

Review

Notch1 in oral squamous cell carcinoma

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Summary. Notch signaling has been reported to be involved in several types of malignant tumors. However, the role and activation mechanisms of Notch signaling in oral squamous cell carcinoma (OSCC) remain poorly characterized. The present review focuses on the dual role of Notch signaling in OSCC. A number of expression and functional analyses demonstrated that Notch1 plays a crucial role in development and progression of OSCC. On the other hand, a tumor suppressive role of Notch1 was also suggested from studies, based on deep sequencing of cancer genomes. Interestingly, although some Notch1 mutations overlap in tumors from Caucasian and Asian patients, the overall spectrum of such mutations is vastly different between these cohorts. Accumulating evidence suggests that variation of Notch1 mutation signature may determine the role of Notch signaling in OSCC. As Notch is thought to act as an oncogene in a subset of OSCC, but also has a tumor suppression role, the role of Notch in OSCC seems to be highly context dependent.

Key words: Notch signaling, Oncogene, Tumor suppressor gene, Oral squamous cell carcinoma

Introduction

Oral cancer, predominantly oral squamous cell carcinoma (OSCC), is one of the most common cancers (Siegel et al., 2012), and the survival rate of patients with oral cancer has not improved, despite

improvements and innovations in diagnostic techniques and treatments (Gupta et al., 2009; Shah and Gil, 2009; Dansky Ullmann et al., 2012). To date, the multistep development of the squamous cell carcinoma of the head and neck (HNSCC) including oral cavity is well recognized. In the model, accumulation of multiple genetic alterations, influenced by a patient's genetic predisposition and environmental influences, are needed for carcinogenesis. These changes in mucosal epithelia lead to the appearance of precancerous lesion of oral cavity (Califano et al., 1996; Rothenberg and Ellisen, 2012). Therefore, a better understanding of the molecular mechanisms underlying the development of OSCC should add to the improvement of prevention, early detection and prognosis of this disease.

The Notch signaling pathway is critical for cell proliferation, differentiation, development and homeostasis (Artavanis-Tsakonas et al., 1999; High and Epstein, 2008; Ranganathan et al., 2011). Therefore, the perturbations in Notch signaling pathway are closely associated with various mammalian diseases, including several inherited syndromes and cancers (Artavanis-Tsakonas et al., 1999; Gridley, 2003; Fortini, 2012; Louvi and Artavanis-Tsakonas, 2012). Although the oncogenic role of Notch signaling was initially demonstrated in T-ALL (Robbins et al., 1992), a common theme for the role of Notch signaling in tumorigenesis remains unclear. In many solid tumors; including lung (Westhoff et al., 2009), breast (Reedijk et al., 2005), pancreas (Miyamoto et al., 2003) and prostate (Zayzafoon et al., 2004), Notch1 signaling seems to play a crucial role. On the other hand, Notch1 signaling also appears to have tumor suppressor roles in murine skin (Nicolas et al., 2003), human pancreatic (Hanlon et al., 2010) and hepatocellular carcinomas (Viatour et al., 2011.) and small-cell lung cancer (Sriuranpong et al., 2001). Taken together, these observations indicate that aberrant

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activation of the Notch1 signaling pathway plays various roles in solid tumors. Moreover, the cellular reactions and outcomes of the overexpression of Notch1 activity are highly dependent on contextual cues, such as interactions with the tumor microenvironment and crosstalk with other signaling pathways. In this review, we discuss the evidence for both oncogenic and tumor suppressor roles of Notch 1 signaling in OSCC and suggest that researchers should take into account the bifacial function of Notch1 when considering OSCC.

Notch signaling

It has been reported that a small number of evolutionarily highly conserved signaling mechanisms operate across multicellular organisms, and are critical for development and homeostasis in most tissues. This group includes the WNT, Sonic hedgehog, Bone morphogenetic protein (BMP)–Transforming growth factor- β (TGF- β) and Notch signaling pathways (Richards and Degnan, 2009). Notch signaling has been highly conserved through the evolution from worm to man, and plays an important role in cellular patterning and differentiation during development (Artavanis-Tsakonas et al., 1999). Mammals have four Notch receptors (Notch1-4), that are transmembrane receptors harboring an extra-cellular domain responsible for the binding of their specific ligands, and an intra-cellular domain involved in transcriptional regulation. Notch receptors can receive signals from neighboring cells, which express transmembrane-type ligands such as Delta-like ligand (Dll) and Jagged (JAG) (Gray et al., 1999). Ligand binding to Notch receptors leads to the activation of common intra-cellular signaling pathways, which results in cleavage of the intra-cellular Notch domain (NICD). Engagement of Notch receptor with its

