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DOI: https://doi.org/10.1007/978-3-030-55031-8_7

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-197111>

Book Section

Accepted Version

Originally published at:

Porcheri, Cristina; Mitsiadis, Thimios A (2021). Notch in Head and Neck Cancer. In: Reichrath, Jörg; Reichrath, Sandra. Notch Signaling in Embryology and Cancer. Cham: Springer, 81-103.

DOI: https://doi.org/10.1007/978-3-030-55031-8_7

Notch in Head and Neck Cancer

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Abstract

Head and neck cancer is a group of neoplastic diseases affecting the facial, oral and neck region. It is one of the most common cancers worldwide with an aggressive, invasive evolution in the late stages of malignancy. Due to the heterogeneity of the tissues affected, it is particularly challenging to study the molecular mechanisms at the basis of these tumors, and to date we are still lacking accurate targets for prevention and therapy. Notch signaling is involved in a variety of tumorigenic mechanisms, such as regulation of the tumor microenvironment, cell-to-cell communication and metabolic homeostasis. Here, we provide an up-to-date review of the role of Notch in head and neck cancer and draw parallels with other types of solid tumors where the Notch pathway plays a crucial role in emergence, maintenance and progression of the disease. We therefore give a perspective view on the importance of the pathway in neoplastic development in order to define future lines of research and novel therapeutic approaches.

Keywords

Notch pathway; Oral Epithelium; Oral Mucosa; Epithelial Notch; Oral cancer; Carcinoma; Squamous Cell Carcinoma; Cell-to-Cell Interaction; Intercellular Communication;

List of abbreviations

ADAM	A Disintegrin And Metalloproteinase
AKT	Protein Kinase B
APC	Adenomatous polyposis coli
CAF	Cancer-associated fibroblast
CCL	Chemokine (C-C motif) ligand
CCND1	Cyclin D1
cK	Cytokeratin
COX2	Cyclooxygenase-2
CSC	Cancer stem cell
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
CRC	Colorectal cancer
DC	Dendritic cells
DLL	Delta-like
EGF(R)	Epidermal growth factor (receptor)
EMT	Epithelial-to-mesenchymal transition
FBXW7	F-box and WD repeat domain containing 7
GSI DAPT	Gamma-secretase inhibitors
Hes	Hairy and enhancer of split-1
Hey	Hairy/enhancer-of-split related with YRPW motif protein 1
Hif1	Hypoxia-inducible factor 1
HNSCC	Head and neck squamous cell carcinoma
HPV	human papillomavirus
IL	Interleukin
ILC	Innate lymphoid cell
LAC	Lung adenocarcinoma
LNR	Cysteine-rich Lin-12/Notch Repeats
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MALM	Mastermind-like
MET	Mesenchymal-to-epithelial-transition

MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
NCR	Natural cytotoxicity receptor
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NK	Natural killer cells
NLS	Nuclear localizing sequence
Oct4	Octamer-binding transcription factor 4
OSCC	Oral Squamous Cell Carcinoma
PTEN	Phosphatase and tensin homolog
PI3K	Phosphatidylinositol 3-kinase
RBPj	Recombination signal binding protein for immunoglobulin kappa J region
SCC	squamous cell carcinoma
Shh	Sonic hedgehog
Sox2	Sex determining region Y-box 2
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophages
T-ALL	T cell acute lymphoblastic leukemia
TCF	T-cell factor
TGF- β	Transforming growth factor- β
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor alpha
VEGF(R)	Vascular endothelial growth factor (receptor)
α -SMA	α -smooth muscle actin

Introduction

Head and neck cancers (HNSCCs) represent a group of neoplastic diseases affecting different tissues and organs in the head and neck anatomical region. An estimated 5.5 million people are affected by HNSCCs with a poor prognosis of survival (379.000 deaths in 2015). More than 90% of HNSCCs are carcinomas that mainly emerge from the epithelial wall of the oral cavity, although several other tissues, such as the nasal cavity, pharynx, larynx and salivary glands, can also be affected [1–4]. Carcinogenesis is often triggered by chronic exposure to tobacco or alcohol, but it might also be associated with viral infections, commonly the human papillomavirus (HPV) infection [5–7]. Alcohol dissolves the lipid barrier in the external part of the epithelium and is metabolized into acetaldehyde, which in turn reacts with DNA molecules to induce damage (including adducts, single- and double-strand breaks, and point mutations) [5, 8]. Similarly, tobacco-derived carcinogens (such as benzopyrenes, aromatic amines, and nitrosamines) covalently bind DNA, inducing gene mutations [9]. Finally, chemical exposure reduces the local immune-surveillance, and local inflammation increases susceptibility to cancer development [5, 7]. Thus, chemically-promoted DNA damage often results in an aberrant repair process, consequently causing uncontrolled proliferation and dysplasia of the epithelial layers [8].

Conversely, infections with HPV require the presence of a preexisting wound, which allows the virus to reach the basal cell layer and integrate its dsDNA into the DNA of the host cell, ultimately exploiting the proliferative machinery of the epithelial tissue [6, 10]. Abrasion through mastication is a common cause of wound occurrence, and therefore tongue and oral mucosa are the most common sites of cancer development [11, 12].

Less common neoplasms of the head and neck region include odontogenic tumors and tumors of the salivary glands. Salivary glands tumors represent 6% of all HNSCCs and mainly occur in the parotid gland. A substantial risk factor for the development of a salivary gland tumor is

exposure to ionizing radiation, which is typically used in radiotherapy. Odontogenic tumors are a heterogeneous group arising specifically in the jaw and are classified based on their peculiar ability to generate aberrant interconnection between ectomesenchyme and epithelium. Malignant odontogenic tumors are quite rare, however their benign counterparts are able to infiltrate surrounding structures, alter their architecture and function, and ultimately evolve into malignancy. Of the most common types of odontogenic tumors, the ameloblastoma and intra-osseous carcinoma have epithelial origin and involve progression from altered odontogenic epithelium or an odontogenic cyst [13].

