q24.3	*PTK2*					■				■				■	■	
11q13.3	*CCND1*	■		■						■						
11q13.3	*FGF4*	■		■						■						
11q13.3	*FADD - exon 1*	■		■		■		■		■						
	FADD - exon 2	■		■				■		■						
11q13.3	*CTTN*	■		■						■						
11q22.3	*ATM*															
11q23.3	*H2AFX*					■				■					■	
11q24.2	*CHEK1*	■														
13q13.1	*BRCA2*															
13q14.2	*RB1*															
13q14.3	*KCNRG*							■								
18q21.1	*SMAD2*								■	■						
18q21.2	*SMAD4*									■						
18q23	*GALR1*	■														
1p21.3	*DPYP**															
2p11.2	*RPIA**															
2p16.1	*PEX13**			■		■									■	
2q37.3	*LRRFIP1*														■	
6p12.3	*PKHD1**															
12q24.22	*NOS1**															
14q24.3	*POMT2**															
15q21.1	*SPG11**	■					■	■								
19q13.42	*PRPF31**	■		■	■	■	■	■							■	
21q21.1	*USP25**					■			■							
22q11.21	*PPIL2**							■							■	

4.3 Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) Assay

Eight fresh-frozen tumour tissue samples and seven non-tumour samples were also analysed by MS-MLPA using *SALSA MS-MLPA probemix ME001-C2.*

The analyses of electropherograms given by MS-MLPA assay is similar to the one described in MLPA. However, for each MS-MLPA reaction, two eletropherograms are generated: one corresponding to CNVs (Figure 17) and the other corresponds to methylation profile of the samples which were digested with HhaI (Figure 18).

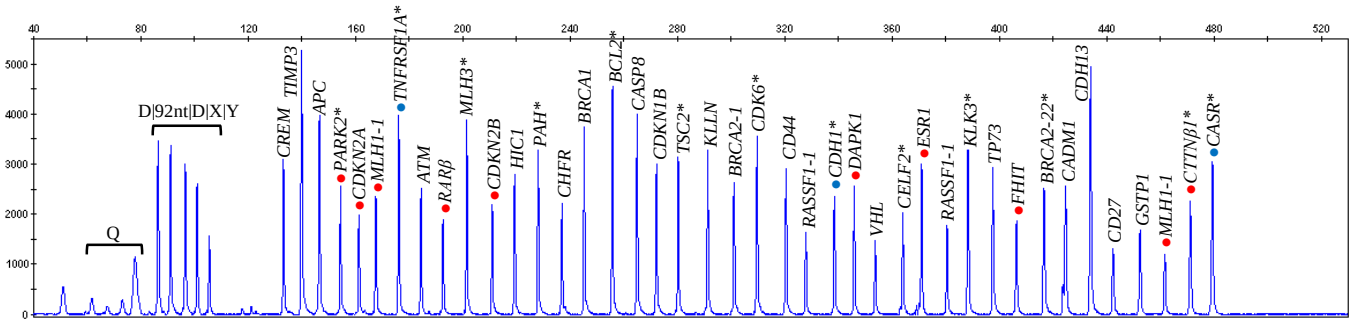

Figure 17 – CNVs electropherogram of a LCSS sample from a male patient analysed by MS-MLPA using *SALSA MS-MLPA probemix ME001-C2*. (●)-Gain of genetic material; (●)-Loss of genetic material; *-reference probe.

Regarding the methylation electropherogram, only the gene probes which do not have restriction site for HhaI or the methylated genes are amplified in PCR reaction and so, they are able to create a peak (Figure 18).

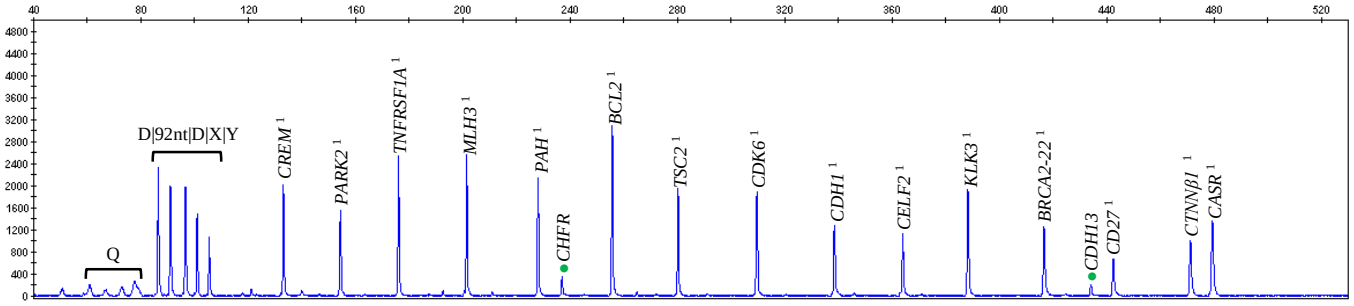

Figure 18 - Methylation electropherogram of a LCSS sample from a male patient analysed by MS-MLPA using SALSA MS-MLPA probemix ME001-C2. (●) – Methylated genes; 1-Gene probes without restriction site for HhaI.

4.3.1 Copy Number Variations

4.3.1.1 Tumour Samples

Regarding the eight tumour samples, all of them presented several genetic imbalances. Among all 41 gene probes (Table 10), six of them did not present any change (Table 10): *CASP8* (2q33.1), *CD44* (11p13), *BRCA2 – exon 1* and *BRCA2* – exon 22 (13q13.1), *CHFR* (12q24.33) and *HIC1* (17p13.3).

Overall, the genetic profile of these tumour samples was characterized mainly by losses of genetic material, especially on chromosomes 3p (6/8), and 9p (5/8). The gain of genetic material occurred mainly on chromosomes 12p (6/8) and 17q (5/8).

The most common genetic alteration was the gain of *TNFRSF1A* (12p13.2) which was present in 75% (6/8) of the patients. Although *CDKN2A* was also altered in six patients, it did not present only one type of genetic alteration, being lost in five patients and gained in one patient. The second most common exclusive variation was the gain of *BRCA1* (17q21.31) in 62,5% (5/8) of the patients. The genes *CTNNβ1* (3p21), *FHIT* (3p14.2) and *CDKN2B* (9p21) were lost in 50% (4/8) of the patients while *CADM1* (11q23.2) and *RARβ* (3p24.2) were lost in 37,5% (3/8) of the patients. On the other hand, the gain of *CDKN1B*, (12p13.1-p12) and *CD27* occurred in 50% of the patients while the gain of *TIMP3* (22q12.3) and *CDH1* (16q22.1) occurred in 37,5% (3/8) of the patients (Figure 19). Regarding *VHL* (3p25.3), three of the patients (5, 7 and 8) presented loss of genetic material while another one (patient 4) present gain of it (Table 11).

