Universidad de Oviedo

PROGRAMA DE DOCTORADO EN BIOMEDICINA Y

ONCOLOGÍA MOLECULAR

LA RUTA DE SEÑALIZACIÓN DE NOTCH EN LOS CARCINOMAS EPIDERMOIDES DE CABEZA Y CUELLO

NOTCH SIGNALLING PATHWAY IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS

TESIS DOCTORAL

GIANLUIGI MARIANO GRILLI

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Doctorando	Director de la tesis	Tutor
Gianluigi Mariano Grilli	Dr. D. Juan Pablo Rodrigo Tapia	Dr. D. César Alvarez Marcos
	Co-Directora	
	Dr. D. Juana María García Pedrero	

Universidad de Oviedo

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: **LA RUTA DE SEÑALIZACIÓN DE NOTCH EN LOS CARCINOMAS EPIDERMOIDES DE CABEZA Y CUELLO**	Inglés: **NOTCH SIGNALLING PATHWAY IN HEAD AND NECK SQUAMOUS CELL CARCINOMAS**

2.- Autor	
Nombre: **Gianluigi Mariano Grilli**	DNI/Pasaporte/NIE:
Programa de Doctorado: **Biomedicina y Oncología Molecular**	
Órgano responsable: **Centro Internacional de Postgrado**	

RESUMEN (en español)

Objetivos: La función de la señalización de NOTCH (oncogénica u oncosupresora) sigue siendo controvertida en los carcinomas de células escamosas de cabeza y cuello (HNSCC). El propósito de este trabajo es investigar el papel de la ruta de NOTCH en el pronóstico de HNSCC.

Métodos: La expresión inmunohistoquímica de NOTCH1 y HES1 se evaluó conjuntamente y se correlacionó con otros objetivos de NOTCH1, p21 (WAF1/Cip1) y Ciclina D1, utilizando una cohorte de 372 pacientes con HNSCC negativos para HPV tratados quirúrgicamente.

Resultados: Se detectó expresión de NOTCH1 membranoso en 197 (61%) de 324 muestras tumorales evaluables y expresión de NOTCH1 nuclear en 91 muestras (28 %). Se encontró expresión de HES1 nuclear en 224 (67%) casos. La expresión de NOTCH1 membranosa y nuclear se correlacionó de forma consistente y significativa con la expresión de HES1 nuclear ($P < 0,001$) y p21 ($P = 0,03$), pero no con la Ciclina D1. La expresión de NOTCH1 se asoció significativamente con estadios tempranos (I-II), enfermedad no recurrente y mejores tasas de supervivencia general (SG) y enfermedad-especifica (DSS) ($P < 0,001$). Además, los casos triple-positivos (NOTCH1+/HES1+/p21+) exhibieron DSS ($P < 0,001$) y OS ($P = 0,004$) significativamente mejorados, lo que refuerza la asociación de la activación de la ruta NOTCH con un mejor pronóstico en HNSCC. El análisis multivariado demostró, además, que la expresión de NOTCH1 membranoso constituye un predictor independiente sólido de mejor DSS (HR = 0,554; 95 % IC 0,412–0,745; $P < 0,001$) y mejor SG (HR = 0,640; 95 % IC 0,491–0,835; $P = 0,001$).

Conclusión: Estos hallazgos muestran la asociación de la activación de la ruta de NOTCH con un mejor pronóstico en pacientes con HNSCC y también revelan la expresión de NOTCH1 membranoso como un predictor independiente sólido de una mejor supervivencia. En consecuencia, estos resultados sugieren un papel supresor de tumores en lugar de oncogénico para la vía NOTCH en HNSCC.

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RESUMEN (en Inglés)

Objectives: The function of NOTCH signaling (oncogenic or oncosuppressive) remains controversial in head and neck squamous cell carcinomas (HNSCC). The purpose of this work is to investigate the role of NOTCH pathway in HNSCC prognosis.

Methods: Immunohistochemical NOTCH1 and HES1 expression was jointly evaluated and correlated with other NOTCH1 targets, p21 (WAF1/Cip1) and Cyclin D1, using an unbiased cohort of 372 surgically treated HPV- negative HNSCC patients.

Results: Membranous NOTCH1 expression was detected in 197 (61%) out of 324 evaluable tumor samples, and nuclear NOTCH1 expression in 91 samples (28%). Nuclear HES1 expression was found in 224 (67%) cases. Membranous and nuclear NOTCH1 expression were consistently and significantly correlated with nuclear HES1 ($P < 0.001$) and p21 ($P = 0.03$) expression, but not with Cyclin D1. NOTCH1 expression was significantly associated to early stages (I-II), non-recurrent disease, and better disease-specific (DSS) and overall survival (OS) rates ($P < 0.001$). Moreover, triple-positive cases (NOTCH1+/HES1+/p21+) exhibited significantly im- proved DSS ($P < 0.001$) and OS ($P = 0.004$), thus reinforcing the association of NOTCH pathway activation with a better prognosis in HNSCC. Multivariate analysis further revealed membranous NOTCH1 expression as a robust independent predictor of better DSS (HR = 0.554; 95% IC 0.412–0.745; $P < 0.001$) and better OS (HR = 0.640; 95% CI 0.491–0.835; $P = 0.001$).

Conclusion: These findings show the association of NOTCH pathway activation with a better prognosis in HNSCC patients, also revealing membranous NOTCH1 expression as a robust independent predictor of improved survival. Accordingly, these results suggest a tumor suppressive rather than an oncogenic role for NOTCH pathway in HNSCC.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOMEDICINA Y ONCOLOGÍA MOLECULAR

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I. INTRODUCTION

1.1 NOTCH SIGNALING PATHWAY

Notch signaling pathway has been extensively characterized as a regulator of cell fate decisions in a variety of tissues and organisms. It mediates short-range cell interactions via juxtacrine signaling during the development of all metazoa thereby inducing distinct cell fate decisions by activating different programs depending on signal strength and dynamics (1,2). Hence, Notch controls cell fate decisions in a binary mode, and its activity can favor one fate over the other in two different ways: in the first one a cell can adopt a new fate or remain in its original state, similar to a stem cell development. In the second one, which is usually associated with differentiative cell division, the daughter cells can adopt one of two fates (*3*,*4*). The pleiotropic functions of Notch signaling shown in different contexts are based on the ability to influence developmental choices between neighboring cells during the development of the organism and during the maintenance of self-renewing adult tissues. Thus, Notch signals can promote or suppress cell proliferation, cell death, acquisition of specific cell fates, or activation of differentiation programs. Given the critical role Notch plays in all these fundamental processes in a wide range of tissues, it is not surprising that aberrant gain or loss of various Notch signaling components has been directly linked to multiple human disorders.

1.1.1 NOTCH RECEPTOR STRUCTURE

The Notch receptor family comprises a group of four receptors (named NOTCH1–4 in humans) sharing similar protein structure and modular arrangement of domains (Figure 1). A "canonical" Notch receptor consists of a single pass type I transmembrane molecule coded by a single precursor that becomes a non-covalently linked heterodimer consisting of an N-terminal extracellular (NEC) fragment and a C-terminal intracellular subunit (NICD) involved in signal transduction as a result of cleavage by a furin-like protease in the trans-Golgi network. The NEC structure is unique to the Notch receptor family: it is composed of up to 36 tandemly arranged epidermal growth factor (EGF)–like repeats, followed by three similarly arranged Lin12-Notch (LN) repeats. The NTM contains the RBPJk-associated molecule (RAM) region in the juxtamembrane region, followed by seven ankyrin repeats (ANK), a putative transactivating domain (*5*), and a C-terminal PEST motif (rich in proline, glutamine, serine, and threonine). EGF-like repeats and LN repeats have different functions. The first contain the receptor ligand binding sites (*6,7,8*), while the LN repeats are involved in preventing ligand-independent signaling (*9,*10,*11*). The entire intracellular part of the receptor, the NICD, is involved in relaying signal to the nucleus (*12,13,14*) (Figure 1).

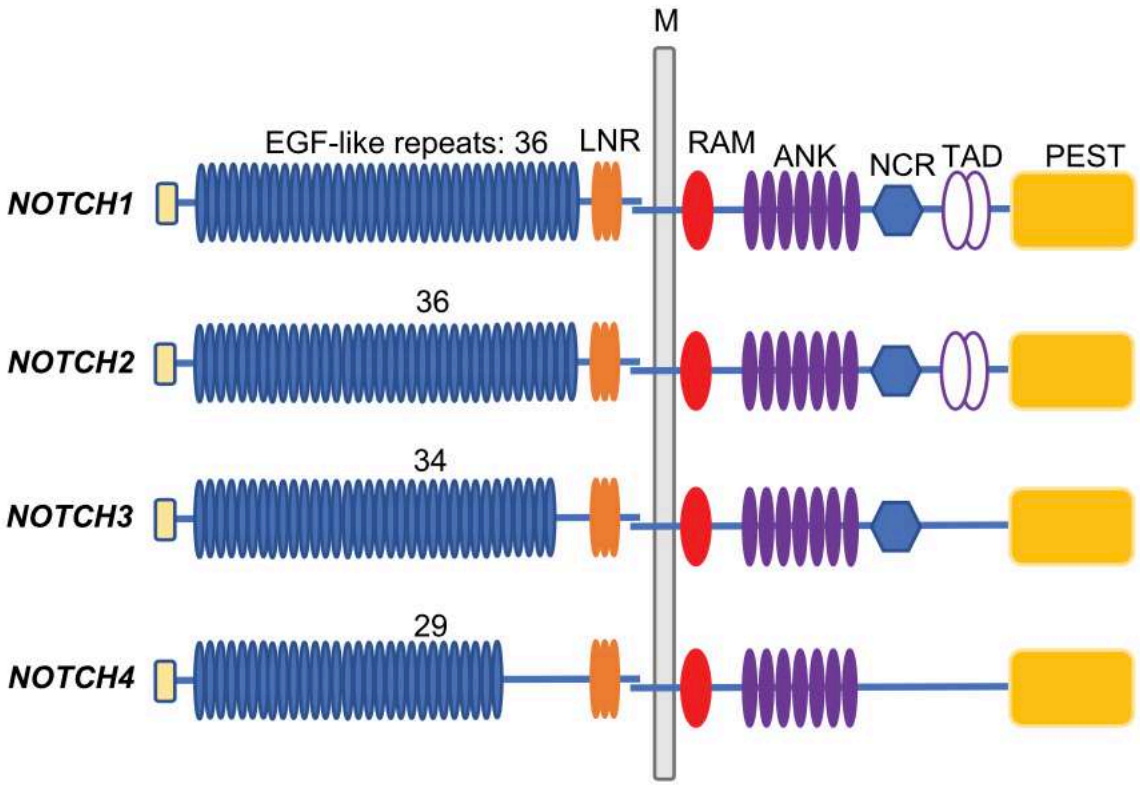

Figure1. Structure of human NOTCH Family receptors. Schematic representation of the conserved protein domains in the four members of NOTCH family - EGF: epidermal growth factor-like repeats; LNR: lin12-Notch repeats; M: membrane; RAM: RBPJk-associated molecule; ANK: ankyrin repeats; NCR: Notch cytokine response region; TAD: trasactivating domain; PEST: C-terminal PEST motif.

(Fukusumi T, Califano JA. The NOTCH Pathway in Head and Neck Squamous Cell Carcinoma. J Dent Res. 2018; Jun;97(6):645-653)

1.1.2 THE NOTCH PATHWAY

The Notch pathway directly couples events at the cell membrane with the regulation of transcription (15). The receptor-ligand interaction at the cellular membrane induces sequential cleavages of the Notch receptor. In particular, ADAM10/17 metalloproteases cause an S2 cleavage in the receptor, followed by a third cleavage (S3 cleavage) mediated by the presenilin–γ-secretase complex, composed of presenilin 1 (PSEN1), PSEN2, nicastrin (NCSTN), presenilin enhancer 2 (PEN2), and anterior pharynx-defective 1 (APH1) (16). The Notch intracellular domain (NICD) is then released in the signal-receiving cell and reaches the nucleus (Figure 2). Notch receptors possess a regulatory region that avoids uncontrolled proteases receptor cleavage while ligand endocytosis induces a *trans* conformation of the receptor that exposes cleavages sites (17). NICD interaction with CBF-1/ Su(H)/LAG1 (CSL) transcription factor regulates the recruitment of the transcriptional co-activator (Co-A) Master-mind-like (MAML) and other transcriptional Co-As in place of transcription co-repressors (Co-Rs) (15). In mammals, Notch ligands comprise three delta-like ligands (Dll1, Dll3, and Dll4) and two jagged ligands (Jag1 and Jag2), which are all transmembrane proteins of the Delta/Serrate/LAG-2 (DSL) family. The different fate of the signal-sending and the signal-receiving cells is directly connected to a trans-activation ability expressed by ligands of neighbor cells, while ligands expressed in *cis* may have an inhibition ability (referred to as *cis* inhibition). Each cell type determines the list of different target genes regulated by Notch pathway, which can include genes whose products are involved in fundamental aspects of cell biology, such as cellular differentiation, cycle regulation and metabolism (18,19,20). Common targets of this pathway include the HES and HEY (21,22,23) families of transcription repressors as well as MYC transcription factor (24,25) (Figure 2). Notch binding and its action on DNA appears to be a rapid and dynamic process controlled by the ubiquitin ligase Fbxw7 and the kinase CDK8, leading to Notch

phosphorylation, ubiquitination, and its subsequent proteasomal degradation (26,27,28,29), which shuts off the pathway.

