Universidad de Oviedo

Programa de Doctorado en Biología Molecular y Celular

Identificación de biomarcadores con utilidad clínica en biopsia líquida y de tejido de pacientes con carcinoma epidermoide de cabeza y cuello

Identification of biomarkers with clinical utility in liquid and tissue biopsies of patients with head and neck squamous cell carcinomas

TESIS DOCTORAL

Rocío Granda Díaz

Enero de 2023

Universidad de Oviedo

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Identificación de biomarcadores con utilidad clínica en biopsia líquida y de tejido de pacientes con carcinoma epidermoide de cabeza y cuello	Inglés: Identification of biomarkers with clinical utility in liquid and tissue biopsies of patients with head and neck squamous cell carcinomas

2.- Autor	
Nombre: Rocío Granda Díaz	DNI/Pasaporte/NIE:
Programa de Doctorado: Biología Molecular y Celular	
Órgano responsable: Centro Internacional de Postgrado - Universidad de Oviedo	

RESUMEN (en español)

Los carcinomas escamosos de cabeza y cuello (CECC) comprenden un complejo y heterogéneo grupo de neoplasias que, a su vez, presentan una amplia diversidad de alteraciones genéticas y moleculares. Sin embargo, dichas características ofrecen grandes oportunidades para identificar potenciales biomarcadores de interés clínico. España, y en especial Asturias, presenta una de las más altas incidencias a nivel mundial, siendo un importante problema de salud pública. La mortalidad continúa siendo elevada debido principalmente al diagnóstico tardío, las limitadas opciones terapéuticas y las recurrencias. En esta tesis hemos combinado diversos análisis moleculares y celulares, junto con una amplia variedad de tecnologías (inmunohistoquímica, western blot, RT-PCR cuantitativa para análisis de ARNm y miARN, análisis in silico, metagenómica, formación de colonias, MTS, invasión 3D) con el objetivo de afrontar los principales retos actuales de esta enfermedad, e identificar: 1) biomarcadores tempranos fiables que permitan determinar el riesgo de desarrollo de cáncer, y prevenir o intervenir en las primeras fases de la enfermedad; 2) alteraciones moleculares y vías de señalización involucradas en la progresión tumoral, el desarrollo de enfermedad metastásica y/o recurrente, y nuevas herramientas para mejorar la estratificación de los pacientes y la selección de tratamiento; 3) biomarcadores cuantificables no invasivos en biopsia líquida de saliva aplicables a la detección de cáncer, seguimiento de la enfermedad y riesgo de recurrencia.

Entre los principales hallazgos de esta tesis, cabe destacar la relevancia clínica de la expresión y amplificación génica de SOX2 en etapas tempranas de la tumorigénesis laríngea. Nuestros resultados revelan por primera vez la potencial aplicación clínica de la expresión de SOX2 como potente predictor independiente del riesgo de cáncer de laringe, superior a la actual clasificación histológica de la OMS. También demostramos que la amplificación y sobre-expresión de genes localizados en 11q13 (CTTN, CCND1 y ANO1) es frecuente y característica en CECC VPH-negativos, mientras que son infrecuentes en tumores VPH-positivos. Estas alteraciones moleculares podrían contribuir a las diferencias observadas en el pronóstico de ambos grupos de pacientes y, por lo tanto, servir como base para diseñar estrategias terapéuticas personalizadas. De forma análoga, la expresión del oncogén p-SRC (Tyr419) y varios efectores de la vía de SRC, como la quinasa de adhesión focal (FAK) y la proteína activadora de Arf GTPasa (ASAP1), mostraron un impacto diferencial en el pronóstico de los pacientes con CECC dependiendo de la localización tumoral, siendo predictores específicos de mal pronóstico en la laringe. Estos hallazgos podrían tener implicaciones importantes para los ensayos clínicos en curso con inhibidores de SRC, ya que estos nuevos criterios podrían ayudar a mejorar la selección y estratificación de pacientes que podrían beneficiarse de estos tratamientos. Esta tesis también proporciona valiosa información sobre el papel clínico y biológico de la desregulación del miR-301a en CECC. La caracterización tanto a nivel funcional como mecanístico ha permitido demostrar que el miR-301a promueve el crecimiento y la invasión de células tumorales, revelando las vías de señalización de PI3K y

ERK como ejes centrales de estas funciones mediadas por el miR-301a. Es destacable que dos inhibidores farmacológicos de las vías de PI3K y ERK demostraron su efectividad contrarrestando y revirtiendo los efectos protumorales y proinvasivos del miR-301a. Por último, hemos realizado un amplio estudio prospectivo en 125 pacientes con CECC para explorar y descifrar cambios bacterianos salivales asociados con la localización tumoral, la respuesta al tratamiento y el desarrollo de recurrencias.

RESUMEN (en Inglés)

Head and neck squamous cell carcinomas (HNSCC) are a complex and heterogeneous group of malignancies, harboring multiple and very diverse genetic and molecular alterations. However, this also offers vast opportunities for the identification of novel clinically-relevant biomarkers. Spain and, in particular, Asturias has one of the highest incidence rates worldwide, therefore representing an important problem of public health. Mortality remains high, mainly due to late diagnosis, limited treatment options and tumor recurrence. In order to face and overcome these major disease challenges, this thesis combines molecular and cellular analyses and a wide range of techniques (immunohistochemistry, WB, mRNA and miRNA expression by quantitative RT-PCR, CNA by qPCR, in silico analyses, metagenomics, colony formation, MTS, 3D invasion), aimed to identify: 1) early reliable biomarkers for cancer risk assessment to enable detection of cancer at an early and more curable stage; 2) molecular alterations and signaling pathways involved in tumor progression, development of metastatic and/or recurrent disease, and better prognosticators to improve patient stratification and treatment selection; 3) non-invasive quantifiable biomarkers in saliva liquid biopsy applicable to cancer detection, disease monitoring and recurrence risk.
As main findings, we highlight that this thesis evidences for the first time the clinical relevance of SOX2 expression and gene amplification in early stages of laryngeal tumorigenesis. Our results unprecedentedly revealed the clinical application of SOX2 expression as an independent predictor of laryngeal cancer risk in patients with precancerous lesions beyond current WHO histological grading. We also demonstrated that various genes mapping at 11q13 amplicon (i.e. CTTN, CCND1 and ANO1) are frequently co-amplified and overexpressed in HPV-negative HNSCC and correlated with reduced patient survival, while absent or very rarely expressed in HPV-positive tumors. Therefore, these molecular alterations could contribute to the distinct clinical outcomes of these two HNSCC entities and serve as the basis to design more personalized therapeutic strategies for these patients. Similarly, the expression levels of the oncogene p-SRC (Tyr419) and various downstream SRC effectors such as the focal adhesion kinase (FAK) and the Arf GTPase-activating protein ASAP1 showed a differential impact on HNSCC prognosis depending on the tumor site and were specific predictors of poor prognosis in the larynx. These findings could have important implications for ongoing clinical trials, as these new criteria could help to improve the selection and biomarker-based stratification of patients who may benefit from treatment with SRC inhibitors. This thesis also provides valuable information on the clinical and biological role of miR-301a dysregulation in HNSCC. In depth functional and mechanistic characterization further contributed to demonstrate that miR-301a promotes tumor cell growth and invasion, and PI3K and ERK signaling pathways as central nodes of miR-301a-mediated functions. Strikingly, two pharmacologic inhibitors of PI3K and ERK pathways effectively targeted/counteracted the growth-promoting and pro-invasive effects of miR-301a. Besides, we performed a large prospective study in 125 HNSCC patients to explore and decipher salivary bacterial changes associated to tumor site, treatment response, and development of tumor recurrence.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGIA MOLECULAR Y CELULAR

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Autora	Directores	Tutor
Rocío Granda Díaz	Juana María García Pedrero	Santiago Jesús Cal Miguel
	Juan Pablo Rodrigo Tapia	

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SUMMARY

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RESUMEN

Los carcinomas escamosos de cabeza y cuello (CECC) comprenden un complejo y heterogéneo grupo de neoplasias que, a su vez, presentan una amplia diversidad de alteraciones genéticas y moleculares. Sin embargo, dichas características ofrecen grandes oportunidades para identificar potenciales biomarcadores de interés clínico. España, y en especial Asturias, presenta una de las más altas incidencias a nivel mundial, siendo un importante problema de salud pública. La mortalidad continúa siendo elevada debido principalmente al diagnóstico tardío, las limitadas opciones terapéuticas y las recurrencias. En esta tesis hemos combinado diversos análisis moleculares y celulares, junto con una amplia variedad de tecnologías (inmunohistoquímica, western blot, RT-PCR cuantitativa para análisis de ARNm y miARN, análisis in silico, metagenómica, formación de colonias, MTS, invasión 3D) con el objetivo de afrontar los principales retos actuales de esta enfermedad, e identificar: 1) biomarcadores tempranos fiables que permitan determinar el riesgo de desarrollo de cáncer, y prevenir o intervenir en las primeras fases de la enfermedad; 2) alteraciones moleculares y vías de señalización involucradas en la progresión tumoral, el desarrollo de enfermedad metastásica y/o recurrente, y nuevas herramientas para mejorar la estratificación de los pacientes y la selección de tratamiento; 3) biomarcadores cuantificables no invasivos en biopsia líquida de saliva aplicables a la detección de cáncer, seguimiento de la enfermedad y riesgo de recurrencia.

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ABBREVIATIONS

ACRV1: Acrosomal Vesicle Protein 1

Ago2: Argonaute RISC Catalytic Component 2

AJCC: American joint committee on cancer

AKT: Protein kinase B

ANO1: Anoctamin-1

ARF: ADP-ribosylation factor 1

ASAP1: Arf GTPase-activating protein

ASRs: Age-standardized rates

AUC: Area under de curve

AZGP1: Alpha-2-Glycoprotein 1, Zinc-Binding

BCL2: Apoptosis Regulator BCL2

BSA: Bovine seroalbumine

CagA: Cytotoxin-associated gene A

CA15-3: Cancer Antigen 15-3

CCND1: Cyclin D1

CCNL1: Cyclin L1

CDKN2A: Cyclin Dependent Kinase Inhibitor 2A

CEIC: Comité de Ética de Investigación Clínica

cMYC: MYC Proto-Oncogene

CNA: Copy number alteration

COL7A1: Collagen Type VII Alpha 1 Chain

CPT1A: carnitine palmitoyltransferase 1A

CRT: Chemoradiotherapy

CSC: Cancer stem cell

CSF: Cerebrospinal fluid

CT: Chemotherapy

CTCs: Circulating tumor cells

CtDNA: circulating tumor DNA

CTTN: Cortactin

DFS: Disease-free survival

DLC1: DLC1 Rho GTPase Activating Protein

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dsDNA: Double-stranded DNA

DPM1: Dolichyl-Phosphate Mannosyltransferase Subunit 1

EBV: Epstein-barr virus

EDTA: Ethylene diamine tetra acetate

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

EIA: Enzyme-immuno-assay

EMT: Epithelial-mesenchymal transition

ERK: Mitogen-Activated Protein Kinase

ESCC: Esophagus squamous cell carcinoma

FAK: Focal adhesion kinase

Fap2: Fibroblast activation protein 2

FDA: Food and drug administration

FDR: False discovery rate

FFPE: Formalin-fixed paraffin-embedded

FXR1: Fragile X mental retardation syndrome-related protein 1

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

HDL: high-density lipoprotein

HE: Hematoxylin and eosin stain

HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

HER2: Erb-B2 Receptor Tyrosine Kinase 2

HERG1: Potassium Voltage-gated channel subfamily member

HHV-8: Human herpes virus type 8

HIF-1: Hypoxia Inducible Factor 1

HNSCC: Head and neck squamous cell carcinoma

HP: Haptoglobin

HPV: Human papillomavirus

HR: Hazard ratio

HSP27: Heat shock protein 27

HTLV-1: Human T-lymphotropic virus type 1

IACR: International Agency for Cancer Research

IHC: Immunohistochemistry

IMRT: Intensity-modulated radiation therapy

KRAS: KRAS Proto-Oncogene, GTPase

LncRNA: Long non-coding ribonucleic acid

LOH: Loss of heterozygosity

MAP3K13: Mitogen-activated protein kinase kinase kinase 13

MDSCs: Myeloid-derived suppressor cells

MEM: Minimum Essential Media

MEOX2: Mesenchyme Homeobox 2

MiRNA: MicroRNA

MOI: Multiplicity of infection

MSK: Mitogen and stress activated protein kinase

mTOR: Mechanistic target of rapamycin kinase

MUC4: Mucin 4

MUC20: Mucin 20

NaCl: Sodium cloride

NANOG: Homeobox Transcription Factor Nanog

NGS: Next generation sequencing

NIH: National Institute of Health

NPC: Nasopharyngeal carcinoma

NPM1: Nucleophosmin 1

OCT4: Octamer-binding transcription factor 4

OPC: Oropharyngeal carcinomas

ORAOV1: Oral cancer-overexpressed protein 1

ORF: Open reading frame

OS: Overall survival

OPSCC: Oropharyngeal squamous cell carcinoma

OSCC: Oropharyngeal squamous cell carcinoma

OTUs: Operational Taxonomic Units

PAHs: polycyclic aromatic hydrocarbons

PCoA: Principal coordinate analysis

PD1: Programmed cell death 1

PDL1: Programmed death-ligand 1

PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PFS: Progression-free survival

PPFIA1: Protein tyrosine phosphatase receptor type F polypeptide-interacting protein alpha-1

RB: Retinoblastoma-associated protein

PRKC1: Protein Kinase C Iota

PTEN: Phosphatase and tensin homolog

qPCR: Quantitative polymerase chain reaction

RISC: RNA-induced silencing complex

ROC: Receiver operating characteristic curve

RPS6KC1: Ribosomal protein S6 kinase C1

RT: Radiotherapy

RUNX3: Runt-related transcription factor 3

SCC: Squamous cell carcinoma

SD: Standard deviation

SDS - PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SHANK2: SH3 and multiple ankyrin repeat domains 2

SMAD4: Mothers against decapentaplegic homolog 4

SOCS6: Suppressor of cytokine signaling 6

SOX2: SRY-box transcription Factor 2

SRC: Proto-oncogene tyrosine-protein kinase Src

STAT3: Signal transducer and activator of transcription 3

TCGA: The cancer genome atlas

TGF-β: Transforming growth factor-beta

TGFBRS: Transforming growth factor beta receptor 1

TH: Tyrosine Hydroxylase

THPO: Thrombopoietin

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TIMP2: Tissue inhibitor of metalloproteinases 2

TMA: Tissue microarray

TINCR: terminal differentiation–induced noncoding RNA

TNM: Tumor-node-metastasis

TP53: Tumor protein p53

TP63: Tumor protein 63

Tris-HCl: Tris-Hydrochloride

TSC1: TSC Complex Subunit 1

UICC: Union for international cancer control

UTR: Unstranlated region

VacA: Vacuolating cytotoxin A

VGLL4: Vestigial Like Family Member 4

WHO: World health organization

YAP1: Yes-associated protein 1

1. INTRODUCTION

1.1 Head and neck squamous cell carcinoma

Head and neck cancer encompasses a complex and heterogeneous group of aggressive neoplasias [1]. These cancers include several anatomical locations and multiple sublocations, leading to variable clinical behavior, prognosis and treatment. Head and neck squamous cell carcinomas (HNSCC) represent over 90% of cases, primarily originated from the mucosa lining of the upper aerodigestive tract [2,3]. The major tumor sites are the oral cavity, pharynx and larynx (**Figure 1**). Regarding the sublocations, the oral cavity is constituted by the lips, the anterior part of the tongue, the floor of the mouth, the hard palate, the upper and lower gingiva, and the retromolar trigone. The pharynx encompasses three sublocations: nasopharynx, oropharynx (tonsils, vallecula, base of tongue, soft palate, and posterior pharyngeal wall), and hypopharynx (pyriform sinuses, posterior surface of larynx, postcricoid area, and lower throat). Finally, the larynx comprises the supraglottis, glottis (vocal cords and anterior and posterior commissures) and subglottis.

Salivary gland tumors, thyroid tumors, as well as other solid tumors such as melanomas, sarcomas, esthesioneuroblastomas and paragangliomas, are usually examined separately. On the other hand, nasopharyngeal carcinomas are distinct in their epidemiology, etiology and clinical behavior [4]. Due to the high prevalence and prognostic impact of human papilloma virus (HPV) in oropharyngeal squamous cell carcinomas, these tumors are now classified as an independent tumor entity by the AJCC/UICC 8th Edition New Staging [5].

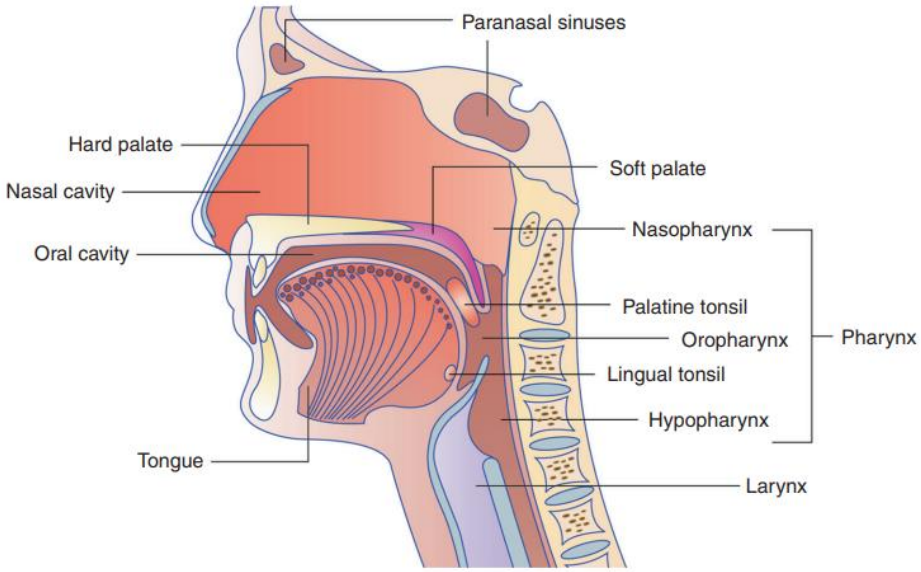


Figure 1. Main anatomical locations of head and neck cancers (figure taken from [6]).

1.2 Epidemiology and etiology

HNSCC is categorized as the sixth most common cancer worldwide, with 900.000 new cases per year, leading to approximately 500.000 deaths (**Figure 2**) [7]. Noteworthy, the distribution of HNSCC cases worldwide results heterogeneous; southern regions from Asia show the highest prevalence of this disease, followed by North America and southern and central Europe. Noteworthy, Asturias is the leading region in cases diagnosed each year in Spain and Europe.

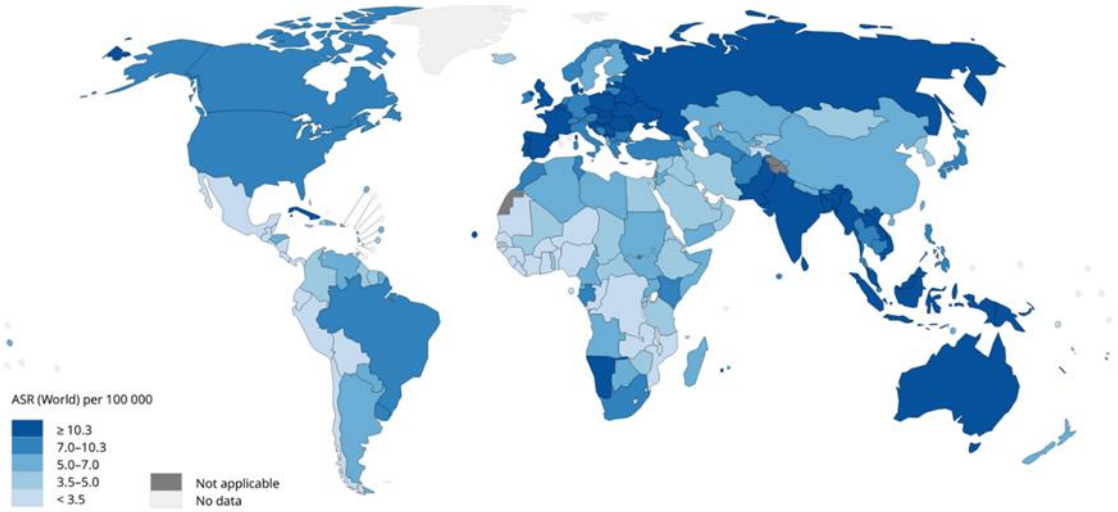


Figure 2. Worldwide incidence of HNSCC (GLOBOCAN 2020). The map was generated using the GLOBOCAN website mapping tool (gco.iarc.fr) by selecting the 'hypopharynx', 'larynx', 'lip, oral cavity', 'nasopharynx' and 'oropharynx' cancer sites. ASRs: Age-Standardized Rates.

Among the etiological factors, smoking and alcohol have been widely described as the main risk factors for this cancer type [8,9]. Tobacco contains more than 7,000 chemical components, including potential carcinogenic compounds, such as polycyclic aromatic hydrocarbons (PAHs), nitrosamines and aldehydes among others [10]. In line with this, 90% of HNSCC patients are habitual smokers [11]. In addition, the alcohol-derived compound acetaldehyde has been linked to DNA damage and generation of DNA adducts, thereby promoting the appearance and accumulation of mutations [12]. Likewise, this metabolite disrupts both DNA synthesis and repair [13]. Besides hepatic alcohol-derived production, oral microbiota can also metabolize ethyl alcohol into acetaldehyde. Therefore, this carcinogenic compound has direct contact with the head and neck mucosa, causing its damage. Importantly, alcohol and tobacco consumption act synergistically, so people who smoke and drink have a > 35-fold risk factor for developing HNSCC [14].

Beyond alcohol and tobacco, other recognized risk factors for HNSCC are ageing, poor oral hygiene, laryngopharyngeal reflux, marijuana consumption, exposure to environmental contaminants and diets with a low percentage of vegetables and high content in animal fats [15-18]. Besides, in some Asian countries it is very common to chew areca nut, including "betel quid" (a mixture of areca nut, betel leaf, slaked lime and tobacco). In particular, China, Taiwan, and India show high incidence rates of oral cancer related with the consumption of these substances. Finally, genetic factors also contribute to HNSCC tumorigenesis. For example, people with Fanconi anemia, a rare genetic disease characterized by impaired DNA repair, have a 500 to 700-fold increased risk of developing HNSCC, mainly oral cancers [19].

On the other hand, infections by oncogenic viruses, such as HPV and Epstein Barr virus, have gained attention in the last years due to their strong contribution to oropharyngeal and nasopharyngeal cancer development, respectively. Importantly, HPV is considered to be responsible of 70% oropharyngeal cancers [20]. HPV infection of mucosal keratinocytes leads to the production of 6 viral early proteins (E1, E2, E4, E5, E6 and E7) and two late proteins (L1, L2) by host cell. The oncogenic role of HPV is related to E6 and E7, which act as oncoproteins through its binding to both p53 and pRB, thus promoting their degradation [21]. To date, it has been classified more than 100 different HPV types, being HPV16 the most common high-risk subtype.

Nowadays, the incidence of laryngeal and nasopharyngeal cancer is decreasing, whereas oropharyngeal cancers are increasing. This trend has been related with the increment of HPV infections, whose transmission is mainly sexual [22,23]. This has prompted oropharyngeal carcinomas to be currently recognized and classified as a separate tumor entity [5].

1.3 Clinical characteristics

Classically, HNSCC has been considered a group of neoplasias mainly affecting middle-aged men (mean age 60 years) with toxic habits like regular tobacco and/or alcohol consumption. However, the prevalence is changing within the last years due to, at least in part, the increment of tobacco consumption in women and HPV infection prevalence [24,25].

Moreover, HPV infection has been mainly correlated with oropharyngeal cancer development. The average of age in these cases are around 45 years and the transmission is through sexual intercourse [2]. Interestingly, being a carrier of HPV is correlated with a better response to radiotherapy and therefore with a better prognosis, but the explanation is still not entirely clear. Several vaccines to hinder HPV infection have been approved by the Food and Drug Administration agency (FDA) and are currently being administered mainly to young girls [26,27]. Remarkably, HPV positive oropharyngeal carcinoma was included in an independent staging

system in 2017 by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) due to the improved prognosis for this subgroup [28]. However, vaccination targeting HPV-16 and -18, the main oncogenic serotypes, is expected to prevent the appearance of cervical cancer and reduce HPV-related HNSCC cases within the next years [29].

The diagnosis of HNSCC greatly varies among tumor locations. However, common symptoms include dysphagia, odynophagia, otalgia, hoarseness, oral pain, or weight loss as well as the presence of lumps in the neck [2]. Hypopharyngeal cancer is usually diagnosed in advanced stage due to the lack of symptoms in initial stages, unlike laryngeal tumors which are usually detected in early phases by hoarseness associated with the vocal cords.

The most relevant prognostic factor in these patients is lymph node involvement. This is an important point to decide the choice of treatment. Likewise, these types of tumors are characterized by a high incidence of recurrences and secondary neoplasias, mainly affecting lungs and esophagus [30,31]. The risk of development a new primary tumor is about 2-4% per year and 10% to 20% for the rest of their life [32]. Additionally, autopsies of these patients have revealed that there are 3-4 times more distant metastases than those reported in the clinic [33].

The mortality rate of these patients is mainly due to the failure of locoregional control of the disease. On the other hand, the overall survival (OS) depends principally on the tumor stage. Thus, stages I and II have a 5-year survival of between 70% and 90%. However, the more advanced stages (III and IV) have a worse prognosis and survival is much lower (around 50%) [34]. Remarkably, survival statistics of these patients has not been improved within the last decades, highlighting the urge of finding novel biomarkers that anticipate cancer development or response to treatments.

1.4 Treatments

The treatment of this disease is multimodal and includes surgical resection, radiotherapy (RT) and/or chemotherapy (CT). However, curation rates depend on tumor location, size, stage and the clinicopathological characteristics of each patient. For this reason, the TNM classification system (tumor, node, metastasis) plays a key role in clinical management of patients with HNSCC.

Surgery and RT alone are recommended for approximately 30%-40% of patients with early-stage disease. Both treatments may result curative and give similar results in terms of survival (70-90%) [35]. Nevertheless, surgery is generally preferred for oral cavity cancers, whereas RT (alone or in combination with CT) is usually the option of choice for nasopharyngeal carcinoma. Besides, Cisplatin is the radiosensitizer of choice when radiation therapy is given with chemotherapy [36]. On the other hand, cetuximab, an anti-EGFR blocking antibody is frequently

used in combination with radiotherapy in patients with HPV-negative HNSCC non-susceptible to undergo chemotherapy [29]. However, all these treatments are usually associated with morbidity and worsened quality of life.

The curative proportion of early stages is high. Unfortunately, more than 60% of cases are diagnosed in advanced stages of the disease. The stages III and IV are characterized by a high local invasion and a greater involvement of regional lymph nodes. Therefore, they have a worse prognosis. In these cases, the selection of the best treatment depends on a series of factors such as tumor location, age, patient preference, toxic effects, associated diseases, and organ preservation, among others [28]. Patients with metastatic disease, locoregional recurrent or unresectable disease who have been previously irradiated, receive generally palliative treatment.