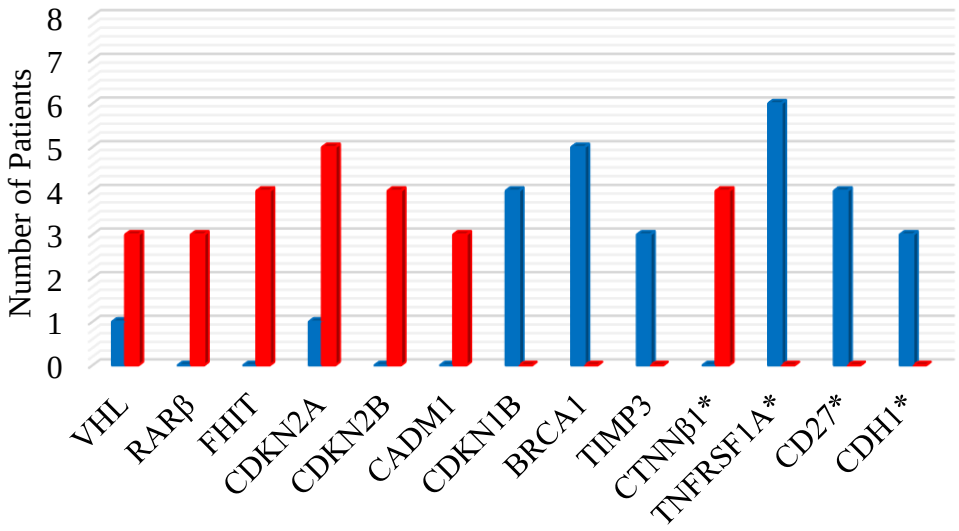

Figure 19 - The most common genetic imbalances in eight patients diagnosed with LSCC detected using SALSA MS-MLPA probemix P428. (■) – Gain of Genetic Material; (■) – Loss of Genetic Material; * – Reference probe

Although *CASR* (3q21.1) and *PARK2* (6q26) showed a genetic alteration rate of 37,5%, both of them showed both gain and loss of genetic material. Taking this into consideration, patients 6 and 8 presented loss of *CASR* while patient 3 presented gain of it. Regarding *PARK2*, patients 1 and 3 present loss of genetic material while patient 6 present gain of it. (Table 11)

In addition, loss of *MLH1* (3p22.2), *RASSF1* (3p21.31), *APC* (5q22.2) and *ESR1* (6q24-q27) was present in 25% (2/8) of the patients while *TP73* (1p36.32), *DAPK1* (9q21.33), *CELF2* (10p14) and *CDH13* (16q23.3) were in 12,5% (1/8) of the patients. On the other hand, *CREM* (10p12.1) and *KLK3* (19q13.33) were gained in 25% of the patients whereas *CDK6* (7q21.3), *ATM* (11q22.3), *MLH3* (14q24.3) and *TSC2* (16p13.3) were gained in 12,5% (1/8) of the patients. (Table 11).

Finally, the genes *KLLN* (10q23.3), *GSTP1* (11q13.2) and *BCL2* (18q21.33) were altered in 25% of the patients. However, all of them presented both gain and loss of genetic material in different patients. Patient 6 had loss of *KLLN* while patient 8 had gain of it, gain of *GSTP1* occurred in patient 4 while its loss occurred in patient 5 and finally, *BCL2* was lost in patient one and gained in patient 5 (Table 11).

4.3.1.2 Non-Tumour Samples

Regarding all the seven non-tumour samples, just the one collected from patient 2 did not present any changes (Table 11). Among the 41 gene probes, only 15 present genetic imbalances (Table 10). The most common alteration was the gain of *CREM* and *CD27* in 28,6% (2/7) of the patients. Although the genes *VHL* and *GSTP1* were also altered in 28,6% of the patients, both gain and loss of genetic material were detected. Regarding *VHL*, patient 3 presented gain of genetic material while patient 4 presented loss of it. *GSTP1* was lost in patient 5 and amplified in patient 8. Finally, *RASSF1*, *FHIT*, *APC*, *TIMP3*, *CDH1* and *KLK3* were gained in 14,3% (1/7) of the patients while *CDKN2A*, *CHFR* and *PARK2* were lost in 14,3% of the patients (Table 11).

As in MLPA, MS-MLPA also revealed that some patients presented with the same alterations in both tumour and non-tumour samples: (1) Patient 1 - loss of *PARK2* and gain of *KLK3*; (2) Patient 3 - gain of *CDH1*; (3) Patient 5 - loss of *GSTP1*; (4) Patient 6 - gain of *CREM* and (5) Patient 8 - loss of *CDKN2A* and gain of *CD27*. In addition, MS-MLPA also revealed that in patient 4 there was a gain of *VHL* in the tumour sample while the non-tumour samples presented loss of it (Table 11).

Table 10 - Summary of genetic imbalances detected by SALSA MS-MLPA probemix ME001-C2. (Chr) – Chromosome; (T)-Tumour Sample; (NT)-Non-tumour sample; * - Reference probe; (■) – Gene probe without any alteration in both tumour and non-tumour samples.

Chr	Gene	Gain of Genetic Material		Loss of Genetic Material	
		T	NT	T	NT
1p36.32	*TP73*			✓	✓
2q33.1	*CASP8*				
3p25.3	*VHL1*	✓	✓	✓	✓
3p24.2	*RARβ*			✓	
3p22.2	*MLH1* - exon 1			✓	
	MLH1 - exon 1			✓	
3p21.31	*RASSF1* - exon 1		✓	✓	
	RASSF1 - exon 1		✓	✓	
3p14.2	*FHIT*		✓	✓	
5q22.2	*APC*		✓	✓	
6q24-q27	*ESR1*			✓	
9p21.3	*CDKN2A*	✓		✓	✓
9p21.3	*CDKN2B*			✓	
9q21.33	*DAPK1*			✓	
10q23.3	*KLLN*	✓		✓	
11p13	*CD44*				
11q13.2	*GSTP1*	✓	✓	✓	✓
11q22.3	*ATM*	✓			
11q23.2	*CADM1*			✓	
12p13.1-p12	*CDKN1B*	✓			
12q24.33	*CHFR*		✓		
13q13.1	*BRCA2* - exon 1				
16q23.3	*CDH13*			✓	
17p13.3	*HIC1*				
17q21.31	*BRCA1*	✓			
22q12.3	*TIMP3*	✓	✓		
3p22.1	*CTNNβ1**			✓	
3q21.1	*CASR**	✓		✓	
6q26	*PARK2**	✓		✓	✓
7q21.3	*CDK6**	✓			
10p14	*CELF2**			✓	
10p12.1	*CREM**	✓			✓
12p13	*TNFRSF1A**	✓			
12p13.31	*CD27**	✓			✓
12q23.2	*PAH**				
13q12.3	*BRCA2- exon 22**				
14q24.3	*MLH3**	✓			
16p13.3	*TSC2**	✓			
16q22.1	*CDH1**	✓			✓
18q21.33	*BCL2**	✓		✓	
19q13.33	*KLK3**	✓			✓