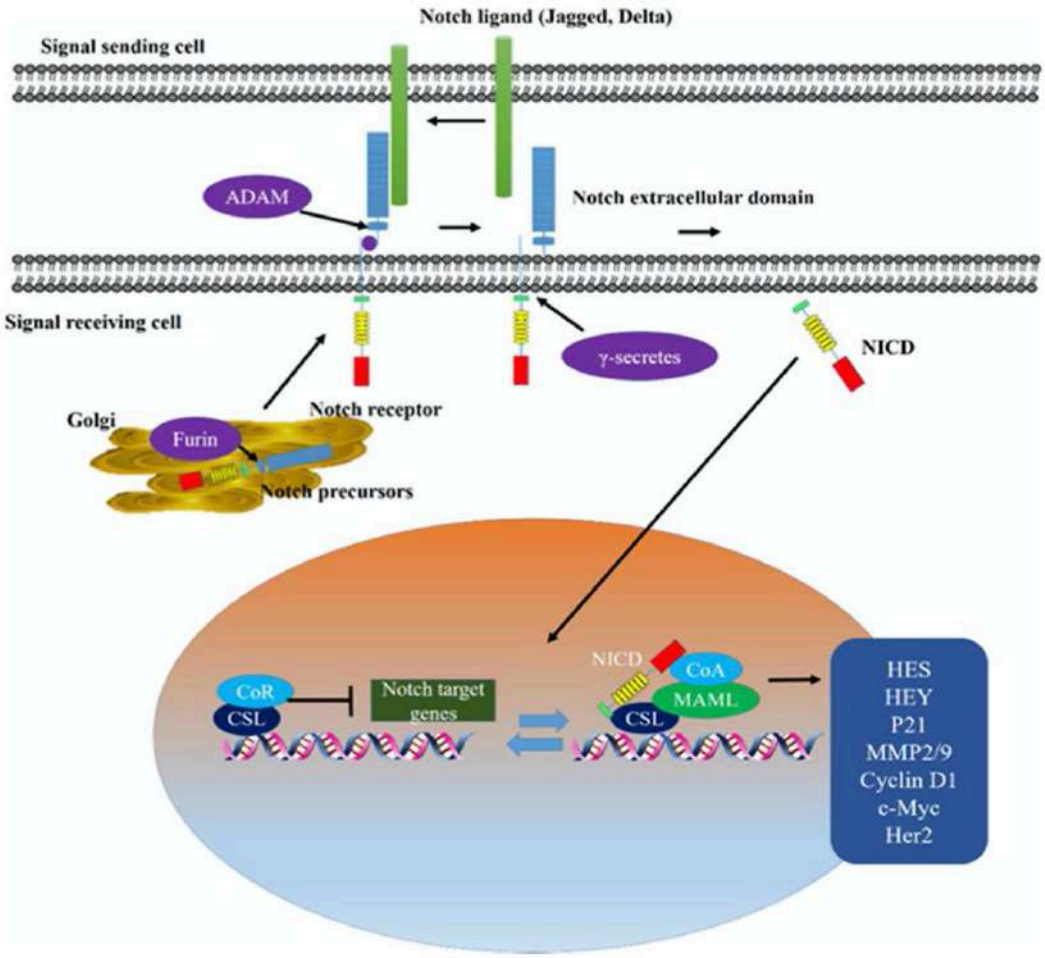

Figure 2. Schematic diagram of the NOTCH signaling pathway.

(Li L, Tang P, Li S, Qin X, Yang H, Wu C, Liu Y. Notch signaling pathway networks in cancer metastasis: a new target for cancer therapy. Med Oncol. 2017 Sep 16;34(10):180)

1.2 NOTCH IN CANCER

Given the fundamental role of Notch in various biological processes in a wide range of tissues, it is not surprising that aberrant gain or loss of different pathway components has been directly linked to multiple human diseases, including cancer. These alterations can lead to either activation or repression of Notch signaling, depending on the cell/tissue context and also the activation status of other potentially oncogenic pathways. Aberrant regulation of Notch pathway and its targets in cancer can occur by multiple and distinct mechanisms. They include receptor/ligand over-expression, epigenetic regulation, activating and inactivating mutations and post-translational modifications, in particular receptor and ligand fucosylation (especially O-fucosylation) (30,31,32,33). Moreover, it has also been demonstrated that the role of Notch in cancer cells dynamically changes over time. As an example, NOTCH1 promotes tumor growth at early stage of cervical cancer, while it inhibits tumor growth at late stages (34). Moreover, Notch may exhibit a dual role either acting as a tumor suppressor or an oncogene, which is determined by the microenvironment and some factors, including the type of Notch receptors, cell type, Notch activation state, and the cross-talk with other signaling pathways. Furthermore, non-mutational activation is often observed in Notch activation processes (35,36), highlighting the role played by heterotypic interactions involving Notch in creating intratumoral heterogeneity (37). The Notch pathway is genetically altered in a large number of hematopoietic and solid tumors. As an oncogene, NOTCH1 is overexpressed in breast cancer (38), gastric cancer (39), pancreatic cancer (40) and colon cancer (41). However, NOTCH1 expression has also been found down-regulated in skin cancer (42), liver cancer (43), prostate cancer (44), non-small cell lung cancer (45) and some breast cancers (46), where it acts as a tumor suppressor.

1.2.1 NOTCH SIGNALING AND THE TUMOR MICROENVIRONMENT

The tumor microenvironment (TME) is nowadays highly recognized as a major regulator of tumor progression. In this context, the role of the Notch signaling in tumor immunity has been extensively investigated over the last decade. Thus, Notch signaling pathway regulates the immunosuppressive environment of tumors by directly modulating the cytotoxic ability of CD8+ T cells and by acting on macrophages and myeloid-derived suppressor cells (MDSC) (47).

In particular, CD8+ T cell infiltration density has a strong prognostic impact in a large variety of solid tumors (e.g. colorectal cancers, breast cancer, head and neck cancer, and melanoma) (48). This anti-tumor infiltrate, characteristically associated with a type I interferon trascriptional signature, activates the innate immunity. However, several regulatory mechanisms such as the recruitment of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) tend to reduce the efficacy of the cytotoxic T cell infiltrate (49). Notch signaling plays a crucial role in regulating the activation of the CD8+ cytotoxic T lymphocytes (CTL), which are considered major players in the antitumor immune response. Mechanisms by which Notch regulates the activity of CD8+ T cells, once the lymphocytes enter the tumor, remain unclear. However, the inhibition of Notch signaling in CD8+ cells from colon cancer patients has shown to increase their cytotoxic activity by decreasing PD-1 expression (50). As a matter of fact, the binding of DLL1 to NOTCH1 or NOTCH2 for the expression of granzyme B and interferon-γ allows the activation of naive CD8+ T cells (51). This explains both why anti-tumor CTL response requires NOTCH2 (52) and why treatments with multivalent DLL1 induce the reduction of tumor growth by eliciting lymphocyte T differentiation and enhancing antigen-specific cytotoxicity (53) (Figure 3).

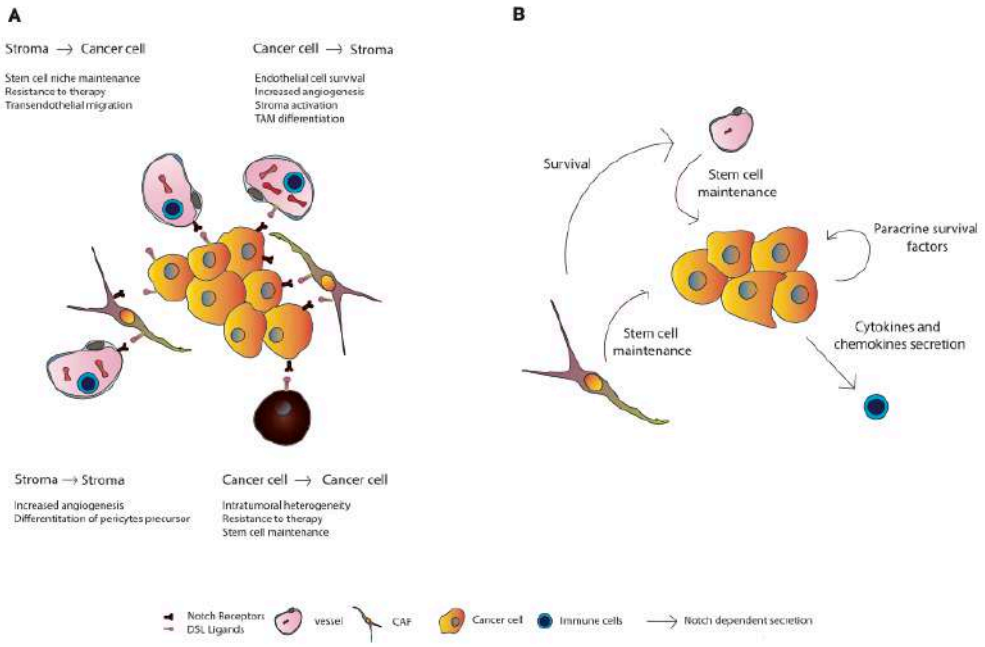

Figure 3. Interaction between Notch signaling and the TME - (A) Juxtacrine signaling between cells belonging to different compartments of the TME involves Notch. Receptors and ligands are expressed by cancer cells, vessels, cancer-associated fibroblasts (CAFs), and immune cells. TAM: tumor-associated macrophage. (B) Autocrine and paracrine signaling between the different compartments of the tumors involves Notch.

(Meurette O, Mehlen P. Notch Signaling in the Tumor Microenvironment. Cancer Cell. 2018; Oct 8;34(4):536-548)

Notch signaling is also important for differentiation of tumor-associated macrophages (TAMs) and for increasing the M1-macrophage phenotype (54,55). In particular, the polarization of macrophages depends on the intrinsic Notch activity, which is influenced by the interaction with other cells expressing Notch ligands in the TME. In fact, endocrine-resistant breast cancer cells may regulate TAM differentiation, highly expressing Jag1, increasing TAM markers in macrophages and being inhibited by γ-secretase inhibitors (56). Moreover, the expression of N1ICD activates Notch pathway and represses tumor growth, inhibiting TAM function (57).

Other immune cells in the TME are affected by Notch signaling. Although no studies explain how Notch affects Tregs, this pathway has been shown to alter Tregs immunosuppressive function (58). Furthermore, Notch signaling helps to orchestrate the interactions between the activated stroma and cancer cells. In this context, cancer-associated fibroblasts (CAFs) represent an important component of TME, being actively involved in the onset, progression, and metastatic dissemination of tumors (59). In fact, CAFs are directly involved in the recruitment of the immune infiltrate and remodeling of the extracellular matrix (59).

The Notch signaling is crucial for the regulation of fibroblast activation in the TME. Indeed, this pathway is typically abolished or reduced in stromal cells adjacent to pre-malignant lesions (60). Moreover, the causal effect of Notch inhibition on tumor formation is demonstrated by the fact that the loss of CSL transcription factor (RBPJ-κ, an effector of canonical NOTCH signaling with an intrinsic transcriptional repressive function) in mesenchymal mice cells induce multifocal epithelial tumors (60), probably associated with CAF activation (61).

Since NOTCH1 is considered a major regulator of the senescence secretome in fibroblasts (62), the loss of NOTCH1 could thus turn the senescence-associated secretory phenotype (SASP) towards a pro-inflammatory phenotype to ultimately induce tumor development. In particular, it has been shown that the loss of NOTCH1 in epithelia causes an increase of immune infiltrate associated with the activation of dermal fibroblasts that express α-SMA, as well as fibroblast-derived epidermal mitogens (63). In colon cancer, instead, Notch signaling allows the activation of bone marrow-derived stromal cells into activated fibroblasts (64). Nevertheless, in some cases, such as prostate cancer, Notch activation rather than its loss is implicated in the activation of fibroblasts, since Jag1 expression promotes the activation of fibroblasts expressing α-SMA and increases tenascin-C and collagen (65). In breast cancer, CAFs promote cancer stem cells (CSC) by paracrine secretion of IL-6 (66) and CCL2 (67). Even in hepatocellular carcinoma (HCC) (68), CSC are induced by NOTCH3. Moreover, the resistance to chemotherapy appears to be influenced by the interaction between cancer cells and CAFs (Figure 3).

Notch signaling also mediates the interaction between CSC and their niche. In fact, Notch signaling has been found to contribute to stem cell maintenance in various cancers (69). On the other hand, TME exerts significant influence on the stem cell niche. Demonstrating this, *in vitro*, the presence of endothelial cells increases the number of CSC (70). Moreover, several studies have demonstrated that ligands of the vascular niche activate Notch pathway (71,72) and, in particular, Jag1 has been pointed as the main Notch ligand implicated in the interaction with the CSC niche. In fact, in B cell lymphomas, colon cancers and breast cancers models, the vascular niche has been involved in the presentation of Jag1 to cancer cells, and thus in the reinforcement of aggressive phenotypes resistant to chemotherapy. (73). Presumably, Jag1 acts by inducing a specific transcriptional program. In head and neck cancer cell lines, Jag1ligand induces Kfl4 expression, which leads to CSC phenotype and resistance to chemotherapy (74). It remains unclear whether other ligands could also be involved in this mechanism.

The expression of Notch ligands and receptors can be dynamically regulated by interplay with other signaling pathways. In this sense, IL-6/ STAT3 regulates Notch pathway in breast cancer cells by the induction of Jag1 by autocrine secretion (75). Moreover, IL-6/STAT3/Notch crosstalk seems to also occur in colon cancer (76) and hepatocellular carcinoma (77). The RIG-1/STAT1 pathway represents another important crosstalk with Notch signaling, found to induce NOTCH3 in breast cancers, which resulted in resistance to therapy (78). Even the transforming growth factor β (TGF-β) pathway is involved in the crosstalk between Notch and TME. In particular, in prostate cancers, the upregulation of TGF-β involves the formation of a reactive stroma (65). Furthermore, Notch activation enhanced TGF-β-induced pSMAD2/3 signaling.

Shaping the tumor vasculature has abnormal features, and this phenomenon is controlled by inducing sprouting of existing vessels. Notch signaling is a major regulator of sprouting angiogenesis (79), involving endothelial tip and stalk cells. In particular, DLL4/Notch controls the emergence of endothelial tip cells, which differentiate in response to pro-angiogenic factors to generate new vessels, while Notch-mediated VEGFR2 inhibition sustains the stalk cell phenotype, thus controlling the vasculature architecture and preventing hypersprouting. Therefore, since DLL4 and Jag1 have opposite functions in controlling sprouting angiogenesis (80), the balance between the endothelial expression of both factors may have a major impact on the tumor vasculature architecture. In particular, Jag1 overexpression in endothelial cells induces an increase in tumor vasculature, whereas a loss of function of Jag1 in these cells leads to decreased vasculature and tumor growth (81). This may be explained by the fact that tumor-induced Notch activation can lead to senescence in the endothelial cells via NOTCH1 activation by tumor and myeloid cells, which in turn induces inflammation and increases metastasis (82), and also because tumor-derived Jag1 is directly linked to the inhibition of NOTCH3-dependent cell death in endothelial cells (83). In addition, endothelial expressed Notch ligands can activate Notch signaling in adjacent cancer cells. This characteristic has been demonstrated in many different cancer types. In colon cancer, Notch activation in cancer cells by adjacent blood vessel cells has also been shown to increase trans-endothelial migration and therefore metastasis (84). It has been reported that Notch activity in glioblastoma cells is higher in the proximity of ECs (72). DLL4 expressed by ECs activates NOTCH3 in T-ALL cells, allowing dormancy escape (85). Also, expression of Jag1 by ECs activates Notch signaling in local pericyte precursor cells to induce pericyte differentiation (86).