However, despite recent advances in the treatment and management of patients with HNSCC, no significant improvements of clinical outcomes have been achieved within the last decades (**Figure 3**). It is worth mentioning that the arise of new therapies, such as immunotherapy, has significantly improved the survival of a subset of patients with HNSCC. Nonetheless, the success of immune checkpoint blockade-based therapies greatly varies among patients with approximately 15-20% responders to date [37]. Specifically, two anti-PD-1 blocking checkpoints, pembrolizumab and nivolumab, have been approved by the FDA for recurrent and metastatic HNSCC [29].

Nonetheless, effective and predictive markers of response to treatment have not been achieved in many cancers. An explanation may be given by the focus of the studies, based largely on the intrinsic characteristics of the tumor [38].

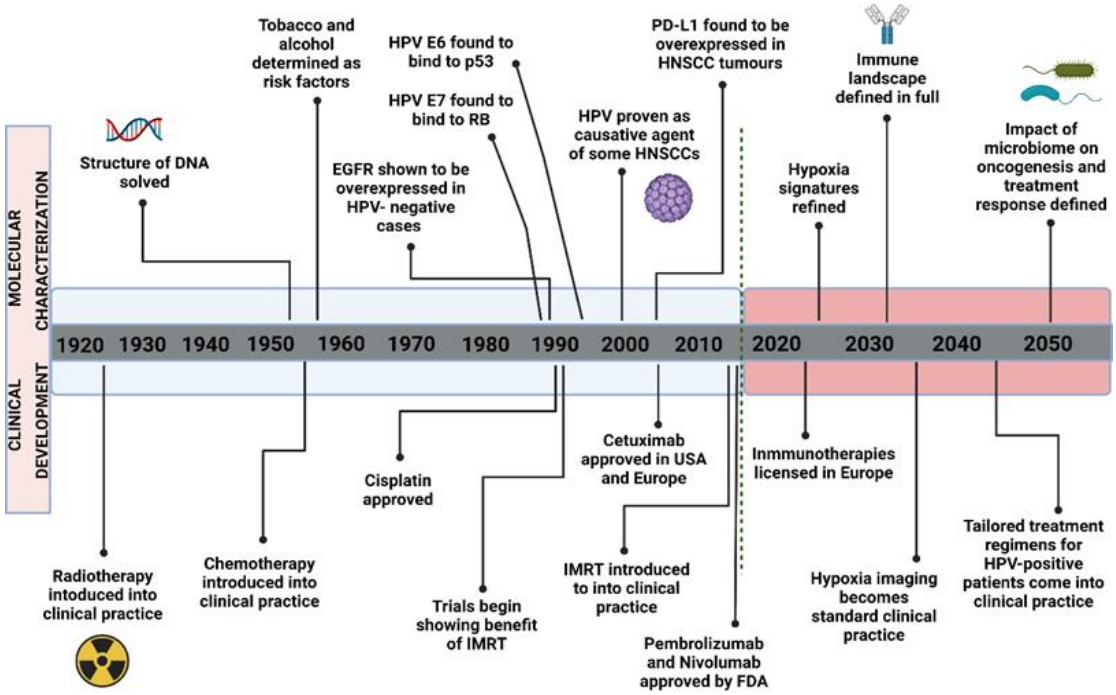


Figure 3. Timeline of principal therapeutic developments and molecular characterization in HNSCC. (Figure modified from [39]). Biorender.

Tumors are heterogeneous entities constituted by a plethora of cellular subpopulations or subclones with different genetic, epigenetic and molecular characteristics. Tumor cells have the ability of evolve and adapt to changes. So, each tumor of patient will have a different behavior. In fact, most cancers commonly exhibit in the early stages of the disease a single clonal origin, but subsequently contain multiple cell populations with different mutations, acquiring the ability to invade other tissues and develop distant metastasis. Experimental evidence suggests that its heterogeneity can change in space and time, determining the development of different clones that evolve independently, but not always divergent [40]. In addition, there are other factors that can affect the response to treatment in HNSCC, such as the microbiome profile [41]. Thus, the discovery of new targeted therapies and the use of precision medicine are the focus of the improvement.

1.5 Cancerization field and progression model in HNSCC

As above-mentioned, advanced stages of the disease have a worse prognosis due to augmented loco-regional lymph node involvement and a higher recurrence rate. Moreover, recurrences are the main reason for therapeutic failure within the first two years. Beyond this point, secondary neoplasias, mainly affecting the upper aerodigestive tract, represent the most frequent cause of mortality for these patients [42]. For this reason, early detection and the availability of predictive biomarkers for patients' stratification is mandatory in order to improve the success against these tumors.

The carcinogenesis of head and neck tumors is a multistage process, which begins with the acquisition of a series of protumoral genetic and epigenetic alterations. This event is explained by the term "field cancerization", proposed by Slaughter in 1953 [43]. A cancerous field is formed by a set of cells that have acquired the necessary tools to develop a carcinoma (**Figure 4**). This process begins before obvious symptoms are observed, such as erythroplasia or leukoplakia. Regarding head and neck cancers, exposure to certain carcinogenic substances, such as alcohol and tobacco, are the main responsible for the alteration of the mucosa of the aerodigestive tract. Another factor is the errors in DNA replication that occur with aging. As a result, a field composed of genetically different clones will be created, where the most suitable phenotypes will predominate [44].

Recurrences are commonly produced by malignant transformation and expansion of preneoplastic cells, usually located at the edges of surgically removed tumors, which differs from secondary neoplasias, considered novel primary tumors [45]. Whether increased rates of new primary tumors are consequence of therapeutic interventions, immune exhaustion or prolonged exposure to certain toxic compounds merits further investigations.

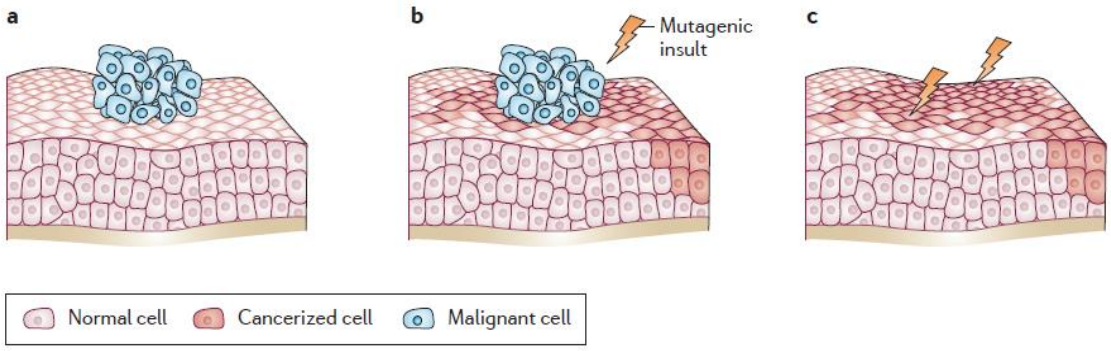


Figure 4. First steps of a cancerization field. **a)** Spontaneous tumor development is characterized by the gain of different mutations or epigenetic alterations compared to healthy tissue. The surrounding mucosa is phenotypically and genotypically normal. **b)** As tumor grows, malignant cells induce phenotypical changes in the surrounding mucosa cells, leading to the formation of a cancer-supportive microenvironment (cancerization field). **c)** Carcinogenic fields origins may also be independent from initial tumors due to mutagenic damage as the induced by ultraviolet light radiation or smoking. Figure taken from [44].

HNSCC tumors are characterized by high genetic instability, based on the loss or gain of certain chromosomal regions [46]. The well-studied carcinogenesis process in HNSCC has permitted the identification of the main events taking place in each phase (**Figure 5**). Firstly, inactivation of tumor suppressor genes and activation of proto-oncogenes initiates the process. Frequently, the loss of 9p21 occurs during the progression of normal epithelial mucosa to the phase of hyperplasia. This chromosomal region harbors important tumor suppressor genes, such as *CDKN2A* and *ARF* (which codes for p14, which is a stabilizer of p53). The transition from hyperplasia to dysplasia is characterized by a loss of heterozygosity of 3p21 and 17p13 [29]. In this phase is inactivated the so-called "guardian of the genome", *TP53*. In fact, somatic mutations for *TP53* have been observed in 60%-80% of patients with HNSCC [45]. Surprisingly, the overall mutation rate and frequency of *TP53* mutations is higher in HPV-negative tumors respect HPV-positive tumors [26]. The next step of the progression model is the transition from dysplasia to carcinoma *in situ*, characterized by the loss of chromosomal regions 11q13, 13q21, and 14q32. The last phase consists in the development of an invasive carcinoma, marked by the loss of 6p, 8, 4q27 and 10q23. Another important tumor suppressor, *PTEN*, is inactivated in this stage [29]. This metastatic profile is associated with the activation of genes involved in the epithelial-to-mesenchymal transition (EMT) process, where the TGF-β pathway has been identified as an important regulator point [47,48].

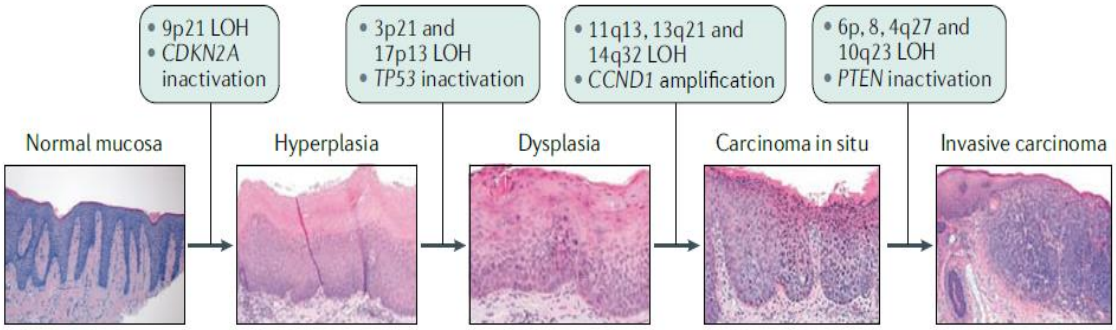


Figure 5. Multi-step progression model for HNSCC with principal genetic events. (Figure taken from [29])

1.6 Principal chromosomic alterations

The expansion of "omics sciences" such genomic, transcriptomic, metabolomic or proteomic have allowed to better understand the biology of cancer. The identification of oncogenes and tumor suppressors allow identifying which signalling pathways are most important to understand the behaviour of tumorigenesis process. Along with other types of cancer, HNSCC is characterized by genomic instability by copy number alteration (CNA) of genes. This process conformed a hallmark of cancer and an instigator of carcinogenesis [49]. Although many CNAs regions have been found in this disease, there is a large proportion unstudied that deserve further investigations [50].

Cytogenetic analyses have revealed frequent genetic and molecular alterations shared by the vast majority of HNSCC (**Figure 6**). In fact, chromosome 3 has been found to be modified more frequently than any other chromosome. The greatest gain has been found in the 3q26 region, where are genes with great oncogenic potential such *as PIK3CA, CCNL1, THPO, MAP3K13, MUC20* or *MUC4* [51]. Besides, the chromosomal region 3q26 has been associated with tumor progression and poor patient prognosis [52]. The *SOX2* gene is also found in this chromosomic location. Similar to other pluripotency-associated transcription factors, *SOX2* has been implicated in sustaining stemness of embryonic stem cells, reprogramming of adult somatic cells to a pluripotent stem cell state and also in multiple tumorigenic processes [53-55]. The role of SOX2 in HNSCC progression and its impact on prognosis and disease outcome has been subject of intense investigation [52,56,57]. However, the role of SOX2 in the early stages of HNSCC tumorigenesis and its possible contribution to malignant transformation and acquisition of an invasive phenotype remains unexplored [58].

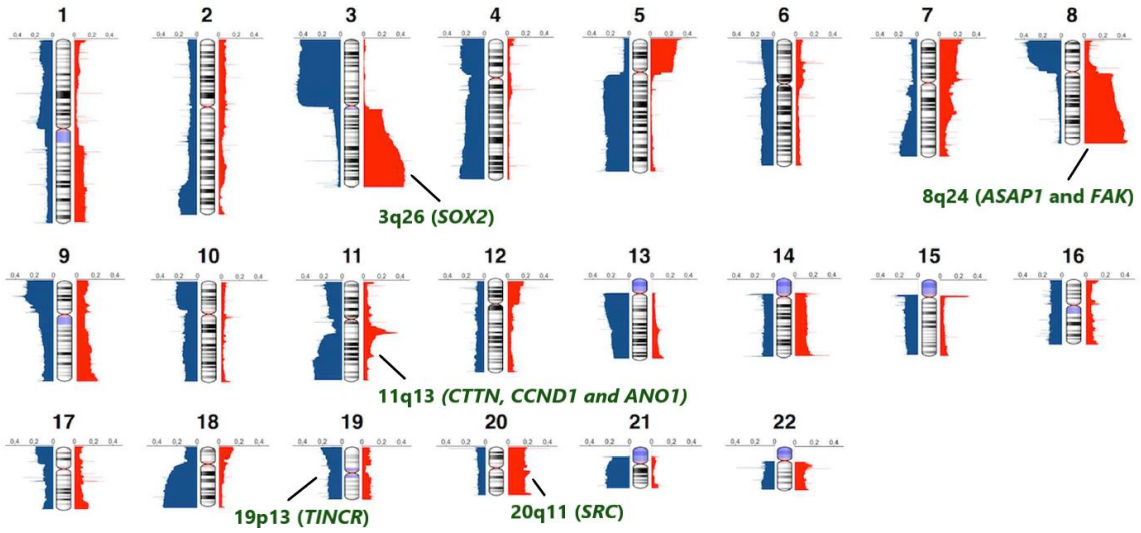


Figure 6. Frequent chromosomic alterations in HNSCC. Modified from [50]. Biorender

The amplification of the 8q23-24 and 11q13 regions are also detected with high prevalence and related to recurrent and metastasic disease in HNSCC. Furthermore, some of the genes found in the 11q13 region, are the oncogenes *CTTN* (Cortactin) *CCND1* (Cyclin D1), and *ANO1* (Anoctamin-1), associated with advanced disease stage and poor prognosis [59]. On the other hand, 8q24 region contains *ASAP1* and *FAK* (Focal Adhesion Kinase) genes. The first gene is correlated with an aggressive phenotype of the disease and diminished overall survival, due to its role in promoting the invasion in laryngeal carcinoma [60]. Further, *FAK* overexpression is associated with invasion and metastatic processes in many cancers [61,62]. In fact, previous results of our group demonstrated the relevant role of *FAK* in the initial stages of the laryngeal tumorigenesis [63].

Other chromosomal regions of interest for HNSCC are 20q11 and 19p13, mapping *SRC* and *TINCR*, respectively. *SRC* is one of the oldest and most investigated proto-oncogenes which modulate multiple oncogenic signalling pathways and biological processes essential for the malignant phenotype, such as proliferation, cell adhesion, motility, invasion, and angiogenesis [64]. On the other hand, Terminal differentiation-induced non-coding RNA (*TINCR*), also known as LINC00036, is involved in control human epidermal differentiation. Recently, the aberrant expression of this Long non-coding RNA (lncRNA) has been suggested to be involved in tumorigenesis of many types of cancers, including as HNSCC tumors [65-67].

1.7 The potential of biomarkers

The complete definition of a biomarker was established by the FDA and the National Institute of Health (NIH) in 2015: "A biomarker is an objectively measurable characteristic that relates to normal biological processes, pathogenic processes (disease status), anatomic measurements, or pharmacological responses to therapeutic intervention". To date, there are many different types of biomarkers, however, no consensus about classification has been achieved (**Figure 7**).

Biomarkers are powerful tools that have been employed in the clinical practice for decades, from determination of glucose blood levels for the diagnosis and control of diabetes to cholesterol for the analysis of cardiovascular risk. The recent importance of biomarker activities has been caused by the updates of new genetic information and best molecular technologies. The term biomarker encompasses a huge range of components. Moreover, the presence or deregulation of genes, proteins, DNA, RNA, metabolites, or metabolic imbalances of several body fluids are indicators of the presence of many diseases. Besides, the set of biomarkers together with the discovery of innovative treatments, such as cell therapies, have the potential to improve patient management [68].

The discovery of potential biomarkers able to predict patients' outcome or response to treatment has gained attention within the last years. This information may be detected and analyzed in tissue, circulation compounds (blood and lymph) and other body fluids like urine, sputum, breast nipple aspiration, etc. Besides, biomarkers may be found even in premalignant cells or in components surrounding the tumor [69].

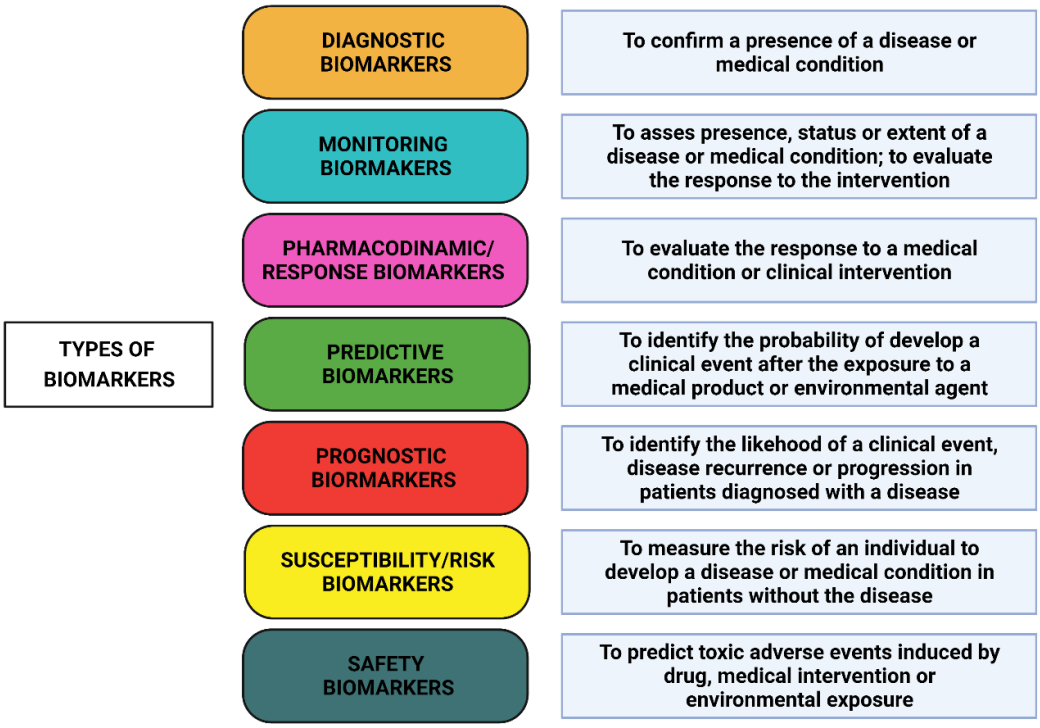


Figure 7. Classification of principal biomarkers. Figure modified from [70].

Our group has made remarkable contributions over the last decade identifying different subsets of clinically-relevant biomarkers, mainly including: early biomarkers for cancer risk assessment [58,63,71-83]; tumor-specific markers for HNSCC diagnosis; biomarkers of tumor progression and prognosticators; risk of metastatic and/or recurrent disease, predictive biomarkers of response/resistance to treatment; non-invasive biomarkers in saliva liquid biopsy. Early detection of cancer is a priority research area in our laboratory. The identification of accurate and reliable predictors for cancer risk assessment will enable to detect cancer at an early and, consequently, more curable stage. Our group has significantly contributed to this field, pioneering the identification of various promising proteins as early cancer risk markers in patients with premalignant lesions [58,71,74,75]. Among them, we unprecedentedly demonstrated the utility of Cortactin and the focal adhesion kinase (FAK) as powerful biomarkers for cancer risk assessment, beyond WHO dysplasia grading (current gold standard) [63]. These results were further validated using large independent multicenter cohorts of patients with laryngeal dysplasias and oral leukoplakias, thus expanding extraordinarily the application of these novel biomarkers [82,83]. Noteworthy, these two novel biomarkers have been transferred into the clinical practice at the HUCA Pathology Department, as the first molecular markers routinely used for cancer risk assessment in patients with a diagnosis of oral and laryngeal precancerous lesions, jointly with histological grading.

1.8 MicroRNAs: discover, biogenesis and function

miRNAs are a non-coding single-stranded RNA of approximately 22 nucleotides in length, derived from long transcript of mRNA and present in the genomes of plants, animals and viruses. However, instead of being translated into protein, miRNAs post-transcriptionally regulates gene expression [84,85].

The discovery of the first miRNA, *lin-4*, arose from the collaboration between Rosalind Lee, Rhonda Feinfaum and Victor Ambros in the mid-1970s. His studies focused on *Caenorhabditis elegans* mutant discovered in Sydney Brenner's lab [86]. At that time, the possibility of the existence of non-coding RNAs was not questioned. Ambros's group started cloning *lin-4* obtaining a 693 bp fragment. They sequenced this fragment but could not find an ORF (Open Reading Frame) in the entire sequence. Despite all the efforts, they were unable to eliminate its function, so they concluded that *lin-4* could not generate any protein, since they did not expect that it could be transcribed to a transcript of only 22 bp. However, they did find that *lin-4* was essential for the post-embryonic development of worms [87]. The second miRNA, *let-7 (LETha1)*, was discovered in the 2000s. This miRNA is also involved in the development of *C.elegans*. Furthermore, *let-7* was found to be phylogenetically conserved in many species, including molluscs and arthropods [88]. These findings stablished a new field of study in molecular biology: gene regulation by small non-coding RNAs [89].

The processing and maturation of miRNAs takes place in several steps. Firstly, miRNA genes are transcribed by RNA Polymerase II (Pol II) leading to long primary transcripts (pri-miRNAs) formation. Then, pri-miRNAs are processed later in the nucleus by a ribonuclease III enzyme, Drosha, to hairpin-shaped intermediates (pre-miRNAs) approximately 60-100 nucleotides long. Subsequently, the pre-miRNAs are transported to the cytoplasm by a Ran-GTP-dependent transmembrane protein, *Exportin 5*. Once in the cytoplasm, the pre-miRNAs are processed by the endonuclease RNase III Dicer, and a double-stranded RNA molecule of about 22 nucleotides is generated. Finally, the mature miRNAs are incorporated into the RISC complex (RNA-induced silencing complex). This complex is the responsible of guiding the miRNA to its targeted mRNA. Usually only one of the strands is incorporated into the complex. The selection is carried out depending on the position of the pre-miRNA and its thermodynamic stability [90,91].

miRNAs have a series of guidelines for nomenclature. The precursors are named "mir" whereas “miR” is the right form when referring to mature miRNA. Likewise, prefixes of 3 or 4 letters are used to define species. For example, hsa-miR-101 is the mature miRNA 101 of *Homo sapiens*, although mmu-mir-101 is the precursor 101 of *Mus musculus*. In addition, a letter is assigned as a suffix when miRNAs that differ by 1 or 2 nucleotides are obtained from paralogous

sequences (e.g., hsa-miR-10a and hsa-miR-10b). Remarkably, in some cases, the same miRNA can be obtained from sequences located in different parts of the genome. For example, miR-142 arise from two precursors, hsa-miR-142-1 and hsa-miR-142-2, which are located at different loci. Finally, depending on the strand of the premiRNA they come from, the mature miRNA is defined as 3p or 5p [92].

The biological functions of miRNAs depend on their ability to silence gene expression, mainly through translational repression and degradation of their target mRNA. This regulation is possible by complementary base pairing with the 3'-untranslated region (UTR) of target messenger RNA, also known as "seed region" [93]. To date, there are numerous studies where regulatory functions of these molecules are described in key processes such as embryonic development, fat metabolism, insulin secretion, hematopoiesis, stem cell differentiation or brain development [94-99].

Within the last years, several studies brought to light the relevance of miRNAs in the field of oncology. More than a half of the miRNAs currently known are located in genomic cancer-related areas (chromosomal regions with a high rate of cytogenetic alterations) [100]. Cancer-related miRNAs, also known as oncomirs, are commonly classified in two groups: oncogenes and tumor suppressors (**Figure 8**). Oncomirs were firstly described in cancer by Carlin *et al.* in patients with chronic lymphocytic leukemia (CLL). Deletions in the 13q14 locus, where miR-15a and miR-16-1 are located, is the most frequent cytogenetic alteration in this disease (68% of cases) and it is generally associated with good prognosis [101]. In line with this, decreased expression of the above-mentioned oncomirs was described in patients with pituitary adenoma. Further, miR-15a and miR16-1 expression correlated with reduced tumor diameter, suggesting a potential role of these oncomirs as tumor suppressors [102]. In agreement, miR-15a/16 cluster targets the *BCL2* oncogene, that encodes the antiapoptotic protein BCL2 broadly increased in leukemia cells. Low levels of these oncomirs correlated with increased expression of the anti-apoptotic gene *BCL2*, thus suggesting a protecting role of miR-15/16 in cancer [103,104].

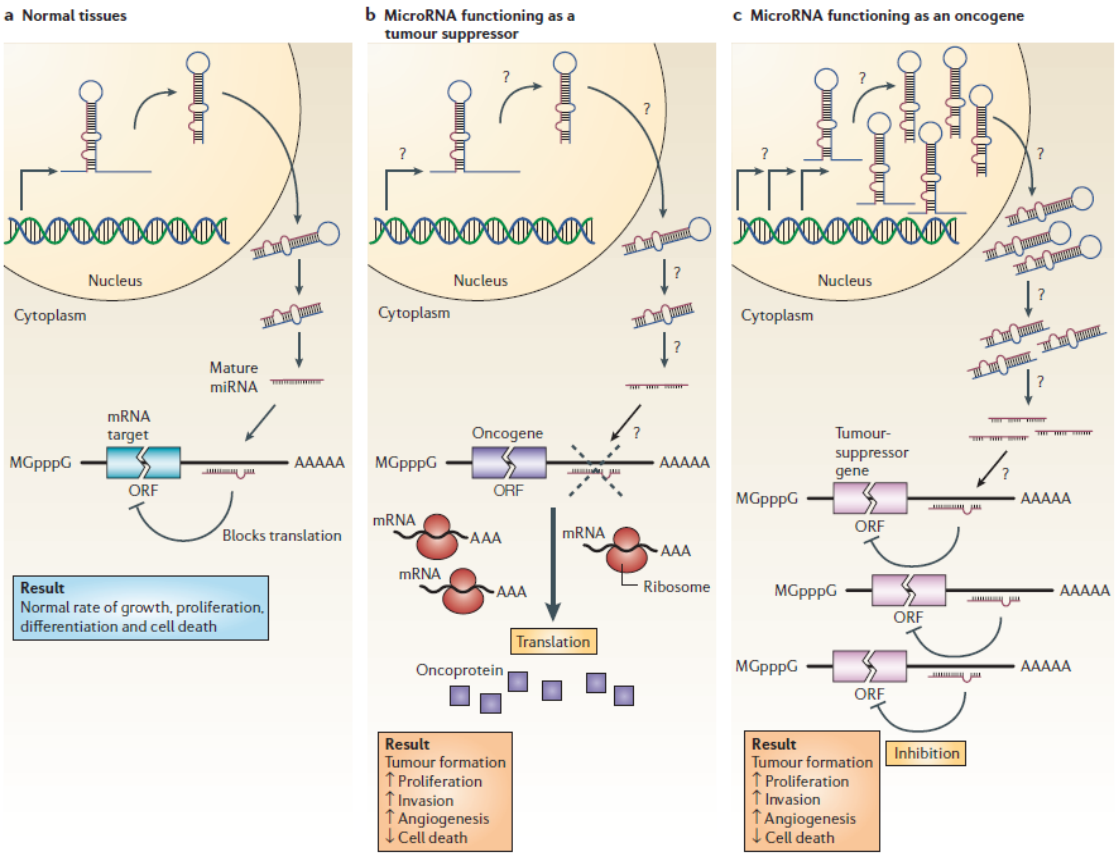


Figure 8. **miRNAs can act as tumor suppressors or oncogenes**. **a)** In normal tissues, all non-coding RNA functions, including transcription or processing of the miRNA are correct, as well as the union between the sequences of the targeted mRNA and the mature miRNA obtained, leading to a blockade of subsequent translation. For this reason, all cellular processes are carried out correctly (proliferation, cell growth, differentiation and apoptosis). **b)** The elimination of a miRNA that acts as a tumor suppressor may lead to tumor formation**.** Specifically, increased proliferation, invasion, and angiogenesis will be promoted. **c)** The overexpression of a miRNA that has a tumor suppressor gene as target, will generate an increase in all the processes that trigger tumor formation (figure taken from [105]).