Table 11 - Summary of genetic imbalances detected by MS-MLPA in all the 8 patients included in this study. (Chr)-Chromosome; (T)-Tumour Sample; (NT)-Non-tumour sample; * - Reference probe; (■) – Gain of Genetic Material; (■) – Loss of Genetic Material

Chr	Gene	Patients															
		1		2		3		4		5		6		7	8		
		T	NT	T	NT	T	NT	T	NT	T	NT	T	NT	T	T	NT	
1p36.32	*TP73*										■		■	■			
2q33.1	*CASP8*																
3p25.3	*VHL1*						■	■	■	■				■	■		
3p24.2	*RARβ*	■		■		■											
3p22.2	*MLH1* - exon 1					■				■							
	MLH1 - exon 1					■				■							
3p21.31	*RASSF1* - exon 1									■		■					■
	RASSF1 - exon 1									■		■					■
3p14.2	*FHIT*	■				■				■		■					■
5q22.2	*APC*			■				■			■						
6q24-q27	*ESR1*	■				■											
9p21.3	*CDKN2A*			■		■		■		■		■			■	■	
9p21.3	*CDKN2B*			■		■				■					■		
9q21.33	*DAPK1*					■											
10q23.3	*KLLN*											■			■		
11p13	*CD44*																
11q13.2	*GSTP1*							■		■	■						■
11q22.3	*ATM*														■		
11q23.2	*CADM1*	■										■		■			
12p13.1-p12	*CDKN1B*			■								■		■	■		
12q24.33	*CHFR*								■								
13q13.1	*BRCA2* - exon 1																
16q23.3	*CDH13*											■					
17p13.3	*HIC1*																
17q21.31	*BRCA1*	■		■				■				■			■		
22q12.3	*TIMP3*	■									■	■			■		
3p22.1	*CTNNβ1**	■		■		■						■					
3q21.1	*CASR**					■						■			■		
6q26	*PARK2**	■	■			■						■					
7q21.3	*CDK6**														■		
10p14	*CELF2**														■		
10p12.1	*CREM**										■	■	■	■			
12p13	*TNFRSF1A**	■		■		■						■		■	■		
12p13.31	*CD27**	■		■			■	■							■	■	
12q23.2	*PAH**																
13q12.3	*BRCA2- exon 22**																
14q24.3	*MLH3**											■					
16p13.3	*TSC2**														■		
16q22.1	*CDH1**					■	■	■				■					
18q21.33	*BCL2**	■								■							
19q13.33	*KLK3**	■	■					■									

4.3.2 Methylation Profile

The cut-off used to define a gene as methylated was having a methylation percentage above 20%. Taking this into consideration, all the reference samples as well as the non-tumour samples revealed to be unmethylated while only 50% of the tumour samples showed one or two genes methylated.

Among the samples with an altered methylation pattern, *CHFR* was the gene more frequently altered (25% of the patients). The genes *RARβ, RASSF1* and *CDKN2A* were methylated in 12,5% (1/8) of the patients.

Table 12 – Summary of the results obtained for the samples with a methylation pattern altered

Chromosome	***Gene***	***Methylation* (%)***	**Alterations [Number of patients (%)]**
3p24.2	*RARβ*	*31%*	1/8 (12,5%)
3p21.31	*RASSF1*	*22%*	1/8 (12,5%)
9p21.3	*CDKN2A*	*61%*	1/8 (12,5%)
12q24.33	*CHFR*	*28%* and *22%*	2/8 (25%)

*Unmethylated gene: (%) < 20%; Methylated gene: (%) ≥ 20%

5 Discussion

Laryngeal squamous cell carcinoma (LSCC) is one of the most common malignancies in head and neck cancer family and despite the recent therapeutic and diagnostic advances, the overall 5-year survival rate did not improved much in the last years. Although there is a clear lack of precise genetic information, it has been accepted that LSCC arises from accumulation of genetic alterations which leads to genomic instability. Taking this into consideration, genetic studies arise as potential tool to understand the disease in order to develop new diagnostic methods to detect the tumour in an early stage and to develop personalized therapeutic strategies.

In order to characterize genetically and epigenetically all the samples of this study, the DNA was extracted and copy number variations was assessed through aCGH, MLPA and MS-MLPA while the methylation profile was analysed through MS-MLPA.

5.1 Assessment of Copy Number Variations

Among the eight fresh-frozen samples analysed, aCGH results revealed that the gain of genetic material was the most common alteration, especially in chromosomes 3q, 6p, 8q, 11q, 14q and chromosome X. Regarding losses, the chromosomes more affected were 3p, 6q, 8p, 9p, 12p, Yp and Yq. However, it is important to know that aCGH do not descrimite potential pathogenic structural variations from common structural variations present in healthy people. In order to identify potential chromosomal alteration that might have a role in laryngeal carcinogenesis, all common variations were excluded from the analyses using the "Database of Genomic Variants: A curated catalogue of human genomic structural variation" (120). Among the seventeen minimal common regions mentioned in Table 6, six alterations were immediately excluded due to the presence of many common variations in healthy controls samples already described: 3q26.1, 6p25.3, del(6)(q24.3q27), 8p23.1, 8p11.22 and 12p13.31.

With the exception of the chromosomal region Yq11.21-Yq11.221, all the other ten regions code for many genes that have been described as having a role in carcinogenesis. All of them will be discussed individually in the following sections.

5.1.1 Chromosome 3

5.1.1.1 Region 3p

In head and neck cancer, the loss of the short arm of chromosome 3 is frequently followed by gain of its long arm, leading to the formation of isochromosome 3q. This alteration has already been seen in head and neck carcinoma as well as laryngeal carcinoma (33,121). However, the presence of isochromosomes can only be confirmed by karyotype or FISH (*Fluorescent In Situ Hybridization*). Nevertheless, the loss of 3p and gain of 3q lead to deletion of tumour suppressor genes and amplification of oncogenes, respectively.

Loss of the short arm of chromosome 3 is one of the earliest and most frequent changes in head and neck carcinoma, being present in dysplasia lesions (33,121). The partial loss of del(3)(p26.3p12.1) was also one of the most common events found in this study. This region codes for several genes whose role in laryngeal carcinogenesis has been already reported. The results of MLPA and MS-MLPA together showed a higher percentage of genetic alterations in *FHIT, CTNNβ1* and *VHL* genes. Among these genes, *FHIT* is commonly found to be deleted and the loss of function leads to abnormal cell proliferation and resistance to apoptosis, thus contributing to the formation of a tumour (33,35,121).