1.2.2 NOTCH SIGNALING IN LEUKEMIA AND LYMPHOMAS

Ellisen and colleagues were the first to demonstrate that alterations in the Notch signaling occur in cancer. In particular, NOTCH1 is constitutively activated in leukemia, as a result of rearrangements occurring between the intracellular part of NOTCH1 (ICN1) and the T cell beta receptor (TRB) locus. (87) ICN1, in fact, represents a strong oncogenic allele. Among the mutations shown in human T-Cell Acute Lymphoblastic Leukemia (T-ALL), there are single amino acid substitutions and insertions or deletions located in exons 26 and 27 of the genetic locus, which code respectively the N-terminal and C-terminal components of the hetero-dimerization domain. These mutations result in independent activation of the ligand or in hypersensitivity of the Notch signaling pathway, due to a lower protection of the S2 cleavage. Notch activation may also increase due to Juxtamembrane Expansion mutations (JME) (88) and PEST domain mutations (20-25% of cases). The latter alterations cause truncation or loss of the domain via frame-shifts or nonsense nucleotide substitutions, which result in proteasomal degradation mediated by ubiquitin ligase FBXW7 and in higher ICN1 cell concentrations (89).

Mutations or deletions in *FBXW7* occur in 15% of T-ALL cases (90) and involve three arginine residues that are critical for the interaction with ICN1. Probably PEST and FBXW7 both contribute to increasing the stability of ICN1. Thus, the mutations affecting these two domains are unlikely to occur simultaneously (91). Since *FBXW7* mutations directly affect cells with leukemia initiation properties (LIC) through MYC stabilization and overexpression, it appears clear that NOTCH and MYC actions are intertwined in cancer cells. *NOTCH1* mutations in T-ALL have been correlated with a favorable prognosis. A study conducted in pediatric patients confirmed that *NOTCH1* and *FBXW7* mutations are associated with improved early chemotherapy response and lower minimum residual disease (MRD) levels (92). Currently, the aim of the

research is to exploit the inhibition of Notch pathway to treat T-ALL, especially in relapsing and refractory diseases. *NOTCH1* activating mutation have also been demonstrated in 10-12% of chronic lymphocytic leukemia (CLL). Again, mutations occur in the PEST domain, with the formation of truncated protein variants with a longer half-life. P2515Rfs represent the prevalent mutation (93,94). These mutations are mutually exclusive with TP53 abnormalities. In both cases, however, survival outcomes are poor (95). *NOTCH1* and *SF3B1* mutations (a splice factor) are associated with reduced overall survival and therefore represent a negative prognostic factor (96). Among the different types of lymphoma, Burkitt lymphoma exhibits recurrent *NOTCH1* mutations with gain-of-function (8-9% of cases) and appears to be associated with MYC upregulation, due to its translocation into the immunoglobulin locus. (97). Follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) are other malignant tumors of B cell origin. FL and the germinal center B-cell (GCB) diffuse large B cell lymphoma (DLBCL) subtype derive from germinal center B-cells, whereas the activated B-cell (ABC) DLBCL subtype derives from cells that have exited the germinal center. *NOTCH2*mutations are found in 8% of DLBCL, (98) generally represented by gain-of-function mutations affecting the PEST domain and copy number alterations (99). NOTCH2 plays a crucial role in the development of B cells in the spleen marginal zone and appears to be implicated in splenic marginal zone lymphoma (SMZL) (100) (20% of cases) (101). These mutations, even in the latter tumors, are associated with adverse prognosis (100).

In Hodgkin lymphoma (102,103), NOTCH1 activates the tumor niche and suppresses genes involved in the B-cell identity, such as E12 / E47 and the early B-cell factor (EBF) (104).

In contrast to the oncogenic role just described, emerging evidences point out that the Notch pathway plays also suppressive roles in several types of tumors (105,106). Indeed, the deletion of nicastrine (Ncstn), a component of the γ-secretase complex, leads to the induction of chronic myelomonocytic leukemia (CMML) (107), characterized by increased extramedullary hematopoiesis, monocytosis, myeloproliferation and frequent progression to acute myeloid leukemia (AML). This is supported by the fact that, in study models, the deletion of FX (the homolog of human GDP-L-fucose synthase) or O-fucosyltransferase 1 results in myeloid hyperplasia (108). The ablation of MAML1 (Mastermind-like transcriptional coactivator 1) can cause similar phenotypes. In fact, the role of Notch as a tumor suppressor is mediated by the direct repression of PU.1 and CEBPa promoters by HES1. MAML1, APH1A and NOTCH2 are mutated and genetically inactivated in 12% of patients with CMML. These mutations are not found in other myeloproliferative diseases such as polycythemia vera (PV) and myelofibrosis (MF). Inactivating *NOTCH* mutations can occur simultaneously with other described myeloid mutations, such as *TET2*, *FLT3* and *ASXL1* (107).

The combined inactivation of NOTCH and TET2 induces acute myeloid leukemia (AML) and the reactivation of Notch signaling in AML results in complete remission of the disease (109,110). The epigenetic silencing, obtained from DNA and histone methylation of target gene promoters / transcriptional start sites, may cause Notch inactivation. Therefore, the use of NOTCH2 activating antibodies or specific agonists emerges as a useful therapeutic strategy for AML (REF??).

Notch signaling also acts as a tumor suppressor in acute B-cell leukemia (B-ALL) (111). As in AML, Notch reactivation inhibits tumor growth and induces apoptosis in human B-ALL cells, in which several Notch targets are suppressed by DNA hyper-methylation of cytosine on their promoters and by trimethylation of histone H3K27 and H3K9 (112). These results support the hypothesis that Notch signaling influences the differentiation of progenitors in the hematopoietic system. Indeed, the

inactivation of Notch pathway can induce CMML and AML, by increasing the development of granulocyte-monocyte progenitors (GMP) (107).

1.2.3 NOTCH SIGNALING IN SOLID TUMORS

The role of the Notch signaling in solid tumors has been extensively investigated in recent years (106,113,114). In breast cancer, Notch may act as a suppressor or an oncogene, depending on the cancer subtype. In fact, the first studies on the role of Notch signaling in solid tumors derive from experiments with mouse mammary tumor viruses (MMTV), where the integration of the MMTV genome alongside "Int-3" locus resulted in an activating mutation of *NOTCH4*, constitutively activated (115,116,117). Since this discovery, several subsequent studies have confirmed that activation of Notch signaling plays an oncogenic role in breast cancer (117,118,119,120). In this cancer, crosstalks with other signaling pathways, including Ras and Wnt, can activate Notch signaling (121,122,123). In this context, NOTCH4 plays a more specific role than other Notch receptors. Conversely, NOTCH3 hyperactivation can induce cellular senescence (124). Interestingly, breast cancer cells respond differently to the expression levels of Notch receptors (125). In fact, even if Notch plays an oncogenic role in breast cancer, in some cases, high levels of Notch activation may exhibit tumor suppressive effects. Accordingly, the concept of "differentiation switch" could better explain the multiple actions of Notch.

Several studies have demonstrated the involvement of Notch pathway in the development of different lung cancer subtypes. In particular, Notch signaling promotes the expansion of cultured lung adenocarcinoma (LAC) cells, one of the major lung cancer subtypes. (126,127,128).In addition, Notch promotes the development and maintenance of LAC *In vivo* (129,130,131) thereby inducing the renewal of tumor propagation cells (132). The expression of JAG2 on the surface of these cells enhances the metastatic potential (133). Concordantly,

Notch activation represents a negative prognostic factor (132,134) and NOTCH3 and / or JAG2 targeting has emerged as a potentially useful treatment for patients with lung cancer. By contrast, in squamous cell lung cancer (SqCCL), Notch signaling acts as a tumor suppressor (135). Loss-of-function *NOTCH1* mutations have been associated with the development of these cancers. These mutations are located in the EGF-like region of NOTCH1 and have the potential to disrupt ligand binding or to produce truncated receptors. Although the underlying mechanisms are not yet fully understood, the inactivation of Notch signaling promotes the development of squamous cell carcinoma in other tissues as well, including skin and head and neck cancers (135,136,137,138,139). In fact, the loss of Notch pathway favors the growth of tumor cells with characteristics of squamous differentiation. As regards small cell lung cancer (SCLC), recurrent mutations in Notch pathway components have not yet been identified (140,141). Indeed, the hyperactivation of Notch signaling blocks the cell cycle (142,143). This effect also occurs in other neuroendocrine tumors, such as medullary thyroid carcinoma (144). However, further studies are needed to understand how Notch activation can block the development or maintenance of SCLC (145,146). Therefore, the Notch system plays an active oncogenic role in LAC, a suppressor role in SqCCL and possibly a suppressor role with no sign of mutations in SCLC.

The role of Notch pathway has also been extensively investigated in hepatocellular carcinoma (HCC) (147,148). Specifically, low levels of Notch were correlated with high Wnt activity, one of the main oncogenic pathways in HCC (149), whereas high levels of active NOTCH1 can inhibit the expansion of HCC cells (150). This suppressive effect could be possible due to the inactivation of the RB pathway (43). However, recent studies have demonstrated an oncogenic role of Notch (151,152,153), probably involved in the development of HCC following hepatitis B virus (154). Therefore, the functional role of Notch signaling in HCC may vary in distinct molecular subgroups. This role appears clearer in cholangiocarcinoma (CCC), where it plays a truly oncogenic role. Thus, NOTCH1 activation leads to CCC development in

mouse models (155). Beyond the apparent contradictory evidence regarding the oncogenic/suppressive role of Notch signaling, the crosstalk with other signaling pathways, the timing of activation and the type of cell / receptor in which this activation occurs could also influence its effects (156). In fact, Notch may exert a suppressive role in the early stages of HCC development and subsequently acquire an oncogenic role. Moreover, Notch activation could promote CCC and suppress HCC (157). Notch may be involved in the control of homeostatic self-renewal in intestinal epithelial stem cell populations and thus in the development of colorectal cancer (CRC) (158,159,160). The intestinal epithelium self-renewal rate is very high and could be linked to a high susceptibility to malignant transformation. In CRC, mutations in regulatory genes cause Notch overexpression or its constitutive activation (161,162,163,164,165). This activation, mediated by Jagged1, has been found to correlate with the activation of Wnt and Hippo / YAP signaling in CRC cells (166,167,168,169,170). Another Notch ligand, DLL4, has been implicated in tumor neoangiogenesis (171,172). Interestingly, Notch signaling potentiates CSC in the early stages of tumor development, while it promotes tumor invasion and metastasis in the later stages (84). Notch activation has also been detected in the early stages of pancreatic ductal adenocarcinoma (PDAC) development, during ductal metaplasia (173). This activation, together with K-Ras, induces dysplastic progression and tumor development (174), whereas genetic inactivation of NOTCH2 counteracts the action of K-Ras (175,176). Furthermore, pharmacological NOTCH inhibition was able to reduce disease progression in animal models (177,178,179). NOTCH1 also promoted the progression of melanomas (180,181), through the induction of melanocyte growth in hypoxic conditions. Although the underlying mechanisms are not yet fully understood, recent studies have shown that γ-secretase inhibitors (GSI) were effective at reducing disease progression. Therefore, in combination with chemotherapy, GSI could constitute a new therapeutic strategy for the treatment of melanomas. (182).

In conclusion, Notch signaling is involved in the development and progression of several cancer types, solid and liquid (hematopoietic) tumors. Further studies are needed to fully understand the specific molecular pathways and major target genes that are involved in different cancers.

1.2.4 THERAPEUTIC TARGETING OF NOTCH

As previously mentioned, γ-secretase inhibitors (GSI) have proved to effectively block NOTCH1 activity in T-ALL cells, since the presenilin / γ-secretase complex proteolytically cleaves Notch receptors. In light of this, GSIs have been proposed as therapeutic approaches for ALL patients (183,184). However, animal model studies and phase 1 clinical trials have shown that systemic Notch inhibition has important gastrointestinal toxic effects, since they result in the accumulation of secretory goblet cells in the intestine. Consequently, treatments based solely on GSI are not the most suitable choice. In contrast, the combinatorial use of glucocorticoids and GSI could have important therapeutic results, since glucocorticoids reduce intestinal toxicity by the expression of cyclin D2 (185)., Other drugs which are currently being tested include alpha-secretase inhibitors (ASI) against the metalloproteases ADAM10 / 17, which mediate the cleavage of the S2 receptor (186). Moreover, highly specialized antibodies against NOTCH1 and NOTCH2 have been developed, which mechanism of action is represented by the stabilization of the negative regulatory region (NRR) of receptors and protection from proteolytic cleavage, resulting in inhibition of ICN1 / 2 production (187). These antibodies are associated with lower gastrointestinal toxicity and fewer side effects than using GSIs. In preclinical models, selective blockade of NOTCH1 inhibits tumor expansion by reducing tumor cell growth and angiogenesis. Moreover, in study models NOTCH1 decoys decrease tumor cell viability. However, since a DLL1 decoy can have an activating or inhibitory role (188) depending on the different conditions, it is necessary to precisely

decipher their function before being able to consider decoys as potential therapeutic targets. Synthetic peptides mimicking MAML1 without active domains have also been developed. In particular, a synthetic alpha-helical peptide (SAHM1) that blocks the recruitment of MAML1 and Notch-mediated transcription has been developed (189) but needs further testing before application.

New approaches do not target the Notch pathway itself but focus on its signaling targets, for example targeting the NOTCH1-induced IKK kinase complex, which has a crucial role in controlling the NF-kB activation, the CyclinD:CDK4/6 kinase complex or the bromodomain-containing protein BRD4 (190). A thienodiazepine molecule that inhibits the binding of BRD to the acetylated residues of histone H4 has recently been proposed (191). This drug is associated with the complete remission of diseases such as T-ALL. Since NOTCH1 binding has recently been connected to the loss of H3K27me3 on target promoters and in particular H3K27me3, demethylation inhibitors could be useful for treating NOTCH1-induced T-ALL (or LLC).

The impact of Notch inhibition on tumor angiogenesis has been widely described. Jag1 induces blood vessel maturation downstream of DLL4/ Notch regulating sprouting angiogenesis (192). Since Jag1 and DLL4 ligands have opposite effects on angiogenesis, the effects of targeting Jag1 will therefore differ from targeting DLL4 on the tumor-associated vasculature. Indeed, anti-DLL4 antibodies induce non-productive angiogenesis (193). On the other hand, treatment with GSI affects the interactions of both ligands and receptors (DLL4 / Notch and Jag1 / Notch, for example) and reduces tumor angiogenesis (194). These pleiotropic effects could explain why GSIs decrease tumor angiogenesis, whereas anti-DLL4 treatments induce massive non-productive angiogenesis (193). Moreover, the latter treatments might force dormant tumor cells (DTCs) (195) to re-enter the cell cycle. Contrary to this, anti-Jag1 antibodies may antagonize the consequences of Jag1 overexpression and normalize angiogenesis.