Histology of craniofacial epithelium

Epithelium lining the respiratory and digestive tract are often formed by squamous epithelium, of the simple or stratified type. Simple squamous cells line the air sacs of the lungs and heart, blood and lymphatic vessels, and are characterized by a single layer of epithelial cells resting on the basal lamina. Stratified squamous cell epithelium characterizes the epithelium of the oral and nasal cavity, the esophagus and also skin and vaginal walls. In contrast to the simple version of squamous epithelium, the stratified epithelium is composed of several overlapping layers of flattened cells, of which the first layer is in direct contact with the basement membrane. Cells are bound together by tight junctions with very limited or absent intercellular space. These structures allow resistance to constant abrasion and are normally found in areas where the physical barrier of the body meets with the external environment. The continuous exposure to sheering forces induces a constant elimination of older cells from the external layers. As a result, stem and progenitor cells located in the deep basal layers undergo a proliferation stage and progress further in their maturation process, replenishing the outer layers of the stratified epithelium. Therefore, this type of epithelium faces a fast turnover, with

cycles of stem cell activation, increased proliferation, and differentiation occurring in a finely coordinated manner.

In the head and neck region, the protective layers of a stratified squamous cell epithelium are the major protective structure of the tongue, oral mucosa, internal portion of the lips, larynx, and pharynx (Figure 1). Similarly, within specialized skin such as the palm of the hand or the sole of the foot, the oral mucosa is additionally protected by a keratinized external layer. Cytokeratins (cK) are fibrous structural proteins abundant in epithelial cells, and the expression of specific keratins within a tissue determines both the type of cell and the function of the tissue. High levels of keratins increase the endurance of epithelium to mechanical stresses, and at the same time preserve hydration of its deeper layers. The basal epithelial cell layer, called stratum basale, contains slow-cycling stem cells that are anchored to the basement membrane. The larger portion of the stratified epithelium is composed by several layers of proliferating progenitors that are generated from stem cells via asymmetric cell divisions and passively displaced toward the surface. When proliferative progenitors differentiate further they acquire an elongated shape, leading to a tightening of the intercellular space by expression of modified desmosomes in the stratum granulosum. The most external layer of the squamous stratified epithelium is composed of maturing cells that produce and accumulate large amounts of protein aggregates containing keratin filaments. The large amount of aggregates promotes the collapsing and flattening of the corneocytes, while the synthesis of other proteins (involucrin, trichohyalin, and other small proline-rich proteins) continues. Proteins integrate into the plasma membrane, where they physically interact with membrane lipids (ceramides and cholesterol) to provide a waterproof barrier. Once desquamation is induced by abrasion, desmosomes are degraded, cells detach and are thus released from the epithelial structure.

More specialized epithelia characterize other head and neck regions, such as the ciliated pseudostratified columnar epithelium of the nasal cavity, trachea, and upper respiratory tract. Here, a single layer of ciliated columnar epithelial cells is positioned in direct contact with the basal lamina (Figure 1). They possess cilia, projections extruding from the cell membrane, which help to trap particles brought in through respiration. Finally, the secretory function of the salivary glands is supported by cuboidal and columnar epithelium, which are differentially distributed within acini and ducti, and are functionally important for the distinct levels of permeability of these structures to ions and water (Figure 1) [14].

Epithelial turnover varies greatly depending on the region analyzed (50 to 75 days in the skin, 4 to 14 days in the gut, 40 to 55 days in gingiva, and 25 days in the oral mucosa of the cheek) and correlates with epithelial maturation. Cancer treatment severely affects the dynamics of epithelium maturation and regeneration, as it interferes with mitosis regulation. Following chemotherapy for oral cancers, patients often develop ulcers, experience painful conditions, and have difficulty in maintaining oral hygiene, eating, and drinking.

Epithelial Notch from embryo to cancer

Mammalian epithelial tissues derive from all three embryonic germ layers and form different anatomical structures (i.e., epidermis derives from the ectoderm, inner epithelial wall from mesoderm, and gut epithelium derives from the endoderm). The epithelium of the head and neck region has a heterogeneous nature from the beginning of its development. The oral mucosa is subdivided into epithelial walls composing palate, floor of the mouth, inner lips and gingiva, all structures that are derived from the ectoderm. Conversely, the epithelium of the tongue is derived from endoderm and mesoderm [15, 16]. Embryonic epithelial structures often constitute organ primordia, and aid in shaping future functional units as a result of cell-to-cell interactions that coordinate specific molecular program activation, mechanical stimuli, and

induction of maturation. During early embryonic stages (gestational week four in humans) neural crest cells migrate to the first and second pharyngeal arches to instruct the local epithelial structure in forming bone and cartilage of the face and neck, but also pigment and cranial nerves. Once within the pharyngeal arch, neural crest cells surround a specific portion of the epithelium and give rise to odontoblasts, small bones of the middle ear and thymic cells [17].

The molecular mechanisms involved in the specification of craniofacial epithelium are largely unknown, but the essential role of cell-to-cell communication appears to play a major role in orchestrating maturation and dynamics of epithelial organs. In regards to intercellular communication, the Notch pathway plays an essential role in both inside-out and outside-in molecular activation [18]. In mammals, four Notch receptors interact with specific ligands (three of the delta-like type: Dll1, Dll3; Dll4 and two of the jagged type: Jagged1, Jagged2) to regulate fate determination, survival, proliferation and regulation of transcription (Figure 2). Upon receptor interaction with the ligand, the intracellular portion of Notch is cleaved and transmits into the nucleus, where it recruits a transcription complex responsible for regulating the expression of specific target genes (among others of the Hes and Hey family: Hes1-7 and Hey1, Hey2, HeyL).