Loss of *CTNNβ1* has been associated with tumour invasion and metastasis (37,39,41). Álvarez-Marcos et al. (2012) established a positive relationship between loss of *CTNNβ1* and cytoplasmic β-catenin overexpression. They suggested that mutations or dysregulation of the gene might lead to change of protein location which ultimately might promote loss of cell-cell adhesion. This alteration was observed in laryngeal epithelial precursor lesions which may suggest that loss of *CTNNβ1* has a role in malignant transformation phenotype (122).

Zhang et al. (2014) found a significant correlation between *VHL* (von Hippel-Lindau tumour suppressor, E3 ubiquitin protein ligase) loss and epithelial-mesenchymal transition in oral squamous cell carcinoma, thus affecting the prognosis of the patient. Through the re-expression of *VHL* in cells, Zhang et al. (2014) demonstrated that the cells have a lower capacity of proliferation, migration and invasion. Taking this into consideration, the *VHL* may play a role in invasiveness and metastasis. In healthy people, *VHL* is involved in degradation of HIF-1α (hypoxia-inducible factor 1α). In hypoxic tumours, the loss of *VHL* may lead to accumulation of HIF-1α which is followed by synthesis of HIF-1 that is involved in angiogenesis (123,124).

Loss of *MLH1*, *RARβ* and *RASSF1* was detected more frequently by aCGH than by MLPA and/or by MS-MLPA. This fact may be explained by the different sensitivities of the three techniques in the analyses of tumour samples which are contaminated by normal cells. The loss of *MLH1, RARβ* and *RASSF1* has already been stablish in several types of tumours, including head and neck carcinoma and some of them in laryngeal carcinoma (33,121). Marcos et al. (2011), who also used MLPA to analyse LSCC samples, found out that loss of *MLH1* was one of the most common alterations in patients diagnosed with LSCC (31).

Lee et al. (2010) was the first to identify *ALS2CL* (ALS2 C-terminal like) as a potential suppressor tumour gene in head and neck cancer. In previous studies, this alteration was reported in breast and colorectal cancers (125).

Gollin et al. (2014) also, through a meta-analysis study, identified the loss of *ESRG* (embryonic stem cell related (non-protein coding)), *LRTM1* (leucine-rich repeats and transmembrane domains 1) and *GBE1* (glucan (1,4-alpha-), branching enzyme 1) in chromosome 3p (121). Although the first two genes do not have a known function, *GBE1* has a role in carbohydrate metabolism and its loss has been associated with chemoradioresistance in breast cancer and so to a poorer prognosis (126).

Although loss of *CHL1* (cell adhesion molecule L1-like) has not been associated with laryngeal carcinoma, this variation has already been reported in oral, esophageal and breast squamous cell carcinomas (127,128). *CHL1* [Uniprot: O00533] belongs to the family of L1 neural cell adhesion molecules that plays a role in nervous system development and in synaptic plasticity. However, this gene has been considered as a potential suppressor tumour gene since its overexpression seems to suppress cell proliferation and invasion while its knockdown leads to an increased proliferation and invasion *in vitro* as well as promotion of tumour formation *in vivo* (128). Uchida et al. (2011) proposed that loss of *CHL1* might be and indicator of aggressiveness of oral cancer (127).

5.1.1.2 Region 3q

Alongside with loss of 3p, the gain of 3q is also one of the most frequent alterations in HNSCC. Within 3q, two smaller fragments were considered to be minimal common regions that were found to be amplified in aCGH: dup(3)(q26.33q27.3) in 6 out of 8 patients and 3q29 in 7 out of 8 patients. Moreover, these alterations have been associated with a poor clinical outcome (121).

The gain of the genes *MCCC1* and *PIK3CA*, which are mapped within 3q26.33-q27.3, was a frequent event detect by MLPA and MS-MLPA and, overall, the results are supported by aCGH. *PIK3CA* is an oncogene whose gene product is able to stimulate AKT signalling which is involved in growth factor independent growth, cell invasion and metastasis (121). Redon et al. (2002) observed *PIK3CA* amplification in precancerous oral dysplasia, suggesting that this event may be involved in early head and neck cancer (129). Gain of *PI3KCA* leads to *PI3K*-Akt-mTOR aberrations which will promote a malignant phenotype by supressing immune system and inflammation and by promoting angiogenesis, survival, invasion and metastasis (130). Regarding *MCCC1*, its amplification has been seen in lung and oral squamous cell carcinomas (131,132)

Other candidate genes within 3q26.33-3q27.3 which are found to be amplified in head and neck cancer include *DCUN1D1, LAMP3* and *EIF4G1*.

Sarkaria et al. (2006) suggested that *DCUN1D1* was a candidate oncogene due to its ability to transform cells fibroblastic and keratinocytic lineage. Also, they used shRNA against *DCUN1D1* which resulted into apoptosis of cancer cell lines carrying the amplified gene, suggesting that *DCUN1D1* overexpression may also has a role in maintenance of malignant phenotype (133).

The expression of *LAMP3* (Lysosomal associated membrane protein 3), which is a downstream target of TP53, has been associated with hypoxia-induced metastasis and poor overall survival in both cervical and breast cancers (134,135). In addition, Nagelkerke et al. (2011) suggested also that the overexpression of LAMP3 may be a biomarker for hypoxia mediated treatment resistance in breast cancer. Although the amplification of *LAMP3* has already been reported in laryngeal carcinoma (32), its role in laryngeal carcinogenesis is still needed to be clarified.

EIF4G1 (eukaryotic translation initiation factor 4 gamma, 1), a member of the translational initiation factor family, is over-expressed in head and neck cancer, including nasopharyngeal and hypopharyngeal carcinoma as well as in lung squamous cell carcinoma (136–138). In nasopharyngeal carcinoma, Tu et al. (2010) found a significant association between *EIF4G1* over-expression and lymph node involvement, suggesting that this alteration was a poor prognosis factor in nasopharyngeal carcinoma. In addition, they found that *EIF4G1* promoted tumourigenesis *in vivo* and they proposed that *EIF4G1* may play a role in cell proliferation, cell cycle, migration and invasion (137).

Finally, the last chromosomal segment identified by aCGH as a minimal common region amplified in chromosome 3 is 3q29. Although both MLPA and MS-MLPA do not have gene probes for this regions, several genes have been identified as having a role in head and neck carcinogenesis (*MUC4, MUC20* and *TNK2*).