Since Notch pathway has a global impact on the immunosuppressive environment of the tumor, the use of GSIs induces a reduction in TAM, MDSC and Treg populations (196). This effect could be due to an inhibition of tumor growth or to a reduced M1 polarization of macrophages. In fact, compared to wild-type macrophages, Notch-deficient macrophages are less efficient at reducing tumor growth of B16 or LLC1 syngeneic grafts (55). Multivalent forms of DLL4 also induce T lymphocyte differentiation and elicit antigen-specific cytotoxicity, thus enhancing anti-tumor immunity (53). Even the inhibition of Notch signaling, with anti-Jag1/2 antibody, enhanced the anti-tumor response, by the accumulation and tolerogenic activity of MDSCs within the tumor (197). From this point of view, Tregs also appear to be involved in the enhanced regulatory phenotype in graft- versus-host disease (58).

1.3 GENERAL AND EPIDEMIOLOGICAL FRAMING OF OROPHARYNGEAL, HYPOPHARYNGEAL AND LARYNGEAL CANCER

1.3.1 OROPHARYNGEAL CANCER

The oropharynx is the central portion of the pharynx bounded cranially by the posterior edge of the hard palate and distally by the valleculae and hyoid bone. Posterior and lateral limits are represented by the muscular pharyngeal wall, while the circumvallate papillae and palatoglossal muscle mark the anterior borders. Oropharynx consists of four subsites (Figure 4): the posterior pharyngeal wall, the soft palate, the tonsillar complex (tonsil, tonsillar fossa, and pillars) and the base of the tongue [198]. Although minor salivary tumors, primary lymphoid tumors, undifferentiated tumors, various sarcomas, and mixed cellularity neoplasms also develop primarily in the oropharynx, the vast majority of primary oropharyngeal tumors are squamous cell carcinomas (SCCs) [198]. In retrospective analyses across all anatomic subsites, approximately 60% of oropharyngeal SCCs have been found to be moderately differentiated, 20% well differentiated, and 20% poorly differentiated [198].

In the United States, approximately 5000 new cases of oropharyngeal cancer are diagnosed annually, of which 85–90% are SCCs [199]. The incidence of oropharygeal SCC is closely correlated with tobacco and alcohol abuse. The last, in particular, appears not only to be an independent risk factor for oropharyngeal SCC but also seems to potentiate the carcinogenic potential of tobacco smoke in the oropharynx. Moreover, alcohol and tobacco carcinogenic effects on the oropharynx appear to function in dose-dependent manners. Although SCC of the oropharynx is diagnosed predominantly in people

over the age of 45 years, Western European and American studies suggest an increasing incidence of the disease in people less than 45 years of age, over the past 20–30 years [200].

Human papilloma virus (HPV) plays an important role in the oncogenesis of oropharyngeal SCC. In particular, patients with HPV seropositivity and/or oral HPV have shown an increased relative risk for oropharyngeal SCC. These neoplasms possess particular features: the risk appears to be higher in younger populations, although different patterns of sexual behaviors may partially account for this trend, tonsil is the most affected oropharyngeal subsite and HPV-associated oropharyngeal cancers may be less aggressive than those not associated with the virus, showing much better survival rates. Although HPV-18 and HPV-16 are associated with genital cancers, the vast majority (84%) of HPV-related head and neck cancers are only associated with HPV-16.

The role of inheritable predispositions in the development of oropharyngeal cancer is still debated. It has been demonstrated that individuals with Fanconi anemia have a 500 – 700-fold increase in the risk of head and neck SCC, the majority of which are related to HPV. A family history positive for head and neck SCC confers a 2 – 4-fold increase in the risk of developing head and neck SCC across all anatomic sites, including the oropharynx. The risk is also increased in people with a positive family history of alcohol and/or tobacco abuse [200].

1.3.2 HYPOPHARYNGEAL CANCER

The hypopharynx is the most inferior part of the pharynx (Figure 4). It extends in continuity with the oropharynx, limited by the the hyoid bone cranially and the cricopharyngeus muscle and the cricoid cartilage caudally. It can be divided into three sub-sites: the pyriform sinuses, the postcricoid region and the posterior pharyngeal wall, including the inferior aspect of the middle constrictor. The hypopharynx sits behind the larynx, limiting anteriorly the retropharyngeal space.

More than 95% of patients presenting malignant hypopharyngeal tumors are proven to be SCC, sharing with oropharyngeal cancer tobacco and alchool abuse as the main risk factors. Cancers of the hypopharynx are generally aggressive in their behavior and demonstrate a natural history characterized by diffuse primary tumor with mucosal and submucosal local spread, early cervical nodal metastasis, and a relatively high rate of distant spread. Most (80%) of all hypopharyngeal carcinomas arise from the piriform sinuses [201], with primary tumors of the posterior pharyngeal wall, postcricoid region and the esophageal inlet accounting for >10%, in most reported series. The incidence of carcinoma is much higher in men than in women, but in the postcricoid region, the reverse is true in the developed world (frequently in association with sideropenic anemia). Aggressive invasion is a common feature, and tumors in the neck may spread along muscle or fascial planes for a variable distance from the visible primary mucosal lesion. Bone and cartilage usually act as a barrier to spread, and these structures generally are spared during the initial tumor growth and when invasion is present signifies a late event of the disease process (202).

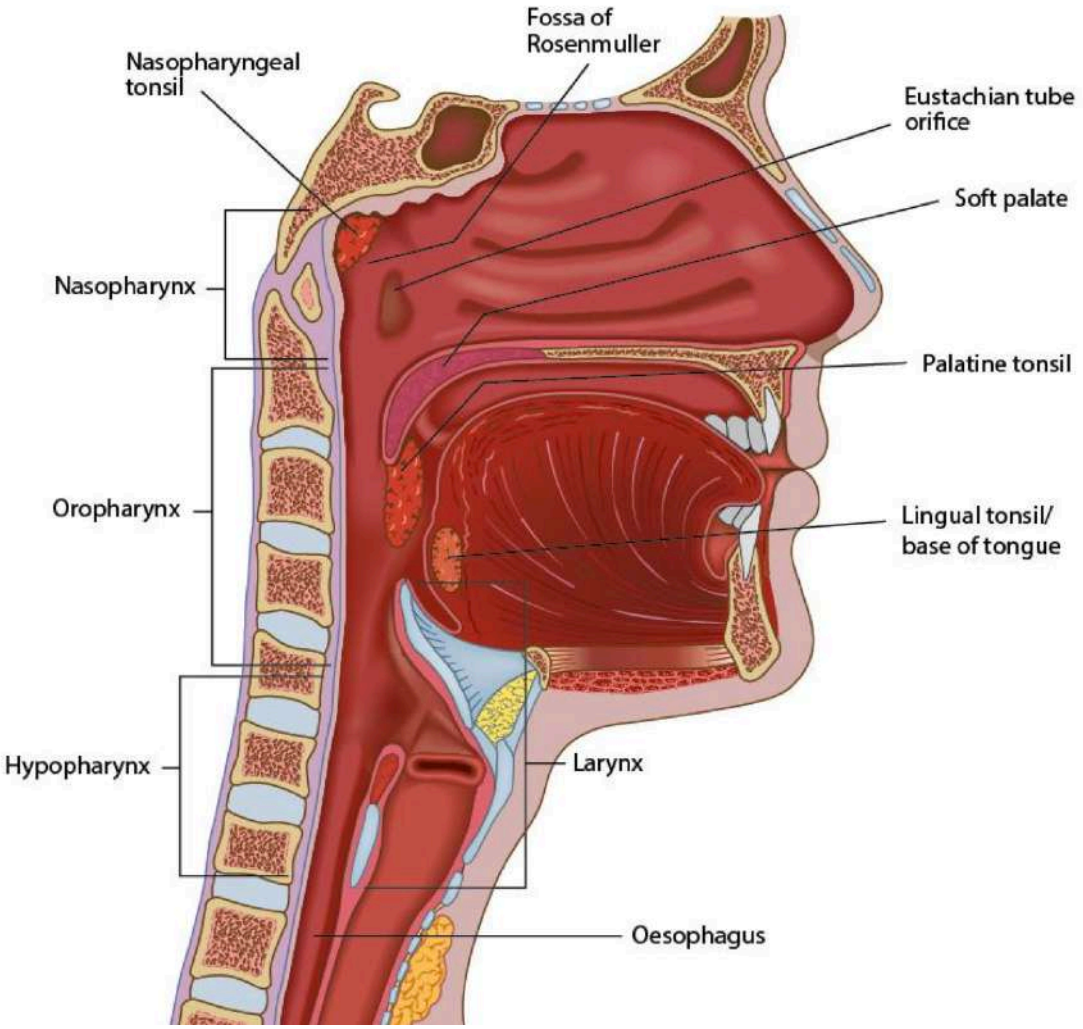

Figure 4. Pharyngeal structure and subsites (ICCR - International Collaboration on Cancer Reporting)

1.3.3 LARYNGEAL CANCER

The larynx is divided into three subsites partially based on embryologic development (Figure 5). The supraglottis extends from the tip of the epiglottis to an artificial horizontal plane extending bilaterally across the apex of the laryngeal ventricles. It includes five separate subsites, the epiglottis, false vocal folds, ventricles, arytenoids, and aryepiglottic folds. The glottis includes the true vocal folds and anterior and posterior commissures. The subglottis extends below the true vocal folds to the lower margin of the cricoid cartilage.

SCCs represent up to 98% of laryngeal cancers. It occurs more commonly in men than in women (5.8 cases per 100,000 vs 1.2 per 100,000, respectively) (203) associated with racial disparities: African Americans present it at a younger age, having a higher incidence and mortality compared with Caucasians (204,205,206). Approximately 60% of patients present with advanced (stage III or IV) disease at diagnosis (207). Several risk factors have been implicated in the pathogenesis of laryngeal cancer. The most significant of them are tobacco and alcohol consumption. Tobacco use has been shown to have a linear association with the development of laryngeal cancer, with a risk for smokers that is 10 to 15 times higher than the risk for non-smokers, and the heaviest smokers have as much as a 30 times greater risk (208,209). Research has also demonstrated a linear relationship between the amount of alcohol consumed and the risk of laryngeal cancer (210). Moreover, alcohol and tobacco exhibit a multiplicative effect on the risk of laryngeal cancer (211). Exposure to several other environmental factors is thought to potentially increase the risk of laryngeal SCC, such

as asbestos, polycyclic aromatic hydrocarbons, and textile dust (212,213). In addition, the role that both gastroesophageal and laryngopharyngeal reflux play in the disease process is still controversial and under investigation (214,215). Although HPV is a proven major driver of oropharyngeal cancers , it was initially thought that HPV did not play a role in laryngeal cancer. However, new research is emerging that demonstrates the presence of HPV and/or the surrogate marker p16 in a minority of laryngeal tumors, although the biologic and prognostic relevance of this finding is as of yet unclear. It is estimated that the prevalence of HPV ranges from 20% to 30% in laryngeal cancer; however, this percentage varies widely between studies, underlining that more work is needed to determine the clinical relevance of HPV/p16-positive status in laryngeal cancer (216,217,218).

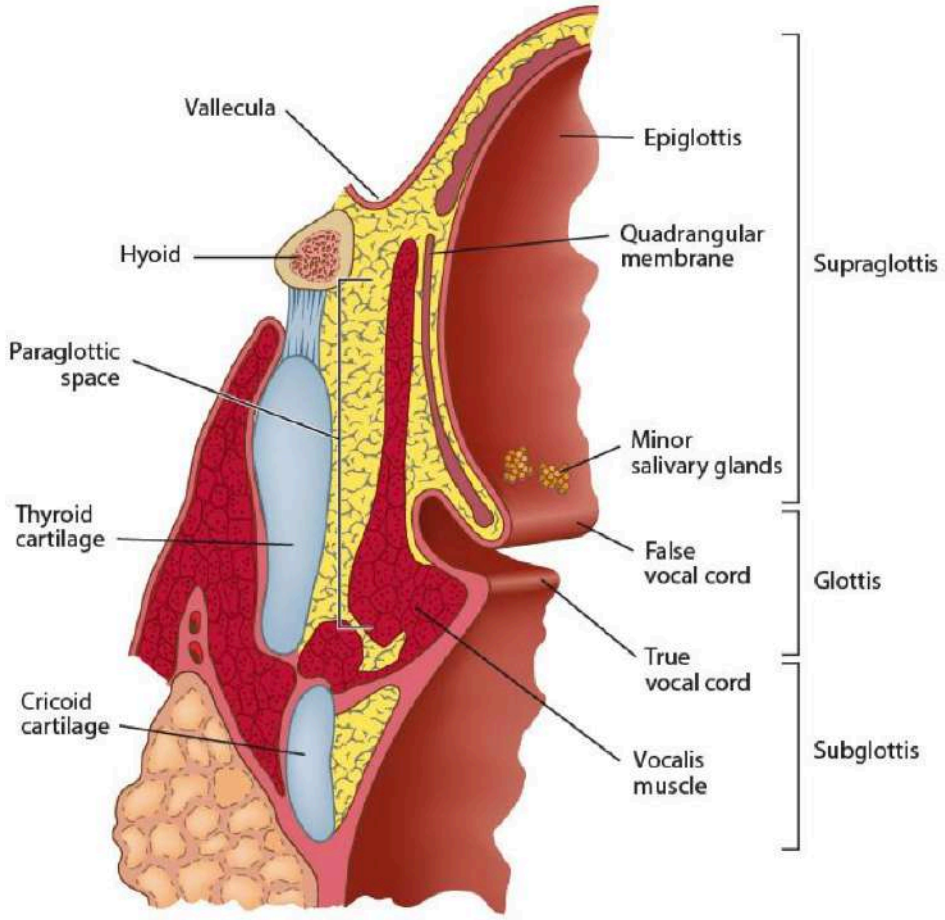

Figure 5. Laryngeal structure and subsites (ICCR - International Collaboration on Cancer Reporting)

1.4 OBJECTIVE OF THE STUDY

To date, the clinical significance of the expression of Notch pathway components has only barely been studied using small HNSCC patient cohorts, and raising contradictory results (219,220). Given the strong evidence pointing to a critical role for Notch signaling in HNSCC pathogenesis, efforts aimed at elucidating clinically relevant alterations of this pathway are fundamental to identify novel biomarkers as well as the key components to successfully develop novel molecular targeted therapies.

The aim of this study was to investigate the expression status of two main members of the pathway, NOTCH1 and HES1, together with the NOTCH1 targets p21 (WAF1/Cip1/CDKN1A) and Cyclin D1 using a large cohort of surgically treated HPV-negative HNSCC patients, and to ascertain the prognostic role of Notch pathway activation in this cancer type.

Our specific objectives were:

1. To evaluate the protein expression of NOTCH1,HES1, and two Notch targets p21 (WAF1/Cip1/CDKN1A) and Cyclin D1 in a large series of HPV-negative HNSCC compared to normal tissue counterparts.

2. To analyze the correlations between NOTCH1 and HES1 expression and the targets p21 and Cyclin D1.

3. To determine the prognostic relevance of NOTCH1 and HES1 expression individually or in combination, and also in relation to the downstream targets p21 and Cyclin D1.