An important role of miRNAs has been widely in solid tumors. Chan *et al*. observed that miR-21 was highly expressed (5 to 100-fold) in glioblastoma compared to healthy tissue. In line with this, enhanced expression of miR-21 has been also described in mammary tumors and renal carcinoma [106-108]. This miRNA promotes cell growth, cell cycle progression and prevents apoptosis by regulating BCL2.

Since its discovery, a large number of different miRNAs have been disclosed. In addition, miRNA expression patterns between cancer and normal are different. It has been observed that miR-143 is the most expressed miRNA in normal tissues, decreasing in different types of tumors

(11-33% of all cases) [109]. Despite this, many of their characteristics (especially with their regulation) remain unclear. The reason is that is a relatively young field of study.

As mentioned above, the main function of miRNAs is post-transcriptional gene regulation. Noteworthy, these molecules can be detected found in different body biofluids, such as saliva, urine, breast milk, seminal liquid or blood, thus suggesting a potential role in intercellular signalling [110,111]. About 10% of these miRNAs, also known as circulating miRNAs, can be found into exosomes. The other 90% are bonded to proteins, like argonaute 2 (Ago2), nucleophosmin 1 (NPM 1) or high-density lipoprotein (HDL) thus preventing RNases-mediated digestion [111]. Consequently, these molecules own ideal conditions as biomarkers, such as accessibility or high sensitivity, suggesting their potential as novel non-invasive biomarkers for cancer prognosis and diagnosis. In line with this, the presence of increased levels of miR-155, miR-210 and miR-21 in the sera from patients with leukemia led, in 2008, to establish for the very first-time circulating miRNAs as cancer biomarkers by Lawrie *et al* [112]. In the literature, there are several studies evaluating several miRNAs as early diagnosis biomarkers [113-116].

1.8.1 MiRNAs in HNSCC

It is widely known that the most important etiological factors of HNSCC are tobacco, alcohol and HPV status. Even so, the non-coding regions of the genome play an important role in the formation and development of this type of tumors. Over the last few years, many miRNAs related to prognosis, development and tumor progression have been described in different types of HNSCC patients' fluids, suggesting a potential role of these molecules as biomarkers **(Table 1)**. miRNAs may provide new therapeutic opportunities for those patients who have few or no treatment alternatives. Thus, miRNA-based therapy, also known as miRNA replacement therapy, could be used to detect and suppress the expression of an oncogene. Further, normal expression of a tumor suppressor gene can also be restored through suppression of an oncomiR, through a type of therapy named miRNA inhibition therapy [117]. As above-mentioned, HPV-positive patients generally show better prognosis than HPV-negative tumors. Favorable outcomes of tumors bearing HPV antigens correlates with increased immune infiltrate and greater response to immunotherapy, radiotherapy and chemotherapy [118,119]. However, there is a lack of predictive biomarkers of treatment response in HPV-negative patients. Despite advances in the landscape of HNSCC treatment, little improvement in overall survival has been achieved within the last three decades due to, at least in part, chemo and radiotherapy resistance.

In this context, several miRNAs have been proposed as potential tools to predict prognosis and response to treatment in HNSCC, in both in virus-related and HPV-negative tumors.

For example, miR-9 enhanced levels correlated with more radio-sensitive tumors in patients with HPV-positive HNSCC [120]. In agreement, decreased levels of miR-9 in plasma were detected in patients with recurrent or metastasic nasopharyngeal carcinomas [121]. Precancerous lesions are an important control point for this type of cancer. In line with this, Alvarez-Teijeiro *et al*. described that miR-196a and miR-196b are increased in the 95 to 100% of laryngeal precancerous lesions. Noteworthy, these levels were similar or even increased in patient-matched invasive carcinomas subsequently developed. Besides, these miRNAs were also detected in saliva, thus providing non-invasive biomarker detection. Altogether, these data sustain the potential utility of miR-196 a/b as biomarkers for early HNSCC detection and/or disease surveillance [71].

Table 1. Principal miRNAs as biomarkers in head and neck squamous cell carcinoma (HNSCC).

miRNA	Cancer type	Sample material	Main Outcome-Association	Reference
miR-9	NPC/ NPC/ OSCC	Plasma/ Plasma/ Serum	lower expression in metastatic patients/ higher expression at posttreatment and lower at recurrence or metastasis/ higher expression associated with longer OS and DFS	[121-123]
miR-20b	OSCC	plasma	higher expression in patients with lymph node metastases	[124]
miR-21	OSCC/ LSCC/ HNSCC/ HNSCC	Whole blood/ serum derived exosomes/ plasma/ plasma	higher expression associated with positive lymph node status/ higher expression associated with higher stage and positive lymph node status/ expression turning back to normal after surgery in patients with good prognosis; not changing in patients with poor prognosis/ expression higher in HNSCC tissue and decreasing in plasma after tumor excision	[125-128]
miR-22	NPC	serum	higher expression associated with shorter OS	[129]
miR-26b	HNSCC	plasma	expression turning back to normal after surgery in patients with good prognosis; not changing in patients with poor prognosis	[127]
miR-31	NPC/ OSCC	Whole blood/ saliva, plasma	lower expression associated with local lymph node metastasis, but not distant metastasis/ lower expression after tumor excision	[130,131]
miR-92b	OSCC	plasma	lower expression associated with recurrence	[132]
miR-93	HNSCC	saliva	higher expression 12 months post-radiotherapy than at baseline	[133]
miR-99ª	HNSCC	plasma	expression lower in HNSCC tissue and increasing in plasma after tumor excision	[128]
miR-106	OSCC	plasma	higher expression in patients with lymph node metastases	[124]

miR-124	NPC	plasma	Higher expression at posttreatment and lower at recurrence or metastasis	[122]
miR-130b	OSCC	plasma	lower expression associated with positive lymph node status	[124]
miR-139	TSCC	saliva	expression lower in TSCC patients and turning back to normal levels after tumor excision	[134]
miR-142	HNSCC	plasma	higher expression associated with worse locoregional control	[135]
miR-184	TSCC	plasma	lower expression after the surgical removal of primary tumor	[136]
miR-186	HNSCC	plasma	higher expression associated with worse locoregional control	[135]
miR-195	HNSCC	plasma	higher expression associated with worse locoregional control	[135]
miR-196a	OSCC	plasma	higher expression in patients with recurrence during follow-up	[137]
miR-196b	HNSCC	saliva	Higher expression in early HNSCC tumorigenesis stages	[71]
miR-200a	HNSCC	saliva	higher expression 12 months post-radiotherapy than at baseline	[133]
miR-221	LSCC	plasma	expression higher in LC patients than in healthy controls and turning back to normal after tumor excision	[138]
miR-222	OSCC	plasma	lower expression associated with lymph node metastases and higher disease stage; expression decreasing with tumor progression	[139]
miR-223	HNSCC	plasma	expression higher in HNSCC tissue and decreasing in plasma after tumor excision	[128]
miR-296	OSCC	plasma	higher expression in patients with lymph node metastases	[124]
miR-301a	OSCC	plasma	lower expression in patients with lymph node metastases	[124]
miR-374b	HNSCC	plasma	higher expression associated with worse locoregional control	[135]
miR-375	OSCC	plasma	lower expression associated with recurrence	[132]
miR-423	OSCC	plasma	lower expression associated with lymph node metastases and higher disease stage; expression decreasing with tumor progression	[139]
miR-483	OSCC	serum	higher expression associated with shorter OS, higher disease stage and lymph node metastases	[140]
miR-486	OSCC	plasma	lower expression associated with recurrence	[132]

miR-572	NPC	serum	higher expression associated with shorter OS	[129]
miR-574	HNSCC	plasma	higher expression associated with worse locoregional control	[135]
miR-626	OSCC	serum	higher expression associated with shorter OS	[141]
miR-638	NPC	serum	higher expression associated with shorter OS	[129]
miR-892b	NPC	plasma	posttreatment and lower at recurrence or metastasis	[122]
miR-1234	NPC	serum	higher expression associated with shorter OS	[129]
miR-3651	OSCC	Whole blood	higher expression associated with lymph node metastases, higher tumor grade and stage	[142]
miR-3676	NPC	plasma	Higher expression at posttreatment and lower at recurrence or metastasis	[122]
miR-5100	OSCC	serum	higher expression associated with shorter OS	[141]

NPC—nasopharyngeal carcinoma; OSCC—oropharyngeal squamous cell carcinoma TSCC—squamous cell carcinoma of the tongue; qRT-PCR—quantitative real-time PCR; OS—overall survival; DFS—disease-free survival; PFS – progression-free survival; LSCC—laryngeal squamous cell carcinoma.

(Table modified from [143])

1.8.2 Role of miR-130 family in cancer

The miR-130 gene family is composed by four miRNA precursors: mir-301a (located at chromosome 17), mir-130a (chromosome 11), mir-130b and mir-301b (both located at chromosome 22). All of them are vertebrate-specific and share the same seed region (**Figure 9**). These miRNAs are considered an oncogenic superfamily due to their capability to target tumor suppressor genes, including *PTEN*, *TGFBRS* and *SMAD4* [144].

miR-130 gene family has been described to be increased in tumor cells and sera from patients with bladder cancer [145,146]. Moreover, *miR*-130 gene family (*miR*-130b, *miR*-301a and *miR*-301b) promote *in vitro* tumor invasion and migration in bladder tumors through FAK and p-AKT signaling, regulated by PTEN, thus suggesting that these miRNAs may become promising therapeutic targets in this disease [145]. In line with this, several studies support the oncogenic role of this family of miRNAs in proliferation, invasion, migration and EMT in many solid tumors [147-152].

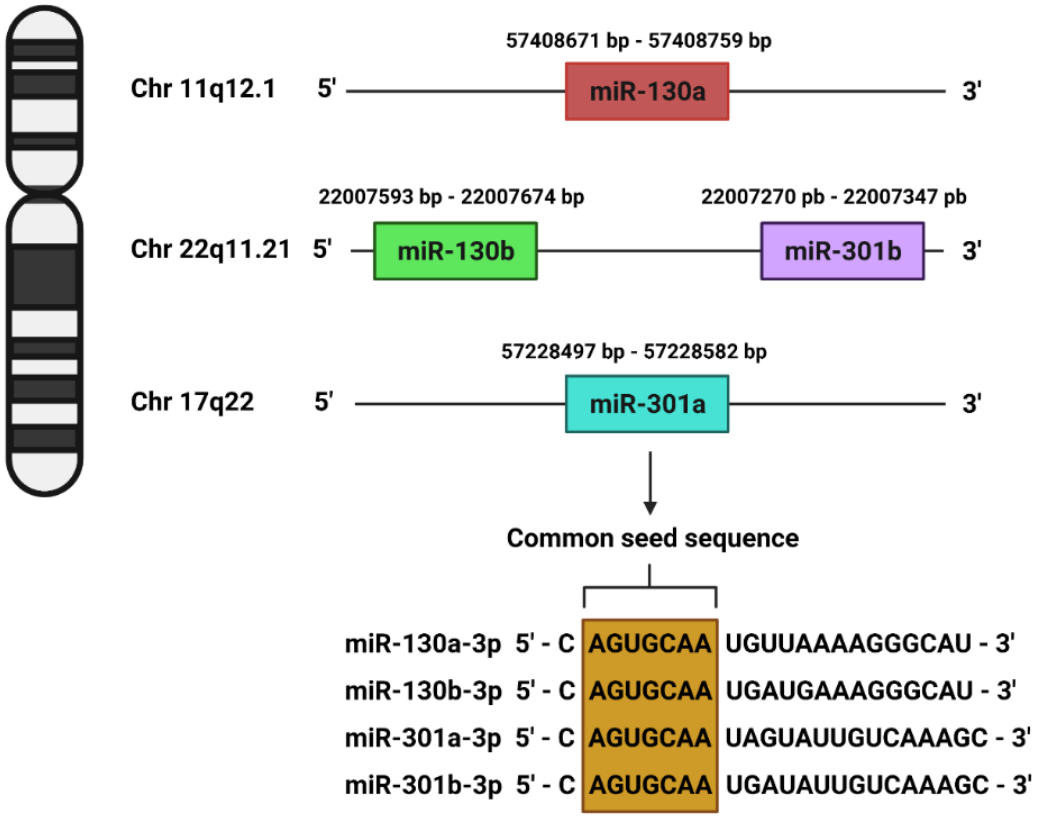


Figure 9. Genomic localization of *miR*-130 family. Figure modified from [145] using Biorender.

Besides their role in cancer progression, these miRNAs could also be useful as biomarkers for clinical applications in diagnosis, prognosis or prediction of treatment efficacy [153-155]. For example, the miR-301a expression in breast cancer was correlated with an aggressive behaviour of the tumor, poor prognosis and overall survival [156].

miR130 family members have been described as predictive biomarkers in HNSCC. In fact, low levels of miR-130b were associated with higher response rates to radiotherapy, as well as improved progression-free and overall survival in HPV-negative oropharyngeal squamous cell carcinoma patients [157]. Upregulation of p63, a p53 family member, has been proposed as the underlaying mechanisms of the radioresistance-promoting role of miR-130b in oral cancer among others [158-160].

The oncogenic role of another member of this family, the miR-130a, has been recently reported in OSCC. It is widely described that alterations in the PI3K/AKT/mTOR pathway deeply correlate with the pathogenesis of head and neck cancers. In consonance with this, TSC1, a component of this signaling pathway, is downregulated in oral carcinomas and associates with unfavorable outcome. miR-130a post-transcriptionally regulates TSC1 expression, thus leading to increased cell proliferation, growth and invasion capabilities. Moreover, downregulation of miR-

130a led to lower tumor volume in murine models of OSCC [161]. Finally, RBP Fragile X Mental Retardation protein-1 (FXR1) promotes tumorigenesis through the stabilization of miR301a-3p in oral cancer. This miRNA targets p21, a tumor suppressor protein, supporting tumor growth and associating with poor prognosis in patients with HNSCC [78,162,163].

1.9 Liquid biopsy: saliva

Tissue biopsy has always been considered a key tool to determine the histological characteristics of the tumor. Moreover, tissue was the only way to study mutations in this disease. Despite advances in obtaining tissue samples, biopsies harbor real limitations such as technical difficulties caused by the absence of a visible tumor or organ-related restrictions [164]. Other limitations are the patient comorbidities, availability logistics and high cost. Besides, it is also necessary to consider the impossibility of repeating it frequently, which discard it as a good monitoring method. This biological process is marked by formation and expansion of the tumor mass. The clones diverge and form different subpopulations or subclones over time that cause tumor heterogeneity. Its diversity is not represented in a tissue sample [165]. By contrast, one of the tools that can overcome these barriers is the so-called liquid biopsy [166].

The term “liquid biopsy” was coined by Klaus Pantel and Catherine Alix-Panabières in 2010 [167]. They studied circulating tumor cells (CTCs) in the blood of patients but is currently extended to study circulating tumor DNA (ctDNA), RNA, proteins and even disease information contained in exosomes (**Figure 10**). We could define liquid biopsy as the analysis of tumor material, cells or nucleic acids, obtained with non-invasive or minimally invasive methods. The efficacy of this tool has been demonstrated in different tumors including lung, head and neck or breast cancers, among others [168-170]. Liquid biopsy is becoming a promising tool for early detection of cases that symptoms do not appear until the tumor is in a very advanced stage.

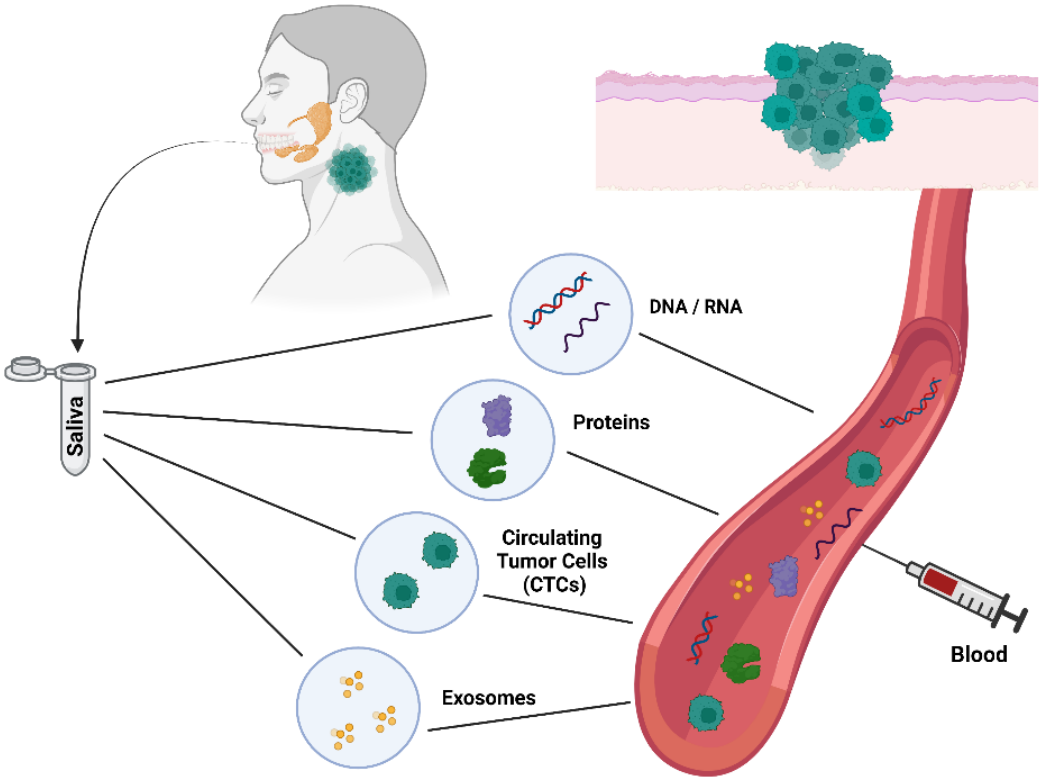


Figure 10. Principal components of liquid biopsy. Biorender.

Regarding liquid biopsy, it is generally referred to the blood. However, it encompasses any biological fluid containing tumor material that we can molecularly characterize. So, for the clinical management of cancer patients we can use other fluids such as urine, seminal fluid, saliva, or cerebrospinal fluid (CSF) [171-174]. The choice of the fluid type depends on the cancer study. In the case of head and neck cancer, saliva is the best option.

Saliva is a slightly acidic (pH=6) and hypotonic biological fluid, as it is made up mostly of water (94-99%). However, it also presents organic and inorganic substances, proteins (such as antibodies), and numerous cellular elements, such as bacteria, among others. Saliva is mainly produced by three principal glands: parotid, submandibular and sublingual. Even so, there are between 300 and 400 minor glands located in our oral mucosa that also contribute to its formation. Saliva participates in important functions as chewing, lubrication, immunity or digestion [175,176]. In addition, it is very easy and fast to obtain, and its collection does not cause any harm to the patient.

It is important to mention that when we talk about saliva samples, we include both stimulated and unstimulated saliva samples. The unstimulated occurs at repose and the stimulated occurs in response to stimuli such as eating [177]. Changes in composition can be

identified between both types of saliva, so it is important to take this into account when making an analysis.

The analysis of saliva is an emerging study field, named as “salivaomics” [178]. It has been possible to isolate all kinds of molecules, such DNA, miRNAs, or proteins. Strikingly, approximately 27% of salivary proteins coincide with plasma proteins [179]. However, 73% of differential salivary proteins are currently considered to be a unique opportunity to unveil tumor markers. For example, in lung cancer, a panel of three proteins (HP, AZGP1 and Calpropectin) was identified to be significantly increased in saliva samples from patients compared to healthy individuals with a sensitivity and specificity close to 90% [180]. In line with this, salivary EGF, p53, Erb2 and CA15-3 levels has been proposed as monitoring tools to guide the clinical management of patients with breast cancer [181]. At transcriptomic level, different salivary components with value as tumor markers have also been described. Zhang *et al.* identified a panel of four salivary mRNAs (KRAS, MBD3L2, ACRV1, and DPM1) that can diagnose localized pancreatic cancer with a sensitivity and specificity of 90% and 95%, respectively [182].

1.9.1 Microbiome and cancer

Bacteria were firstly discovered in 1683, when Anton Van Leeuwenhoek was able to observe some creatures under a microscope that he baptized at that time as "animalcules". For this reason, Leeuwenhoek is considered the father of microbiology [183]. Almost two centuries later, in 1861, Louis Pasteur discovered anaerobic lactic acid bacteria, thus refuting the theory of spontaneous generation according to which it was established that certain forms of animal and plant life arose spontaneously from inorganic, organic matter or a combination of both [184,185]. Pasteur demonstrated with his experiments that it was the microorganisms that generated the putrefaction of food, and not the other way around. Later, Robert Koch was the responsible of demonstrating that bacteria were disease-causing agents. Koch unveiled that tuberculosis and carbuncle were caused by infection with *Mycobacterium tuberculosis* and *Bacillus anthracis*, respectively [186,187]. Likewise, the study of microorganisms has led to great discoveries, such as the case of penicillin by Alexander Fleming, in 1928 [188]. Noteworthy, in the last few years the influence of microorganisms in human diseases such as cancer has gained attention.

The term *microbiome* was coined in 2001 by Joshua Lederberg. The Nobel Prize in Medicine affirmed that humans and symbiotic microorganisms form a great metabolic unit, where bacteria that inhabit our organism are really protecting us [189]. Many microorganisms are capable of metabolize certain nutrients in the diet that are inaccessible for us. This is the case of

the short-chain fatty acids which are microbial metabolites obtained during the colonic fermentation of indigestible fibers food. These components play an important role in homeostasis [190].

The microbial kingdom including bacteria, viruses, archaea, fungi and protists have coevolved with the human system for many years leading to a plethora of host-microbiome interactions thus correlating with several physiological pathways [191]. As a consequence, alterations in human microbiota (commonly known as *dysbiosis*) promotes a wide of diseases such as immune disorders, neurological alterations, metabolic affliction, and cancers development [192,193].

Nowadays cancer is the second death factor worldwide and it was estimated to be responsible of 1 of 6 overall deaths per year [194]. Although cancer is a heterogeneous group of diseases where genetic, human habits and environmental effects are key players, microorganisms might play a crucial role in tumorigenesis. Noteworthy, approximately 20% of human cancers are related to infections by microorganisms, even more in underdeveloped countries [195].

The Earth harbours 3.7 $x10^{30}$ of microorganisms, which colonize all kinds of ecological niches. Importantly, the International Agency for Cancer Research (IACR) has catalogued 10 of them like carcinogenic to human: *Helicobacter pylori (H. pylori),* hepatitis B virus (HBV), hepatitis C virus (HCV), *Opisthorchis viverrini, Clonorchis sinensis*, human papillomavirus (HPV), Epstein-Barr virus (EBV), human T-cell lymphotropic virus type 1 (HTLV-1), human herpes virus type 8 (HHV-8) and *Schistosoma haematobium* [196]. Further, some other microorganisms have been related to cancer development. The yeast *Candida albicans* might promote tumorigenesis through activation of epidermal growth factor (EGFR) pathway by the release of a cytolytic toxin, known as candidalysin. [197]. The EGFR factor is a transmembrane protein dysregulated in a broad type of cancers, such as head and neck, breast, lung, or colon cancers [198]. In agreement, these types of tumors are more frequent in regions where *Candida albicans* infection is more common. *C. albicans*-related tumorigenes has been also related to activationg of MAPK signalling pathway, which is involved in proliferation, apoptosis, stress induction, angiogenesis, loss of E-cadherin and occludin expression (observed in EMT), and consequently, it is correlated with poor survival [199-203].

The tumor microenvironment is constituted by cells, molecules, and blood vessels that surround and feed the tumor. Likewise, bacteria are also part of this construction [204]. The set of bacteria that live in the tumor is known as “tumor microbiome” and it has an important interaction with the host immune system. Recently, a study that included many samples from seven different solid tumors (breast, lung, ovary, pancreas, melanoma, bone, and brain tumors) suggested that tumor-associated microbiota is specific for anatomical location. The results

demonstrated that microbiota present in breast tumors is broader and more diverse than other types. Likewise, the authors concluded that this microbial specificity is involved in tumor development [205].

Microorganisms have been developed different pro-oncogenic mechanisms (**Figure 11**). The best-known example of bacteria responsible of cancer is *Helicobacter pylori*, identified as one of the most important risk factors for gastric adenocarcinoma development. Each year, this pathogen is responsible of more than 60% of all cases [206]. This type of Gram-negative bacteria has flagella which help to its motility into the stomach. Another virulent factor is the lipopolysaccharides present on its surface, that facilitates the adherence to mucosal gastric cells. However, the most important component is an enzyme present of the surface too, known as urease. This enzyme can convert urea and water into ammonia and carbon dioxide. Ammonia is an alkaline molecule, thus neutralizing the acid pH of the stomach. Likewise, *H. pylori* secretes exotoxins named vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) into the host cell cytoplasm, inducing tissue damage. The VacA toxin is involved in blockade of T-cell proliferation, interruption of normal mitochondrial function, and apoptosis process [207,208]. On the other hand, CagA toxin disrupt cellular adhesion through the E-cadherin/β-catenin complex disruption. Alterations in β- catenin pathway encourage the transcription of different oncogenes such as *c-Myc* and *CyclinD-1*, favouring carcinogenesis and tumour progression [209].

Since intestine has the greatest number of microbes of the human body (10^{12} bacteria/g feces), colorectal tumour-associated microbiota has been widely studied [210]. Importantly, among the huge number of microorganisms inhabiting human digestive tract, three procarcinogenic microbes stand out from the rest. Firstly, *Escherichia coli*, producer of a genotoxin (colibactin) that is responsible of damaging the double-stranded DNA of human cells [211]. On the other hand, *Bacteroides fragilis* generate a multi-step inflammatory cascade mediated by its toxin [212]. Finally, *Fusobacterium nucleatum* diminishes T cell and Natural Killer cell-mediated antitumor responses through Fap2. This adhesin acts as a ligand for the inhibitory immune checkpoint TIGIT, thus decreasing NK cells cytotoxicity and dampening cytokine production. Likewise, *F. nucleatum* encourages the recruitment of myeloid-derived suppressor cells (MDSCs) through the release of short peptides and short-chain fatty acids, thus leading to decreased T cell proliferation and enhanced apoptosis [213]. Altogether, these data bring to light the relevance of tumor-related microbiota in the regulation of antitumor responses mediated by the immune system.