MUC4 (mucin 4) and *MUC*20 code for membrane-bound glycoproteins which are expressed in epithelial cells and they play a role in protection, differentiation and renewal of epithelium as well as in cell adhesion, cell signalling and immune response. Hamada et al. (2012) found an association between aberrant over-expression of *MUC4*, nodal metastasis, diffuse invasion and tumour progression in oral squamous cell carcinoma. In addition, patients with over-expression of *MUC4* appeared to have a worse overall and disease-free survival. *MUC4* promotes tumour progression by repressing apoptosis, promoting tumour metastasis and escaping from immune response by masking the surface epitopes (139). Regarding *MUC20*, its overexpression has been associated with poor prognosis in colorectal and endometrium carcinomas (140,141). Xiao et al. (2013) showed that over-expression of *MUC20* promoted metastasis while knockdown of the gene attenuated migration and invasion in colorectal cells.

Over-expression of *TNK2* (tyrosine kinase, non-receptor, 2) has already been reported in lung, breast and prostate cancer. Its activation is associated with progression to a metastatic phenotype and its inhibition seems to lead to cell cycle arrest, apoptosis and sensitization to ionizing radiation (142).

The gene probemix used in MLPA also target other genes outside the minimal common regions identified by aCGH, namely *CCNL1* (3q25.32) and *TP63* (3q28). Although genetic imbalances in both genes were not the most frequent alterations in MLPA, the chromosomal region in which *CCNL1* and *TP63* are mapped is, according to aCGH results, amplified in 37,5% and 50% of the patients, respectively. *CCNL1* [Uniprot: Q9UK58] is involved in the regulation of pre-mRNA splicing as well as RNA polymerase II. Its amplification and over-expression is associated with lymph node metastases and shorter overall survival in HNSCC, including in laryngeal carcinoma. However, the role of amplification of *CCNL1* in carcinogenesis remains unclear (121,143). *TP63* is commonly over-expressed in head and neck carcinoma and it is associated with poor prognosis. According to Orzol et al. (2014), it was suggested that *TP63 locus* is a rare site for *HPV* integration in lung cancer (144).

5.1.2 Chromosome 8

The dup(8)(q23q24) is one of the most frequent copy number alterations in early head and neck cancer (121,145). Array CGH allowed to identified the amplification of 8q24.21 as one of the minimal common regions affected in the LSCC patients included in this study. Within this region, *MYC* is one of the genes whose amplification has already been associated with laryngeal carcinoma (33,47,48). *PVT1* (Pvt1 oncogene (non-protein coding)) is frequently co-amplified with *MYC* and acts as an oncogene by up-regulation of anti-apoptotic genes and down-regulation of genes whoso product has pro-apoptotic functions, thus favouring an apoptotic phenotype (146). Although *PVT1* has not been associated with laryngeal carcinoma yet, its amplification has been seen as a poor prognosis factor in patients with colorectal cancers (147). *PVT1* locus contain a cluster of microRNAs, such as *MIR1204* (microRNA 1204) whose depletion seems to promote the expression of anti-apoptotic genes (146).

ASAP1 (ArfGAP with SH3 domain, ankyrin repeat and PH domain 1), also mapped in 8q24, plays a role in actin cytoskeletal remodelling and focal adhesions. Li et al. (2014) showed that up-regulation of *ASAP1* in LSCC primary tumours was associated with lymph node metastasis and its down-regulation lead to a decreased in the invasive potential (148).

Other frequent alterations in chromosome 8 that were identified by MS-MLPA include loss of the *MTUS1* and *GATA4* as well as gain of genes *WISP1* and *PTK2*.

WISP1 and *PTK2* are also mapped in the long arm of chromosome 8, close to the locus of *MYC* and *PVT1*. aCGH showed that the region where these genes are mapped were amplified in 62,5% of the patients. Jarvinen et al. (2006) found that *WISP1* was simultaneously amplified and over-expressed in laryngeal carcinoma (32). However, its role in laryngeal carcinogenesis remains unclear. In oral squamous cell carcinoma, *WISP1* seems to promote cell migration (149). Regarding *PTK2*, which is involved in adhesion and growth-regulatory signal transduction, its over-expression was shown to be present in early stages of laryngeal carcinoma (121,145). Also Rodrigo et al. (2011) observed that patients carrying over-expressed *PTK2* in dysplastic lesions have a higher cancer incidence. In addition, they showed that the frequency of *PTK2* over-expression increased with the grade of dysplasia, which may suggest a role in malignant progression (145).

MTUS1 and *GATA4* are mapped on short arm of chromosome 8, which is found to be deleted in HNSCC(121). The reduced expression of *MTUS1*, a tumour suppressor gene that

inhibits cell proliferation, has been observed in colon, ovarian, pancreatic and tongue oral cancers. According to Ye et al. (2007), a previous mutation analysis of *MTUS1* in hepatic cancer showed that the gene is susceptible to point mutations or small deletions which may explain the fact that, in this study, the tree different exons analysed by MLPA revealed different alteration rates. Regarding oral tongue carcinoma, loss of *MTSU1* expression seems to be associated with an advance stage of this cancer (107). Regarding *GATA4*, its role in human cancers is unclear. The loss of this gene has been implicated in colorectal cancer, where Hellebrekers et al. (2009) showed that the introduction of *GATA4* leaded to inhibition of cell growth, migration and invasion, thus suggesting a tumour suppressor function (150).

5.1.3 Chromosome 9

In head and neck carcinoma, loss of the band 9p21 is one of the most frequent genetic early stage changes. aCGH results showed that loss of 9p21.3 happened in 37,5% of patients. *CDKN2A* and *CDKN2B* are mapped in this sub-band.

As mentioned, the loss of suppressor tumour gene *CDKN2A* has already been reported in head and neck cancer, as well as in laryngeal carcinoma (29,33,49). In addition, MS-MLPA also showed that *CDKN2A* was deleted in 62,5% of the patients, being one of the most common genetic imbalances detected.

As *CDKN2A*, *CDKN2B* also promotes cell cycle arrest at G0/G1 checkpoint, thus being a negative regulator of cell cycle. Swellam et al. (2008) found that, in LSCC, deletion of both genes was significantly associated with increased telomerase activity and this correlation was associated with poor prognosis (151).

5.1.4 Chromosome 11

Amplification of 11q13, which was one of the most common amplifications detected by aCGH, has already been associated with poor prognosis in head and neck cancer. In this region are mapped several oncogenes and genes candidates involve in laryngeal carcinogenesis, namely *ANO1*, *CCND1*, *CTTN*, *FADD*, *FGF4* and *PPFIA1*. The amplification of *CCND1*, *CTTN* and *FADD* has already be shown in laryngeal carcinoma (31,33,51,60,55). MLPA also revealed that those genes were frequently amplified in LSCC samples included in this study. In addition, MLPA also detected the amplification of *FGF4* which has a role in regulation of embryonic development, cell proliferation and differentiation. However, its role in human carcinogenesis needs further studies (51).