II. MATERIALS AND METHODS

2.1 PATIENTS AND TISSUE SPECIMENS

Surgical tissue specimens from 382 patients who were diagnosed of HNSCC at the Hospital Universitario Central de Asturias between 1991 and 2010 were retrospectively collected, in accordance to approved institutional review board guidelines. All experimental protocols were approved by the Institutional Ethics Committee of the Hospital Universitario Central de Asturias and by the Regional CEIm from Principado de Asturias (date of approval May 14th, 2019; approval number: 141/19, for the project PI19/00560). Informed consent was obtained from all patients. All patients were surgically treated for a single primary tumor and received no treatment prior to surgery. No patient had distant metastasis at the time of diagnosis. Clinical, demographic and follow-up data were collected from the medical records. The tumors were staged according to the TNM system of the International Union Against Cancer (7th Edition).

Tissue sections were obtained from archival, formalin-fixed paraffin-embedded (FFPE) blocks provided by the Principado de Asturias BioBank (PT17/0015/0023), included into the Spanish National Biobanks Network. The histological diagnosis was confirmed by an experienced pathologist. Three 1-mm cylinders were taken from each FFPE tumor block to construct tissue microarray (TMA) blocks (221), containing a total of 249 oropharyngeal, 65 hypopharyngeal and 68 laryngeal SCC. In addition, each TMA included three cores of normal epithelium (pharyngeal and laryngeal mucosa obtained from non-oncologic patients).

Only 10 (3%) HPV-positive tumors (8 oropharyngeal, 1 laryngeal and 1 hypopharyngeal) were detected using p16-immunohistochemistry, high-risk HPV DNA detection by *in situ* hybridization and genotyping by GP5+/6+-PCR, as previously reported (221,222). To have a homogeneous cohort in terms of HPV, those cases were excluded from the subsequent analyses.

2.2 IMMUNOHISTOCHEMISTRY

The TMAs were cut into 3-µm sections and dried on Flex IHC microscope slides (Dako). 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) using the following monoclonal antibodies for 30 min:

- Anti-NOTCH1 (clone D1E11, Cell Signaling) at 1:400 dilution.

- Anti-HES1(clone D6P2U, Cell Signaling) at 1:200 dilution.

- Anti-Cyclin D1 (clone DCS-6,Santa Cruz Biotechnology, Inc. sc-20044) at 1:100 dilution.

- Anti-p21 monoclonal antibody (clone 4D10; Leica Biosystems NCL- L-WAF-1) at 1:10 dilution.

Immunodetection was carried out using the Dako EnVision Flex + Visualization System (Dako) and diaminobenzidine as chromogen. Counterstaining with hematoxylin was the final step.

Immunohistochemistry staining was evaluated by two independent observers, blinded to clinical data. For NOTCH1 and HES1 expression, a semiquantitative scoring system based on staining intensity and the percentage of stained tumor cells was applied. NOTCH1 and HES1 immunostaining was respectively scored from 0 to 2 if 0% to 10%, 11% to 50%, and > 50% of tumor cells showed either membranous NOTCH1 staining or nuclear HES1 staining. The staining intensity was scored from 0 to 2 scale (0 = negative, 1 = weak, 2 = strong). The raw data were then converted to a Immunoreactive Score (IRS) by multiplying the quantity and staining intensity scores. Theoretically, the scores could range from 0 to 4. For statistical purposes, these scores were dichotomized as negative expression (score 0) *versus* positive expression (scores 1–4). Since the NOTCH1 antibody recognizes both

the full-length and the intracellular domain NICD, according to NOTCH1 function, staining into the nucleus was also separately evaluated as a surrogate of NOTCH1 activation. Nuclear NOTCH1 staining was scored in a binary fashion, as positive *versus* negative depending on the presence or absence of stained tumor cells, respectively.

For p21 and Cyclin D1 proteins, nuclear staining was evaluated and dichotomized as negative expression (0–10% stained cells) *versus* positive expression (> 10% stained tumor cells). A high level of inter-observer concordance (> 90%) was achieved.

2.3 IN SILICO ANALYSIS OF NOTCH1 AND HES1 mRNA EXPRESSION USING THE CANCER GENOME ATLAS (TCGA) HNSCC DATABASE

mRNA expression analysis was performed using transcriptomic data from a TCGA cohort of 530 HNSCC patients (223). mRNA levels of NOTCH1 and HES1 were compared between primary tumors (n = 520) and normal tissue samples (n = 44) using the UALCAN web tools (http://ualcan.path.uab.edu/) (224). Correlations with clinicopathological parameters and patient survival were assessed in a subset of 445 HNSCC patients with available data using the platform cBioPortal (http://cbioportal.org/) (225).

2.4 STATISTICAL ANALYSIS

Chi-squared and Fisher's exact tests were used for comparison between categorical variables. For time-to-event analysis, Kaplan-Meier curves were plotted. Differences between survival times were analyzed by the log-rank method. Cox proportional hazards models were utilized for univariate and multivariate analysis. 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.

III. RESULTS

3.1 PATIENTS CHARACTERISTICS

The main clinical and pathological features are summarized in Table 1. Only 16 patients were women, and the mean age was 59 years (range 30 to 86 years). 360 patients were habitual tobacco smokers, 198 moderate (1–50 pack-year) and 162 heavy (> 50 pack-year), and 341 were alcohol drinkers. Twenty tumors were stage I, 24 stage II, 64 stage III, and 264 stage IV. The series included 147 well, 148 moderately and 76 poorly differentiated tumors. 230 (62%) of 372 patients received postoperative radiotherapy. The mean and median follow-up times were 34.66 and 21.5 months, respectively, for the whole series. Tumor recurrence was found in 224 (60.2%) cases. The mean and median follow-up times were respectively 71 and 67 months for the patients without recurrence, and 18 and 13.5 months for the patients died of tumor. The five-year disease-specific (DSS) and overall survival (OS) rates were 39% and 29.7%, respectively.

Table 1. Clinicopathological characteristics of the 372 HNSCC patients selected for study

Characteristic	Number of cases (%)
Gender	
- Male	356 (95.7%)
- Female	16 (4.3%)
Localization	
- Larynx	67 (18%)
- Oropharynx	241 (64.8%)
- Hypopharynx	64 (17.2%)
Tumor classification	
- T1	38 (10.2%)
- T2	77 (20.7%)
- T3	125 (33.6%)
- T4	132 (35.5%)
Nodal classification	
- N0	103 (27.7%)
- N1	46 (12.4%)
- N2	183 (49.2%)
- N3	40 (10.8%)
Disease Stage	
- I	20 (5.4%)
- II	24 (6.5%)
- III	64 (17.2%)
- IV	264 (71%)
Histological grade	
- G1	147 (39.5%)
- G2	148 (39.8%)
- G3	76 (20.4%)
- Unknown	1 (0.3%)
Postoperative Radiotherapy	
- No	142 (38.2%)
- Yes	230 (61.8%)
Recurrence	
- No	148 (39.8%)
- Local	57 (15.3%)
- Regional	30 (8.1%)
- Distant metastasis (DM)	67 (18%)
- Locoregional (LR)	35 (9.4%)
- LR+DM	35 (9.4%)
Follow-up	
- Alive without disease	88 (23.7%)
- Dead by the tumor	202 (54.3%)
- Dead by other causes	63 (16.9%)
- Lost to follow-up	19 (5.1%)

3.2 NOTCH1 AND HES1 EXPRESSION IN NORMAL EPITHELIA AND HNSCC SPECIMENS

In normal laryngeal and pharyngeal epithelium, NOTCH1 showed a weak cytoplasmic and nuclear staining in basal and suprabasal cell layers, with absence of expression in the most superficial layers (Figure 6A). HES1 expression was also detected in normal epithelium, with a nuclear staining pattern in suprabasal cell layers (Figure 7A).

Membranous NOTCH1 expression was successfully evaluated in 324 out of 372 tumor samples. Only cases with adequate tumor tissue integrity and/or representability were considered, and the staining was scored. Thus, 127 tumors (39%) showed negative expression (IRS = 0), 68 (21%) low expression (IRS = 1), 82 (25%) moderate expression (IRS = 2), and 47 (15%) strong expression (IRS = 4). Membranous NOTCH1 expression was also concomitantly accompanied by cytoplasmic staining in some cases. Representative examples of NOTCH1 staining are shown in Figure 6B-E. Nuclear NOTCH1 expression was positive in 91 (28%) of the 324 evaluable tumor samples (Figure 6F).

There was a significant positive correlation between nuclear and membranous NOTCH1 expression (Spearman's Rho = 0.502, $P < 0.001$). All the cases with positive nuclear staining showed some degree of membranous staining.

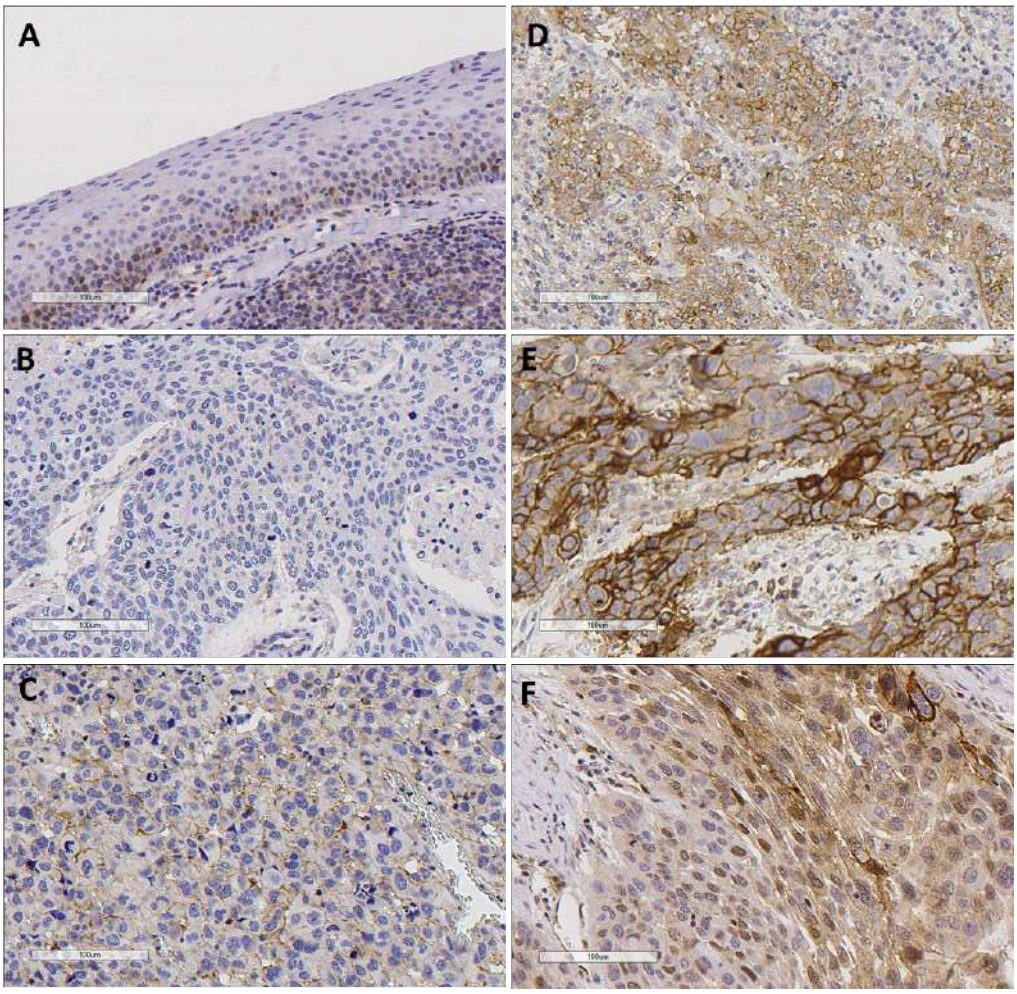

Figure 6. Immunohistochemical analysis of NOTCH1 expression in HNSCC tissue specimens. Representative images of NOTCH1 expression in normal epithelium (A), a tumor showing negative NOTCH1 expression (B), examples of tumors with low (C), moderate (D), or strong membranous NOTCH1 expression (E), and a tumor with nuclear NOTCH1 expression (F). Scale bars, 100 μm

Immunohistochemical analysis of HES1 expression in HNSCC samples showed a nuclear pattern, with negative expression (IRS = 0) in 110 tumors (33%), low expression (IRS = 1) in 81 (24%), moderate expression (IRS = 2) in 86 (26%), high expression (IRS = 4) in 57 (17%), and 38 non-evaluable cases (Figure 7B-C).

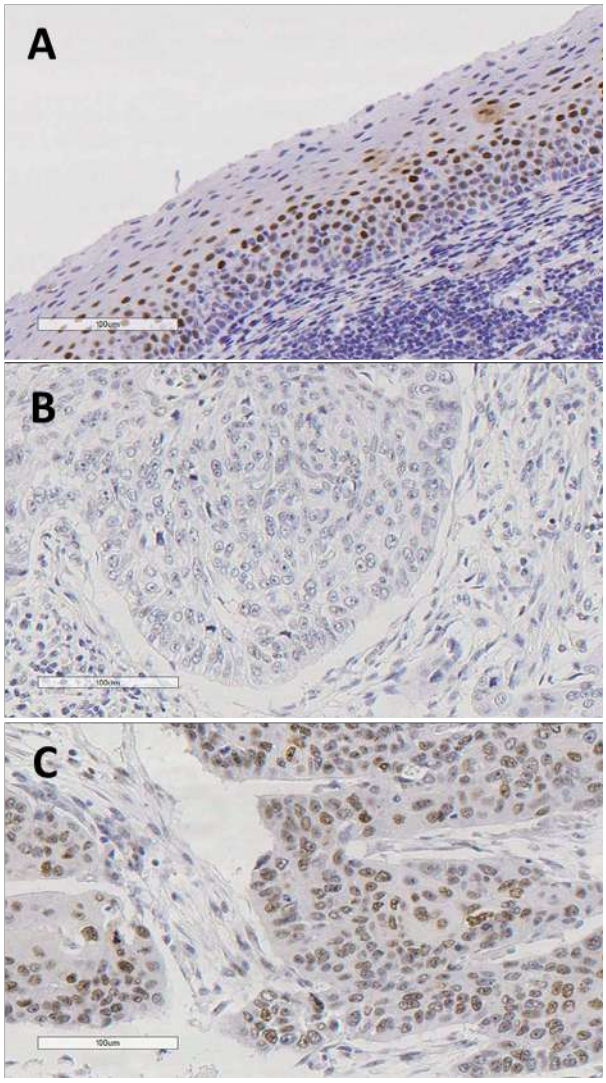

Figure 7. Immunohistochemical analysis of HES1 expression in HNSCC tissue specimens. Representative images of HES1 expression in normal epithelium (A), a tumor showing negative HES1 expression (B), and a tumor with nuclear HES1 expression (C). Scale bars, 100 μm

We found that membranous and nuclear NOTCH1 expression were both significantly correlated with nuclear HES1 expression (Spearman's Rho = 0.259, $P < 0.001$, and Spearman's Rho = 0.241, $P < 0.001$, respectively; Table 2).