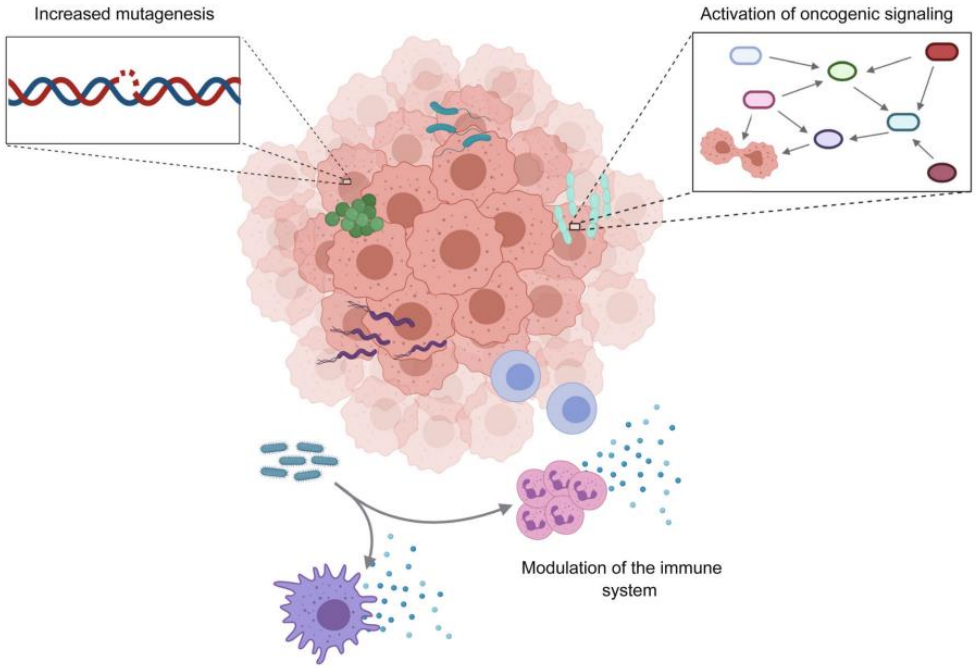


Figure 11. Crosstalk between microbiota and tumor. Many aspects of the role of the microbiota in the tumor remains unclear. However, microbial procarcinogenic mechanisms have been grouped into three categories: promotion of carcinogenesis induced by mutagenesis, activation of oncogenic signaling mediated by toxins or other bacterial metabolites, and modulation of the immune system. (Figure taken from [204]).

Traditional culturing methods present evident limitations for microorganisms' detection; only 70% of the taxa present in the human oral cavity can be isolated and identificated by these techniques [214]. However, the arise of "omics", including the development of Next-Generation Sequencing (NGS), has allowed to detect the presence of microorganisms in a sample that would not be feasible with traditional culture methods. In this line, the prokaryotic ribosome is assembled by two main components: the large subunit (50S) and the small subunit (30S). Each subunit is made up by one or two RNAs and several proteins. The large ribosomal subunit has two rRNAs (23S and 5S) and 34 proteins. In contrast, the small ribosomal subunit contains 21 proteins and a single rRNA: 16S. **(Figure 12)**. The genes that code for ribosomes are highly conserved. The choice to sequence the 16S rRNA is based on several reasons: the gene is comparably short, being about 1500 bp and it presents 10 conserved and 9 hypervariable regions [215]. Among these hypervariable regions, the V3 to V4 region confer the most discriminating power for analyzing the bacterial samples [216]. However, it is difficult to determine if the microbes detected in many cases are causal agents or not.

Currently, there are no conclusive data that allow us to use the microbiome in clinical practice as a diagnostic or prognostic biomarker in cancer. Further, other studies are required to determine whether microbes have the potential to successfully discern which patients show increased risk of cancer development [217]. However, infections are the second leading cause of death in patients with cancer [218]. So, if we add this fact to the enormous molecular heterogeneity of tumors, it leads us to consider combined therapy as one of the best clinical approaches for this disease. Elimination of microbial pathogens can lead to an improvement in response to treatment. For instance, the impact of *Fusobacterium nucleatum* on the response to chemotherapy in patients with esophageal carcinoma has also been studied. The presence of this bacteria confers chemoresistance by modulating autophagy process in these patients. The combination of antibiotics and chemotherapy cycles have been suggested as a good therapeutic strategy [219]. In line with this, it has also been studied that the intestinal microbial composition influences the response to anti-PD1 therapy in patients with melanoma [220,221].

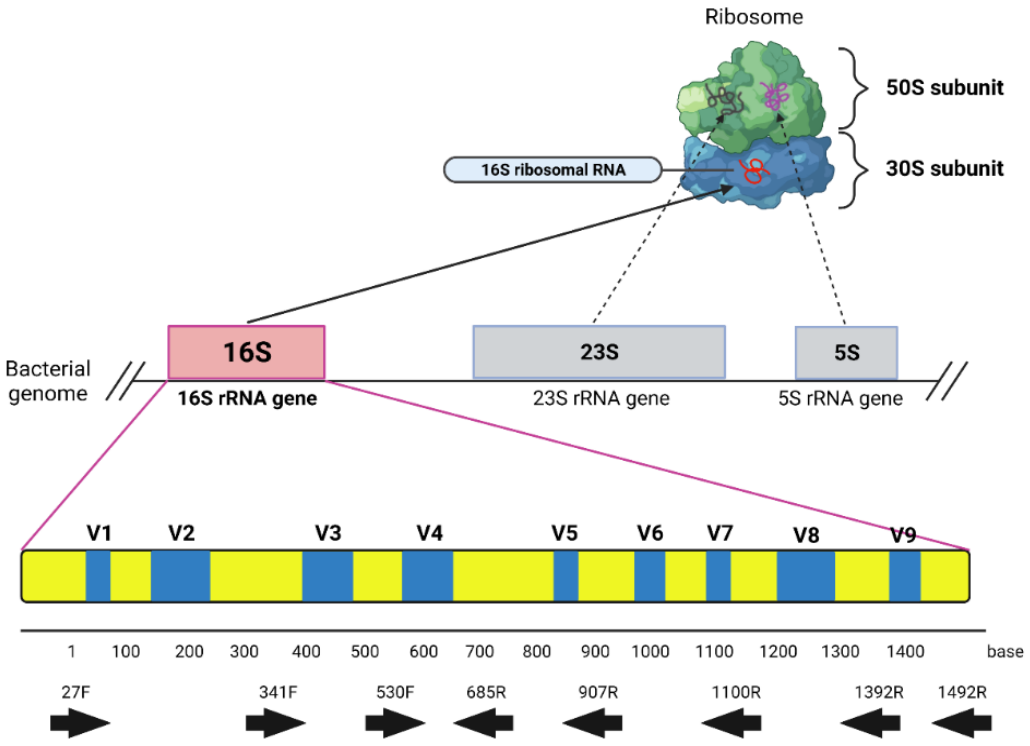


Figure 12. Representation of ribosome structure and 16S rRNA gene. The yellow and blue boxes are conserved regions and hypervariable regions, respectively. The black arrows indicate approximate positions of universal primers on 16S rRNA gene sequence of *Escherichia coli.* Modified from [215].

1.9.2 Microbiota and HNSCC

Regarding head and neck cancers, it is well established that viral infections by Epstein-Barr virus (EBV) and human papillomavirus (HPV) are etiological agents of nasopharyngeal carcinomas (NPC) and oropharyngeal carcinomas (OPC), respectively [222,223]. However, it seems that bacteria may play an important role in the development of these types of tumors [224]. To date, the oral cavity presents the vast number of microbiome studies. Nevertheless, this type of location is very complex to analyse because it encompasses more than 700 different bacterial species [225]. Since the characteristics of saliva (37ºC, pH= 6.5, etc.) provide to bacteria an excellent microenvironment, oral cavity microbiota encompasses more than 700 different species, including commensal bacteria with protective role against the development of OSCC. Specifically, *Kingella* and *Corynebacterium* participate in biodegradation and/or metabolization processes of tobacco and alcohol carcinogens [226,227].

The oropharyngeal cavity shares a similar microbial profile than the esophageal cavity. For example, both locations have elevated levels of *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. These types of bacteria are anaerobic and potentially pathogenic. *Porphyromonas gingivalis* is one of the main bacteria associated with periodontal diseases. However, it has been shown to promote the development of oral cancers by activating oncogenic pathways and immune evasion mechanisms [228,229]. Likewise, Song *et al* analysed the role of this bacteria in murine xenografts of OSCC, concluding that it also induces resistance to paclitaxel [230]. Regarding esophagus squamous cell carcinoma (ESCC), the presence of this type of bacteria has been correlated with metastasis [231]. Furthermore, the presence of *Fusobacterium nucleatum*, both in HNSCC and ESCC, is correlated with advanced stages of the disease [232,233]. On the other hand, a recent meta-analysis study revealed the remarkable enrichment of oral bacteria in colorectal cancer tissue. These data unveil the close microbial relationship between both locations [234].

Recently, Frank *et al*. identified and the microbial profiling of HNSCC tumors (larynx, pharynx and oral cavity) validated in a cohort of 43 saliva untreated patients and 78 healthy donors. These authors found that the relative abundances of *Lactobacillus spp.* and *Neisseria spp.* are increased and diminished, respectively, in HNSCC cases. Likewise, they demonstrated that the elimination of microbiota delays oral tumorigenesis development in a mouse model of oral carcinoma. By contrast, the transference of microbiota from mice with oral cancer to mice that lacked associated microbiota favoured tumorigenesis process [225]. Altogether, these data support further investigations about the potential role of microbiome as predictive or prognostic biomarkers in patients with HNSCC.

2. HYPOTHESIS

Advances in 'omics' technologies have revealed the great complexity and heterogeneity of HNSCCs, harboring multiple and very diverse genetic and molecular alterations. However, this also offers vast opportunities for the identification of novel clinically-relevant biomarkers. Ideally, information from all available studies, including traditional clinical, radiologic and morphologic findings together with novel molecular data would be applied to define a patient's disease profile. These new tools based on the individual molecular and biological characteristics of patients will allow the development of personalized medicine strategies. This will result in a better, more precise and individualized selection of treatments, avoiding side effects and unnecessary toxicities. The major goals we expect to achieve are: 1) early reliable biomarkers for cancer risk assessment to enable detection of cancer at an early and more curable stage; 2) molecular alterations and signaling pathways involved in tumor progression, development of metastatic and/or recurrent disease, and better prognosticators to improve patient stratification and treatment selection; 3) non-invasive quantifiable biomarkers in saliva liquid biopsy applicable to cancer diagnosis and dynamically monitoring of disease status; and 4) to provide potential molecular targets to develop novel therapeutic strategies for HNSCC patients. Unfortunately, very few recent scientific advances have so far been translated into significant improvement of disease diagnosis and patient care. Biomarkers and targets for personalized therapy are still lacking. It is hoped that successful completion of this thesis project will provide a catalogue of novel biomarkers and therapeutic targets to improve diagnosis and clinical management of HNSCC patients.

3. OBJECTIVES

The overall goal of this thesis was to identify novel valuable biomarkers to improve diagnosis, prognosis and treatment of HNSCC patients. To accomplish this, the following specific aims were pursued:

1.- To investigate the role of SOX2 protein expression and gene amplification in the early stages of HNSCC tumorigenesis to ascertain their potential clinical application as cancer risk markers in patients with laryngeal precancerous lesions.

2.- To assess the expression and amplification status of *CTTN*, *CCND1* and ANO1 genes mapping at the 11q13 amplicon in relation to the HPV status in HNSCC patients, and the potential prognostic implications.

3.- To analyze the expression and prognostic significance of SRC activation and downstream SRC effectors (CTTN, FAK and ASAP1) in HNSCC tissue specimens, and to establish correlations with clinicopathological data and the possible impact on patient survival.

4.- To evaluate the expression and clinical significance of the microprotein TINCR in HNSCC patient samples.

5.- To characterize the pathobiological role of miR-301a dysregulation in HNSCC.

6.- To perform a metagenomic analysis in a large series of saliva samples prospectively collected before/after treatment from HNSCC patients and healthy donors to identify changes in microbiota composition associated to tumor site, treatment response, and development of tumor recurrence.

4. MATERIAL AND METHODS

4.1 Patients data

4.1.1 Laryngeal precancerous lesions

Surgical tissue specimens from patients who were diagnosed with laryngeal dysplasia at the Hospital Universitario Central de Asturias between 1996 and 2010 were retrospectively collected, following the approved institutional review board guidelines. All experimental procedures were conducted in accordance with the Declaration of Helsinki and approved by Institutional Ethics Committee of the Hospital Universitario Central de Asturias (HUCA), and by the Regional CEIC from Principado de Asturias (date of approval: 18 July 2013; approval number: 81/2013) for the project PI13/00259. Informed consent was obtained from all patients. Patients must meet the following criteria to be included in the study: (i) pathological diagnosis of laryngeal dysplasia; (ii) with lesions of the vocal folds (iii) no previous history of head and neck cancer; (iv) complete excisional biopsy of the lesion; (v) a minimum follow-up of five years (or until progression to malignancy occurred); and (vi) patients with a diagnosis of laryngeal dysplasia who developed cancer within the next six months were excluded from the study. A total of 94 patients who met these criteria were included in this study.

All the patients were treated with macroscopically complete excisional biopsy of the lesion, either with CO_2 laser or with cold instruments. Microscopically surgical margins were not assessed. No other treatments were administered. Follow-up with the patients occurred every two months in the first six months after completing the treatment, every three months until the second year, and every six months thereafter. Representative tissue sections from the original biopsy material were obtained from archival, paraffin embedded blocks and the histological diagnosis and epithelial dysplasia grade was confirmed in all the cases by an experienced pathologist. The sections selected for study also contained normal epithelia as internal controls. The premalignant lesions were classified into the categories of low-grade and high-grade dysplasia following the WHO classification [235]. All patients were men, with a mean age of 65 years (range 36–86 years). All but two patients were active or old smokers. The mean tobacco consumption was 50 packs-year (range 15–150 packs-year). After the diagnosis, all the patients that were active smokers received smoking cessation advice; however, 14 of them continued smoking. Of the 94 patients included in the study, 14 (15%) lesions were classified as low-grade dysplasia, and 80 (85%) as high-grade dysplasia. During the follow-up period, 29 (31%) of 94 patients developed an invasive carcinoma at the biopsy site. The mean time to cancer diagnosis in the cases that progressed was 27 months (range 8 to 66 months). No significant differences attributable to age were observed (p = 0.501) between the patients who developed cancer (mean, 64 years) and

those who did not (mean, 64 years). The mean tobacco consumption for patients who developed an invasive carcinoma was 58 packs per year, compared to 53 packs per year for those who did not develop cancer (p = 0.819).

4.1.2 HNSCC tissue specimens

Surgical tissue specimens from 392 patients with HNSCC who underwent tumor resection at HUCA and Hospital Sant Pau between 1990 and 2010 were retrospectively collected, in accordance to approved institutional review board guidelines. All experimental protocols were conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committees of the Hospital Universitario Central de Asturias and Hospital Sant Pau and by the Regional CEIC from Principado de Asturias (approval number: 81/2013 for the project PI13/00259). Informed consent was obtained from all patients. Representative tissue sections were obtained from archival, paraffin-embedded blocks, and the histological diagnoses were confirmed by an experienced pathologist. Clinical, sociodemographic, follow-up, and risk factors information was collected from the medical records.

All patients had a single primary tumor, microscopically clear surgical margins, and received no treatment prior to surgery. Only 19 patients were women, and the mean age was 60 years (range 30 to 89 years). All but 22 patients were habitual tobacco smokers—202 moderate (1–50 pack-year) and 159 heavy (>50 pack-year)—and 353 were alcohol drinkers. Nineteen tumors were stage I, 25 stage II, 69 stage III, and 279 stage IV. The series included 152 tonsillar, 116 base of tongue, 62 hypopharyngeal, and 62 laryngeal carcinomas. A total of 147 tumors were classified as well-differentiated, 151 as moderately differentiated, and 94 as poorly differentiated. In total, 232 (59%) patients received postoperative radiotherapy.

On the other hand, to assess the impact of SRC expression on the prognosis of patients with HNSCC, surgical tissue specimens were collected from 122 patients with laryngeal or hypopharyngeal squamous cell carcinoma surgically treated at HUCA between 1996 and 2005, in accordance with approved institutional review board guidelines. Experimental procedures were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. Formalin-fixed paraffin-embedded (FFPE) tissue samples and data from donors were provided by the Principado de Asturias BioBank (PT17/0015/0023), integrated in the Spanish National Biobanks Network, and histological diagnosis was confirmed by an experienced pathologist. Samples were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees of the HUCA and the Regional CEIC

from Principado de Asturias for the project PI16/00280 (approval number: 70/16; date: 5 May 2016).

All patients had a single primary tumor and microscopically clear surgical margins. Patients did not receive any treatment prior to surgery. Only five patients were women. The mean age was 60 years (range 38 to 86 years). Of the 122 patients, 119 were habitual tobacco smokers, 71 moderate (1–50 pack-years) and 48 heavy (>50 pack-years), and 107 were habitual alcohol drinkers; further, 66 (54%) of the 122 patients received postoperative radiotherapy.

The stage of the tumors was determined according to the TNM system of the International Union Against Cancer (7th Edition).

4.1.3 *In silico* analysis using transcriptomic data from The Cancer Genome Atlas (TCGA) HNSCC Database

miR-301a expression was evaluated from publicly available RNAseq data from The Cancer Genome Atlas (TCGA) (TCGA-Firehose Legacy dataset) using the online webtool UALCAN (http://ualcan.path.uab.edu/). Transcriptomic data included 482 samples from HNSCC patients and healthy tissue (n=44).

4.1.4 Saliva samples from patients with HNSCC

Saliva from patients diagnosed with HNSCC at HUCA between 2016 and 2021 were retrospectively collected. All experimental procedures were conducted in accordance with Declaration of Helsinki and approved by Institutional Ethics Committee from HUCA. Informed consent was obtained from all patients. A total of 125 patients with laryngeal and pharyngeal carcinoma were included in the study. Further, 65 healthy saliva samples were collected from HUCA and Hospital de Jarrio. The clinicopathological characteristics are shown in **Table 2**. All mouthwashes (0.9% saline solution) were collected before breakfast. None of the patients were undergoing any treatment with antibiotics at least one week before sampling. Patients who accomplished any of the following criteria were excluded from the study: (i) previous history of head and neck cancer (ii) presence of secondary HNSCC tumor at collection time; (iii) patients diagnosed with dysplasia (iv) non-malignant diagnosis after pathological anatomy lab confirmation, and (v) samples with presence of antibiotics.

Table 2. Characteristics of patients and controls included in microbiome study.

		Cases		
	Controls	**Total**	**Pharynx**	**Larynx**
N	65	125	38	87
Males	56 (86.36%)	114 (91.2%)	36 (94.7%)	77 (88.5%)
Females	9 (13.63%)	12 (8.8 %)	2 (5.7%)	10 (11.5%)
Age (mean ± sd; yrs)	58.7 ± 10.3	64.7± 9.1	63.4 ± 9.6	65.3 ± 8.9
Saliva before treatment	Na	125	37	87
Saliva after treatment	Na	79	31	48
Saliva during follow-up	Na	51	21	30
Treatment: CRT	Na	18 (22.78%)	10 (32.25%)	8 (16.66%)
Treatment: Surgery	Na	61 (77.2%)	21 (67.74%)	40 (83.33%)
Tumor Size	Na	T0: 3 (2.4%) T1: 32 (25.6%) T2: 33 (26.4%) T3: 34 (27.2%) T4: 34 (27.2%)	T0: 0 (0%) T1: 7 (18.4%) T2: 11 (28.9%) T3: 10 (26.3%) T4: 10 (26.3%)	T0: 3 (3.4%) T1: 25 (28.7%) T2: 22 (25.28%) T3: 24 (27.58%) T4: 24 (27.58%)
Recurrence	Na	31 (39,06%)	16 (42.1%)	15 (17.1%)

Na. Non applicable; sd. Standard deviation; CRT. Chemoradiotherapy.

4.2 Tissue microarray (TMA) construction and DNA extraction

Five morphologically representative areas were selected from each individual tumor paraffin block: two for DNA isolation and three for the construction of a TMA. To avoid cross-contamination, two punches of 2 mm diameter were taken first, using a new, sterile punch (Kai Europe GmbH, Solingen, Germany) for every tissue block, and stored in eppendorf tubes (Sigma Aldrich, Saint Louis, MO, USA) at room temperature. Subsequently, three representative tissue cores (1 mm diameter punches) were selected from each tumor block and transferred to a recipient 'Master' block in a grid-like manner using a manual tissue microarray instrument to construct TMA blocks, as described previously [74,236]. In addition, each TMA included three cores of normal epithelium (tonsil) as an internal negative control and three cores of a HPV-positive cervix carcinoma as a positive control. A section from each microarray was stained with hematoxylin and eosin and examined by light microscopy (Leica Microsystems, Wetzlar, Germany) to check the adequacy of tissue sampling. The protocol for DNA extraction from paraffin-embedded tissue sections has been described elsewhere [236].

4.3 Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues were cut into 3-µm sections and dried on Flex IHC microscope slides (Dako, Glostrup, Denmark). The sections were deparaffinized with standard xylene and hydrated through graded alcohols into water. Antigen retrieval was performed using Envision Flex Target Retrieval solution, high pH (Dako). Staining was done at room temperature on an automatic staining workstation (Dako Autostainer Plus, Dako, Glostrup, Denmark) with the antibodies summarized in **table 3**. Counterstaining with hematoxylin was the final step.

SOX2 staining was evaluated as the percentage of cells with nuclear staining in the dysplastic epithelium. The optimal cut-off value for SOX2 staining calculated by ROC analysis using progression to cancer as endpoint was 12.5% (**Figure 13**). Scores were classified as negative or positive staining based on values below or above cut-off value of 12.5%.

CCND1 staining was evaluated as the percentage of cells with nuclear staining, scored as 0–2 according to the semiquantitative scale (0–10, 10–50, or >50% positive tumor cells). CCND1 staining scores were classified as negative or positive staining on the basis of values below or above the median value of 10%. Since ANO1, SRC, ASAP1, CTTN, and FAK staining showed a homogeneous distribution, a semiquantitative scoring system was applied, and staining intensity was scored as negative (0), weakly (1+), moderately (2+), or strongly positive (3+). Scores of ≥2 were considered as positive expression. Scores ≥ 1 were considered positive ANO1 expression.

Table 3. Antibodies used in immunohistochemistry analysis

Antibody	Dilution	Manufacture
Anti SOX2 rabbit polyclonal antibody	1:1000	Merck Millipore (# AB5603)
mouse anti-Cyclin D1 monoclonal antibody DCS-6	1:100	Santa Cruz Biotechnology (Inc. sc-20044)
rabbit polyclonal Anti-TMEM16A	1:500	Abcam # ab53212
mouse anti-cortactin monoclonal antibody Clone 30	1:200	BD Biosciences Pharmingen (# 610049)
Anti-FAK monoclonal antibody Clone 4.47	1:250	Merck Millipore (# 05-537)
Anti-ASAP1	1:750	Abcam (# 11011)
phospho-SRC (Y419)	1:80	Invitrogen (# 44-660G)
Total SRC	1:100	Santa Cruz Biotechnology (# sc-8056)
TINCR rabbit polyclonal antibody	1:1000	In-house antibody

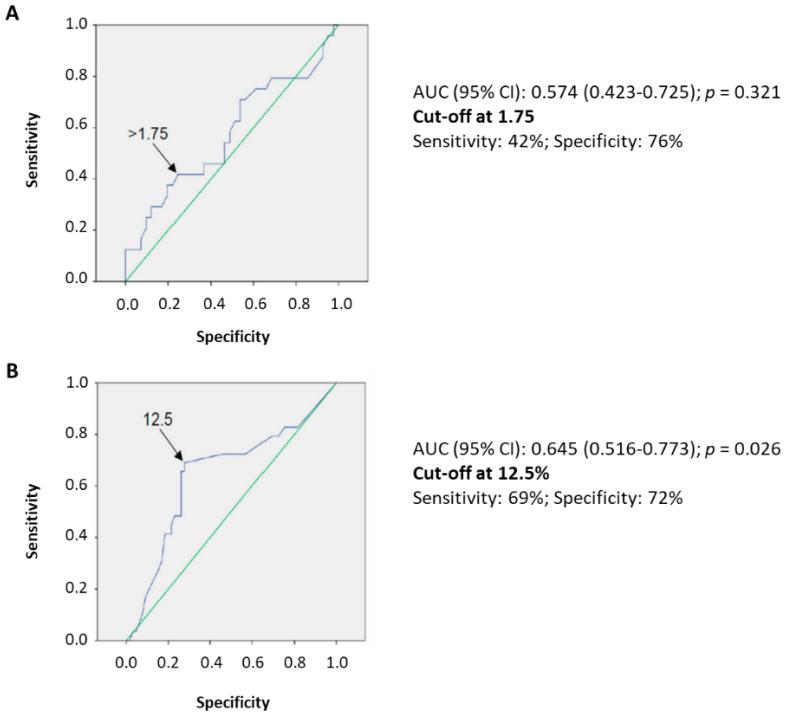


Figure 13. **Determination of the optimal cut-off values for SOX2 amplification and SOX2 expression by receiver operating characteristic (ROC) curve analysis.** ROC curves for SOX2 amplification (**A**) and SOX2 expression (**B**) (as percentage of positive nuclei). The optimal cut-off values calculated were 1.75 for SOX2 amplification and 12.5% for SOX2 expression. The area under de curve (AUC), with 95%CI, the p-values, and the sensitivity and specificity calculated for each optimal cut-off are shown.

4.4 Gene amplification analysis

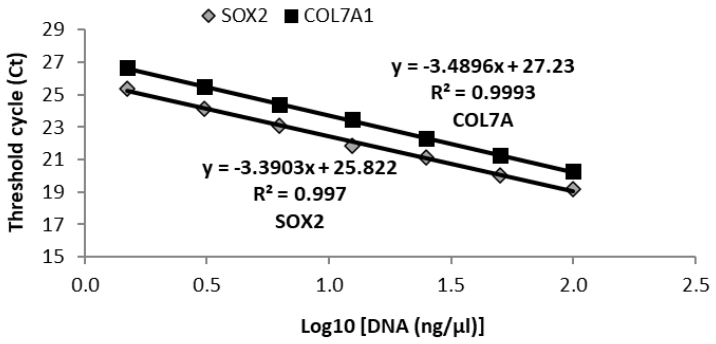
Gene amplification was evaluated by real-time PCR (qPCR) in an ABI PRISM 7500 Sequence detector (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix and oligonucleotides with the sequences included in the **table 4**. Samples were analysed in triplicates and template-free blanks were also included. The relative gene copy number was calculated using the $2^{-\Delta\Delta CT}$ method, as we previously described [63]. Calibration curves for the reference gene (*COL7A1*) and the target gene (*SOX2*) showed parallel slopes and comparable amplification efficiency across the linear range (1.5–25 ng) (**Figure 14**). The ΔΔCT represents the difference between ΔCT of dysplasia - ΔCT of normal mucosa, with ΔCT being the average CT for the target gene minus the average CT for the reference gene. The optimal cut-off value for SOX2 amplification was identified via a receiver operating characteristic (ROC) curve analysis, using progression to cancer as an end point, and patients were categorized into positive SOX2 amplification (≥1.75) and negative amplification (<1.75). TH (Tyrosine Hydroxylase, located at 11p15) was the gene reference to *CCND1, ANO1* and *CTTN*. In correlation with this, relative copy

numbers ≥ 2-fold were considered gene gain, and relative copy numbers ≥ 4-fold were considered positive for gene amplification.