ANO1 (anoctamin 1, calcium activated chloride channel) codes for a calcium-dependent chloride channel whose exact role on tumourigenesis appears to be controversial(121,152). According to Ayoub et al. (2010), over-expression of *ANO1* stimulates cellular attachment, metastasis and invasion, but not cell proliferation (153). However Duvvuri et al. (2012) showed that *ANO1* seems to stimulate cell proliferation (154). Ruiz et al. (2012) proposed that *ANO1* enhances cellular motility and migration, thus facilitating the appearance of metastasis (152).

PPFIA1 (protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 1) may regulate the disassembly of focal adhesions. Its function in laryngeal carcinogenesis is poorly understood (32,51). Tan et al. (2008), through siRNA and *in vitro* invasion assays, found that when *PPFIA1* levels were reduced, invasion of HNSCC cells was increased, thus suggesting a potential role in invasion (155).

CADM1 was found to be deleted in 37,5% of the patients. Marcos et al. (2010) found that loss of *CADM1* was one of the most frequent events in laryngeal squamous cell carcinoma group (31). In addition, Lu et al. (2012) found that over-expression of this gene seemed to inhibit cell proliferation, reduce cell invasion and to induce apoptosis. Taking this into consideration, *CADM1* appears to have tumour suppression functions in LSCC (156).

Regarding *ATM*, *CHEK1* and *CD44*, which are gene analysed by MLPA and MS-MLPA, their frequency of genetic imbalances was low (0%-12,5%) among the samples included in this study and so, it is probable that they do not have a relevant role in laryngeal carcinogenesis. In addition, because *GSTP1* and *H2AFX* presented both genetic gains and losses (12,5%-25%), they also might not have a significate role in the same pathological process.

5.1.5 Chromosome 14

In chromosome 14, a small region was found to be amplified in 62,5% of the patients: 14q13.1. In this sub-band is mapped the gene *SNX6* (sorting nexin 6) and little is known about its physiological function. *SNX6* [Uniprot: Q9UNH7] is involved in intracellular trafficking as well as in *EGFR* and E-cadherin degradation.

Currently, Rivera et al. (2010) establish the only association between *SNX6* and cancer. They identified *SNX6* as a binding partner of *BRMS1* (breast cancer metastasis suppressor 1), a member of growing metastasis suppressors family which reduces breast and

melanoma metastasis without affecting primary tumour growth. In addition, *SNX6* is capable of interact with TGFβ which has been largely associated with human cancer (157).

Cetuximab (ErbituxTM, C225, ImC-225; ImClone Systems, Inc.) is a chimeric human/murine monoclonal antibody of the IgG1 isotype that binds to the EGFR with higher affinity than its natural ligands, preventing proliferation, angiogenesis, anti-apoptotic signalling, invasion and metastasis(158). Cetuximab is the only EGFR-targeting therapy approved by the Food and Drug Administration and if *SNX6* degrades *EGFR*, perhaps patients with amplification and over-expression of *SNX6* have a worse prognosis. Thus, *SNX6* could be a therapeutic biomarker.

Regarding *POMT2* and *MLH3* mapped in 14q and reference probes in MLPA and MS-MLPA, the results revealed a low frequency rate (0%-12,5%) of genetic imbalance, suggesting that they might not be important in laryngeal carcinogenesis.

5.1.6 Chromosome X

In chromosome X, two regions were found to be frequently amplified in the patients included in this study: Xp22.31 and Xq21.1.

VCX3A is mapped on Xp22.31 and belongs to human testis-specific gene family known as VCX/Y. Although *VCX3A* [Uniprot: Q9NNX9] function is poorly understood, it may play a role in spermatogenesis and in sex ratio distortion. Taguchi et al. (2014) observed that *VCX3A* was overexpressed in lung and colon cancer cell lines (159).

MAGT1, a gene which codes for a magnesium transporter, is mapped on Xq21.1 A few studies have been conducted in patients with XMEN (X-linked immunodeficiency with magnesium defect, EBV infection, and neoplasia) disease which may be caused by loss of function of *MAGT1*. Chaigne-Delalande et al. (2013) showed that magnesium has a major role in anti-tumour immunity. In addition the association between its decrease inside the cell and the predisposition to lymphoma onset seems to be controversial (160,161). However, the amplification of *MAGT1* has never been reported to be associated with cancer.

5.1.7 Chromosome Y

The human Y chromosome is made by two pseudoautosomal regions (PAR1 and PAR2), which recombine with chromosome X during meiosis, and one Y-specific region (YSR). Genes located in chromosome Y are involved in cell cycle control, signal transduction, cell proliferation, protein degradation and gene expression (162).

Partial loss of chromosome Y and Y nullisomy has already been reported in head and neck cancer (162,163). However, its role in head and neck carcinogenesis remains poorly understood. Silva Veiga et al. (2012) showed that, although head and neck cancer has higher incidence in older male people, the loss of chromosome Y is independent of the onset age of the disease (162).

In most of the male samples (6/7), total or partial loss of chromosome Y was observed, especially del(Y)(q11.21q11.221) and del(Y)(p11.32p11.2). The first region has no known genes mapped in it while in the second one, *CD99,* which is located in PAR1 (Xp22.32 and Yp11.3), has been associated with human carcinogenesis. Regarding the only female patient included in this study, she present amplification of Xp22.32.

CD99 is a glycoprotein present in cell surface whose role in human physiology remains unclear. *CD99* has been associated with cell adhesion, morphology and death as well as diapedesis of leukocytes. Its loss has been associated with poor prognosis in several types of tumours, including osteosarcoma, gastric adenocarcinoma, pancreatic tumours and carcinoma of gall bladder (164,165). Jung et al. (2007) also showed that down-regulation of *CD99* was associated with cell proliferation and migration in gastric adenocarcinoma (165).

5.1.8 Other Genetic Imbalances

In MLPA, the reference probe *PRPF31* was found to have a high frequency amplification (62,5%) in tumour samples. Regarding *PRPF31,* which is involved in pre-mRNA splicing, there is no reported association between this gene and human cancer yet. However, two patients who presented amplification of this gene in tumour sample, also had this alteration in the non-tumour sample, which may suggest that amplification of *PRPF31* may be an early event in laryngeal carcinogenesis.

In MS-MLPA, other gene and reference probes were also found to have a high frequency amplification in tumour samples: *TNFRSF1A* (75%), *BRCA1* (62,5%), *CD27* and *CDKN1B* (50%) and *CDH1* (37,5%).