Specifically, the Notch target family of Hes genes is thought to be involved in the definition of a variety of head and neck structures, as mutations in this gene produce defects in frontal bone agenesis, cranial base formation, proper size definition of lower and upper maxilla and are involved in palate cleft. These malformations are associated with an uncontrolled migration and directional role of the neural crest stem cells, which might utilize Hes1 as a regulator of local morphogenesis. Similarly, Hey1 was reported to be expressed in the branchial arches from early to late facial development, where a clear expression is confined to the epithelium of the nasal pit [19].

During embryonic development, several epithelial structures express Notch3 and Notch1 receptors, together with the ligands Jagged1 and 2, and are involved in craniofacial morphogenesis [20]. Alagille syndrome patients have mutations in the gene coding for the Jagged1 ligand, resulting in facial hypoplasia and craniosynostosis. Depletion of the Jagged2 ligand results in altered tooth morphogenesis, mainly due to aberrant ameloblast differentiation and poor enamel deposition. Additionally, mutations of the Jagged 2 gene result in the abnormal fusion between palatal processes and the tongue, causing cleft palate. The Notch pathway has a specific role in the regulation of tongue morphogenesis, where the crosstalk between Notch activity and the canonical Wnt signaling pathway directs the boundary formation between epithelium and mesenchyme, inducing the formation of the lamina propria during tongue organogenesis [20].

Due to the involvement of Notch signaling in epithelial tissue definition and its role in balancing stem cell maintenance and differentiation, its activation is common to a variety of embryonic epithelial structures [21]. In particular, the tongue epithelium shares strong similarities with the keratinized epithelium of the skin. From E8.5 in the mouse embryos, ectodermal cells activate the expression of cK5 and cK14 as differentiation markers of keratinocyte fate acquisition [22, 23]. Ectodermal cells at specific locations become columnar (epidermal placode) and interacting mesenchymal cells start to secrete extra-cellular matrix molecules that will provide an initial lamina upon which the epithelial stratification is built [24–28]. Ectodermal progenitors that acquire keratinocyte fate start to express the differentiation marker p63 first, followed by keratin 14. Notch signaling is activated upstream of these markers, as blockage of the Notch signaling leads to a failure of keratinocyte differentiation in the mouse embryos [29, 30].

Various events occurring during development are recapitulated during tumorigenesis, such as the establishment of a supportive niche for undifferentiated cells, epithelial-to-mesenchymal

transition and change of balance between stem cell maintenance and differentiation (Figure 3). The Notch pathway is involved in the regulation of common embryonic and oncogenic processes, and it is therefore not surprising that aberrant expression of Notch receptors and ligands are hallmarks of several solid tumors, including head and neck tumors [31]. Epithelial Notch has been specifically linked to the maintenance of cancer stem cells, the increase in invasion capabilities promoting epithelial-to mesenchymal transition (EMT) and the constitution of a supportive tumor microenvironment (TME) (Figure 3).

Notch in cancer stem cells

Cells with stem cell characteristics have been identified in a variety of cancers, including colon, pancreatic, lung and prostate carcinomas. Due to their low rate of proliferation, undifferentiated cells inside the tumor can escape chemotherapy and radiotherapy treatments and activate primordial programs for tissue homeostasis. Notch signaling is one of the major pathways involved in preserving undifferentiation of stem cells in both healthy and cancerogenic conditions [21], and has been specifically linked to cancer stem cell (CSC) self-renewal [32–34]. The Notch pathway increases stem cell survival in a variety of cancers (such as glioma and medulloblastoma [35]) and its activity results in induced de-differentiation of committed cells into more undifferentiated progenitors, as well as expansion of the stem cell pool by proliferation (i.e. mammary stem cells and mammospheres [36, 37]). A similar role of Notch is preserved in non-epithelial tumors, such as acute myeloid leukemia, where blocking the Notch pathway affects the survival of CD34+/CD38- undifferentiated populations [38].

Epithelial Notch operates as a stem cell keeper in a variety of tissues, with a few notable exceptions. In the head-neck region and specifically in the oral epithelium, stem cells located in the basal layer express the Notch1 receptor, although genetic depletion of Notch1 was

reported to have limited effects on the maturation of normal mucosa that displays unaltered morphology and expression of differentiated keratinocytes markers such as cK13 and cK15 [39, 40]. In oral squamous cell carcinomas, the level of Notch expression correlates with tumor development and severity of dysplasia. In-vitro assays based on HNSCC-derived spheres showed high expression of Notch1 and a direct role for Notch1 in regulating their self-renewal. Overexpression of cleaved Notch induces the expression of classical stem cells markers, such as Oct4, Sox2 and CD44, while knock-out of Notch1 inhibits tumor formation and increases sensitivity to chemotherapy [41].

As previously noted, the Notch signaling pathway has diverse, context-dependent functions. For example, in the adult gut, Notch preserves proliferation and undifferentiation via interaction with Dll1 and Dll4 ligands [42]. Notch1 upregulation results in maintenance of an undifferentiated state in colon cancer, mainly by interaction with Jagged1 and activation of Hes1 [43–47]. In epidermal tumors, Notch works as a tumor-suppressor, promoting differentiation of uncommitted progenitors in the hair follicle, sebaceous glands and interfollicular epidermis [48, 49]. During the development of embryonic epidermis, Notch activity regulates the expression of p63, an important transcription factor involved in stem cell maintenance, and its upregulation in the basal layer prompts progression of differentiation [29, 50–52]. Consistently, Notch deletion induces the development of spontaneous squamous cell carcinoma in the skin, probably via the Jagged1 ligand [52, 53].