Table 4. Primers used for real-time PCR

Gene		Primers
SOX2	Fw	CTCCGGGACATGATCAGC
	Rv	CTGGGACATGTGAAGTCTGC
COL7A1	Fw	ACCCAGTACCGCATCATTGTG
	Rv	TCAGGCTGGAACTTCAGTGTGT
CCND1	Fw	GGAAGATCGTCGCCACCTG
	Rv	GAAACGTGGGTCTGGGCAAC
ANO1	Fw	CAAAGGCAGGTGCTTTGCA
	Rv	TCTACGGGCCTCTGCTCACT
CTTN	Fw	GATCTCATTTGACCCTGATGACATC
	Rv	CGTACCGGCCCTTGCA
TH	Fw	TGAGATTCGGGCCTTCGA
	Rv	GACACGAAGTAGACTGACTGGTACGT

A



B

Gene	Slope	Efficiency
SOX2	-3,390	1,97
COL7A	-3,489	1,93

Figure 14. *SOX2* real time PCR analysis. Standard curve showing the Log10 DNA amount, in ng per µL, plotted against threshold cycle (Ct) for different dilutions of genomic DNA in PCR-grade water by real-time PCR (**A**). The table shows the efficiency of amplification for each primer pair, calculated using the slope of the corresponding standard curve (**B**).

4.5 HPV detection

The algorithm used to detect the presence of HPV in the patients has been previously described in detail [236,237]. Briefly, the presence of HPV was assessed by p16-immunohistochemistry in all cases, and those cases showing p16-positive immunostaining (any nuclear and or cytoplasmic staining) were subjected to high-risk HPV DNA detection and genotyping by GP5+/6+-PCR with an enzyme-immuno-assay (EIA) read-out for detection of 14 high-risk HPV types. Subsequent genotyping of EIA-positive cases was performed by bead-based array on the Luminex platform. In addition, in situ hybridization (ISH) with biotinylated HPV DNA probes considered to react with HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (Y1443, DakoCytomation, Glostrup, Denmark) was performed on all carcinomas, using 3 µm tissue sections of the TMAs, according to the manufacturer's instructions.

4.6 Cell lines and culture conditions

The head and neck cancer-derived cell lines UT-SCC2 (mouth floor, T4N1M0), UT-SCC29 (glottic larynx, T2N0M0), UT-SCC38 (glottic larynx, T2N0M0), UT-SCC40 (tongue, T3N0M0), UT-SCC42A (supraglottic larynx, T4N3M0), and UT-SCC42B (neck metastasis from same patient as SCC-42A) were kindly provided by Dr. Reidar Grenman (Department of Otolaryngology, University Central Hospital, Turku, Finland) [238]. FaDu cells (hypopharynx) were purchased to the ATCC.

Cell lines were grown in DMEM (Corning 10-017-CV) supplemented with 10% fetal bovine serum (Corning Media Tech 35-079-CV), 100 U/mL penicillin (Biowest, Nuaille, France), 200 mg/mL streptomycin (Biowest, Nuaille, France), 2mM L-glutamine, 20 mM HEPES (pH 7.3) (Biowest, Nuaille, France) and 100 mM MEM non-essential amino acids (Biowest, Nuaille, France). Cells were maintained at standard conditions, 37°C, 5% CO2 in humidified atmosphere.

4.7 Drugs

BYL-719 (Selleckchem, Houston, TX) and PD98059 (MedChemExpress, NJ, USA) were aliquoted as 10 mM stock solutions in sterile DMSO and maintained at -20 ^{o}C until use at the indicated concentration in each case.

4.8 Lentivirus production and transduction

Lentiviral infections were performed as previously described [239,240]. Cells were infected with miR-301a (InteRNA technologies) and miR-301a-3p inhibitor carrying lentiviruses (lenti miRNA inhb hum hsa-miR-301a-3p, ref HLTUD044, Sigma) and were selected in the presence of 1 µg/ml

puromycin for 3 days and then maintained with 0.1 µg/ml puromycin (Sigma). Cells were infected at a MOI of 1, o/n.

4.9 RNA extraction and RT-qPCR analysis

Total RNA was extracted from HNSCC-derived cell lines using Trizol (Life Technologies). miRNA reverse transcription (RT) was carried out using Taqman Megaplex miRNA primer pool A (Thermo Fisher Scientific), and U48 as control gene as previously described [241]. Reactions were carried out in triplicate on an Applied Biosystems 7900HT PCR System. The mean Ct value of each triplicate was used for analysis, and the ΔCt method (ΔCt = mean Ct of U48 – mean Ct of miR-301a) was applied.

4.10 Cell viability assay (MTS)

HNSCC cell lines were seeded into 96-well tissue culture plates at a density of 1.500 – 2.000 cells *per* well and incubated overnight. Cell viability was measured at 24 hours, in quadruplicates, by MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay from Promega, Madison, WI, USA) reading absorbance at 490 nm using a Synergy HT plate reader (BioTek, Winooski, VT, USA).

4.11 Colony formation assay

HNSCC cell lines were seeded at a low confluence (3,000-5,000 cells) in 12-well culture plates. After 9 to 12 days, cells were fixed with methanol at room temperature, and subsequently stained with crystal violet (0.1% w/v) for 2 minutes and thoroughly washed. Afterwards, colony formation was analyzed using a GelCount scanner (Oxford Optronix Ltd) and quantified by Image J analysis software. Data were normalized to the scramble control condition. As control of initial time, an MTS assay was performed in order to determine absolute cell count in each condition.

4.12 Three-dimensional spheroid invasion assay

Invasion assays using 3D tumor spheroids were performed as previously described [82]. HNSCC cells were suspended in DMEM plus 5% Methyl cellulose (Sigma, St Louis, MO, USA) at 80.000 cells/mL to form tumor cell spheroids by serial pipetting into a non-adhesive Petri dish (2.000 cells/spheroid), followed by overnight incubation in an inverted position. Afterwards, each tumor spheroid was individually transferred into a 96-well culture plate, embedded with 70 µL of bovine collagen matrix (Advanced Biomatrix PureCol), and filled with 100 µl of complete media. Drug

treatments were added to the 100 µL of complete media used to fill each individual collagen-embedded tumor spheroid. Cell invasion was monitored using a Zeiss Cell Observer Live Imaging microscope (Zeiss, Thornwood, NY, USA) coupled with a CO_2 and temperature-maintenance system. Images were acquired every 15 min for 24 h using a Zeiss AxioCam MRc camera. The invasive area was calculated as the difference between the final area (t = 24 h) and the initial area (t = 0 h) using image J analysis program, and data were normalized to control condition (either scramble or vehicle-treated cells, as indicated). At least three independent experiments were performed using quadruplicates for each condition.

4.13 Phosphoproteome array

Total protein was extracted from UT-SCC38 cells transduced with miR-301a mimic, miR-301a inhibitor and scramble control using lysis buffer (R&D Systems). 600 µg of each cell lysate was applied to the Proteome Profiler Human Phospho-Kinase Array Kit (ARY003C; R&D Systems, Minneapolis, MN), which allows simultaneous analysis of of 37 kinase phosphorylation sites and 2 related total proteins. Following the manufacturer's instructions, a cocktail of biotinylated detection antibodies, streptavidin–horseradish peroxidase and chemiluminescent detection reagents were used to detect phosphorylated proteins. Membranes were scanned with Odyssey Fc Dual-Mode Imaging System (LI-COR Biosciences), and signal density analysis was performed using Image Studio Lite software (LI-COR Biosciences). Based on the output data, a heatmap was built using Morpheus software (https://software.broadinstitute.org/morpheus). Individual validation of specific phosphorylated proteins was carried out by Western blot.

4.14 Western blotting analysis

Cells were lysed in Laemmli sample buffer and sonicated before centrifugation. Protein concentration was measured by Pierce BCA protein assay kit (Thermo Fisher, Rockford, IL). Then, protein lysates were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Amersham Protran, GE Healthcare, Pittsburg, PA, USA). Upon blockade with 5% bovine seroalbumine (BSA) for 1 hour, the membranes were incubated with specific primary antibodies at 1:1000 dilution overnight. The antibodies employed are listed in **table 5**. Secondary antibodies anti-Rabbit and anti-Mouse IRDye 800CW and IRDye 680RD (LI-COR Biosciences) were used at 1:10000 dilution for detection. Odyssey Fc Dual-Mode Imaging System (LI-COR Biosciences) was employed to scan the membranes using the red (700 nm) and green (800 nm) channels, and signal analysis was performed using Image Studio Lite software (LI-

COR, Nebraska). Proteins were normalized to β-actin GAPDH as loading control, depending of study.

Table 5. Primary antibodies used in Western Blotting.

Primary antibody	Dilution	Manufacture
phospho- P44/42 MAPK (Erk 1/2)	1:1000	Cell Signaling (#4370)
total ERK	1:1000	Cell Signaling (#9102)
PTEN	1:1000	Cell Signaling (#9552)
phospho-Akt (T308)	1:1000	Cell Signaling (# 4056)
phospho-Akt (S473)	1:1000	Cell Signaling (#9271)
total Akt	1:1000	Cell Signaling (#9272)
phospho-S6 (S240/244)	1:1000	Cell Signaling (#5364)
S6 Ribosomal Protein (5G10)	1:1000	Cell Signaling (#2217)
phospho-SRC (Y419)	1:1000	Invitrogen (# 44-660G)
total SRC	1:1000	Santa Cruz Biotechnology (# sc-8056)
β-actin	1:10000	Sigma Aldrich #AC15
Anti-GAPDH clone 6C5	1:10000	Merck Millipore # MAB374

4.15 *In vivo* orthotopic HNSCC model

Female athymic NMRI-nude mice of 6 weeks old (Envigo) were inoculated sublingually with 300.000 UT-SCC38 cells from three groups (scramble, miR-301a mimic and miR-301 inhibitor, n=7 in each group). After 17 days, all mice were euthanized. At sacrifice, tissue sampling was conducted jointly collecting the mandible and tongue from each animal. After decalcification, tissue samples were embedded in paraffin, cut into 3-4 µm slides, stained with hematoxylin and eosin (H&E), and examined histopathologically. The tumor infiltration depth (infiltration depth and extent of epithelial disruption in the tongue) and mitotic count rates (by Ki67 staining) were evaluated. All experimental protocols were performed in accordance with the institutional guidelines of the University of Oviedo and approved by the Animal Research Ethical Committee of the University of Oviedo prior to the study (date of approval 2 September 2015; approval number PROAE46/2015).

4.16 Microbial DNA extraction and quantification

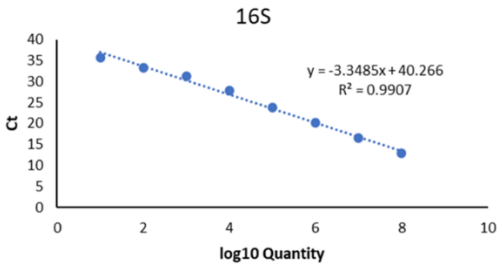
The sample collection was homogenized using 1 mL of saline rinse. Firstly, samples were centrifuged for 10 minutes at maximum speed (13,000 rpm). The supernatant was removed to subsequently resuspend the pellet in 0.5 mL of lysis buffer (200 mM Tris-HCl pH 7.0, 25 mM EDTA, 250 mM NaCl), supplemented with 20 mg/mL of lysozyme (Sigma-Aldrich), 5 µg/mL of lysostaphin (Sigma-Aldrich), and 40 U/mL of mutanolysin (Sigma-Aldrich) for 1.5 hours at 37°C. Subsequently, SDS was added to a final concentration of 0.5% (w/v) and the entire volume was transferred to Precellys tubes to carry out mechanical disruption in a homogenizer (Precellys 24 tissue homogenizer, Bertin instruments). The lysate was treated with an RNAse cocktail (Thermo Scientific) for 15 minutes at 37°C and proteinase K (Sigma-Aldrich) for 1 hour and a half at 37°C. Right afterwards, 100 µL of 1.5M NaCl was added and then, DNA was extracted with phenol/chloroform/isoamyl alcohol (Sigma-Aldrich). DNA precipitation was assessed with 0.1 volumes of sodium acetate pH 5.2 and 2.5 volumes of pure ethanol. The DNA was washed with 70% ethanol and resuspended in 20 µL of sterile nuclease-free water (Sigma-Aldrich). Finally, the concentration of total DNA and the 260/280 ratio were quantified in a spectrophotometer (Nanodrop 2000, Thermo Scientific), which was generally around 80 ng/µL and the 260/280 ratio > 1.7. Besides, this concentration was verified in parallel in a Qubit fluorometer with dsDNA assay kits (Thermo Fisher Scientific). DNA samples were stored at -20°C.

4.17 Determination of prokaryotic and eukaryotic ratio

Quantification of eukaryotic and prokaryotic DNA from saliva samples were carried out by qPCR using specific primers to the 18S rRNA gene of eukaryotic cells (Fw: CGGCGACGACCCATTCGAAC and Rv: GAATCGAACCCTGATTCCCCGTC) and the 16S rRNA gene of prokaryotic microorganisms (Fw: CGGCAACGAGCGCAACCC and Rv: CCATTGTAGCACGTGTGTAGCC). Amplification reactions were performed in 96-well optical plates (Applied Biosystems, Foster City, CA, USA) in a 7500 Fast Real- Time PCR System (Applied Biosystems). Amplifications were done in triplicate in a final volume of 25 µl containing 2× SYBR Green PCR Master Mix (Applied Biosystems), 0.2 µM of each primer, and 1 µl of DNA obtained from saliva. Then, the standard curve was calculated by plotting the Ct values against the numbers of cells corresponding to serial dilutions of cultures of *L. lactis* strain NZ9000 as a reference for prokaryotic DNA and HT-29 cell line as a reference for eukaryotic DNA. The prokaryotic 16S rDNA/eukaryotic 18S rDNA ratio was found to be adequate, and the estimated amounts of bacterial DNA were in the range of 10^7-10^8 **(figure 15)**.

A

Target Name	Task	Cт Mean	Cт SD	Quantity
16S	STANDARD	12.86	0.107	100000000
16S	STANDARD	16.55	0.113	10000000
16S	STANDARD	20.15	0.053	1000000
16S	STANDARD	23.77	0.094	100000
16S	STANDARD	27.86	0.059	10000
16S	STANDARD	31.34	0.301	1000
16S	STANDARD	33.38	-	100
16S	STANDARD	35.64	-	10
16S	NTC	36.96		
16S	NTC	36.96		

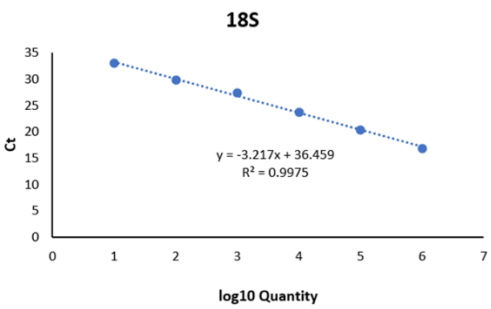


Sample	Target Name	Cт Mean	Cт SD	Quantity Mean	Quantity SD
Saliva 1	16S	15.37	0.062	2.51E+07	1.06E+06
Saliva 2	16S	15.53	0.071	2.26E+07	1.07E+06
Saliva 3	16S	17.12	0.139	7.81E+06	7.42E+05
Saliva 4	16S	15.17	0.069	2.87E+07	1.36E+06
Saliva 5	16S	15.49	0.042	2.31E+07	6.56E+05
Saliva 6	16S	18.57	0.094	2.94E+06	1.86E+05
Saliva 7	16S	16.15	0.024	1.49E+07	2.41E+05
Saliva 8	16S	16.36	0.042	1.29E+07	3.63E+05

	Sample	Final Quantity
16S	Saliva 1	2.51E+08
	Saliva 2	2.26E+08
	Saliva 3	7.81E+07
	Saliva 4	2.87E+08
	Saliva 5	2.31E+08
	Saliva 6	2.94E+07
	Saliva 7	1.49E+08
	Saliva 8	1.29E+08

B

Target Name	Task	Cт Mean	Cт SD	Quantity
18S	STANDARD	16.89	0.108	1000000
18S	STANDARD	20.33	0.085	100000
18S	STANDARD	23.78	0.096	10000
18S	STANDARD	27.31	0.046	1000
18S	STANDARD	29.90	0.040	100
18S	STANDARD	32.96	-	10
18S	NTC	37.07		
18S	NTC	Undetermined		



Sample	Target Name	Cт Mean	Cт SD	Quantity Mean	Quantity SD
Saliva 1	18S	22.05	0.149	3.02E+04	3.30E+03
Saliva 2	18S	27.44	0.068	6.55E+02	3.21E+01
Saliva 3	18S	27.63	0.209	5.80E+02	8.27E+01
Saliva 4	18S	20.66	0.040	8.09E+04	2.35E+03
Saliva 5	18S	28.80	0.170	2.52E+02	3.08E+01
Saliva 6	18S	29.14	0.482	2.04E+02	7.45E+01
Saliva 7	18S	24.08	0.075	7.16E+03	3.87E+02
Saliva 8	18S	21.72	0.076	3.80E+04	2.05E+03

	Sample	Final Quantity
18S	Saliva 1	3.02E+05
	Saliva 2	6.55E+03
	Saliva 3	5.80E+03
	Saliva 4	8.09E+05
	Saliva 5	2.52E+03
	Saliva 6	2.04E+03
	Saliva 7	7.16E+04
	Saliva 8	3.80E+05

C

Ratio 16S / 18S	
Saliva 1	829.88
Saliva 2	34557.70
Saliva 3	13467.24
Saliva 4	354.56
Saliva 5	91618.12
Saliva 6	14404.77
Saliva 7	2078.85
Saliva 8	339.17

Figure 15. Determination of prokaryotic-eukaryotic ratio in mouthwashes samples by qPCR assay. Prior to our study, and in order to evaluate the convenience of using oral rinsed-derived samples and not total saliva, prokaryotic-eukaryotic ratio was tested in 8 oral mouthwashes-derived samples. **A)** 18S calibration curve, **B)** 16S calibration curve and **C)** 16S:18S. ratio. Mouthwashes samples were diluted 1:10.

4.18 Sequencing of 16S rRNA gene amplicons and analysis

16S rDNA gene sequences were amplified from 320 saliva samples (125 patients and 65 controls). Microbial Genomic DNA (5 ng/µl in 10 mM Tris pH 8.5) was used to initiate the protocol (**Supplementary Figure S1**). The first step was run 1 µl of the PCR product on a Bioanalyzer DNA 1000 chip to verify the size, the expected size on a Bioanalyzer trace is ~550 bp. DNA was fragmented and subsequently prepared with the Next era XT Index kit (Illumina, San Diego, CA, USA). The libraries were sequenced using a 2 x 300pb paired-end run (MiSeq Reagent kit v3) on a MiSeq Sequencer according to manufacturer's instructions (Illumina). The amplification and sequencing of the gene encoding 16S rRNA was performed at FISABIO (Valencia), with the support and collaboration of Dr. Alejandro Mira. The V3-V4 region of the 16S rRNA gene was amplified using the universal primers reported by Klindworth *et al.* [242]:

Fw: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Rv: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

For the processing and analysis of the sequences, a custom script was used in QIIME software. Briefly, reads shorter than 260bp were removed. Subsequently, the sequences were dereplicated, the paired-end ends were joined, and the chimeras were eliminated. The Operational Taxonomic Units (OTUs) were defined with 100% sequence homology, which allows a highly distinctive taxonomic classification, allowing OTUs to be differentiated by a single nucleotide. The readings were classified in the lowest possible taxonomic rank using the SILVA v.138 database.

4.19 Statistical analyses

For statistical analysis of clinical data from patients, the χ^2 and Fisher's exact tests were used for comparison between categorical variables, and t Student test for parametric continuous variables using the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA). Correlations between protein expression, gene amplification, and HPV status were estimated by Kendall's tau correlation test. For time-to event analysis, Kaplan-Meier curves were plotted. Differences between survival times were analysed by log-rank method. Cox proportional hazards models were employed for univariate and multivariate analyses. The hazard ratios (HR) with 95% confidence interval (CI) and *p*-values were reported. All tests were two-sided. *p* values of ≤ 0.05 were considered statistically significant.

For statistical analysis of molecular data, the GraphPad Prism software version 8.0 (Graphpad Software Inc, La Jolla, CA, USA) was used. Data are represented as the mean ± standard deviation (SD) of at least three independent experiments unless otherwise stated. The student's unpaired t-test with two-tailed distribution was employed for comparison across two groups,

whereas one/two-way ANOVA was used for multiple samples/variables comparison. Significance was set at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

The microbiome statistical analysis was carried out using the SPSS v. 22.00 (IBM, Armonk, NY, USA) and the free software R (www.r-project.org). Comparisons of the relative abundances of bacterial taxa were corrected using the non-parametric Mann-Whitney U test and Benjamini-Hochberg Post Hoc, with a False Discovery Rate (FDR) of 0.25. Alfa diversity was performed by Shannon index and observed species mean. Differences in community composition were assessed using permutational multivariate analysis of variance (PERMANOVA), using Adonis test, in the Vegan R package. Principal coordinate analysis (PCoA) was performed to visualize the differences or similarities between groups composition. Results were considered statistically significant with $p < 0.025$. Bacterial analyses were plotted using the GraphPad Prism version 8.0.

5. RESULTS

5.1 Role of SOX2 in early stages of laryngeal tumorigenesis

5.1.1 SOX2 protein expression in laryngeal precancerous lesions

SOX2 protein expression was evaluated by immunohistochemistry in 94 laryngeal dysplasias. Nuclear SOX2 expression was detected in 38 (40%) dysplasias, whereas stromal cells and normal adjacent epithelia showed negative expression (**Figure 16**).

SOX2 protein expression was significantly correlated with the histopathological classification: 2 (14%) of the 14 low-grade dysplasias, and 36 (45%) of the 80 high-grade dysplasias exhibited SOX2-positive expression (Fisher's exact test, $p = 0.039$).

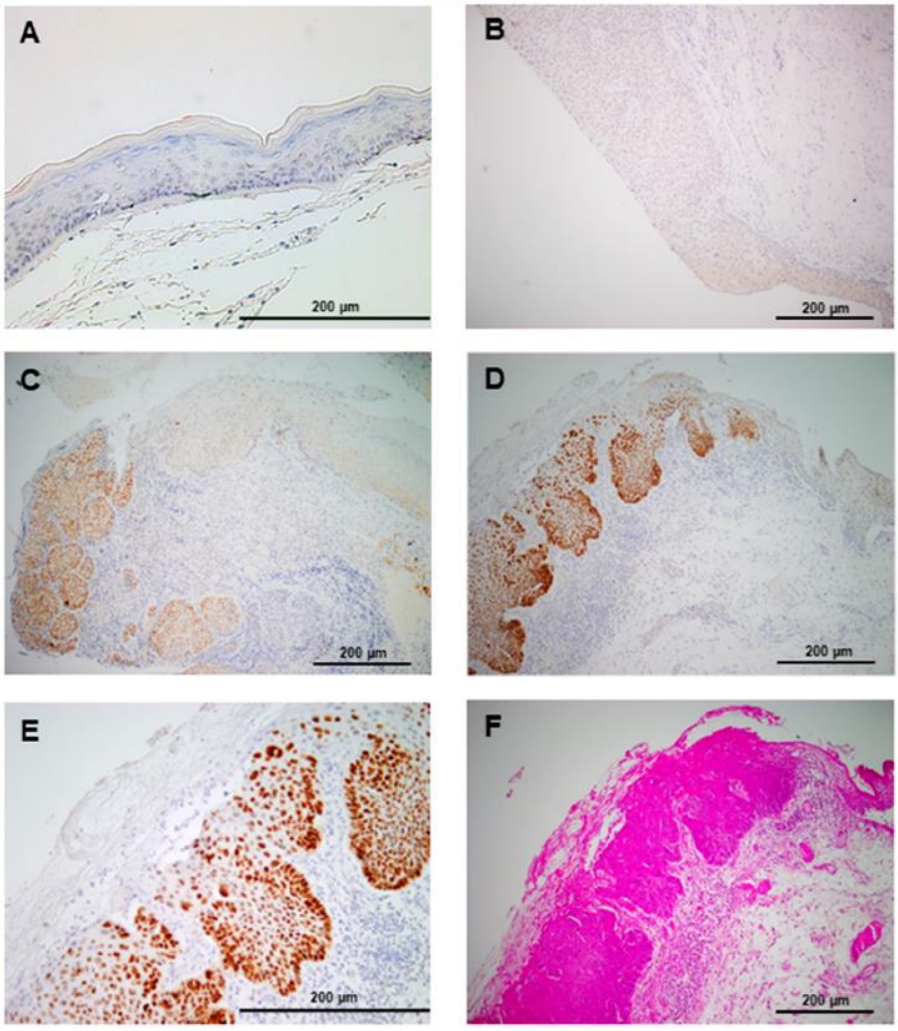


Figure 16. Immunohistochemical analysis of SOX2 expression in laryngeal precancerous lesions. Normal adjacent epithelia showed negative staining (**A**). Representative examples of laryngeal dysplasias showing negative (**B**), and positive nuclear SOX2 staining (**C**, **D**), compared to the negative expression in normal-adjacent epithelia (*right side*). (**E**) Higher magnification from D. (**F**) H&E staining from D.

5.1.2 *SOX2* gene amplification during laryngeal tumorigenesis

SOX2 gene maps at 3q26 a region frequently and recurrently amplified in HNSCC, and other cancers. Therefore, *SOX2* gene amplification was assessed by real-time PCR (qPCR) on a set of 55 laryngeal dysplasias, using DNA extracted from the same paraffin tissue blocks. *SOX2* gene amplification was detected in 18 (33%) of 55 laryngeal dysplasias, with relative copy numbers ranging from 2-fold to 9-fold (**Figure 17A**). *SOX2* gene amplification increased with the severity of the lesions: 1 (17%) of the 6 low-grade dysplasias and 17 (35%) of the 49 high-grade dysplasias showed *SOX2* gene amplification, although the differences did not reach statistical differences (Fisher's exact test, $p = 0.651$). When analyzing the correlation between *SOX2* gene amplification and protein expression, we found that gene amplification only partially lead to SOX2 protein expression (**Figure 17B**). Thus, even though 22 out of 55 dysplasias were negative for both SOX2 expression and gene amplification, 15 amplification-negative lesions also showed positive SOX2 expression, indicating that additional mechanisms should contribute to the frequent SOX2 expression detected in laryngeal tumorigenesis.