Table 2. Correlations between the protein expression of NOTCH1 and the targets HES1, p21 and Cyclin D1

	HES1 expression			p21 expression			Cyclin D1 expression		
NOTCH1 expression	**Negative**	**Positive**	***P*†**	**Negative**	**Positive**	***P*†**	**Negative**	**Positive**	***P*†**
Membranous:									
- Negative	**58 (47.5%)**	**64 (52.5%)**	**<0.001**	**55 (45%)**	**67 (55%)**	**0.030**	**41 (32%)**	**86 (68%)**	**0.21**
- Positive	**44 (22.6%)**	**150 (77.4%)**		**59 (32.5%)**	**122 (67.5%)**		**50 (26%)**	**144 (74%)**	
Nuclear:									
- Negative	**89 (39%)**	**137 (61%)**	**<0.001**	**90 (41%)**	**128 (59%)**	**0.036**	**72 (31%)**	**159 (69%)**	**0.075**
- Positive	**13 (14%)**	**77 (86%)**		**24 (28%)**	**61 (72%)**		**19 (21%)**	**71 (79%)**	
Total cases	**102**	**214**		**114**	**189**		**91**	**230**	

† Fisher's exact test.

In addition, the expression of two additional NOTCH1 targets that are key cell cycle regulators, Cyclin D1 and p21, was evaluated by immunohistochemistry and correlated with NOTCH1 expression. Cyclin D1 expression was successfully evaluated in 360 HNSCC samples, being positive in 258 (72%) tumors. Positive p21 expression was detected in 204 (63%) out of 324 evaluable tumor samples (Figure 8). Membranous and

nuclear NOTCH1 expression were both significantly correlated with p21 expression, but not with Cyclin D1 expression (Table 2).

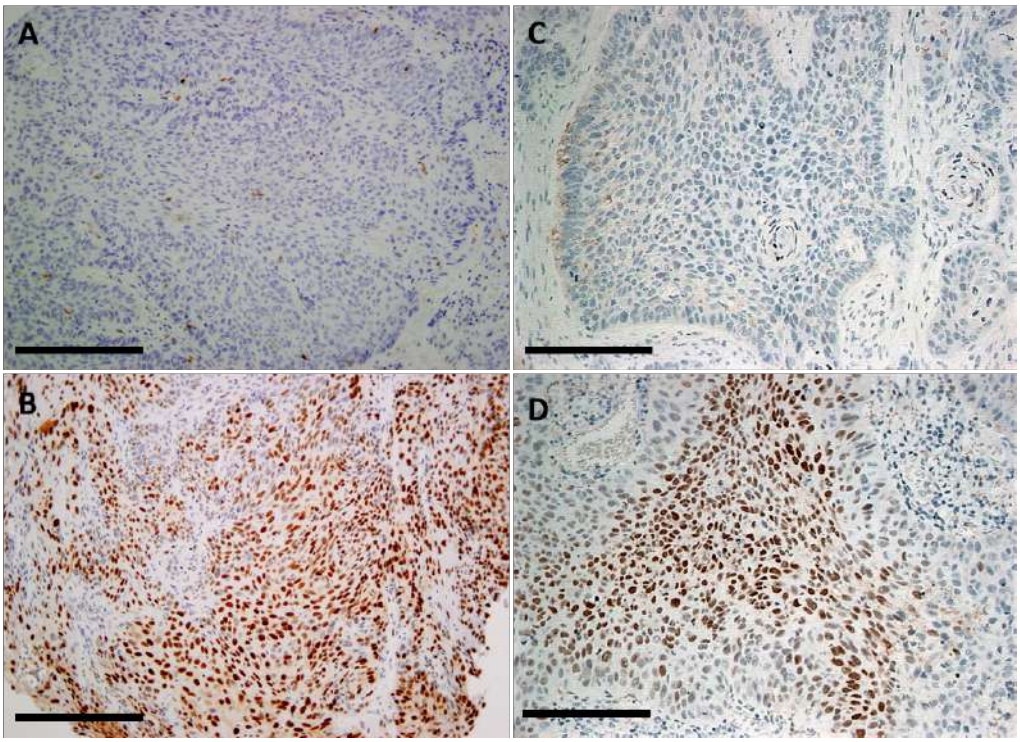

Figure 8. Immunohistochemical analysis of p21 and Cyclin D1 expression in HNSCC specimens. Representative examples of tumors showing negative (A) and positive nuclear p21 staining (B), and negative (C) and positive Cyclin D1 staining (D). Scale bar 100 μm.

On the other hand, *in silico* data set analysis of the TCGA HNSCC cohort (n = 520) showed that *NOTCH1* mRNA levels significantly increased in primary tumors compared to normal tissue samples (P = 0.01) (Figure 9A), whereas *HES1* mRNA levels were similar in tumors and the corresponding normal counterparts (P = 0.94) (Figure 9B). As observed at protein level, *NOTCH1* and *HES1* mRNA levels were positively correlated in the TCGA cohort (Spearman's Rho = 0.182, P < 0.001)

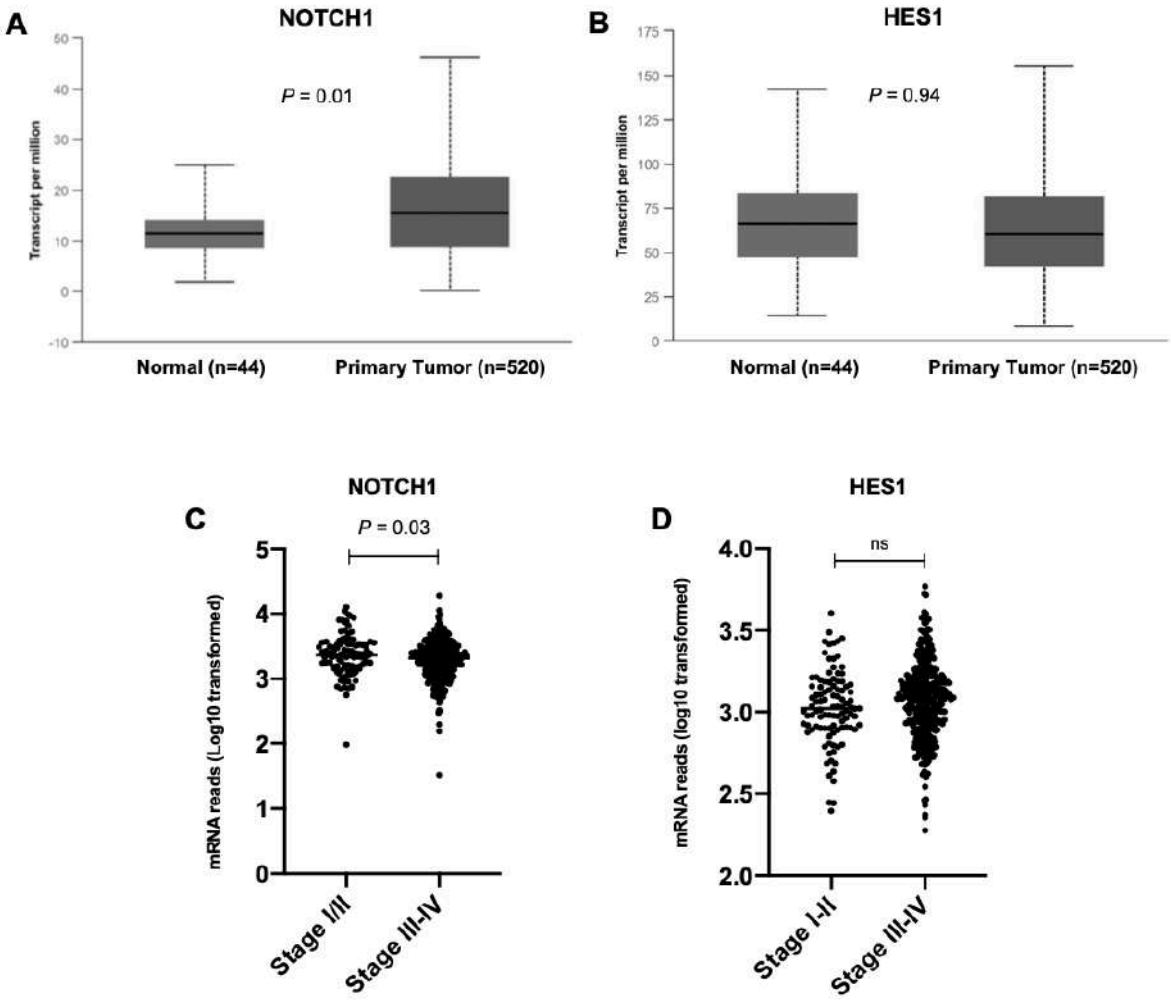

Figure 9. *In silico* analysis of NOTCH1 and HES1 mRNA expression in the TCGA cohort of 530 HNSCC patients. mRNA levels of NOTCH1 (A) and HES1 (B) were compared in normal and tumor samples using UALCAN online resources (http:// ualcan.path.uab.edu/). NOTCH1 (C) and HES1 (D) mRNA levels (RSEM RNAseqV2, log10 transformed) were analyzed in relation to the disease stage and plotted using GraphPad, with *P* values by unpaired t-test.

3.3 CORRELATIONS OF NOTCH1 AND HES1 EXPRESSION WITH CLINICOPATHOLOGICAL PARAMETERS

The expression of both nuclear and membranous NOTCH1 was significantly associated with early stages (I-II) (P = 0.022 and P = 0.007, respectively; Table 3). Nuclear HES1 expression was associated with early pT classification, and with a hypopharyngeal primary site (P = 0.042 and P = 0.001, respectively; Table 3). No other significant correlations of NOTCH1 and HES1 expression with clinicopathological parameters were observed (Table 3).

Table 3. Associations of NOTCH1 and HES1 expression with clinicopathological features.

Characteristic	No. Cases for NOTCH	Membranous NOTCH1 Expression	*P*	Nuclear NOTCH1 Expression (%)	*P*	No. Cases for HES	Nuclear HES1 Expression (%)	*P*
Location								
Oropharynx	216	124 (57)	0.175 #	63 (29)	0.575 #	229	143 (62)	0.001 #
Hypopharynx	54	35 (65)		12 (22)		52	46 (88)	
Larynx	54	38 (70)		16 (30)		53	35 (66)	
pT Classification								
T1-T2	95	66 (70)	0.098 #	32 (34)	0.183 #	97	77 (75)	0.042 #
T3	113	67 (59)		33 (29)		114	81 (68)	
T4	116	64 (55)		26 (22)		123	74 (59)	
pN Classification								
N0	87	61 (68)	0.202 †	30 (34)	0.127 †	85	52 (61)	0.184 †
N1-3	237	143 (59)		61 (26)		249	172 (69)	
Stage								
I-II	34	27 (79)	0.022 #	17 (50)	0.007 #	31	25 (81)	0.205 #
III	55	37 (67)		17 (31)		56	35 (62)	
IV	235	133 (57)		57 (24)		247	164 (66)	
Degree of differentiation								
Well	129	70 (54)	0.128 #	38 (25)	0.882 #	129	87 (67)	0.780 #
Moderately	130	83 (64)		36 (28)		135	88 (65)	
[illegible]	65	44 (68)		17 (26)		70	49 (70)	
Recurrence								
No	121	87 (72)	0.002 †	39 (32)	0.204 †	124	83 (67)	1†
Yes	203	110 (54)		52 (26)		210	141 (67)	
Total cases	324	197 (61)		91 (28)		334	224 (67)	

Chi-squared and † Fisher's exact tests.

Similarly, analysis of the TCGA HNSCC dataset (N = 520) further confirmed that *NOTCH1* mRNA levels were also significantly higher in early disease stages I-II (unpaired *t* test, *P* = 0.03) (Figure 9C), while *HES1* mRNA levels did not show a significant association with stage (unpaired *t* test, *P* = 0.06) (Figure 9D).

3.4 RELATIONSHIP OF NOTCH1 AND HES1 EXPRESSION WITH TUMOR RECURRENCE AND PATIENT SURVIVAL

A significant inverse correlation was found between membranous NOTCH1 expression and tumor recurrence (*P* = 0.002; Table 3). Similarly, nuclear NOTCH1 expression was also more frequent in non-recurrent tumors, although the differences were not significant (*P* = 0.204; Table 3). No correlation between HES1 expression and tumor recurrence was observed (*P* = 1; Table 3).

Patients harboring either membranous or nuclear NOTCH1-positive tumors concordantly showed significantly improved disease-specific survival (DSS) (*P* < 0.001 and *P* = 0.004, respectively) and overall survival (OS) (*P* < 0.001 and *P* = 0.005, respectively) (Figure 10A-D). Notably, significant differences in survival rates were also observed among the different levels of membranous NOTCH1 expression in the tumors, with higher IRS scores showing better survival rates (Figure 11).