Due to the central role of Notch in preserving the more resistant core of cancer stem cells inside the tumor, the Notch signaling pathway represents a major therapeutic target. Radiotherapy treatments were shown to increase the activity of the Notch pathway, particularly in breast cancer and glioma stem cells. The radioprotective role of Notch might be linked with the

Notch-dependent regulation of the AKT/PI3K pathway, which is specifically activated by the subpopulation of undifferentiated cells sustaining tumor survival [54, 55].

Notch in Epithelial to Mesenchymal Transition

It is now widely accepted that epithelial cells can acquire a mesenchymal phenotype by the process of epithelial to mesenchymal transition (EMT). Upon tissue-reorganization stimuli, such as wound healing, embryonic development and cancerogenic conditions, epithelial cells can undergo a remarkable change in morphology, modifying their genetic expression panel to acquire mesenchymal features. Specifically, they downregulate molecules involved in cell-to-cell junctions, such as E-cadherin and γ -catenin, destabilizing the epithelial structure and promoting cell migration [56–58]. An internal reorganization of the actin cytoskeleton together with the upregulation of mesenchymal markers (such as vimentin, fibronectin, α -smooth muscle actin, fibrillar collagen type I and type III and fibroblast-specific protein-1) complete their change of fate [56–58]. Once the cell-to-cell interactions are disassembled, cells in transition start to produce and secrete matrix metalloproteases (MMPs) like MMP-2, MMP3 and MMP9, increasing motility and invasion capabilities [58, 59]. The Notch pathway has been found to be a major regulator in EMT, by regulating E-cadherin regulation of expression, the TGF β pathway and growth factor signaling. Repressors of E-cadherin genes (such as Snail and Slug) bind to the E-cadherin promoter to inhibit gene transcription [60]. Notch directly regulates Snail and Slug, inducing their upregulation in epithelial cells and consequently downregulating E-cadherin expression, a crucial step in the early phase of EMT [61, 62]. Additionally, Notch could control the expression of a hypoxic environment that would stabilize the Snail-1 protein [63].

EMT is an essential event during wound healing and embryonic development and is Notch-dependent. For instance, during cardiac development, the Notch signaling pathway is expressed

in endocardium and cardiac valve maturation and both structures depend on EMT for completion. Similarly, embryos carrying Notch mutations or cardiac explants exposed to the Notch inhibitor DAPT experience impaired EMT, which results in an aberrant cardiac valve formation [61].

In tumorigenic processes, downregulation of E-cadherin with a simultaneous increase of Snail expression is a hallmark of carcinomas [64]. Activation of the EMT program can be triggered at the pre-malignant state of carcinoma, although their prognostic relevance in predicting the formation of new metastases remains to be elucidated [65, 66]. Interestingly, human samples and disease models display an incomplete form of EMT with epithelial and mesenchymal transcripts coexpressed in tumor cells [67, 68]. This partial activation of EMT allows tumor cells to convert back to an epithelial fate via a mesenchymal-to-epithelial-transition (MET) when needed, increasing the level of plasticity of the invasive cancer (Figure 3). Cells from the primary tumor can therefore utilize this great adaptability to establish metastases in distant tissues, increasing their heterogeneity and making their identification via transcriptional signature more challenging [69]. How EMT and MET contribute mechanistically to the spreading of the tumor remains to be understood. Alterations to the surrounding microenvironment may help EMT establishment. Hypoxic conditions often present in poorly vascularized portions of carcinoma can contribute to the establishment of an EMT program. Hif-1, a major hypoxia-activator, indirectly represses the expression of epithelial key factors (such as E-cadherin) and thus promotes a mesenchymal differentiation [70, 71].

EMT is particularly important in conferring drug resistance (specifically to taxol, vincristine and oxaliplatin treatment [72–75]), and may be a useful therapeutic target to prevent the acquisition of an invasive phenotype and the development of metastases. Therefore, understanding the precise mechanism that governs EMT is an essential step for future cancer

therapy, and Notch-dependent regulation of EMT offers a promising target for the molecular dissection of this process in human malignancies.

Notch in tumor microenvironment

A specialized tumor microenvironment (TME) sustains, nourishes and protects tumor cells, which in turn can thrive supported by novel angiogenesis, a cooperative immune system, a modified extracellular matrix. Solid tumors strongly rely on the intercellular communications with the surrounding environment for their maintenance. Cell-to-cell interactions become central in the regulation and generation of a cancerogenic milieu, and the Notch molecular pathway has been implicated in the regulation of various factors shaping the TME [76]. As previously described, the expression of cadherins and integrins might directly depend on Notch regulation, being direct targets of the activated pathway. In parallel, Notch might also coordinate the activity of a subpopulation of resident cells (such as immune and endothelial cells) in the formation of a cancerous-prone environment.