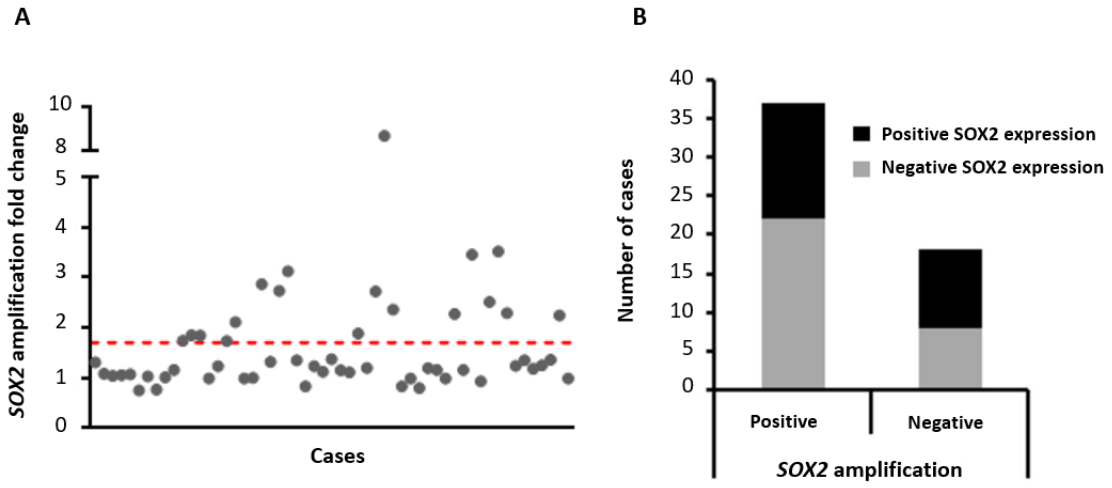


Figure 17. Analysis of *SOX2* gene amplification in laryngeal precancerous lesions. *SOX2* gene copy number was evaluated by qPCR in 55 laryngeal dysplasias. (A) Data are represented as fold-change in the precancerous lesion relative to the normal mucosa. Red dotted line indicates the threshold established to define positive cases (1.75). (**B**) Correlations between *SOX2* gene amplification and protein expression determined by immunohistochemistry.

In silico analysis of *SOX2* gene status and mRNA levels using the TCGA data from 279 HNSCC patients with the platform cBioPortal for Cancer Genomics (http://cbioportal.org/) led to

analogous results, showing that not all the cases harboring *SOX2* gene amplification exhibit increased mRNA expression (**Figure 18**) [46,243].

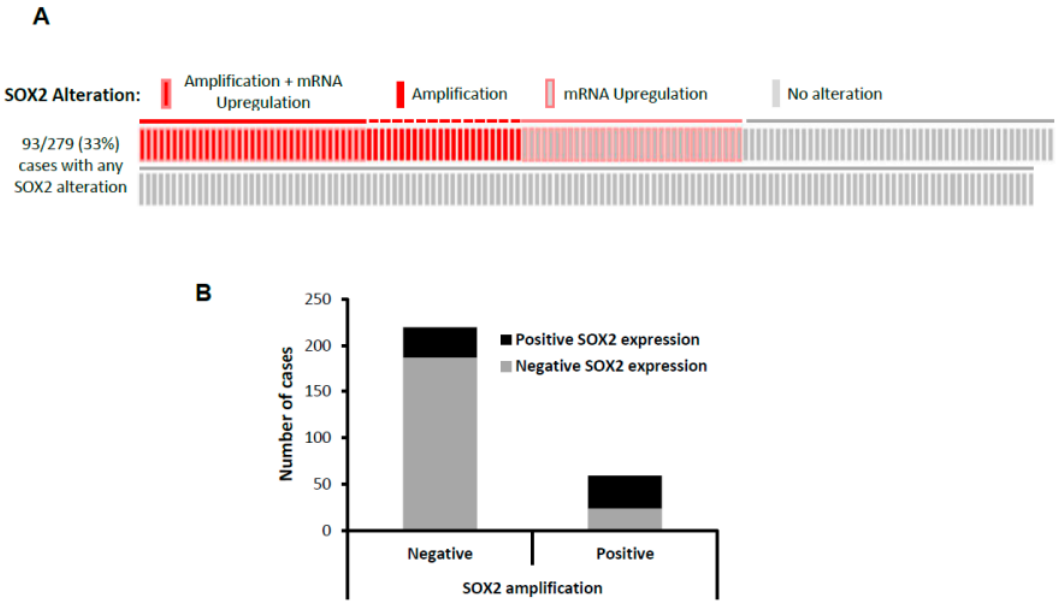


Figure 18. **Analysis of *SOX2* gene amplification and mRNA expression from the TCGA cohort of 279 HNSCC patients using cBioPortal [46,243].** Schematic representation (**A**) showing the number of cases with gene amplification and/or mRNA upregulation or no alteration. Correlations between SOX2 expression and gene amplification in this cohort (**B**).

5.1.3 *PIK3CA* amplification and other genes mapping at 3q26

It has been extensively demonstrated (both experimentally and *in silico*) that 3q26 amplicon harbors numerous genes found to be frequently and concomitantly co-amplified and overexpressed in multiple cancers [244,245]. Various genes have been highlighted as highly significant oncogenic drivers for HNSCC survival, including four known driver genes *PIK3CA*, *PRKCI*, *SOX2* and *TP63*. In order to get further mechanistic insight into the contribution of 3q26 gene amplification in HNSCC, we performed *in silico* analysis of TCGA data from 279 HNSCC patients using the platform cBioPortal for Cancer Genomics (http://cbioportal.org/) [46,243,244]. **Figure 19** illustrates that co-amplification of these four genes occurs frequently in HNSCC, thus showing that 3q26 amplification in HNSCC is not restricted to *SOX2* gene.

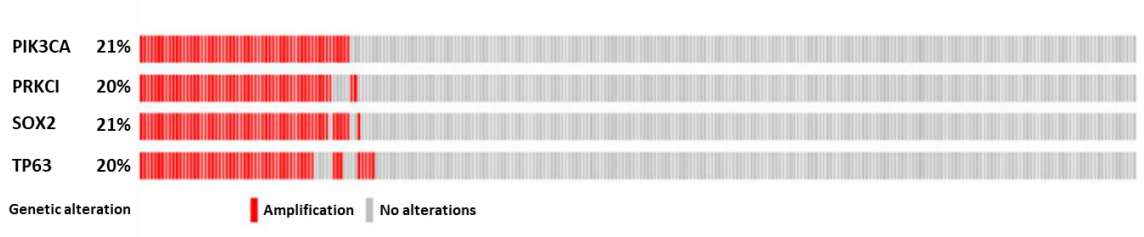


Figure 19. Gene amplification analysis of *SOX2* and other driver genes (*PIK3CA*, *PRKCI* and *TP63*) mapping at 3q26 amplicon using the TCGA cohort of 279 HNSCC patients and the platform cBioPortal [46,243]. Oncoprint illustrates frequent co-amplification of all these genes.

5.1.4 Associations with laryngeal cancer risk

There was no statistically significant correlation in this cohort between the WHO histopathological grade and the risk of progression to laryngeal cancer (p = 0.538; **Table 6**; log-rank test, p = 0.426, **Figure 20A**), although high-grade dysplasias showed a higher cancer risk (HR = 1.615, 95% CI 0.489 to 5.336; p = 0.432; **Table 6**). Even the previous histological classification based in the dysplasia grading (mild, moderate vs. severe dysplasia) did not significantly predict cancer risk (log-rank test, p = 0.438, **Figure 20B**). In marked contrast, we found that *SOX2* gene amplification significantly correlated with an increased laryngeal cancer risk (log-rank test, p = 0.038; **Figure 20C**). Furthermore, SOX2 protein expression showed the most robust association with laryngeal cancer risk (log-rank test, $p < 0.001$; **Figure 20D**). Five years after the patients were diagnosed, 20 (53%) of the 38 patients with SOX2-positive expression developed laryngeal cancer, whereas only 9 (16%) of the 56 patients with SOX2-negative expression progressed to invasive carcinoma ($p <$ 0.001; **Table 6**). Univariate Cox analysis showed that *SOX2* gene amplification and protein expression but not histological grading was significantly associated with laryngeal cancer risk (**Table 7**). In multivariate stepwise analysis including age, tobacco, histology, *SOX2* gene amplification, and SOX2 expression, SOX2 expression (HR = 3.531, 95% CI 1.144 to 10.904; p = 0.028) was the only significant independent predictor of laryngeal cancer development.

Table 6. Evolution of the premalignant lesions in relation to histopathological diagnosis, *SOX2* gene amplification, and protein expression.

Characteristic	No of cases	Progression to carcinoma (%)	*p* †
Histopathological diagnosis			
Low-grade dysplasia	14 (15)	3 (21)	0.538
High-grade dysplasia	80 (85)	26 (32)	
SOX2 gene amplification			
Negative	37 (67)	11 (30)	0.081
Positive	18 (33)	10 (56)	
SOX2 protein expression			
Negative	56 (60)	9 (16)	<0.001
Positive	38 (40)	20 (53)	

† Fisher's exact test

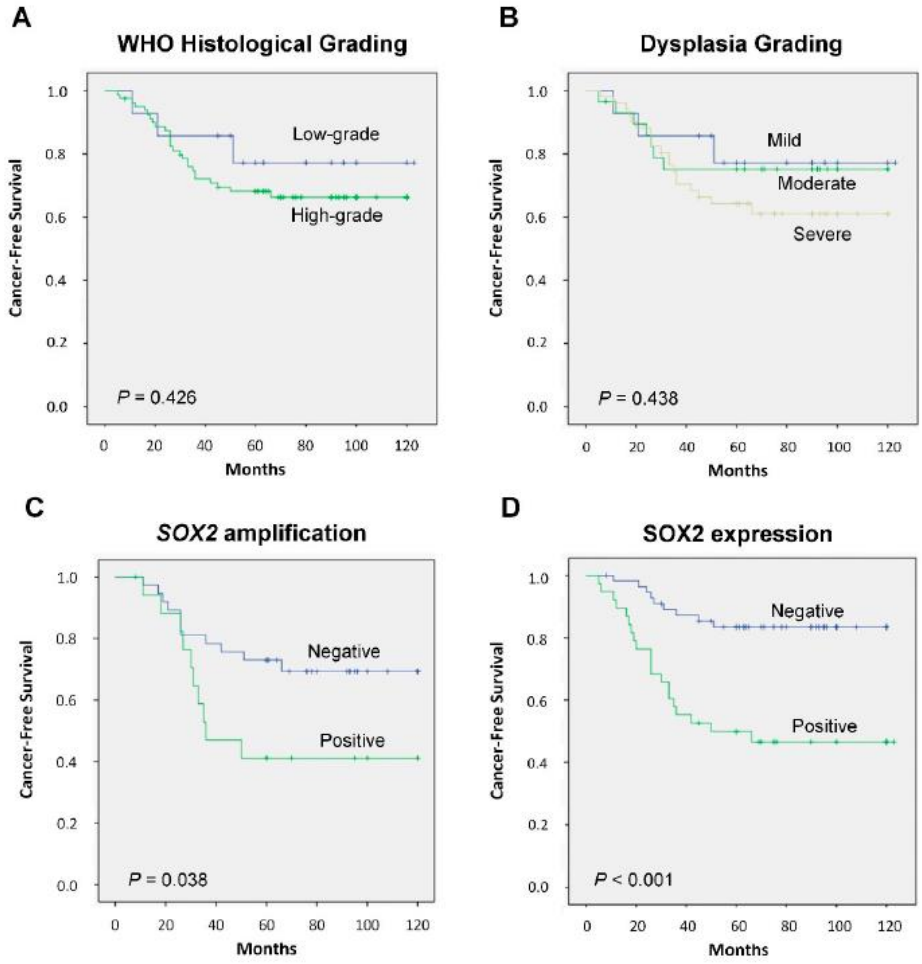


Figure 20. Kaplan-Meier cancer-free survival curves in the cohort of 94 patients with laryngeal dysplasias categorized by WHO histological grading (**A**), dysplasia grading (**B**) *SOX2* gene amplification (**C**) or SOX2 protein expression (**D**). *p* values were estimated using the log-rank test.

Table 7. Univariate Cox proportional hazards model to estimate laryngeal cancer risk.

Characteristic	*p*	Hazard Ratio	95% CI
Age (above vs. below the mean)	0.554	1.254	0.593–2.653
Smoking (above vs. below the mean)	0.618	1.210	0.572–2.558
Histology (high-grade vs. low-grade dysplasia)	0.432	1.615	0.489–5.336
SOX2 amplification (positive vs. negative)	0.046	2.410	1.017–5.710
SOX2 expression (positive vs. negative)	<0.001	4.130	1.878–9.086

5.2 Study of *CTTN, ANO1* and *CCND1* genes mapping at 11q13 in relation to HPV status

HNSCCs exhibit clear molecular and clinical differences depending on the HPV infection status. This study specifically investigated the status of the *CTTN*, *CCND1* and *ANO1* genes mapping at the 11q13 amplicon in relation to the HPV status in a large HNSCC cohort, and their impact on patient prognosis. A flow diagram of the experimental setup is shown in **Supplementary Figure S2**.

5.2.1 Distinctive associations of CCND1, ANO1, and CTTN protein expression with HPV status in HNSCC patients

In order to determine the HPV status in the whole HNSCC cohort, we first analysed by immunohistochemistry p16 expression, a surrogate marker for HPV infection. We found that 67 cases (17%) showed nuclear and cytoplasmic p16 expression. Next, HPV DNA was assessed by GP5+/6+-PCR and by *in situ* hybridization in the 67 p16-positive cases, which resulted in a total of 30 HPV-positive cases (28 oropharyngeal, 1 laryngeal, and 1 hypopharyngeal carcinoma) in our series (all were HPV type 16). Representative images of HPV-positive and HPV-negative cases and also examples of p16 immunostaining are shown in **Figure 21**. We found a total of 267 (68%) positive cases for CCND1 protein expression, 78 (21%) positive cases for ANO1 expression, and 190 (49%) positive cases for CTTN expression (**Table 8**). Representative examples of protein staining are shown in **Figure 22**. We next investigated the relationship between CCND1, ANO1, and CTTN expression and HPV infection status. The expression of these three proteins was strongly and inversely correlated with HPV infection. Notably, all the HPV-positive cases showed negative ANO1 expression, and 28 HPV-positive cases had also negative CTTN expression (**Table 8**).

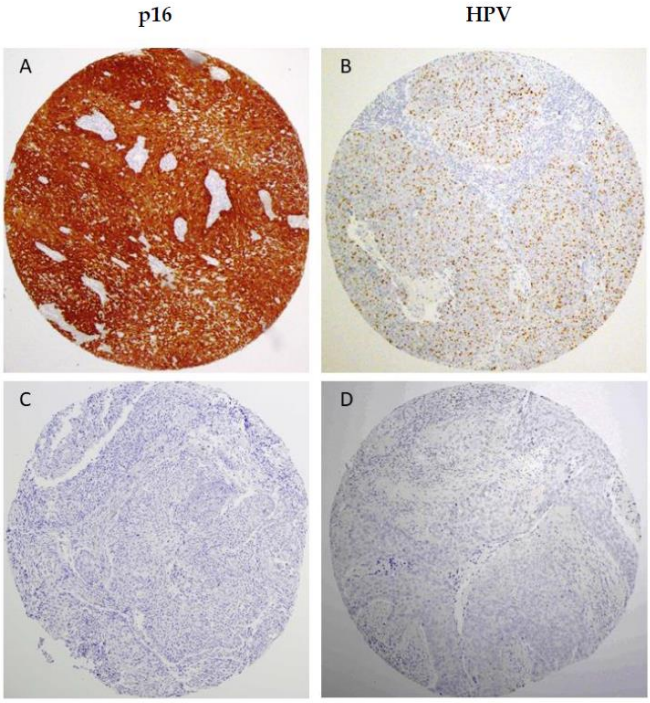


Figure 21. Representative images of p16-positive **(A)** and negative **(C)** immunostaining in the HNSCC tissue microarrays (TMAs) and HPV-positive **(B)** and HPV-negative **(D)** cases detected by *in situ* hybridization. Original magnification x10.

Table 8. Analysis of CCND1, ANO1, and CTTN protein expression in relation to HPV status and p16 expression in 392 HNSCC patients.

Molecular feature	Number	HPV-positive	$p^{\#}$
CCND1 protein expression			
Negative	123	21	(-0.239)
Positive (scores 1-2)	267	9	<0.001
ANO1 protein expression			
Negative	299	29	(-0.147)
Positive (scores 1-2)	78	0	<0.001
CTTN protein expression			
Negative	199	28	(-0.239)
Positive (scores 2-3)	190	2	<0.001
p16 protein expression			
Negative	325	0	(0.634)
Positive	67	30	<0.001

Kendall's tau correlation coefficient with the associated *p* value.

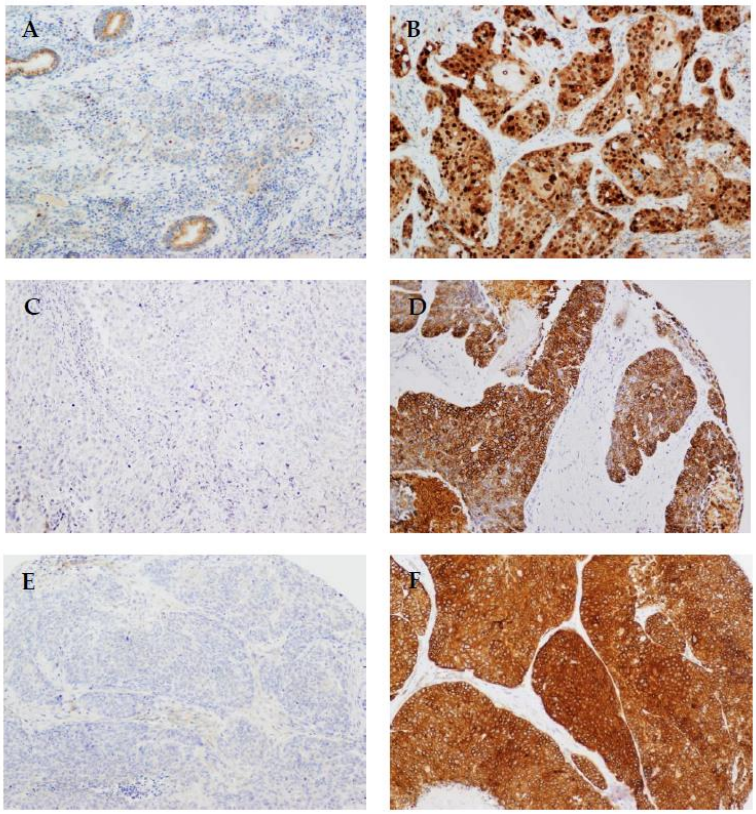


Figure 22. Representative examples of negative and strong positive staining for CCND1 (A, B), ANO1 (C, D), and CTTN (E, F). Original magnification ×20.

5.2.2 Analysis of *CCND1*, *ANO1*, and *CTTN* gene amplification in relation to HPV status in HNSCC patients

CCND1, *ANO1*, and *CTTN* gene gain and amplification was assessed by real-time PCR in 88 cases selected from the same HNSCC tissue blocks, including 26 HPV-positive cases. We found gene gains and amplifications of *CCND1* in 32 (36%) tumors, *ANO1* in 42 (48%) tumors, and *CTTN* in 26 (30%) tumors (**Table 9**), with relative gene copy numbers ranging from 2- to 24-fold. Co amplifications of these three genes were also frequently observed (26 cases, 30%). We also found that tumors harboring gene amplification significantly correlated with higher expression levels of each protein (**Figure 23**). When analyzing the correlations with HPV infection status, we consistently found that *CCND1*, *ANO1*, and *CTTN* gene amplification inversely correlated with HPV status (**Table 9**). Thus, amplifications of *CTTN*, *CCND1*, and *ANO1* were frequent in HPV-negative tumors (ranging from 42 to 61%), while absent in HPV-positive tumors.

Table 9. Analysis of *CCND1*, *ANO1*, and *CTTN* gene gain and amplification in relation to HPV status in 88 HNSCC patients.

Copy Number Alteration	No.	HPV-positive	*p*#
***CCND1* gene**			
Negative	55	24	(-0.430)
Gain (≥ 2 copies)	6	2	<0.001
Amplification (≥ 4 copies)	27	0	
***ANO1* gene**			
Negative	45	22	(-0.472)
Gain (≥ 2 copies)	16	4	<0.001
Amplification (≥ 4 copies)	27	0	
***CTTN* gene**			
Negative	61	26	(-0.422)
Gain (≥ 2 copies)	3	0	<0.001
Amplification (≥ 4 copies)	24	0	
Total cases	88	26	

Kendall's tau correlation coefficient with the associated *p* value.

CCND1		Gene Copy Number			Total
		No Gain	Gain	Amplification	
Protein score	0	34	1	1	36
	1	16	2	7	25
	2	5	3	19	27
Total		55	6	27	88

Spearman Coefficient 0.653, $p < 0.001$.

ANO1		Gene Copy Number			Total
		No Gain	Gain	Amplification	
Protein score	0	40	14	18	72
	1	4	2	6	12
	2	1	0	3	4
Total		45	16	27	88

Spearman Coefficient 0.237, $p = 0.027$.

CTTN		Gene Copy Number			Total
		No Gain	Gain	Amplification	
Protein score	0	22	1	0	23
	1	32	1	0	33
	2	4	1	9	14
	3	3	0	15	18
Total		61	3	24	88

Spearman Coefficient 0.711, $p < 0.001$.

Figure 23. Crosstab to evaluate the correlations between gene gains and amplification and protein staining scores for CCND1, ANO1, and CTTN.

5.2.3 Analysis of CCND1, ANO1, and CTTN mRNA expression in relation to HPV status in 279 HNSCC patients from the TGCA

In order to confirm our results, we performed an analysis of the publicly available TCGA data from 279 HNSCC patients using the platform cBioPortal for Cancer Genomics (http://cbioportal. org/) [46,243]. The clinicopathologic characteristics of this cohort are summarized in **Supplementary Table S1** and **Figure S8**. A total of 36 (13%) patients were positive for HPV infection, most prevalent in the oropharynx (22 cases, 61%), and associated to lower tobacco consumption, lower mutations, improved survival, and a younger age in both men and women. The results also evidenced differential changes in mRNA expression levels of CTTN, CCND1 and ANO1 depending on HPV infection status. Thus, increased mRNA expression levels of these genes were frequently and significantly observed in HPV-negative tumors, while very rare in HPV-positive patients (**Table 10**; **Figure 24A–D**). Similarly, the analysis of copy number alterations of CTTN, CCND1, and ANO1 genes also revealed that both amplifications and gains of these three genes were also highly frequent in HPV-negative tumors, whereas almost absent in HPV-positive tumors (**Figure 24A, E**). Furthermore, the results consistently showed that these three genes were frequently co-amplified (28%) and overexpressed at a higher frequency (39–46%), and, more importantly, these molecular alterations (in particular CTTN and ANO1 overexpression) were associated with a worse clinical outcome in the TCGA cohort of 279 HNSCC patients and also in an extended TCGA cohort which added 251 new HNSCC patients (n = 530) (**Figure 25**).

Table 10. Analysis of CCND1, ANO1, and CTTN mRNA expression in relation to HPV status in 279 HNSCC TGCA patients.

Molecular feature	No.	HPV-positive	*p*#
CCND1 expression			
Normal	206	35	<0.001
UP	73	1	
ANO1 expression			
Normal	207	34	0.002
UP	72	2	
CTTN expresión			
Normal	177	34	<0.001
UP	102	2	
Total cases	279	36	

 # Fisher's exact test.

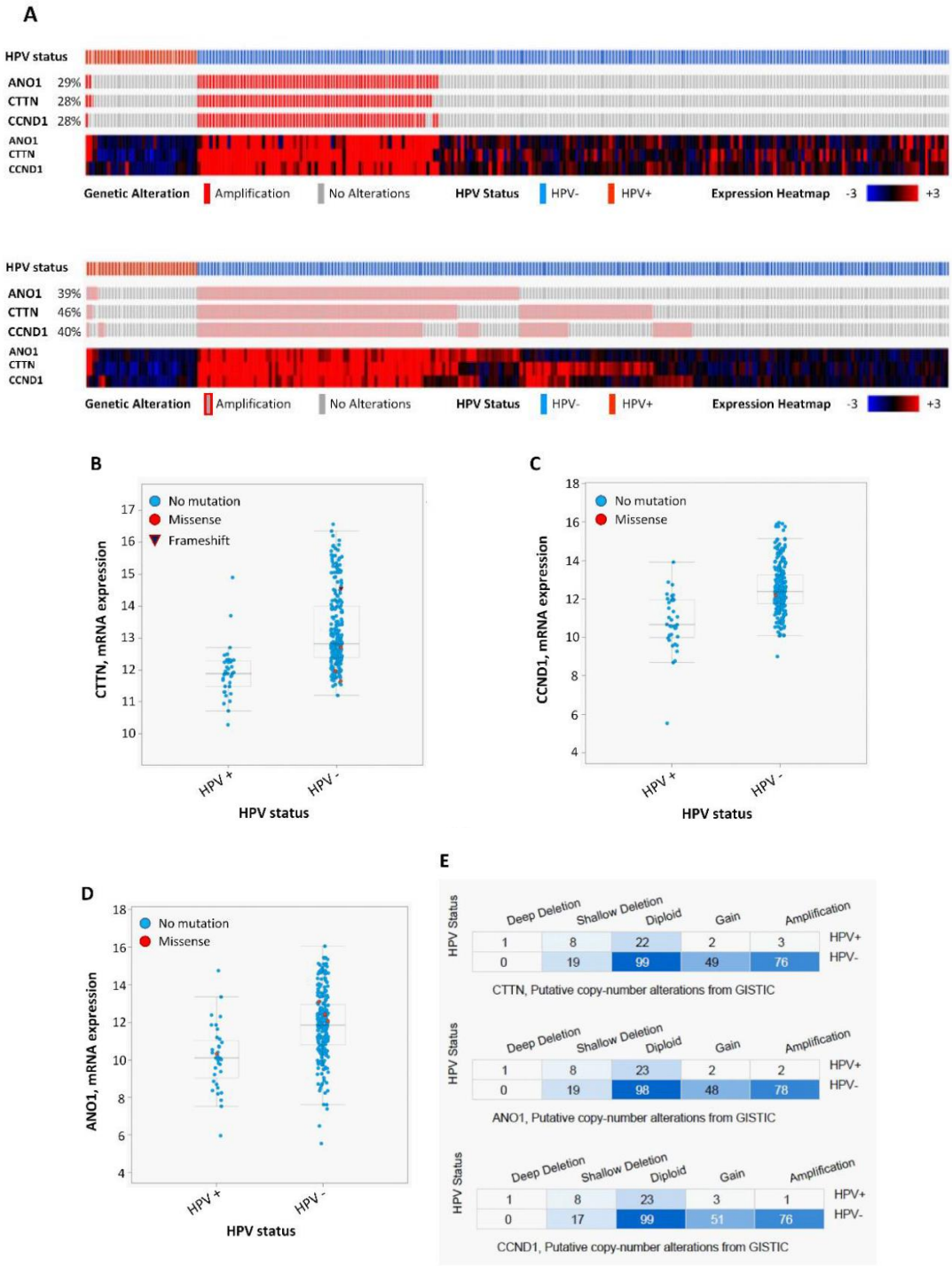


Figure 24. **Analysis of mRNA expression and copy number alterations of *CTTN*, *CCND1* and *ANO1* in relation to HPV status related to the available HNSCC TCGA data (n = 279) obtained from cBioPortal. (A)** Schematic representation and heat map showing the percentage of cases with *CTTN*, *CCND1*, and *ANO1* gene amplification and mRNA expression in relation to the HPV status. **(B)** CTTN, **(C)** CCND1, and **(D)** ANO1 mRNA expression distributed according to the HPV status. mRNA expression (RNA seq V2 RSEM) values were Log2 transformed (y-axis). Whiskers plot (min. to max.) with median values; $p < 0.001$, two-tailed Student t-test. **(E)** Copy number alterations of CTTN, CCND1 and ANO1 according to the HPV status using the GISTIC method.