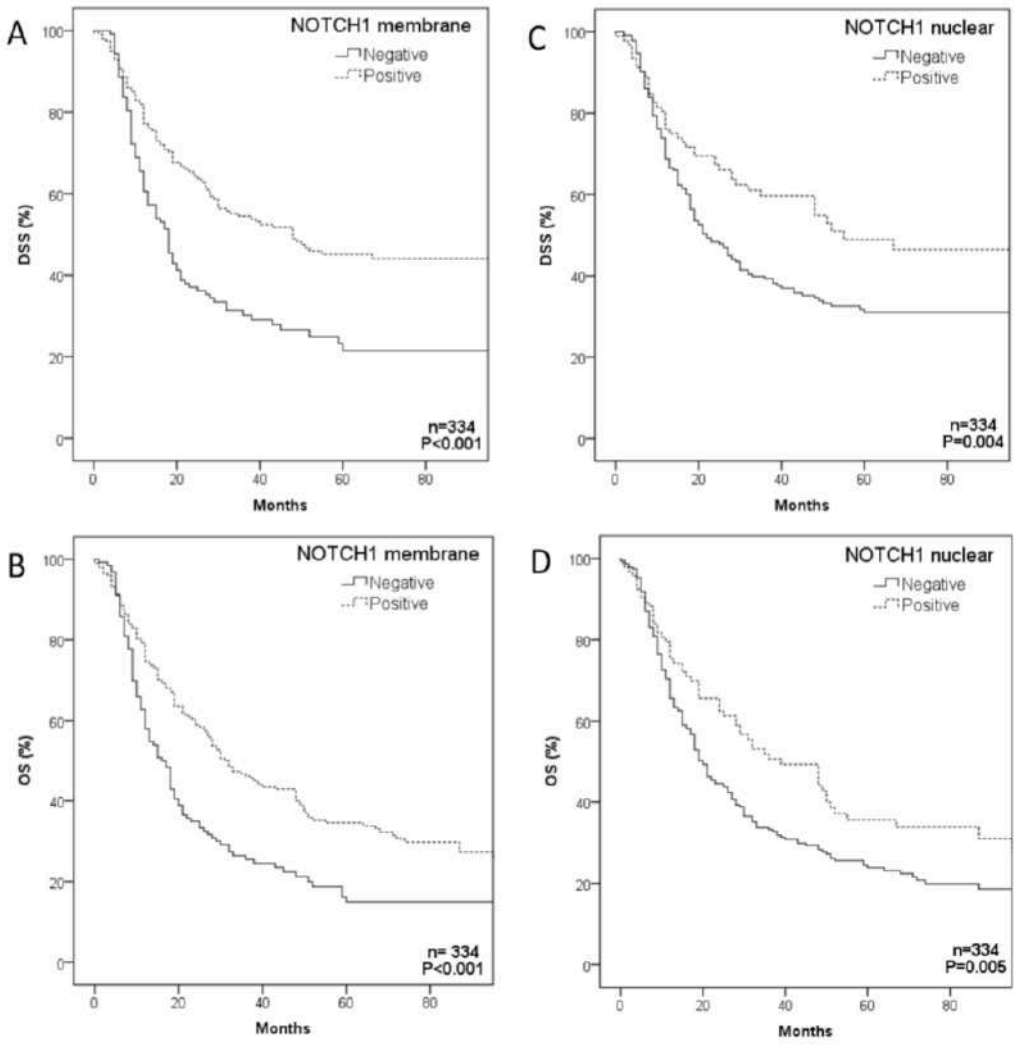

Figure 10 Kaplan-Meier disease specific (DSS) and overall survival (OS) curves categorized according to the expression of membranous NOTCH1 (A, B) and nuclear NOTCH1 (C, D). *P* values were estimated using the Log-rank test.

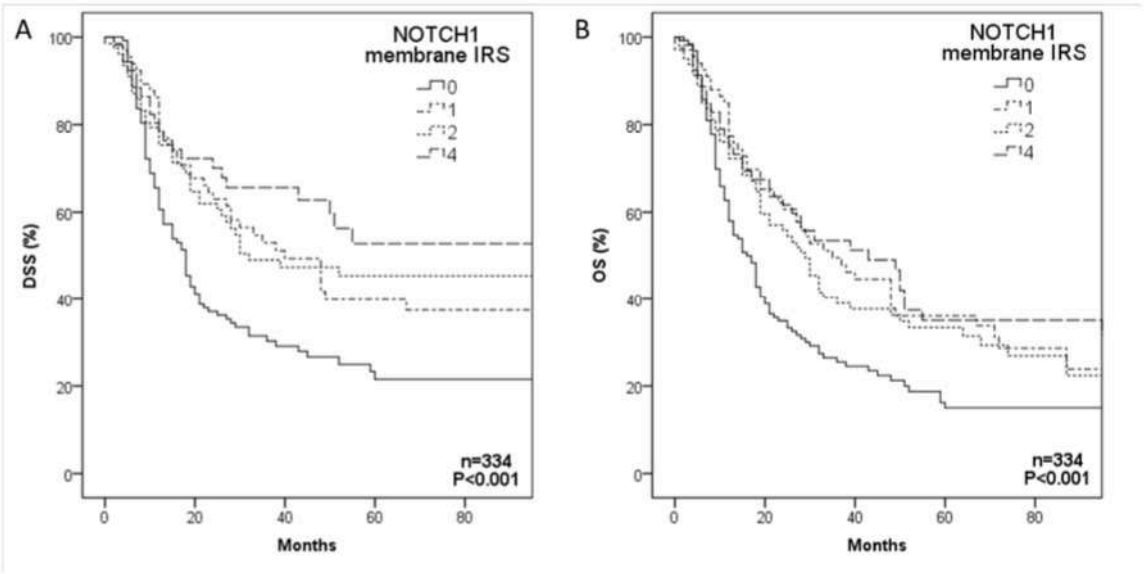

Figure 11. Kaplan-Meier disease specific (DSS) and overall survival (OS) curves, categorized according to the IRS scores (1 to 4) for membranous NOTCH1 expression. *P* values were estimated using the Log-rank test.

Moreover, membranous NOTCH1 expression was associated with better DSS and OS irrespective of the tumor location. Differences were not statistically significant for the laryngeal subgroup of patients, probably due to an insufficient number of cases (Table 4).

Table 4. Five-year disease-specific survival (DSS) and overall survival (OS) according to membranous NOTCH1 expression.

Localization	NOTCH1 positive	NOTCH1 negative	Hazard ratio (95% CI)	*P*
Oropharynx (n=256)				
- DSS	59%	33%	0.536 (0.374-0.766)	0.001
- OS	54%	32%	0.631 (0.461-0.863)	0.003
Hypopharynx (n=54)				
- DSS	30%	0%	0.446 (0.228-0.876)	0.019
- OS	26%	0%	0.424 (0.224-0.803)	0.008
Larynx (n=54)				
- DSS	63%	35%	0.527 (0.227-1.223)	0.129
- OS	53%	32%	0.612 (0.282-1.332)	0.216

DSS: Disease-specific survival; OS: Overall survival; 95% CI: 95% Confidence Interval

By contrast, HES1 expression did not correlate with DSS nor OS (*P* = 0.63 and *P* = 0.67, respectively) (Figure 12A-B). p21-positive cases also showed better DSS although the differences did not reach statistical significance (*P* = 0.058) (Figure 12C-D), whereas Cyclin D1 expression was not associated with survival (*P* = 0.5 and *P* = 0.62 for DSS and OS, respectively) (Figure 12E-F). Correlations between *NOTCH1* and *HES1* mRNA levels and the patients' survival were also further assessed using the TCGA HNSCC data. Consistent with our IHC protein data, high *NOTCH1* mRNA levels were found to associate with a better survival almost reaching significance (*P* = 0.062) (Figure 13A), while *HES1* mRNA levels showed no impact on patients' survival (*P* = 0.56) (Figure 13B).

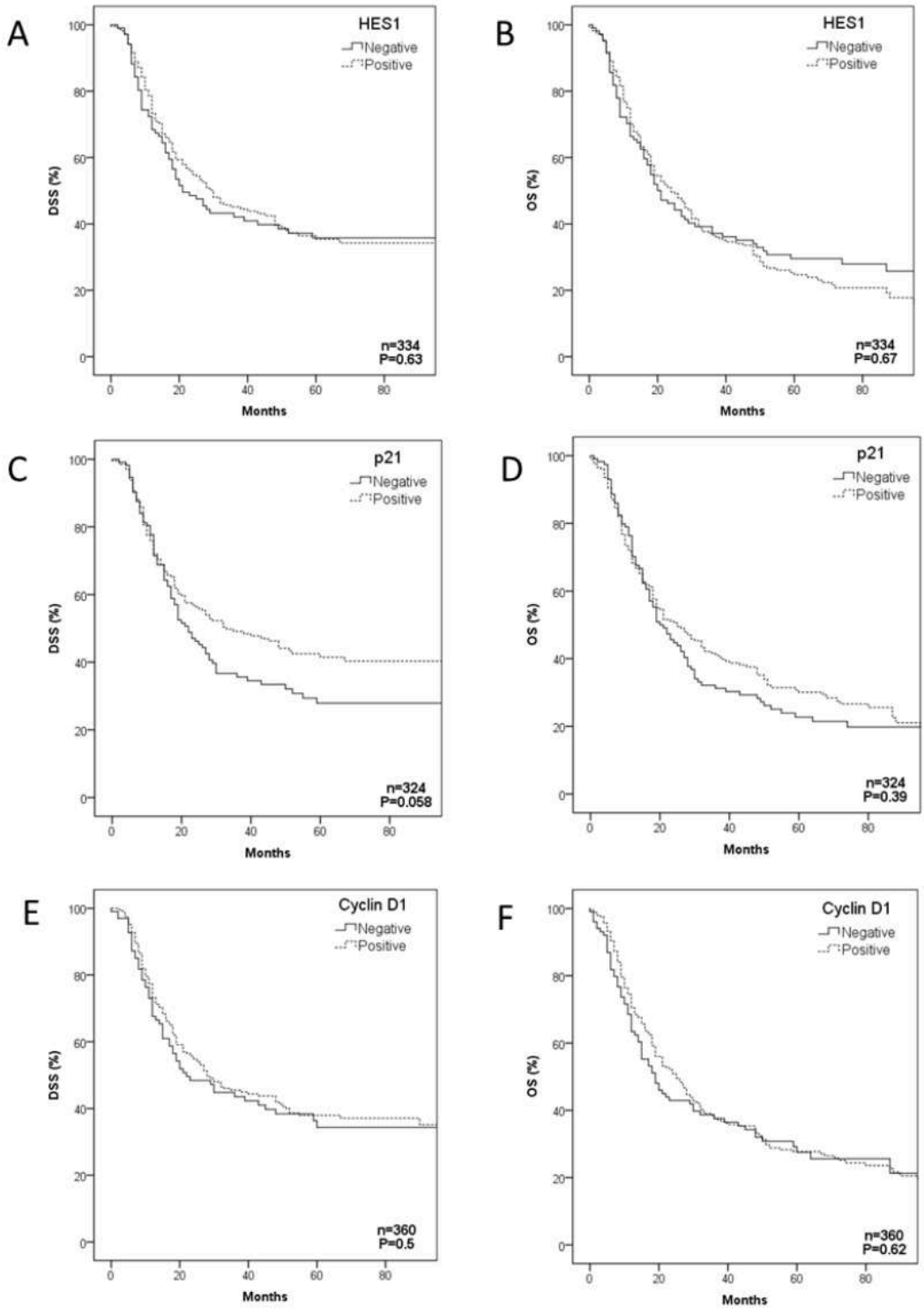

Figure 12. Kaplan-Meier disease specific (DSS) and overall survival (OS) curves, categorized according to HES1 (A, B), p21 (C, D), and Cyclin D1 (E, F) expression. *P* values were estimated using the Log-rank test.

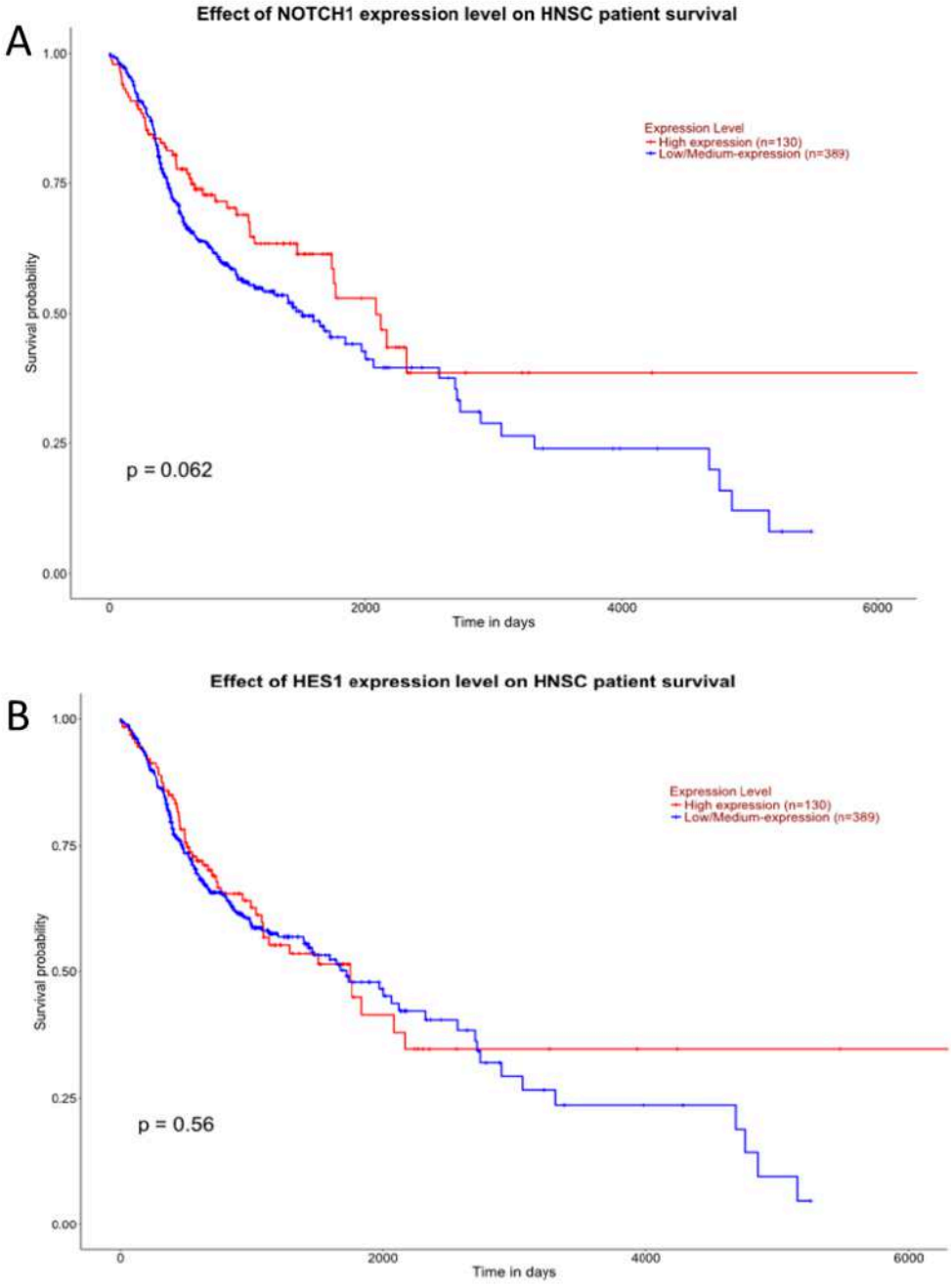

Figure 13. Overall survival curves of 519 HNSCC patients from the TCGA cohort categorized according to NOTCH1 (A) and HES1 (B) mRNA levels (RSEM RNAseqV2) dichotomized as high mRNA levels (above the median) *versus* low mRNA levels (below the median). *P* values were estimated using the Log-rank test.

In an attempt to further investigate the impact of Notch signaling activation on patient prognosis, the effect of combined expression of membranous NOTCH1 and HES1 on survival was explored. We observed that double-positive cases (NOTCH1+/HES1+) clearly exhibited a significantly improved DSS (P = 0.002) and OS (P = 0.03), with single positive cases (NOTCH1+/HES1−, NOTCH1−/HES1+) and double-negative cases (NOTCH1−/HES1−) having similar survival rates (Figure 14A-B).