Notch in tumor macrophages

TME is characterized by very specific immune infiltrates that can be exploited by the cancer itself for local release of tumorigenic factors. A specific subpopulation of bone marrow-derived monocytes give rise to tumor associated macrophages (TAMs) that invade the aberrant tissue. Using the CCL2-CCR2- chemokine pathway, TAMs are recruited by the growing tumor mass whose constant release of trophic cytokines enhances angiogenesis and suppresses antitumoral T-cell response [77]. TAMs have been identified in a variety of solid tumors of epithelial origin, such as breast cancer, bladder, ovarian [78–80] and head and neck cancers [81–83]. Macrophages are generally classified in M1 or M2 subgroups (Figure 3). M1 macrophages are characterized by a pro-inflammatory phenotype, in that they release anti-tumoral cytokines

such as TNF α , IL1b, IL-6 and CXC10, and their polarization is induced upon LPS or TNF α exposure. Tumor cells instead induce the acquisition of the M2 phenotype, characterized by the production and secretion of high levels of IL10, IL4, TGF β , VEGF and matrix metalloproteases to promote tumor survival and invasiveness. In addition to the regulation of the inflammatory milieu, TAMs might also be involved in controlling the EMT process, as suggested by *in-vitro* models [84]. Both subtypes of macrophages express the CD68 markers, while expression of M2 macrophages inside the tumor is specified by the expression of CD163. In oral and oropharyngeal carcinoma, high infiltrates positive for CD68 are a hallmark of the cancerogenic condition already present during the early stages of OSCC [85]. A correlative paradigm between poor survival rate in OSCC and high CD163 expression appears to be present in human samples where the TAM population localizes inside the tumor nest rather than in the tumor stroma [86, 87]. Although the role of the M2 subpopulation seems to be the most relevant in shaping the tumor microenvironment, we cannot exclude a partial role of the M1 macrophages, as sub-distribution of the two populations might be relevant to understanding their mode of action. Additionally, a specific marker for M1 macrophages is still to be found, and their presence in a confined portion of the tumorigenic area cannot be excluded.

The Notch pathway has been largely associated with the induction of differentiation in both central and peripheral lymphoid organs, contributing to the development of the T and B-cell line of lymphocytes. Notch might also be involved in the process of monocytic differentiation starting very early in the progenitor definition and fate acquisition. Gene expression barcoding revealed that Notch controls a myelomonocytic-specific gene signature via inhibition of transcription mediated by Hes1 [88]. Additionally, macrophages and dendritic cells constitutively express members of the Notch family whose expression are regulated by the toll-like receptor (TLR) [89]. TLR upregulates the expression of receptors and Jagged1, Dll1, Dll4

ligands directly promote Notch-pathway activation. During inflammation, cytokines like TNF and IL1b can induce the expression of Notch1, Notch4 and Jagged2 [90]. As both inflammatory cytokines and TLR activate the NFkB pathway, it has been postulated that this pathway functions as a molecular bridge in many systems, including cancer [91]. In contrast with a pro-inflammatory fate acquisition driven by Notch activation, RBPJ depletion in TAMs blocks their differentiation and induces a previously inhibited cytotoxic T-cell response [92]. Therefore, it remains to be clarified whether blockage of Notch signalling involved in the pro-inflammatory induction can dampen the immune activation unfavorable for tumor growth. In epithelial cancers, Notch1 and Notch2 signaling through Jagged1 induces a TAM-anti-inflammatory phenotype [93]. Interestingly, in head and neck squamous cell carcinoma, high levels of the Notch1 receptor are correlated with a high infiltration of CD163⁺ TAMs [92, 94, 95].

Due to their supportive role in tumor growth and evolution, TAMs are under the spotlight as emerging therapeutic targets. Blockage of the CCL2/CCR2 interaction has been shown to be beneficial in human pancreatic ductal adenocarcinoma as it interferes with TAM recruitment [96]. Maturation of monocytes into M2 macrophages depends on IL6, which acts in synergy with other factors (such as COX2-dependent prostaglandin production and STAT3 activation). Current clinical trials are therefore focusing on inhibition of IL-6 and COX2 enzymes with promises to contrast lung and ovarian cancer progression [97, 98].

Notch in tumor angiogenesis

The tumor vasculature is an essential asset providing trophic support in solid tumor masses. Cancer cells secrete endothelial growth factors that sustain sprouting, migration and rearrangement of vessels, in order to regulate the income of oxygen and nutrients enabling tumor growth. Notch signaling is a major regulator of physiological and aberrant angiogenesis,

mainly through the Jagged1 or Dll4 ligands [99]. Vasculature dynamics depend on the coordinated activity of tip and stalk cells: high activation of Notch1 induces acquisition of the stalk phenotype, while low Notch activity is associated with tip-cell fate determination [100]. The difference in activity is directly associated with the type of ligand expressed on the surface of the different cell types, with tip cells expressing high levels of Dll4 and stalk cells expressing low levels of Dll4 and high levels of Jagged1 [101]. This in turns regulates the expression of the VEGF receptor and cell metabolism to drive directional sprouting and new vasculature formation [100].

Tumors modify this balance to create the most suitable environment for their own growth, with high levels of Jagged1 inducing an increase in tumor vasculature and Jagged1 downregulation leading to decreased angiogenesis [102]. Metastatic tumors use the newly generated vasculature to enable spreading of mobilized cancer cells and may directly regulate endothelial cell quiescence [103]. Breast cancer cells, otherwise kept quiescent, proliferate in the presence of newly generated microvasculature derived from lung, brain and bone marrow tumors, suggesting that a stable vasculature contributes to a dormant niche, while sprouting, activated endothelial cells are able to initiate metastatic growth [104]. In line with these findings, high activity of the Notch3 receptor has been found in tumor vasculature [105]. In aberrant conditions, Jagged1 is overexpressed in cancer cells, blocking endothelial apoptosis driven by Notch3 and promoting local angiogenesis [105–108].

Regulation of tumor vascularization is also central in the establishment of a hypoxic microenvironment favored by a subset of carcinomas (i.e. lung, breast, kidney and oral cancer) (Figure 3) [109–113].