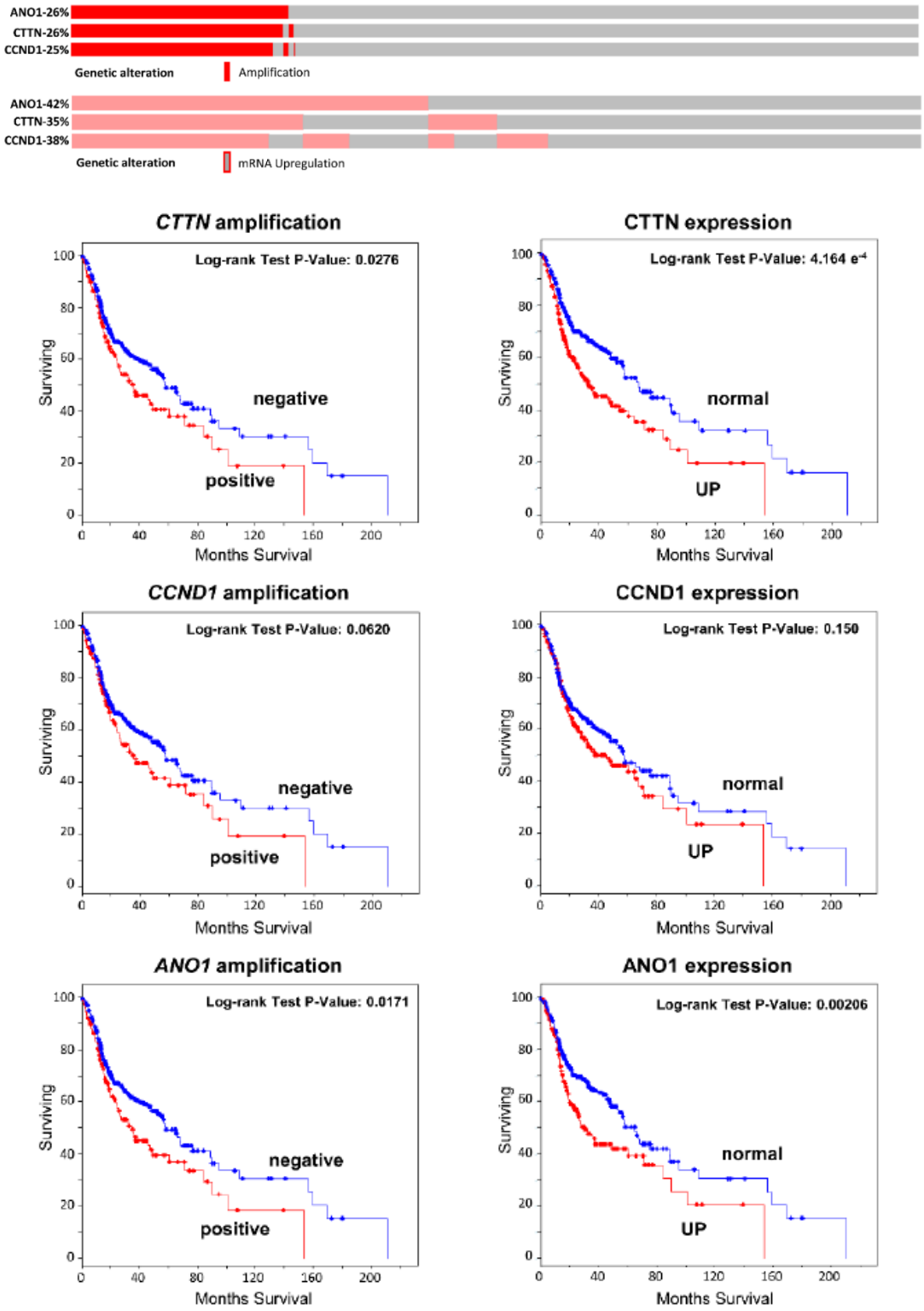


Figure 25. Analysis of *CTTN*, *CCND1* and *ANO1* gene amplification and mRNA expression in the TCGA cohort of 530 HNSCC patients using cBioPortal. Schematic representation showing the percentage of cases with amplification or mRNA upregulation of each gene. Kaplan–Meier survival curves categorized by *CTTN*, *CCND1* and *ANO1* gene amplification dichotomized as positive versus negative; CTTN, CCND1 and ANO1 mRNA expression (RNA seq V2 RSEM, z-score threshold ±2) dichotomized as normal versus upregulation (UP); *p* values estimated using the Log-rank test.

5.3 Analysis of SRC activation and downstream effectors in HNSCC

5.3.1 Analysis of SRC protein expression in HNSCC tissue specimens

Immunohistochemical analysis of p-SRC (Tyr419), a mark of SRC activation, was performed on tissue specimens from 122 HNSCC patients. Positive p-SRC (Tyr419) staining was mainly detected in the nucleus in 98 (88%) of the tumors, and some cases also exhibited cytoplasmic staining (11 tumors) (**Figure 26A-D**). Concordantly, total SRC expression also exhibited both nuclear and cytoplasmic patterns (**Figure 26E-G**) and showed a significant correlation with active SRC expression (Spearman correlation coefficient 0.318, p = 0.001). The expression of p-SRC (Tyr419) was also confirmed by Western blot analysis in a subset of tumor samples compared to patient-matched normal tissues and in a panel of HNSCC-derived cell lines (**Figure 27**). Consistent with the IHC data, p-SRC (Tyr419) levels were increased in tumors compared to patient-matched normal epithelia (Patients 1–3) as well as HNSCC cells. Total SRC expression levels were also found to increase in tumors compared to the normal counterparts (patients 1,2, and 4).

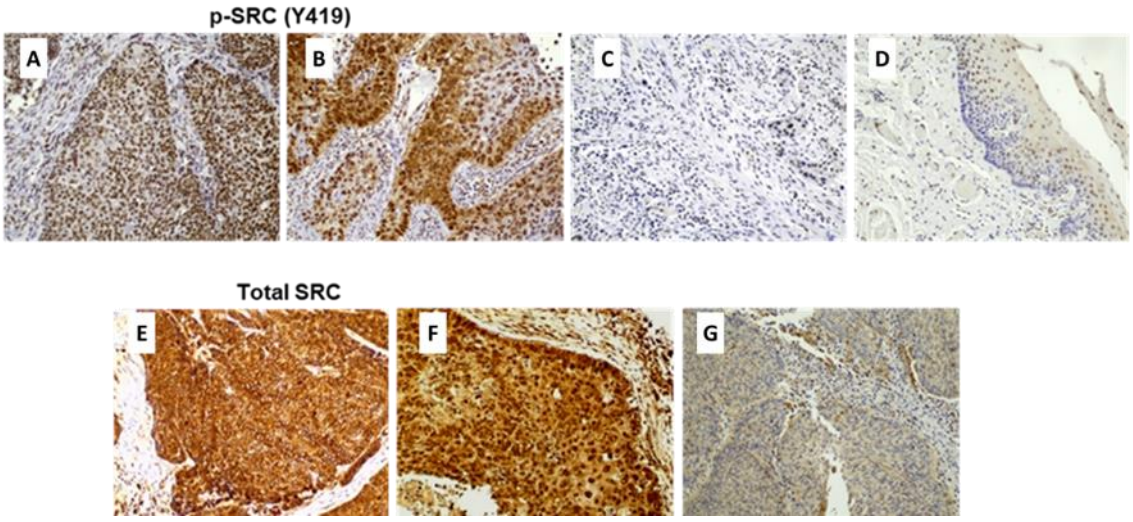


Figure 26. Immunohistochemical analysis of SRC expression in HNSCC tissue specimens. Representative examples of HNSCC showing positive p-SRC (Tyr419) staining (**A**, nuclear and **B**, cytoplasmic and nuclear), negative staining (**C**), and normal adjacent epithelia (**D)**. Representative examples of tumors showing positive total SRC staining (**E, F,** cytoplasmic and nuclear) and negative staining (**G**). Magnification 20x. Scale bars = 50 µm.

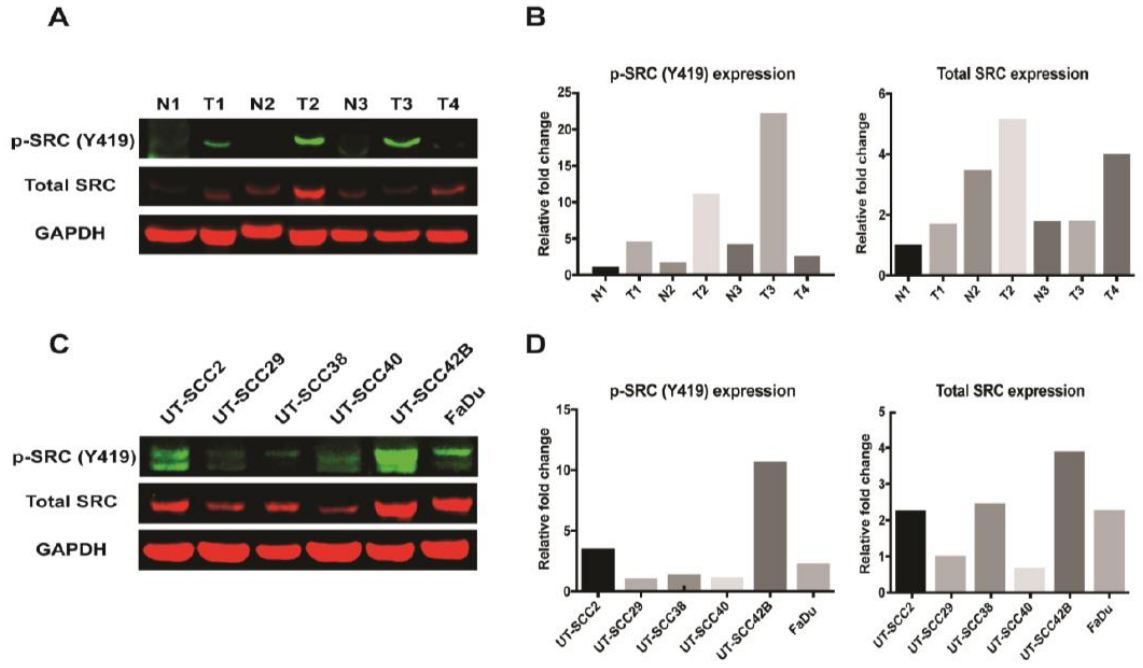


Figure 27. Western blotting analysis and quantification. (A) Western blot analyses of p-SRC (Tyr419) and total SRC in tumor samples (T) and patient-matched normal mucosa (N) from 4 HNSCC patients. Patients #1-3 are examples of positive expression of active SRC by IHC (scored as 2 and 3, moderate to strong), while patient #4 showed negative expression of active SRC (scored 1, low). GAPDH levels were used as loading control. **(B)** Quantification of the infrared fluorescent signals from the Western blot analyses shown in A. Data is relative to N1 **(C)** Western blot analyses of p-SRC (Tyr419) and total SRC in a panel of HNSCC-derived cell lines. **(D)** Quantification of the infrared fluorescent signals from the Western blot analyses shown in C. Data is relative to UT-SCC29.

5.3.2 Immunohistochemical analysis of SRC effectors in HNSCC tissue specimens

We next evaluated by immunohistochemistry the expression of various SRC effectors, such as CTTN, FAK, and ASAP1, known to regulate tumor invasion, metastasis, and aggressive phenotypes in HNSCC and other cancers [75,246-254]. Positive CTTN, FAK, and ASAP1 expression was respectively detected in 59 (51%), 83 (72%), and 64 (55%) tumors in our cohort of HNSCC patients, predominantly cytoplasmic and with membrane enrichment in some cases (**Figure 28**). The expression levels of FAK and ASAP1 were found to significantly correlate with SRC expression (p = 0.015 and p = 0.017, respectively). The potassium channel HERG1 was previously reported to form complexes with FAK and is associated with aggressive tumor behavior and poor prognosis in HNSCC [74,255]. The expression of HERG1 was also found to significantly correlate with SRC expression in our HNSCC cohort (p = 0.011).

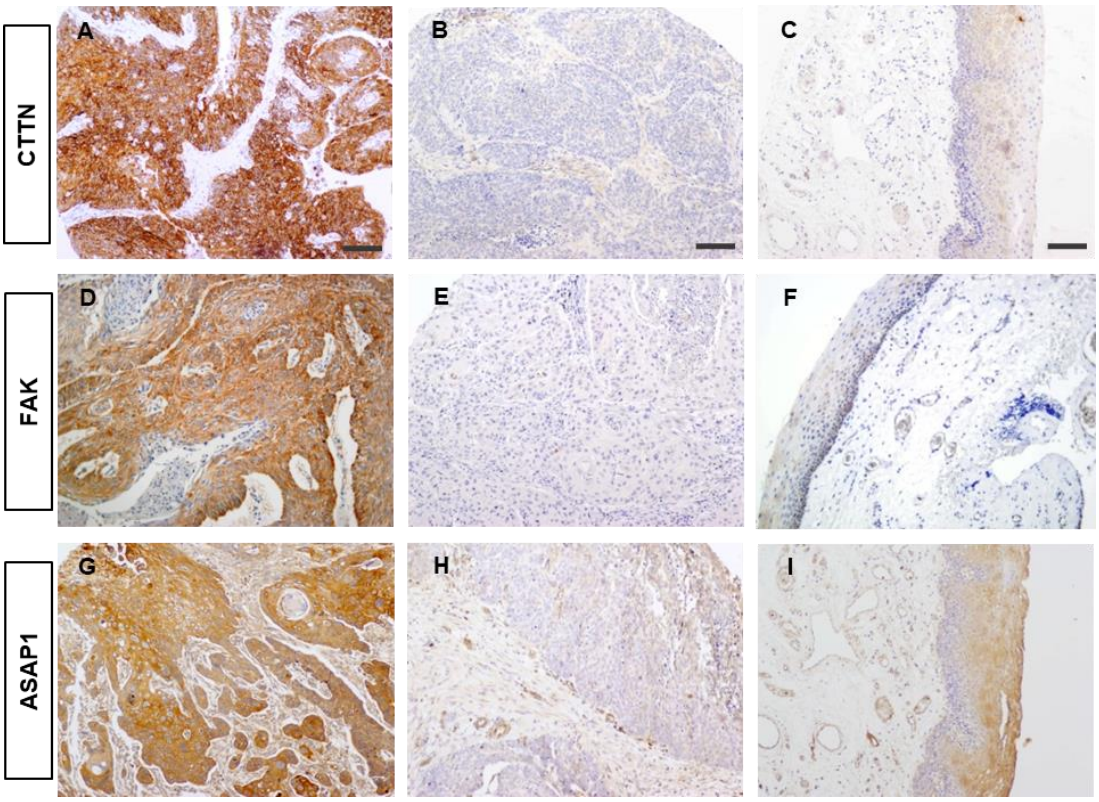


Figure 28. Immunohistochemical analysis of downstream SRC-related proteins in HNSCC tissue specimens. Representative examples of HNSCC showing positive CTTN staining (**A**), negative staining (**B**), and normal adjacent epithelia (**C**). Representative examples of tumors with positive FAK staining (**D**), negative staining (**E**), and normal adjacent epithelia (**F**). Representative examples of tumors with positive staining for ASAP1 (**G**), negative staining (H), and normal adjacent epithelia (**I**). Magnification 10x. Scale bars = 100 μm.

5.3.3 Differential impact of SRC activation on patients' survival

Nuclear p-SRC (Tyr419) was significantly associated with shorter disease-specific survival (log-rank test, $p = 0.043$), with marked differences depending on the tumor site (**Figure 29**).

When examining separately each tumor site subgroup (**Table 11**), we strikingly observed that the correlations between SRC expression and the SRC-related proteins FAK, ASAP1, and HERG1 were specific to the larynx but not the pharynx. Consistent with these results, the expression of the SRC substrates FAK, CTTN, and ASAP1 also showed a distinct impact on patient survival depending on the tumor site (**Figure 30**). Noteworthy, SRC expression was strongly and significantly correlated with tumor recurrences and tumor-associated deaths specifically in the larynx ($p < 0.001$) but not the pharynx ($p = 1.00$).

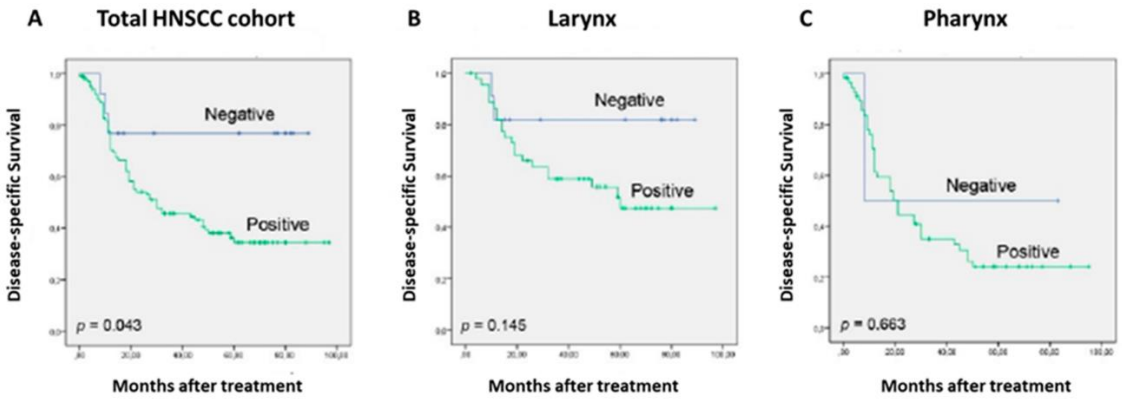


Figure 29. Impact of SRC expression on patient survival. Kaplan–Meier disease-specific survival curves categorized by nuclear p-SRC (Tyr419) in the total cohort of HNSCC patients (**A**) or in the laryngeal (**B**) and pharyngeal subgroups (**C**). *p*-values were estimated using the log-rank test.

Table 11. Associations between SRC expression and SRC-related proteins by tumor site.

Molecular Feature	Pharynx (No. = 58)			Larynx (No. = 58)		
	No.	**SRC (%)**	***p*#**	**No.**	**SRC (%)**	***p*#**
FAK protein expression						(0.326)
Negative	14	11 (79)	(0.063)	19	9 (47)	0.013
Positive	44	37 (85)	0.641	39	31 (79)	
ASAP1 protein expression						(0.230)
Negative	20	15 (75)	(0.149)	32	19 (59)	0.082
Positive	38	33 (87)	0.264	26	21 (81)	
HERG1 protein expression						(0.366)
Negative	5	4 (80)	(0.02)	9	3 (33)	0.006
Positive	52	43 (83)	0.882	46	36 (80)	

Spearman correlation coefficient (in parentheses) with the associated *p* value.

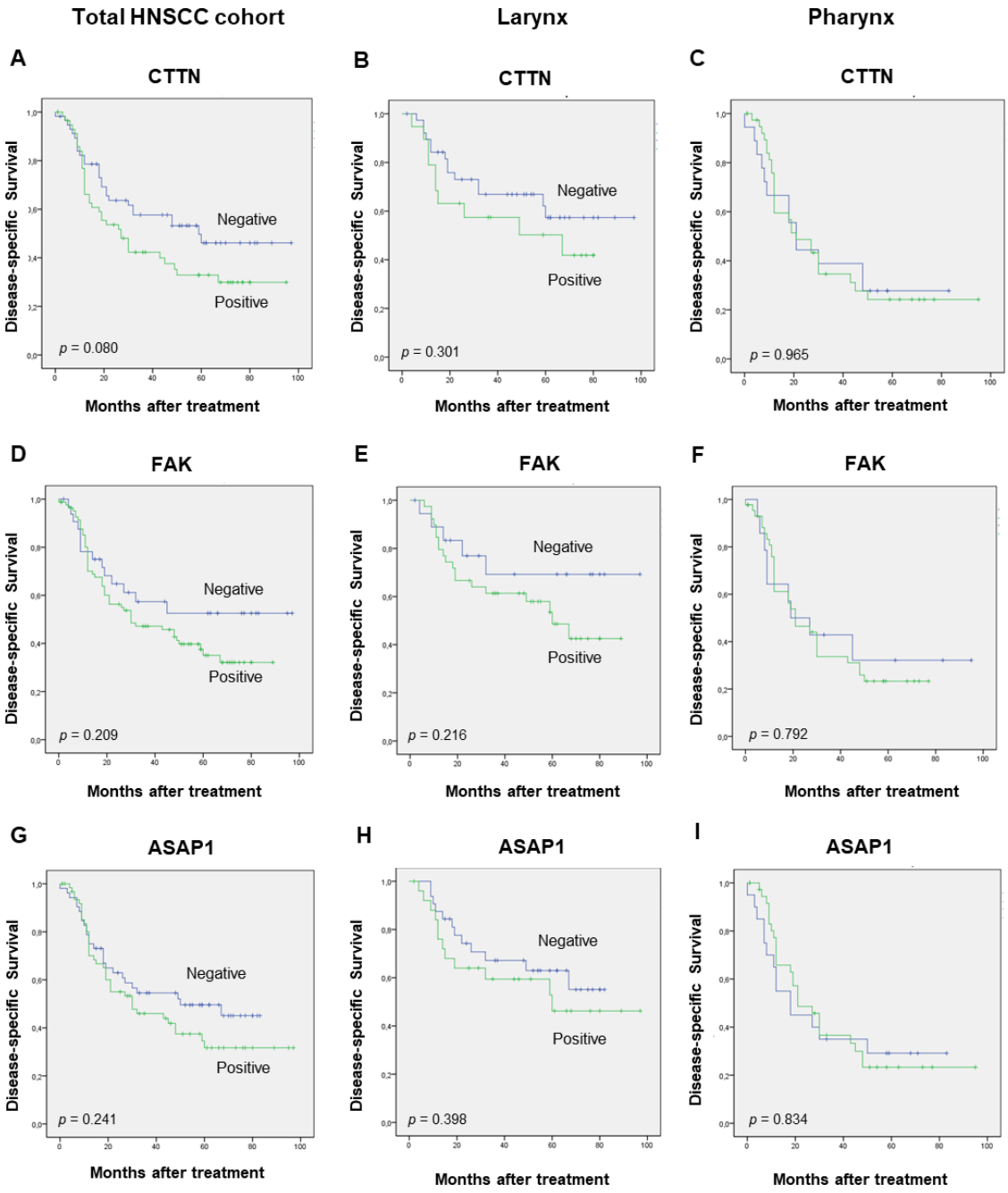


Figure 30. Impact of SRC-related proteins on patient survival. Kaplan–Meier disease specific survival curves categorized by CTTN expression (A–C), FAK expression (D–F), and ASAP1 expression (G–I) in the total cohort of HNSCC patients or in the laryngeal and pharyngeal subgroups. p values were estimated using the log-rank test.

6. DISCUSSION

Early detection biomarkers

There is mounting evidence demonstrating the role of SOX2 in tumorigenesis, and its contribution to tumor progression has been extensively documented in multiple cancers [55-57,259-264]. Accordingly, SOX2 has emerged as a candidate driver gene responsible for the 3q26-associated tumor aggressiveness [52,265-271]. In HNSCC, SOX2 expression has been found to induce cancer stem cell (CSC)-like properties, including increased self-renewal, tumorigenic potential and chemoresistence [272-275]. Accordingly, various studies using large cohorts of HNSCC samples have demonstrated that SOX2 expression significantly correlated with tumor recurrence and poor prognosis [57,274,275]. Although it has also been reported that low expression of SOX2 was associated with reduced survival and poor clinical outcome [276,277]. This study further and significantly extends these data investigating for the first time SOX2 protein expression and gene amplification in early stages of laryngeal tumorigenesis to ascertain its role in malignant transformation. Our findings demonstrate that SOX2 protein expression and gene amplification are frequent events in laryngeal tumorigenesis and, more importantly, both emerge as clinically and biologically relevant features in laryngeal cancer development. Even though SOX2 protein expression and SOX2 amplification were found to increase in high-grade dysplasias, SOX2 protein expression and gene amplification but not histological grading were significantly associated with progression to laryngeal cancer. In particular, patients carrying SOX2-expressing dysplastic lesions exhibited a significantly higher cancer incidence than those with negative expression. In other tumor types, it has been reported that SOX2 antagonizes signals promoting differentiation to maintain stemness in CSC subpopulations [278,279]. On this basis, we could speculate that the increased cancer risk in patients harboring SOX2-expressing lesions may reflect the presence of a larger proportion of cells presenting cancer stem-like properties. Supporting this hypothesis, the expression of other CSC markers such as NANOG or Podoplanin has also been found to increase in precancerous lesions and to associate with a higher risk of malignization [72,280,281].

Podoplanin was identified as a marker of tumor-initiating cells in squamous cell carcinomas [282]. Tumorigenicity and capability of recapitulating human squamous cell carcinomas are by definition properties of CSCs. Thus, it has been interpreted that premalignant lesions with podoplanin expression expanding beyond the basal cell layer may represent truly early neoplastic lesions, enriched in CSC and indeed lesions with such clonal expansion carry a higher risk of progression to invasive cancer. Furthermore, this study uncovers SOX2 expression as a robust independent predictor of laryngeal cancer risk beyond histological evaluation. Histopathological diagnosis of squamous intraepithelial lesions remains the gold standard in clinical practice for cancer risk assessment and decision-making [283]. Quite remarkably, the new

and recently established WHO classification as well as the previous dysplasia grading failed to show a significant role in assessing laryngeal cancer risk in this cohort. This emphasizes the still limited value of histological grading to predict outcome, which is certainly affected by inter- and intra-observer variability [284]. Additional objective and reliable markers are therefore needed to improve patient stratification and to more accurately identify those carrying lesions at higher risk of progression who will require the most intense treatment and follow-up [285]. Our results clearly demonstrate that SOX2 protein analysis may provide valuable additional information beyond histological features. Hence, immunohistochemical evaluation of SOX2 is proposed to be incorporated into the clinical practice as a complementary and relatively simple molecular test for cancer risk assessment and decision-making. Nevertheless, routine implementation of SOX2 expression as biomarker will require confirmation in large prospective studies, while also extending analysis to other subsites in the head and neck area. The present study also revealed important temporal and mechanistic information regarding the early occurrence and frequency of SOX2 expression and gene amplification in laryngeal tumorigenesis. Thus, while both alterations are frequently detected at early stages of tumorigenesis and their frequency increased with the grade of dysplasia, SOX2 gene amplification occurred at a lower frequency and did not perfectly match with protein expression, indicating that additional mechanisms must be contributing to SOX2 expression, such as transcriptional or posttranscriptional regulatory mechanisms. In line with this, it has been demonstrated that various transcription factors frequently altered in HNSCC, such as OCT4, YAP1 (Yes-Associated Protein 1) or the hypoxic factor HIF1 may induce the expression of SOX2 at mRNA level [286,287].