TNFRSF1A codes for a receptor of TNFα which is generally accepted to have a role in human carcinogenesis. Over-expression of TNFα leads to persistent inflammation, DNA damage and increased pro-angiogenic functions. Chronic expression of TNFα has been associated with lymph node involvement and poor prognosis in breast cancer. Regarding *TNFRSF1A*, its blocking seems to impair tumour survival signalling. Although a few functional polymorphisms have been described, the role of *TNFRSF1A* amplification in

human carcinogenesis still remains unclear (166). In addition, *CD27* [Uniprto: P26842] which also is a member of TNF-receptor superfamily, is also amplified. Little is known about its biological function as it may have a role in T-cells survival and apoptosis. The correlation between amplification of *CD27* and human cancer is still unclear.

BRCA1 has tumour suppressor functions due to its role on DNA repair, cell cycle control and maintenance of genomic stability (167). However, our study showed its amplification which has never been reported before.

CDKN1B is an important cell cycle regulator which mediates the progression from G0 to G1 phases. López et al. (2013) found a decreased expression of *CDKN1B* in laryngeal dysplasia lesions (168). In addition, Bodnar et al. (2014) showed that loss of p27 expression was associated in poor prognosis and lymph node involvement in metastatic laryngeal carcinoma (169). Nevertheless, the amplification of *CDKN1B* found in our study has not been reported yet.

TIMP3, a metalloproteinase inhibitor, has reduced expression in colon and thyroid cancers. In addition, Bai et al. (2007), found an association between loss of expression of *TIMP3* and degree of malignancy, infiltration and metastasis of colon carcinoma (170,171). In our study it was observed a loss of genetic material in *TIMP3* which no one has reported it yet.

5.2 Assessment of Methylation Profile

MS-MLPA also allows the study of the methylation profile of several genes. Since this project is a pilot study, only four different controls were used. None of the reference samples presented any genes with a methylation frequency rate above 10% and so they were considered to be unmethylated. In this study, a gene was considered to be methylated when the methylation rate was over 20%. This cut-off was establish based on laboratory experience in cancer research projects as well as in scientific papers: Ozdemir et al. (2012) used 25% as the cut-off for define a methylated gene in ovarian cancer while Verschuur-Maes et al. (2012) and Moelans et al. (2011) used 15% in breast cancer samples (172–174). Nevertheless, is worth noting the importance of increasing the number of controls in this study in the definition of precisely the methylation cut-offs.

MS-MLPA results revealed alterations of methylation pattern in four genes: *RARβ*, *RASSF1*, *CDKN2A* and *CHFR*. The silencing of these genes by promoter hypermethylation has already been associated with laryngeal carcinogenesis. Methylation of *CDKN2A* has

been associated with invasion lymph nodes and cell migration while *CHFR* has been associated with late stage disease (67,68). Regarding *RARβ,* its methylation seems to facilitate cell proliferation and apoptosis resistance (73).

The physiological function of *RASSF1* (Ras association (RalGDS/AF-6) domain family member 1) is involved with regulation of cell cycle, microtubule stabilization, cell adhesion, motility and apoptosis. It is a potential tumour suppressor gene due to its ability to control mitosis and to increase genomic stability (175). Fendri et al. (2009) reported a significant association between methylated *RASSF1* and lymph node metastasis and advanced tumour stage in nasopharyngeal carcinoma (176). Moreover, Park et al. (2007) and Paluszczak et al. (2011) found that *RASSF1* was aberrantly methylated in laryngeal squamous cell carcinoma (73,75). Paluszczak et al. (2011) also suggests that *RASSF1* hypermethylation may promote uncontrollable cell growth as well as resistance to apoptosis (73).

5.3 Comparison between aCGH, MLPA and MS-MLPA

Overall, the results showed that there was an agreement between the three techniques used. In addition, aCGH was not only able to confirm the majority of the alterations detected by MLPA and MS-MLPA but also revealed other genetic imbalances in the genes included in the probemixes used that were found to be normal using MLPA and MS-MLPA assays. It is important to know that the sensitivity of the three techniques is highly dependable on the percentage of tumour and normal cells in the sample. Taking this into consideration, it is possible that different results may be explained by contamination of tumour samples with normal cells. According to Stuppia et al. (2012), it is difficult to detect genetic imbalances by MLPA if the tumour sample contains more than 50% of normal cells (93). Regarding aCGH, Neill et al. (2010) showed that oligo platforms, such as the one used in this study, are able to detect mosaicism of 30% or greater as well as 10% under optimal conditions (177). Regarding the mosaicism, one way to avoid it would be to use laser microdissection to select specific cell populations of interest.

In addition, MLPA and MS-MLPA are techniques directed towards the identification of genetic imbalances in a single exon. Thus, they are not able to identify other alterations outside the gene *probemix* used. In this sense, although aCGH is not able to detect changes in a single exon, it adds the advantage of being able to detect new chromosomal imbalances through all the genome, allowing a complete coverage of the genome. Taking this into consideration, aCGH seems to be a more approachable technique when the main goal is to

identify new genetic biomarkers. However, due to its cost, aCGH is unlikely to be introduced in a diagnostic routine. After the identification of a set of genetic biomarkers with diagnostic, prognostic and therapeutic value, MLPA comes up as a more robust, faster and cheaper technique that allow the simultaneous analyses of multiples samples, representing a valuable tool easy to perform and able to detect different types of genetic alterations, including CNVs, methylation and point mutations, which otherwise would require multiple techniques.

MLPA and MS-MLPA are relative techniques that require reference probes in order to compare with peak pattern obtained by target-specific probes. Since cancer is a very heterogeneous and instable disease, one of the major problems in MLPA and MS-MLPA is defining reference probes which are unrelated with the condition of interest. For instance, in this study, reference probes such as *PRPF31* and *TNFRSF1A* presented a high rate of alterations. The selection of reference probes may be hampered by chromothripsis and chromoplexy events. Chromothripsis is result of random chromosome shattering and reassembly which leads to highly mosaic chromosomes and the pieces that were not incorporated are lost to the cell. A single event may involve a few regions of the genome, leading to high local rearrangement. The difference between chromothrispsis and chromoplexy seems to be the number of breaking points. These events may be caused by chromosome segregation errors, ionizing radiation and exogenous stimuli which normally lead to replication stress and repeated BFB cycles. Chromothripsis may cause loss of genetic material, which leads to disruption of tumour suppressors, and assembly of genomic fragments into highly amplified circular structures (double-minute chromosomes) containing oncogenes, such as *MYC* (178,179).