Mammalian cells typically react to a reduced oxygen availability by activating transcription of HIF-1 factor to trigger angiogenesis [114]. The two existing isoforms of HIF α (HIF1 α and HIF2 α) translocate to the nucleus upon binding with the HIF-1 β form, interact with the hypoxia

binding elements and consequently promote the transcription of selective target genes. Activation of HIF has various implications in promoting tumorigenesis, including changes in metabolism and the production of oxygen radicals, maintenance of undifferentiation and induction of a motile phenotype acquisition via EMT [115]. Specifically, it directly correlates with advanced stages of oral cancer and it has been proposed as significant prognostic marker (Figure 3) [116]. In hypoxic conditions both Notch activity and expression of Notch-dependent target genes are increased. Crosstalk of hypoxia and Notch signaling has been activated implicated in EMT and results in an increase in the invasiveness of oral squamous cell carcinoma, although a full detailed mechanism of action remains to be described [112, 113, 117, 118].

Therefore, Notch signaling is involved in the regulation of normoxia and physiological vasculature growth, with important consequences for therapeutical application. Treatment with cetuximab showed broad effects on both Notch activation and HIF sensitivity, inhibiting tumor-induced angiogenesis in a murine model for HNSCC [118].

Notch in carcinoma: When context matters

The Notch pathway is genetically altered in a large number of hematopoietic and solid tumors, resulting in aberrant activation or repression of the pathway. Different types of mutations interfere with receptor-ligand interaction, molecular regulation, gene targeting and epigenetic regulations [119–122].

In carcinomas, the role of epithelial Notch varies greatly depending on the organ affected [123–125]. Emerging evidence suggests that the Notch signaling network is frequently deregulated in human malignancies, with up-regulated expression of Notch receptors and their ligands in head and neck, cervical, lung, colon, and pancreatic cancer supporting the idea that Notch promotes cancer development [126, 127]. In a limited number of carcinomas, including skin

cancer, human hepatocellular carcinoma and small cell lung cancer, Notch signaling has been shown to be anti-proliferative rather than oncogenic [124]. It is therefore essential to establish the context of analysis to define the function of epithelial Notch as either oncogenic or anti-proliferative [123–125]. In the following paragraphs, we compare the role of Notch in a few relevant carcinomas, where its activity fluctuates significantly with its tumorigenic potential.

Head and neck cancer

Due to the high heterogeneity of the HNSCC and the different types of tissues that group under the same name, the exact function of the Notch pathway in this type of tumor remains to be clarified. All Notch receptors can be found highly expressed in HNSCC samples and OSCC, where they activate downstream signaling through Hey1 [128]. Notch1 mutations are the second most common mutations found in head and neck carcinoma (HNSCC), suggesting an essential role of the pathway in the pathogenesis of the tumor. In an in-vitro model of HNSCC, inhibition of Notch3 decreased sphere formation and proliferation in parallel with the inhibition of the activated target genes Hey1, cMyc and CCND1 [1, 129].

In line with the oncogenic role of Notch1, its upregulation leads to resistance to chemotherapy treatments and inhibition of Notch reduces the undifferentiated portion of cells in the HNSCC [130, 131]. Dll4 overexpression can be found in subtypes of HNSCC, and appears to have a role in vasculature reorganization and risk of metastases, consequently resulting in poor prognosis [132]. Once activated, the Notch pathway increases the expression of target genes between normal mucosa and its dysplastic stage, with Hes1 and Hey1 being key players in the malignant condition [1]. The upregulation of Hes1 seems to also correlate with an increase in undifferentiation or an amplification of the stem cell population, as demonstrated by self-renewal assay in spheres formation [133]. Several studies identified high levels of Notch1 expression in HNSCC, especially when considering the subset of OSCC [117]. Tongue cancer

specifically displays an increased Notch3 expression that correlates with the degree of tumor progression, although cell proliferation does not appear to be altered [134]. High expression of Notch1 additionally correlates with high level of metastasis formation in patients with tongue cancer [135].

The Notch pathway is therefore relevant for HNSCC progression, although the exact mechanism of function remains to be identified.

Breast cancer

Breast cancer is a form of cancer in which the Notch pathway may act as a both tumor suppressor as well as oncogene [136–138]. One of the first indications that Notch signaling may play a role in solid tumors came from experiments with mammary models developed after tissue infection with the mouse mammary tumor virus (MMTV). Integration of the MMTV genome next to the “*Int-3*” locus resulted in an activating mutation of *Notch4*, leading to the constitutive activation of the receptor and subsequent breast cancer development [139–141]. Recent observations indicate that Notch4 may play a more specific role compared to other Notch receptors in breast cancer stem cells [142] through signaling via other oncogenic pathways, such as Ras and Wnt [143–147].

Thus, Notch activation seems to play a pro-tumorigenic role in breast cancer [148–151]. However, recent studies indicate that hyper-activation of NOTCH3 may induce senescence in breast cancer cells and therefore have a detrimental effect on cancer development [152]. This apparent divergence of results might be explained by the fact that mammary epithelial cells respond differently to different levels of activation of the Notch pathway [153]. Consequently, different Notch receptors may have unique signaling outputs in mammary epithelial cells as well as producing alternative responses in different subtypes of breast cancers.