On the other hand, it has also been reported that amplification of PIK3CA and other genes mapping at 3q26 do not necessarily lead to increased expression, indicating that further epigenetic events could be involved in the transcriptional control [288]. Hence, this may explain some cases harboring SOX2-positive amplification that showed negative SOX2 protein expression. These results are in agreement with the TCGA data obtained from 279 HNSCC patients using the platform cBioPortal for Cancer Genomics (http://cbioportal.org/) [46,243]. It has been extensively demonstrated (both experimentally and *in silico*) that 3q26 amplicon harbors numerous genes found to be frequently and concomitantly co-amplified and overexpressed in multiple cancers [244,245]. Various genes have been highlighted as highly significant oncogenic drivers for HNSCC survival, including the four known driver genes PIK3CA, PRKCI, SOX2 and TP63 [244]. *In silico* analysis of TCGA HNSCC data further illustrates that co-amplification of these four genes occurs frequently in HNSCC, thus showing that 3q26 amplification in HNSCC is not restricted to the SOX2 gene. In addition, cytogenetic analyses demonstrated that 3q26 amplification is an early event in

dissemination [321,322]. Given the importance of the 11q13 locus in HNSCC and to contribute to the molecular characterization of these tumors, we conducted a study on a large unbiased cohort of 392 homogeneous surgically treated HNSCC patients to investigate the role of 11q13 amplification in relation to HPV status. This was accomplished by assessing the specific relationship of the protein expression and amplification of the CTTN, CCND1, and ANO1 genes mapping within this locus. Immunohistochemical analysis of CCND1, CTTN, and ANO1 revealed that the expression of these three proteins was strongly and inversely correlated with HPV infection. Noteworthy, all the HPV-positive cases showed negative ANO1 expression, and 28 out of 30 HPV-positive cases had also negative CTTN expression. Likewise, the analysis of gene copy amplification by real-time PCR consistently showed that amplifications of CTTN, CCND1 and ANO1 were frequently detected in HPV-negative tumors (ranging from 42 to 61%), while absent or rare in HPV-positive tumors (0–15%). Mechanistically, we found that the contribution of gene amplification to protein expression varied widely depending on each gene. Even though tumors harboring amplification also concomitantly expressed high protein levels, positive CCND1 and CTTN expression occurred at a higher frequency than gene amplification, while ANO1 protein expression was less frequent than ANO1 gene amplification. Additional regulatory mechanisms (transcriptional and post-translational) should contribute to protein expression, as previously reported [75]. A limitation of our study is the use of tissue microarrays to evaluate protein expression, which may constitute a drawback to assess the possible influence of tumor heterogeneity. To minimize this, three different representative tumor areas were selected from each tissue block and analysed in the TMAs.

Of note, the results showed that these proteins presented homogeneous and highly concordant expression patterns in the three tissue punches from each tumor. These results were further and significantly validated using an independent cohort of 279 TCGA HNSCC patients, thus confirming that the mRNA expression levels of CCND1, CTTN, and ANO1 were significantly increased in a high proportion of HPV-negative tumors, whereas most HPV positive patients exhibited normal mRNA levels of all these genes. Similar observations were obtained by analysing the frequencies of copy number alterations. These findings are also consistent with some studies that reported that 11q13 amplifications and gains were less frequent in HPV-related tumors [1,320]. This is also in agreement with the assumption made by Kostareli *et al.* that, in HPV-positive tumors, a lower number of genetic alterations is required to achieve the malignant phenotype, because of the inactivation of p53 and pRb proteins by the viral E6 and E7 oncoproteins [323]. To our knowledge, this is the first study to assess specifically the protein expression and copy number alterations of various genes mapping at the 11q13 amplicon in relation to HPV infection status using two large independent cohorts of HNSCC patients. Our study unveils important differences

regarding the expression and amplification of the CCND1, CTTN and ANO1 genes between HPV-related and unrelated tumors. It is worth mentioning that the two HNSCC cohorts selected for study are representative, sharing multiple of the unique characteristics reported for HPV-positive tumors , such as a clear prevalence in the oropharynx, low tobacco and alcohol consumption by the patients, lower tumor stage, basaloid histological pattern, a younger patients' age at diagnosis, lower mutations and CNA, and above all, a better prognosis [236]. A recent study by Dixit *et al*. provided the first evidence of a link between ANO1 expression and gene amplification and HPV status using a series of 64 pharyngeal tumors and tissue microarrays for IHC evaluation [324]. Therefore, our results further and significantly strength and validate these preliminary findings on ANO1 protein expression using a large independent cohort of 392 HNSCC patients, as well as on ANO1 mRNA levels in the TCGA cohort of 530 HNSCC patients. Together, these findings suggest that the CCND1, CTTN and ANO1 genes within the 11q13 amplicon could play a significant role in HPV-negative tumors but not in HPV-positive tumors. Given that 11q13 amplification has been associated with poor prognosis in HNSCC patients, and, in particular, these three genes have been involved in tumor progression and resistance to radio, chemotherapy, and EGFR-targeted therapies, the herein found distinctive molecular alterations presumably could contribute to the clinical and biological differences between these two different HNSCC subtypes and explain the better prognosis and response to radiotherapy and chemotherapy associated to HPV-positive tumors [325-331]. In fact, we proved the impact of these molecular alterations on patient survival. In particular, CTTN and ANO1 overexpression, rather than gene amplification, was more frequent and found to associate with a worse clinical outcome in two TCGA cohorts of 279 and 530 HNSCC patients. Nevertheless, given the size of the 11q13 amplicon, it cannot be ruled out that the overexpression of these genes may be secondary and that other genes within the 11q13 amplicon could act as true drivers of HNSCC progression. A recent study has also identified four genes (ORAOV1, CPT1A, SHANK2 and PPFIA1) as important drivers of 11q13 amplification that showed an impact on prognosis [332]. Therefore, various genes within the 11q13 amplicon could cooperatively contribute to the differences in prognosis and clinical outcome between HPV-positive and HPV-negative tumors. In summary, various studies including ours have provided strong evidence demonstrating that HPV-related and unrelated tumors are two different entities; hence, we consider that the management of HNSCC patients should move toward a more personalized approach.

Aberrant SRC expression and activation is frequent in a wide variety of cancers and has been identified as a central node in numerous oncogenic pathways, thereby playing critical roles in tumor formation, progression, and dissemination [333-338]. Hence, targeting SRC has emerged as a promising therapeutic strategy for cancer treatment, and a number of SRC inhibitors have

subsequently been developed and tested [334,339-348]. Overall, SRC inhibitors have demonstrated potent anti-tumor activity in preclinical models, although they are largely ineffective for the treatment of late-stage solid tumors [341,343-347]. In the specific context of HNSCC, dasatinib and saracatinib robustly inhibited cell proliferation, migration, and invasion in preclinical models [349-352]; however, these compounds did not show clinical efficacy as monotherapy in patients with advanced metastatic disease [353,354]. This therefore reflects the need for accurate response markers and adequate patient stratification to guide treatment with SRC inhibitors, which will undoubtedly contribute to improving clinical effectiveness and disease outcome. It seems quite reasonable that those tumors harboring aberrant SRC activation and function are more likely to be effectively targeted by anti-SRC therapies. Indeed, SRC pathway activation has been positively correlated with sensitivity to treatment with dasatinib and saracatinib in different cancer types, suggesting that SRC activation could potentially serve as a biomarker to guide SRC targeting and clinical efficacy [355-357]. The present study investigated the clinical significance of active SRC expression in HNSCC patients, by means of immunohistochemical detection with Clone 28 antibody specifically recognizing the active form of SRC [358,359]. In addition, the expression levels of p-SRC (Tyr419), total SRC, and various downstream SRC-related proteins were also evaluated by IHC and revealed significant correlations with active SRC expression in the total HNSCC cohort and the laryngeal subgroup. Notably, our results evidenced clear differences in the clinical impact of active SRC expression on patient survival depending on the tumor site. Active SRC specifically emerged as an independent predictor of cancer-specific mortality in patients with laryngeal tumors, but not in the pharyngeal subgroup. Consistent with these findings, the expression of p-SRC (Tyr419) and the SRC substrates FAK and ASAP1 also showed specific associations with poor prognosis in the larynx. We also found that CTTN was another specific predictor of poor prognosis in the larynx but not in the pharynx, in agreement with our previous study [75]. However, a significant correlation between CTTN and SRC expression was not observed in our HNSCC group.

Together these observations fit with the well-established oncogenic role of SRC and its downstream effectors favoring aggressive tumor phenotypes by promoting invasion and metastatic dissemination. Specifically, CTTN, FAK, and ASAP1 have been demonstrated to be key regulators of tumor invasion, metastasis, and aggressive phenotypes in HNSCC and other cancers [75,246-254]. In addition, we previously uncovered that the potassium channel HERG1 plays a fundamental role in early stages of HNSCC tumorigenesis and disease progression [74]. HERG1 expression was thus associated with aggressive tumor behavior and poor prognosis. This information was further and significantly extended in the present study, thereby uncovering a differential association of HERG1 expression with SRC expression depending on the HNSCC tumor site. Similarly, SRC expression has been correlated with nodal metastasis, advanced clinical stages,

recurrence, and poor prognosis in patients with oral carcinomas [360]. Increased p-SRC (Tyr419) levels have also been detected in nasopharyngeal carcinomas, both in tissue samples and in plasma, and correlated with tumor aggressiveness, distant metastasis, and unfavorable prognosis [361]. More controversially, SRC inhibitor monotherapy did not demonstrate any significant benefit in patients with metastatic HNSCC. These studies were performed in unselected cohorts of patients. Likewise, the lack of effectiveness could reflect the involvement of SRC in the first steps of the metastatic cascade to facilitate the migration and invasion of tumor cells, but not in the late stage when tumors are already disseminated. In addition, we recently demonstrated that dasatinib and saracatinib enhanced cancer stem cell (CSC) properties in HNSCC models, which could also represent a plausible underlying reason to explain the lack of clinical efficacy as monotherapy in HNSCC patients [362]. Remarkably, while dasatinib was unable to eliminate CSC subpopulations or to exhibit a1ny significant anti-cancer activity in mouse xenografts, the mithramycin analog EC-8042 effectively targeted these deleterious effects and robustly diminished tumor growth in vivo. In line with these findings, it has been reported that dasatinib worsened the anti-neoplastic effects of cetuximab and radiation in mouse HNSCC models [363]. Moreover, saracatinib showed no effect on tumor growth but blocked perineural invasion and nodal metastasis in an orthotopic model of oral cancer [364]. Similarly, the EGFR inhibitor erlotinib, but not dasatinib, was found to significantly reduce tumor size in a randomized trial with operable HNSCC patients [365].

Together these data reinforce a major role for SRC favoring tumor invasion and metastasis rather than sustaining tumor growth, in good agreement with the presented results herein. As an attempt to enhance the clinical benefit of SRC inhibitors, various combinational regimens with other anti-cancer agents have been designed and have shown promising results. Targeting SRC was effective in overcoming trastuzumab resistance and eliminating trastuzumab-resistant tumors *in vivo* [366]. SRC inhibition was also shown to overcome resistance to HER2 inhibitors, restoring lapatinib sensitivity (), and to reduce tumor growth in Met-driven tumors [367,368]. Dual inhibition of SRC and MET led to synergistic cytotoxic effects in HNSCC models, and this combination targeting has also emerged as a promising therapeutic strategy for colon cancer [369,370]. In addition, combination treatment of dasatinib with EC-8042 in HNSCC models demonstrated favorable complementary anti-proliferative, anti-stemness, and anti-invasive effects, suggesting this novel combinational strategy for clinical testing in HNSCC patients [362]. Nevertheless, it should be emphasized that adequate preselection of patients who may respond to and benefit from anti-SRC therapies is fundamental to guide clinical trials, as well as to identify reliable response biomarkers to improve stratification, treatment efficacy, and, ultimately, clinical outcome.

7. CONCLUSIONS

1.- SOX2 expression and gene amplification are relevant features frequently found in early stages of laryngeal tumorigenesis. More importantly, immunohistochemical SOX2 detection emerges as a robust predictor of laryngeal cancer risk in patients with precancerous lesions, beyond current WHO histological grading, which could be easily transferred to clinical routine.

2.- *CTTN*, *CCND1* and *ANO1* genes mapping at 11q13 are commonly co-amplified and overexpressed in HPV-negative HNSCC and correlated with reduced patient survival, while being absent or rarely detected in HPV-positive tumors. Therefore, these molecular alterations could contribute to the distinct clinical outcomes of these two HNSCC entities.

3.- SRC activation and the expression of the downstream SRC effectors FAK and ASAP1 concordantly demonstrate a differential impact on HNSCC prognosis depending on the tumor site, thereby emerging as specific predictors of poor prognosis in the larynx but not the pharynx.

4.- The expression of TINCR is frequently lost in HNSCC patient samples, genetically related to a high prevalence heterozygous chromosomal losses and mutations in the *TINCR* locus. Moreover, negative TINCR expression is more frequently observed in poorly differentiated tumors, hypopharyngeal tumor localization, and patients who developed tumor recurrences.

5.- High *miR*-301a expression levels are frequently found in HNSCC patient samples and derived cell lines. Functionally, miR301a dysregulation exhibits growth-promoting and pro-invasive effects, which could be effectively targeted and abrogated by pharmacologic inhibitors of PI3K and ERK signaling pathways.

6.- We have identified salivary bacterial changes associated to HNSCC tumor site, treatment response, and development of tumor recurrence, which could be potentially useful as non-invasive biomarkers for HNSCC diagnosis and disease monitoring.

8. CONCLUSIONES

1.- La expresión y amplificación génica de *SOX2* son características relevantes y frecuentemente detectadas en estadios tempranos de la tumorigénesis laríngea. Más importante aún, la detección inmunohistoquímica de SOX2 emerge como un robusto predictor del riesgo de cáncer de laringe en pacientes con lesiones precancerosas, más allá de la clasificación histológica actual de la OMS, que sería fácilmente transferible a la rutina clínica.

2.- Los genes *CTTN*, *CCND1* y *ANO1* que mapean en 11q13 están comúnmente coamplificados y sobreexpresados en los CECC VPH-negativos, lo que se asocia con una menor supervivencia de los pacientes; por el contrario, están ausentes o rara vez se detectan en tumores VPH-positivos. Por lo tanto, estas alteraciones moleculares podrían contribuir a la diferente clínica de estas dos entidades de tumores.

3.- La activación de SRC y la expresión de los efectores de SRC FAK y ASAP1 demuestran de forma concordante un impacto diferencial en el pronóstico de los CECC dependiendo de la localización tumoral, emergiendo como predictores específicos de mal pronóstico en laringe, pero no en faringe.

4.- La pérdida de expresión de TINCR es frecuente en pacientes con CECC, lo que está genéticamente relacionado con una alta prevalencia de pérdidas de heterocigosidad y mutaciones en el locus *TINCR*. Además, la expresión negativa de TINCR se observa con mayor frecuencia en tumores pobremente diferenciados, localización tumoral hipofaríngea y pacientes que desarrollaron recurrencias tumorales.

5.- Niveles elevados de expresión del miR301a se detectan con frecuencia tanto en muestras de pacientes con CECC, como líneas celulares derivadas. Funcionalmente, esta desregulación del miR301a promueve el crecimiento tumoral y la invasión. Estos efectos pueden ser revertidos de forma eficaz por inhibidores farmacológicos de las vías de señalización de PI3K y ERK.

6.- Hemos identificado cambios en bacterias salivales asociados con la localización tumoral de los CECC, la respuesta al tratamiento y el desarrollo de recurrencia tumoral, que podrían ser potencialmente útiles como biomarcadores no invasivos para el diagnóstico de CECC y el seguimiento de la enfermedad.

9. APPENDIX

9.1 SUPPLEMENTARY FIGURES

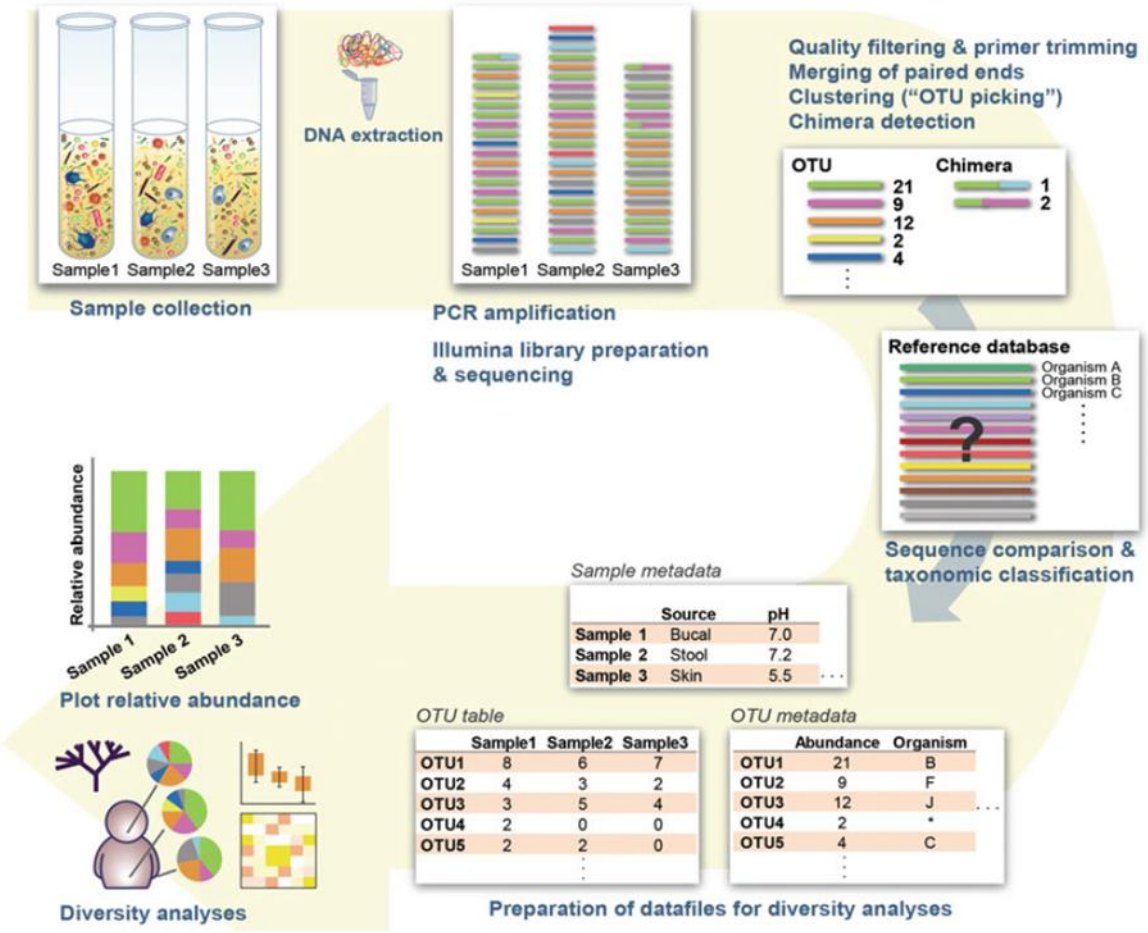


Figure S1. Taxonomic classification of prokaryotic microbes using 16S amplicon sequencing. The guideline includes generation of 16S amplicons, Illumina MiSeq sequence processing, taxonomic classification, and subsequent data visualization and diversity analyses (figure taken from [398]).

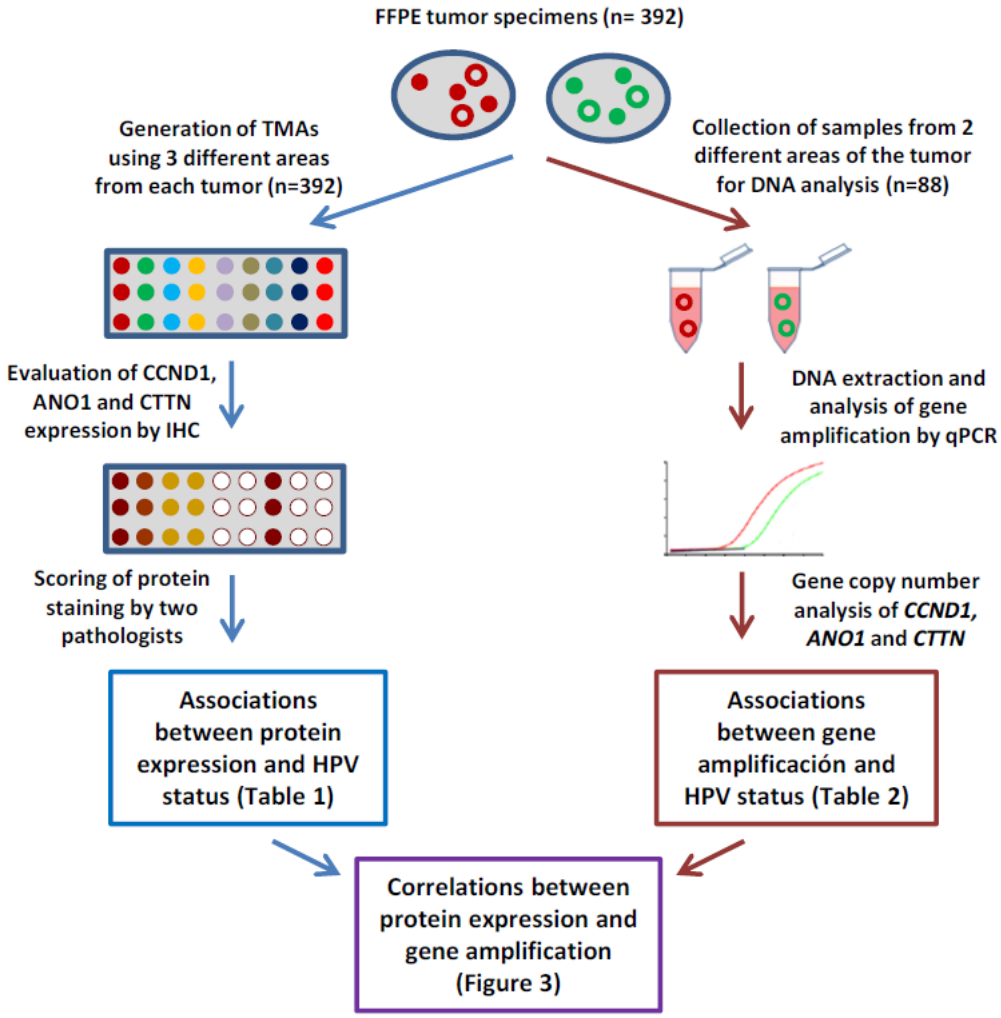


Figure S2. Schematic representation of the experimental setup designed to analyze CCND1, ANO1 and CTTN protein expression and gene amplification in relation to HPV status in a cohort of 392 HNSCC patients.

Table S1: Clinicopathologic features of the validation cohort of 279 HNSCC patients from the TCGA

	HPV -	HPV +
Gender (Age)		
Male	171 (60.4 +/- 11.8)	32 (58.5 +/- 9.9)
Female	72 (65.2 +/- 13.3)	4 (53 +/- 17.6)
Tumor Site (%)		
Oral cavity	160 (66)	12 (33)
Oropharynx	11 (4)	22 (61)
Hipopharynx	1 (<1)	1 (3)
Larynx	71 (29)	1 (3)
Stage (%)		
I	9 (4)	0 (0)
II	49 (20)	8 (22)
III	64 (27)	3 (8)
IV a	113(47)	23 (64)
IV b	5 (2)	2 (6)
Tobacco history (%)		
Life-long non smoker	42 (17)	10 (28)
Current reformed smoker for > 15 years	45 (18)	4 (11)
Current reformed smoker for ≤ 15 years	69 (28)	12 (33)
Current smoker	80 (33)	10 (28)
Alcohol history (%)		
Yes	158 (65)	30 (83)
No	80 (33)	5 (14)
Total patients (%)	243 (87)	36 (13)

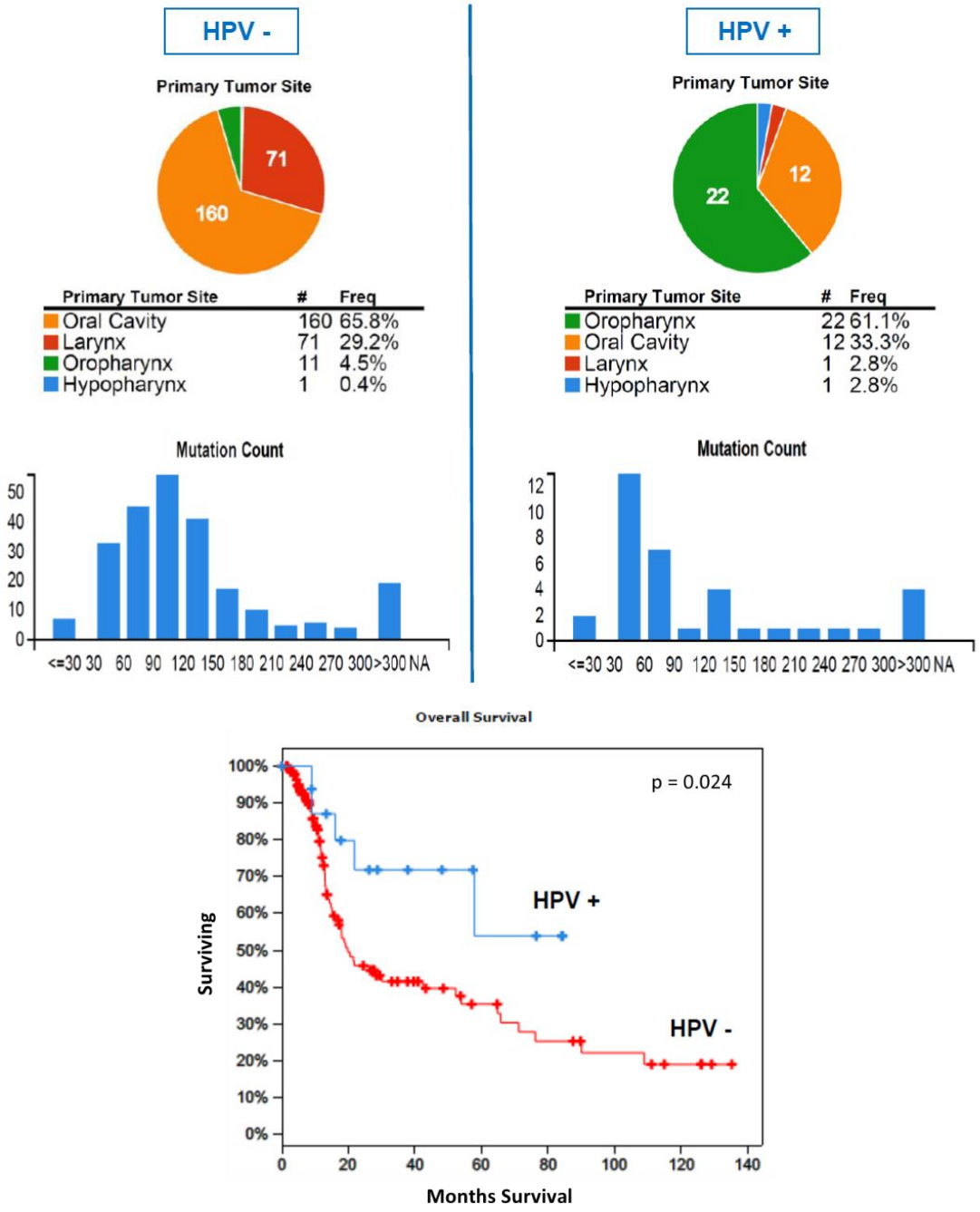


Figure S8. Clinical and molecular features of the TGCA cohort of 279 HNSCC patients, according to the HPV status. Schematic representation showing the HPV incidence by HNSCC subsites, mutation frequencies and Kaplan-Meier survival curves (p = 0.024, Log-rank test).

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