Furthermore, when examining the combined effect of membranous NOTCH1, HES1 and p21 expression, we found that only the triple positive cases (NOTCH1+/HES1+/p21+) were significantly associated with better DSS (P < 0.001) and OS (P = 0.004) (Figure 14C-D). These findings reinforce that Notch pathway activation correlates with a better prognosis in HNSCC patients.

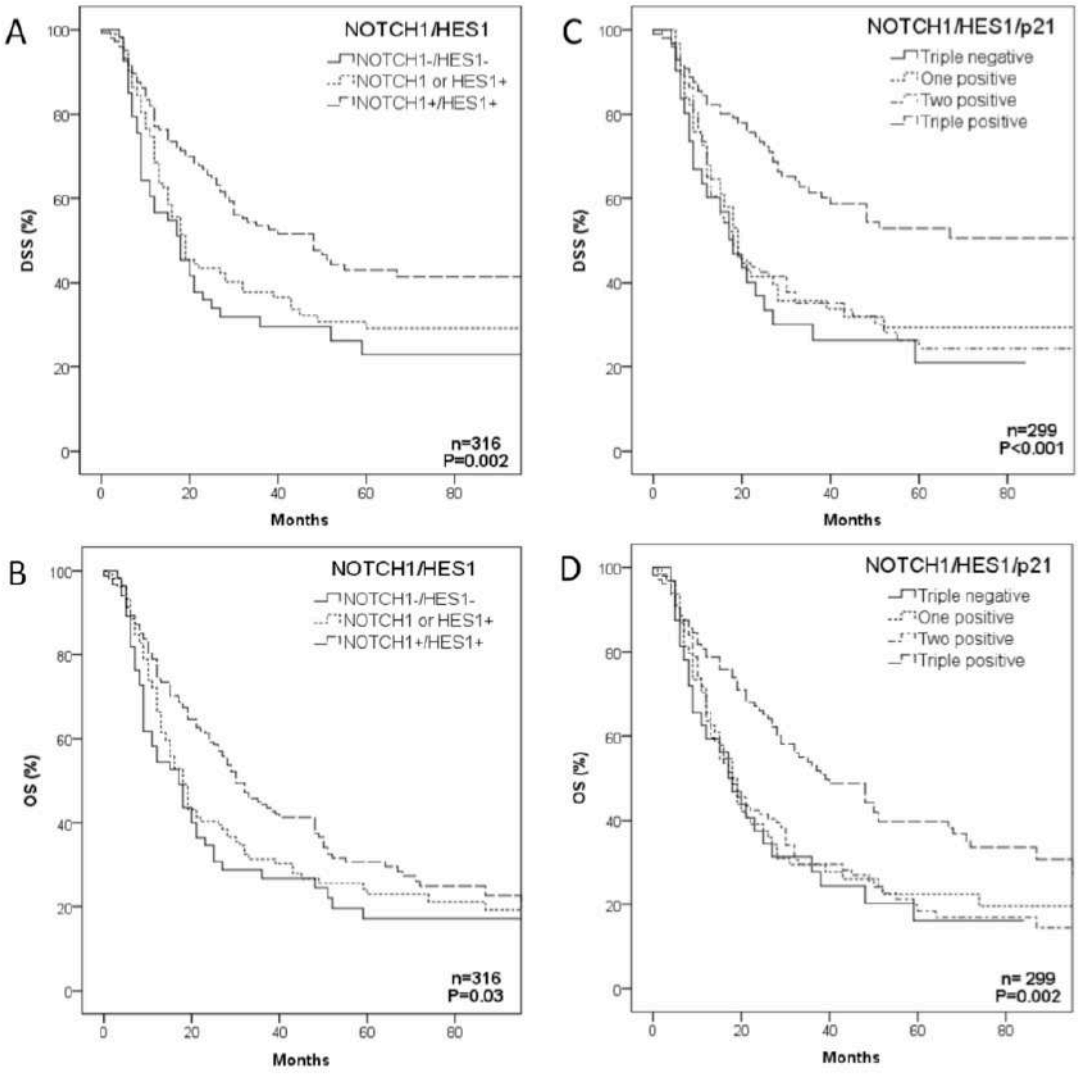

Figure 14. Kaplan-Meier disease specific (DSS) and overall survival (OS) curves according to the combined expression of NOTCH1 and HES1 proteins (A, B), and the combined expression of NOTCH1, HES1 and p21 proteins (C, D). *P* values were estimated using the Log-rank test.

Multivariate Cox analysis, including tumor localization, T classification, N classification, degree of differentiation, membranous NOTCH1 expression, and nuclear NOTCH1 expression showed that the parameters independently associated with a worse DSS were T4 classification, N+ classification and poorly differentiation (Table 5), whereas membranous NOTCH1 expression was an independent predictor of better DSS (HR = 0.554; 95% IC 0.412–0.745; P < 0.001; Table 5). Similarly, T4 and N+ classifications, and a pharyngeal location of the tumor were independently associated with a worse OS, and membranous NOTCH1 expression with a better OS (HR = 0.640; 95% CI 0.491–0.835; P = 0.001; Table 5).

Table 5. Multivariate Cox analysis for disease-specific survival and overall survival

Parameter	Disease-specific survival		Overall survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Localization				
- Larynx	1		1	
- Oropharynx	1.467	0.102	1.603	0.022
- Hypopharynx	(0.927-2.322)	0.067	(1.069-2.404)	0.022
pT classification				
- T1-2	1		1	
- T3	1.203	0.362	1.073	0.685
- T4	(0.809-1.789)	0.001	(0.765-1.504)	0.002
pN classification				
- N0	1		1	
- N+	2.310	<0.001	1.759	0.001
Degree of differentiation				
- Well	1		1	
- Moderately	0.99	0.952	1.053	0.729
	(0.708-1.384)	0.043	(0.785-1.414)	0.085
Membranous NOTCH1				
- Negative	1		1	
	0.554	<0.001	0.640	0.001
Nuclear NOTCH1				
- Negative	1		1	
- Positive	1.050	0.815	1.009	0.960

HR: Hazard Ratio; 95% CI: 95% Confidence Interval

IV. DISCUSSION

There are evidences of both oncogenic and tumor suppressor roles for Notch signaling in different cancers. Notch activity has been involved in tumor progression by activating transcription factors promoting cell survival, motility and angiogenesis (106,226). Moreover, activation of the Notch pathway regulates the expression of target genes, such as HES1, which has been implicated in stemness, metastasis and multi-drug resistance (227). Several other Notch targets are well- known for their relevant roles in tumorigenesis, such as Cyclin D1, c-MYC, and NF-κB (226). Nevertheless, besides this pro-tumorigenic activity of Notch signaling, increasing evidence has also pointed out to a tumor suppressive role for this pathway. Thus, deletions and inactivating mutations in various Notch family members are frequently and commonly detected in a variety of tumor types (228). Specifically, loss-of-function mutations in *NOTCH1* have been reported in HNSCC, cutaneous and lung squamous cell carcinomas, suggesting that NOTCH1 may act as a tumor suppressor of squamous cell carcinogenesis (135,137,229,230). Moreover, a recent report has demonstrated that recurrent but infrequent driver mutations found in HNSCC converge onto the Notch signaling pathway, resulting in inactivation of NOTCH signaling in 67% of patients, thus emerging as one of the most commonly dysregulated pathways in HNSCC (231).

Our results also evidence this conflicting role of Notch in HNSCC carcinogenesis. We have found higher levels of NOTCH1 protein expression in tumors than in the normal epithelium, and these data were confirmed at the *NOTCH1* mRNA expression level in the TCGA database analysis, suggesting an oncogenic role for NOTCH1. Contrary to this, our results also show a lower NOTCH1 expression in advanced tumor stages, suggesting that NOTCH1 expression/activity decreases along disease progression, therefore suggesting a tumor suppressor role for this protein. Hence, increased expression of NOTCH1 in early stages of the disease may represent an attempt to impair malignant progression, as suggested in other solid tumors (228).

In HNSCC, the expression of Notch pathway components has only been examined in small patient cohorts (mostly in Asian populations) with differing results (220,232). Some studies implicated NOTCH1 as pro-oncogenic, and described the association of NOTCH1 expression with poor prognosis (233,234), lymph node metastasis (235), poor differentiation and resistance to chemotherapy (236). In contrast, other studies correlated high NOTCH1 expression with improved survival in oropharyngeal squamous cell carcinoma patients (237) and HNSCC patients (238). Our results show that the expression of NOTCH1 is independently associated with better disease control and survival rates. In addition, *NOTCH1* mRNA expression analyses in the TCGA database also shows a tendency for a better survival in patients with elevated *NOTCH1* mRNA levels. Interestingly, most of the reports that correlate NOTCH1 expression with poor prognosis are focused on subsets of Asian patients, whereas the association of NOTCH1 expression with better prognosis has been reported in studies that included Caucasian patients (220,237,238). In the same way, the studies that found inactivating mutations of *NOTCH1* included mostly Caucasian patients (229,239), whereas studies including Asian patients have shown that more than half of the *NOTCH1* mutations are activating ones (240,241). These findings suggest that NOTCH1 promotes distinct tumorigenic mechanisms in patients from different ethnical populations.

HES1 is one of the few known gene targets indicating Notch pathway activation (242). As expected, we have found a close correlation between NOTCH1 and HES1 expression. However, HES1 expression in tumor samples was slightly more frequent than NOTCH1 expression. Interestingly, although HES1 expression alone was not related with the prognosis of HNSCC patients, only the cases that were NOTCH1+/ HES1+ have a better survival. As these cases probably represent the cases with true Notch pathway activation, this reinforces that the activation of Notch pathway is in fact associated with a better prognosis.

We have also found that HES1 expression is present in normal epithelium, and the analysis of the TCGA shows no statistically significant difference in HES1 expression between HNSCC and control tissues, suggesting that Notch pathway is activated in normal epithelium. This activation of Notch pathway in normal epithelium is well-known in the epidermis and associated with differentiation, supporting the tumor suppressor role of this pathway in the squamous epithelia (243). Few other studies have analyzed the expression of HES1 in HNSCC. A comprehensive study of Notch signaling pathway status reported overexpression of HES1 and/or HEY1 in 30% of HNSCC samples, which was correlated with NOTCH1 overexpression, and significantly lower HES1/HEY1 expression in tumors with inactivating *NOTCH1* mutations (244). This study proposes a bimodal pattern of Notch pathway alterations in HNSCC, with a subset of tumors harboring inactivating *NOTCH1* receptor mutations while a larger subset exhibits other NOTCH1 pathway alterations, such as increased HES1 and/or HEY1 expression driving downstream pathway activation. However, this study did not address the prognostic significance of Notch pathway activation. Another study also found HES1 overexpression in oral squamous cell carcinomas but was not correlated with the expression of NOTCH intracellular domain (NICD) (245). In that study, HES1 expression was associated with a poorer prognosis, but only when associated with c-MYC-positive expression (245). Taking together these studies and our results suggest that Notch pathway is activated in normal epithelium and remain activated in a subset of tumors, depending the clinical significance on the genetic background of the individual tumors.

In this way, one of the mechanisms by which Notch signaling may act in an anti-tumorigenic manner include the induction of p21, which promotes cell cycle arrest (228,246). In agreement with this hypothesis, we found a positive correlation between NOTCH1 and p21 expression in our HNSCC cohort, as described in other tumor types (150,247). Given the anti-proliferative function of p21 (246), this finding could provide a plausible explanation for the better prognosis observed in NOTCH1-

positive patients. In fact, a better survival was observed in the subset of patients with simultaneous NOTCH1+/HES1+/p21+ expression.

In contrast, NOTCH1 expression was not correlated in our series with Cyclin D1 expression, which favors cell cycle progression. NOTCH1 signaling has been associated with either increased (248,249) or reduced (250) Cyclin D1 in different tumor types, suggesting that the regulation of Cyclin D1 expression by Notch signaling may depend on specific cell/tissue contexts.

This study represents the most comprehensive analysis and the largest HNSCC cohort reported to date directed at establishing the clinical and prognostic relevance of Notch signaling activation in HNSCC, thereby bringing together valuable data to improve patient stratification and guide treatment based on the expression of various NOTCH1 signaling components. Patients carrying high expression of NOTCH1 showed a reduced relapse risk and significantly improved prognosis. In particular, the subset of patients harboring NOTCH1+/ HES1+/p21+ tumors exhibited the highest survival rates. Inhibitors of Notch signaling are already in clinical testing in other malignancies such as breast, ovarian, pancreatic and small-cell lung cancers (69,251). However, according to our findings, Notch inhibition seems less promising in HNSCC, since patients with high NOTCH1 expression clearly and consistently demonstrated a better survival in our study. These findings should therefore be highly relevant for clinical evaluation of NOTCH targeting therapies in HNSCC patients.

V. CONCLUSIONS

1. NOTCH1 protein expression (both membranous and nuclear) and the expression of its target nuclear HES1 are both frequently detected in over 60% HNSCC tissue specimens.

2. The expression of nuclear NOTCH1 and nuclear HES1 is detected in basal and suprabasal layers of normal epithelium.

3. Membranous and nuclear NOTCH1 expression is consistently and significantly correlated with nuclear HES1 and p21 expression, but not with Cyclin D1 expression.

4. Membranous and nuclear NOTCH1 expression significantly correlated with early disease stages (I-II), lower tumor recurrences, and better disease-specific (DSS) and overall survival (OS) rates.

5. The subset of patients harboring triple-positive tumors (NOTCH1+/ HES1+/p21+) exhibited the highest survival rates, further suggesting that the activation of Notch signaling pathway is associated to a favorable prognosis in HNSCC.

6. Membranous NOTCH1 expression emerges as a robust independent predictor of better disease-specific and overall survival in multivariate analysis.

CONCLUSIONES

1. La expresión de la proteína NOTCH1 (tanto membranosa como nuclear) y la expresión de su objetivo nuclear HES1 se detectan con frecuencia en más del 60% de las muestras de tejido HNSCC.

2. La expresión de NOTCH1 nuclear y HES1 nuclear se detecta en las capas basales y suprabasales del epitelio normal.

3. La expresión de NOTCH1 membranosa y nuclear está consistentemente y significativamente correlacionada con la expresión de HES1 nuclear y p21, pero no con la expresión de Ciclina D1.

4. La expresión de NOTCH1 membranosa y nuclear se correlacionó significativamente con los estadios tempranos de la enfermedad (I-II), recurrencias tumorales menos frecuentes y mejores tasas de supervivencia cáncer-específica (DSS) y general (OS).

5. El subgrupo de pacientes con tumores triple positivos (NOTCH1+/ HES1+/ p21+) exhibió las tasas de supervivencia más altas, lo que sugiere ulteriormente que la activación de la vía de señalización de Notch se asocia con un pronóstico favorable en HNSCC.

6. La expresión membranosa de NOTCH1 emerge como un sólido predictor independiente de una mejor supervivencia general y específica de la enfermedad en el análisis multivariante.

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