Colorectal cancer

The intestinal epithelium possesses an unprecedented self-renewal rate that appears to be linked to a high susceptibility to malignant transformation [154, 155]. Notch signaling has been known to be involved in both the control of homeostatic self-renewal in stem cell populations and the development of colorectal cancer (CRC) [156–158]. While mutations of *NOTCH* genes are rare, regulators of the pathway are often affected (including *FBXW7*), resulting in an overall overexpression or uncontrolled activation of Notch signaling in CRC [155, 159–162]. In addition, Notch activation has been linked to activation of Wnt signaling and Hippo/YAP signaling in CRC cells, although the various levels of crosstalk between these pathways are still not fully understood [46, 47, 156, 163–166]. In particular, Jagged1, expressed on tumor cells themselves or produced from endothelial cells, is thought to be a key ligand for Notch activation in CRC cells [47, 108, 163]. Another Notch ligand, DLL4, plays a non-cell autonomous role in CRC development, in large part by controlling the development of blood vessels necessary for tumor growth [167, 168]. Expression of miR-34a in CRC stem cells may help to control Notch output and generate a bimodal Notch response [169]. Finally, Notch signaling may play a crucial role not only in the early stages of CRC development by controlling the fate of stem cells and cancer stem cells, but also at the later stages of tumor invasion and metastasis [103].

Cutaneous Squamous Cell Carcinoma (cSCC)

Tumors arising from keratinized squamous epithelium can have different epidemiological associations, however they all derive from a disrupted differentiation of the basal progenitors, resulting in dysplastic epithelium and increased proliferation [170, 171]. Cutaneous squamous cell carcinomas (cSCC) often occur as a result of exposure to UV radiation, which results in genetic aberration, mostly ending in TP53 loss. The Notch pathway has been found to be

inactive in cutaneous SCC malignancies, and instead works as a tumor suppressor under the control of p53 [52, 170]. Notch1 and Notch2 mutations affecting the EGF repeats have been mapped in human cSCC and are linked to a dominant-negative phenotype [172, 173]. Similarly, murine models of conditional Notch1 or Notch2 deletions result in structural defects and tumor formation [174, 175].

The molecular mechanism triggered by Notch in this context is still partially unresolved, as are the elements tuning its activation towards a negative regulation of tumor growth, in contrast to other carcinomas, where the same pathway bears an oncogenic function.

Lung adenocarcinoma

Lung adenocarcinoma (LAC) is a major subtype of lung cancer [176–178]. In-vitro studies initially identified the Notch signaling pathway as a promoter of LAC cell proliferation [179–181]. In parallel with these observations, in-vivo modelling confirmed the relevance of the Notch pathway in preserving LAC development and maintenance [176, 182, 183]. Specifically, the NOTCH3 receptor is crucial in regulating the self-renewal of LAC tumor-propagating cells [178]. LAC cells express the Jagged2 ligand on their surface and support the metastatic potential of LAC stem cells [184]. Thus, despite the absence of Notch mutations in LAC screenings, activation of Notch may be important in LAC growth and Notch activity correlates significantly with the worsening of survival in lung cancer patients [177, 178, 185].

The squamous cell lung carcinoma (lung SCC) is the second major type of non-small cell lung cancer. Upon mapping of human-derived lung-SCC samples, several loss of functions mutations were identified in the EGF-like repeats of the Notch1 receptor and result in a

truncated, non-functional receptor. Therefore, in contrast to LAC, Notch may play a tumor suppressor role in the lung-SCC subtype of malignancy [170, 186–189].

Interestingly, as observed for cutaneous tumors, loss of Notch activity appears to be needed for growth of tumors in tissues derived from squamous differentiated epithelium. Thus, different subtypes of lung cancer display strikingly different roles for Notch signaling in cancer development, possibly depending on the type of cells involved, the crosstalk with other molecular pathway or the fine tuning of Notch activation in different biological context.

Notch as therapeutic target

Notch signaling is implicated in a variety of processes leading to cancer initiation, growth and progression, and has therefore been a focus for the development of novel therapies in the recent years.

As the Notch pathway requires a proteolytic cleavage by γ -secretase for the generation of its active intracellular form, small γ -secretase inhibitor (GSI) molecules have been developed to interfere with Notch1 activity (Figure 2). Unfortunately, testing in animal models and clinical trials has revealed a high gastrointestinal toxicity due to the accumulation of goblet cells in the intestine upon Notch-dependent induction of differentiation [190, 191]. Alternatively, a combination of treatment for GSI blockage and glucocorticoids mitigate intestinal side effects and control goblet cell metaplasia [192]. Other proteolytic enzymes, such as ADAM10/17, participate in pathway activation and are used as target molecules for α -secretase inhibition (Figure 2) [193, 194]. A promising approach involves specific blockage of the receptor-ligand interaction, for which a strong knowledge of the basic biological processes is necessary. Antibodies against Notch1 and Notch2 receptors protect the intracellular domain from its own cleavage, inhibiting the release of NICN1 and NICN2 active molecules (Figure 2) [195].

Importantly, the molecular specificity of these antibodies reduces the intestinal side effects and, in particular, a blocking antibody against Notch1 showed promising results in inhibiting tumor growth [196]. As the function of the Notch pathway strongly relies on the specific interaction between receptor and ligand, whose type varies from system to system, the efficiency of blockers strongly depends on the analyzed tissue [197]. Similarly to the blocking antibodies, several synthetic peptides have been developed to inhibit Notch activation. They are mainly used for basic research studies, although a blocker of the Notch-coactivator MALM1 was found to have useful applications in the treatment of several models of human T-ALL by interfering with cell proliferation and leukemia progression (Figure 2) [198]. Finally, it might be of interest to alter the regulation of Notch turnover by interfering with its trafficking in the cancer cell secretory pathway (Figure 2) [199, 200].