6 Conclusions

Overall, the aims proposed for this study were accomplished. Eight fresh-frozen tumour samples and seven non-tumour were genetically and epigenetically characterized by aCGH, MLPA and MS-MLPA. All the techniques used have proved to be complementary in the genetic study of cancer and revealed that:

- Overall and as expected, aCGH was able to detect more genetic imbalances than MLPA or MS-MLPA and this may be explained by the difference in sensitivity of the techniques;
- Gain of genetic material occurred mainly in chromosomes 3q, 8q, 11q, 14q13.1 and X while loss of genetic material happened mainly in chromosomes 3p, 9p23.1 and Y;
- Among the tumour samples, the gain of *MYC* and *TNFRSF1A* were the most frequently genetic imbalances detected. Although the role of amplified *MYC* is well established in laryngeal carcinoma, the gain of *TNFRSF1A* in laryngeal carcinogenesis is still unclear;
- The presence of the same genetic alteration in both tumour and non-tumour samples may indicate the presence of an early event in LSCC;
- Regarding the methylation profile, methylation in *RASSF1*, *CDKN2A* and *CHFR* have already been reported as having an important role in laryngeal carcinogenesis. However, the role of methylated *RARβ* in LSCC remains controversial;
- Overall, *SALSA MLPA probemix P428* and *SALSA MS-MLPA probemix ME001-C2* revealed to be suitable for detection of genetic imbalances in laryngeal squamous cell carcinoma. However, some reference probes were frequently altered while a few gene probes did not present any change. Taking this into consideration, it is mandatory to continue the studying of genetic imbalances in LSCC in order to create a new probemix containing genes that have been considered biomarkers of LSCC.

7 Future Perspectives

Head and neck squamous cell carcinoma is the sixth most common malignant tumour in the world and the larynx is the second most common affected organ. Laryngeal malignancies are mainly diagnosed in a late stage and since they are aggressive tumours that have a fast growth and a high tendency to develop metastasis, they have a high mortality rate that has not changed in the last years. Taking this into consideration, it is mandatory to continue the study of genetic progression that leads to development of laryngeal carcinoma in order to improve the life quality of the patients. This pilot study was initiated in an attempt to identify genetic and epigenetic biomarkers with diagnostic and prognostic meaning in laryngeal squamous cell carcinoma. The major limitation of this study is related with the reduced number of samples, both tumour, non-tumour and controls. The increase of samples number is mandatory in order to differentiate laryngeal carcinoma from the other tumours that belong to head and neck cancer family as well as to build a genetic progression model. Ultimately, the main goal would be to establish an association between genetic alterations and clinical data.

MLPA, MS-MLPA and aCGH revealed to be complementary in genetic study in the field of cancer. However, the use of one probemix for MLPA and another for MS-MLPA is not enough to establish a genetic alteration pattern in LSCC patients. Taking in consideration the probemixes available, a new one should be created in order to detect genetic imbalances in LSCC with more accuracy which, ultimately, will help to identify a specific set of genes with diagnostic, prognostic and therapeutic value in order to reduce the incidence and mortality rates. In addition, since DNA hypomethylation contributes significantly to oncogenesis, the study of hypomethylated oncogenes should also be included in the analyses of the samples.

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APPENDICES

Appendices I - Abstract regarding oral presentation in VIII Conferences of Genetic and Biotechnology

Detection of Chromosomal Alterations in Laryngeal Squamous Cell Carcinoma Using Array Comparative Genomic Hybridization

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Keywords: Laryngeal Squamous Cell Carcinoma, Array Comparative Genomic Hybridization.

Introduction:

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumour in the world and it can arise from ten anatomic sites. Among them, the larynx is the second most common affected organ. It is generally accepted that solid tumours, such as laryngeal squamous cell carcinoma (LSCC), results from a multistep process in which genetic alterations are accumulated. However, since genetic alterations in LSCC are usually studied as part of head and neck cancers, there is not much information about molecular progression that allow a clear differentiation between laryngeal malignancies and the other types of cancer included in head and neck family.
In most cases, laryngeal carcinoma have origin on the vocal cords in the glottic region or in supraglottic region, thus affecting vital functions such as breathing, swallowing and speaking. Taking this into consideration, the optimal management of patients diagnosed with laryngeal carcinoma is critical. Also, 5-year survival rate has not improved in more than two decades. One of the main challenges is to identify specific tumour markers that will help to improve survival rates and preserve the function of the larynx.

Material and Methods:
DNA was extracted from eight fresh-frozen tissue samples of laryngeal tumors, collected from patients with LSCC, after surgery. Copy number variations were accessed by Array Comparative Genomic Hybridization and one sample from palatine uvula was used as control.

Results:
The results showed several structural rearrangements which were most frequently located in the chromosomes 3, 8, 9, 11 and Y. Among this alterations, the most common imbalances were loss of chromosomal regions 3p, 8p, 9p, Yp and Yq while the most common gains were located in 3q, 8p, 8q and 11q. Smaller alterations were also found, being the gain in 14q13.1 one of the most frequent ones.

Conclusion:
Our study revealed several chromosomal alterations that may be implied in the development of laryngeal carcinoma. The correlation between genetic alterations and clinic-pathological data has the power to identify putative biomarkers with possible diagnostic and prognostic value.

Appendices II - Abstract regarding poster presentation in XXXIX Portuguese Genetics Conference

Genetic and Epigenetic Characterization of Laryngeal Squamous Cell Carcinoma Using Array Comparative Genomic Hybridization and Methylation-Specific Multiplex Ligation-dependent Probe Amplification

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Introduction:
Head and neck squamous cell carcinoma (HNSCC) is the sixth most common malignant tumour in the world and it can arise from the larynx. The presence of a tumour in this region leads to impairment of vital anatomical structures. Also, laryngeal tumours are usually diagnosed in a late stage.
Solid tumours, such as laryngeal squamous cell carcinoma (LSCC), result from a multistep process where genetic alterations play a major role. However, those alterations are usually studied as part of HNSCC and so one of the major challenges is to identify tumour markers that will help to distinguish laryngeal tumours from other cancers included in head and neck family and to improve its survival rates.

Material and Methods:
DNA was extracted from eight fresh-frozen tissue samples of laryngeal tumours, collected from patients with LSCC, after surgery. Copy number variations (CNV) were accessed by Array Comparative Genomic Hybridization (aCGH) and one sample from palatine uvula was used as control.
Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) was used to access CNV and to analyse the methylation profile of the same samples. For each MS-MLPA reaction, three different controls, which were also extracted from palatine uvula, were used.

Results:
aCGH results showed mainly gains of genetic material, especially on chromosome 3q, 8q, 11q and 14q13.1. The most common losses were located on chromosomal regions 3p, 8p, 9p, 12p, Yp and Yq. Overall, MS-MLPA results support the alterations found by aCGH. Regarding the methylation profile, no genes were found to be significantly methylated.

Conclusion:
Although no significant epigenetic changes were found, our study revealed several chromosomal alterations that may be implied in molecular progression of laryngeal cancer. The correlation between genetic alterations and clinic-pathological data has the power to identify putative biomarkers with possible diagnostic and prognostic value.