An additional level of complication in the development of anti-Notch therapies is the condition in which Notch works as tumor-suppressor, as this varies significantly depending on the tissue analyzed, the type of receptor-ligand interaction and the degree of tumorigenic progression. Additional studies are needed to support the most adequate therapy in each case. In head and neck cancer the heterogeneity of the tissues affected increases the level of complexity in predicting which Notch receptor and ligand are most relevant to sustain the tumor. Mapping the expression and the level of activation of the pathway might be a valuable initial screening to determine the relevant molecules in each condition and their clinical interest. Finally, Notch agonists and antagonists could be used in combination with existing therapies to contrast tumor development.

Conclusions

To summarize, the Notch pathway plays an essential role in regulating major aspects of tumor emergence, maintenance and evolution into a more aggressive phenotype. It is involved in

preserving the cellular elements that sustain carcinoma, the formation of a supportive microenvironment and their bilateral synergistic interaction. Although its effect varies greatly from system to system, Notch is central in head and neck malignancies, particularly in the most common and aggressive squamous cell carcinoma subtype. In order to improve our therapeutic approaches, we need a deeper molecular understanding of the function of the Notch pathway, including detailed knowledge of the pattern of expression in the heterogeneous population of tumor cells, genetic changes, transcript signatures and fine tuning of activity levels in a dynamic view that takes into consideration the evolution of the disease over time.

Figure Legends

Figure 1. Epithelia in the head and neck region

Various types of epithelial tissue line the walls of head and neck structures (A). Mesenchyme and epithelia are divided by the ECM-rich basement membrane (brown line), in direct contact with the first layer of immature keratinocytes. Depending on the type of cells found, epithelial tissue is classified as stratified squamous epithelium, cuboidal epithelium, and columnar epithelium. Epithelia exposed to a continuous mechanical stress, such as masticatory mucosa and tongue epithelium (upper and middle left panels), contain an external layer of deposited keratins to function as protective layer. Simple columnar epithelium is found in the striated ducts of salivary glands (right lower panel), while the intercalated ducts are mainly formed by cuboidal epithelium (right middle panel). A specialized form of columnar epithelium is found in the respiratory epithelium of the nasal mucosa, where cilia work as both a physical barrier to pathogens and are actively engaged in their removal via beating motion.

Figure 2. The Notch signalling: controlling steps at a glance

The Notch signalling pathway is a complex pathway with various levels of regulation and fine-tuning. Initially, the immature Notch receptor faces a step-wise process of cleavage and glycosylation in the golgi apparatus, before being exposed to the plasma membrane as a functional molecule. On the cell surface, it interacts with a member of the Delta or Jagged family present on the surface of the juxtaposed cell. Ligands present on the surface of the same cell exposing Notch participate in a more complex level of regulation of the pathway, known as *cis-inhibition* (not depicted, for reviews [21, 201]). Activation of the pathway occurs upon receptor-ligand physical interaction, when the receptor undergoes a series of cleavages for the intracellular release of its active intracellular domain (NICD), which then transmigrates to the nucleus to join the complex for the activation of transcription of Notch-target genes. In the

absence of the NICD, the complex works as a repressor of transcription. The NICD is additionally regulated by its transport in the endosomes and final lysosomes degradation, under the control of specialized inhibitor of Notch activity (such as NUMB).

Figure 3: Notch regulates major elements in the head and neck tumor microenvironment

The Notch pathway is involved in the maintenance of a healthy epithelium homeostasis (A) as well as central tumorigenic elements (B). It controls the balance between self-renewal and differentiation of stem cells in healthy conditions, as well as regulating the amplification of undifferentiated progenitors in the cancer core. It also participates in the process of EMT and its reverted MET, at the basis of invasiveness and metastases formation. Finally, it modulates the formation of an inflammatory milieu, inducing macrophage subtype specification and oxygen levels establishment in the tumour microenvironment. Depicted blue cells highlight the elements where Notch operates.

Acknowledgements

CP, and TAM were supported by the UZH (University of Zurich). CP is a recipient of the Olga Mayenfisch Stiftung grant. The author would like to thank Christian Thomas Meisel and Alexandra Rose Noble for constructive criticism on the manuscript.

Conflict of interest: The authors declared no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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A

A

B

Healthy Microenvironment

This diagram illustrates a healthy microenvironment. It features a wavy, undulating layer of cells, likely representing the intestinal mucosa. The cells are depicted as light yellow, elongated, and somewhat rectangular, with a darker yellow or orange nucleus. A single cell in the center of the wavy layer is highlighted in blue, and it is shown dividing into two daughter cells, indicated by two curved arrows pointing away from the cell. Below the wavy layer, the background is a solid light gray. In the lower right area, there are two small, dark gray, irregularly shaped cells, one of which is a star-like shape. In the lower left area, there is a small, dark gray, circular cell. The overall scene represents a normal, healthy state of the microenvironment.

The diagram illustrates the Tumor Microenvironment and the process of Epithelial-Mesenchymal Transition (EMT). It shows a transition from a structured epithelial layer (orange cells) to a disorganized, invasive cell mass (purple cells). Key factors and processes labeled include:

- EMT (Epithelial-Mesenchymal Transition):** Indicated by arrows showing cells moving from the epithelial layer into the stroma. Associated with $\uparrow$ Snail/Slug and $\downarrow$ E-Cad.
- Metastasis:** Cells are shown migrating to form a secondary tumor in the **distal tissue**. This process is regulated by **MET** (Metastasis-Inducing Factor).
- Stromal Factors:**
 - TNF α , IL1 β , IL6:** Secreted by **M1** (pro-inflammatory) macrophages.
 - TGF β , IL10, IL4:** Secreted by **M2** (anti-inflammatory) macrophages.
- Hypoxia:** Indicated by $\downarrow$ O $_2$ leading to **HIF1** activation, which promotes tumor progression.