ligand induces a conformational change, exposing the S2 cleavage site to an ADAM family metalloprotease, tumor necrosis factor- α -converting enzyme (TACE). Following S2 cleavage, Notch receptor is accessible to a third cleavage (S3), that is mediated by the γ -secretase complex, which is composed of presenilin, nicastrin (NCSTN), presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1). Following this final cleavage, the NICD translocate into the nucleus. In the nucleus, NICD binds to the CSL (RBP- $\text{J}\kappa$) complex and converts the complex from a transcriptional repressor to an activator, by displacing the co-repressor complex (Kao et al., 1998) and recruiting a co-activator complex, composed of mastermind-like 1 (MAML1) (Kovall, 2008). Previously, a number of Notch target genes have been identified, and among them, hairy enhancer of split (HES) and hairy-related transcription factor (HEY) families are often used as indicators of activation status of Notch signaling (Wu et al., 2002) (Fig. 1).

Notch signaling in cancer

A role for Notch1 signaling pathway in tumorigenesis was initially noted in a subset of T-cell acute lymphoblastic leukemia (T-ALL) (Ellisen et al., 1991). Most T-ALL cases harbor activating mutations in the *Notch1* locus (Weng et al., 2004). These mutations generally result in increased stability of the NICD, because of ligand-independent proteolytic cleavage of Notch1 (Malecki et al., 2006). In addition, recent studies have also identified activated *Notch1* mutations in chronic lymphocytic leukemia (CLL) (Puente et al., 2011). In solid tumors, Gallahan et al. (Gallahan et al., 1987; Gallahan and Callahan, 1997) reported the initial evidence for the oncogenic role of Notch signaling pathway in mammary epithelial cell, and Capobianco

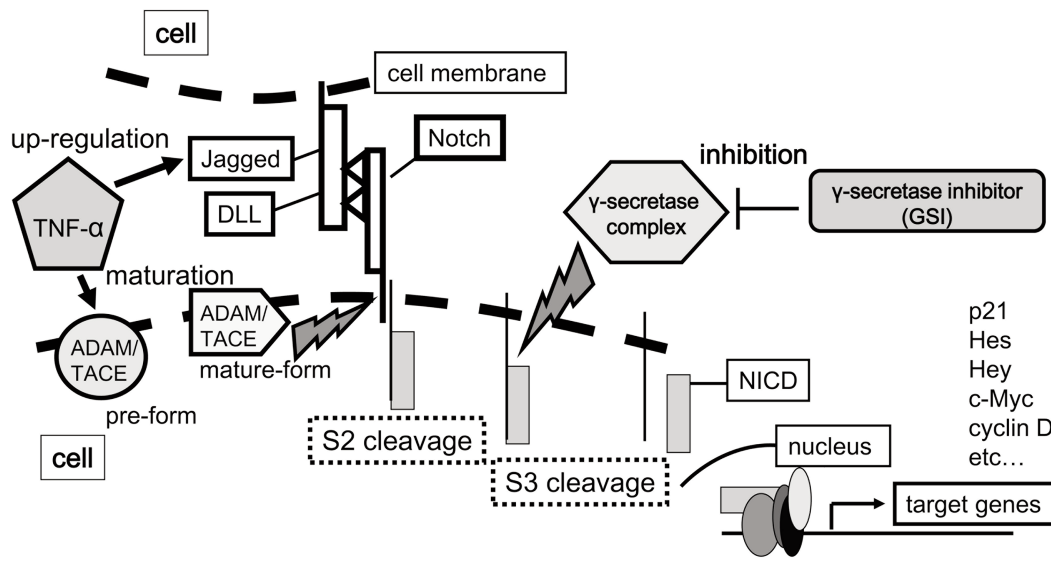


Fig. 1. An overview of Notch signaling pathway. The Notch protein sits like a trigger spanning the cell membrane, with part of it inside and part outside. Ligand proteins binding to the extracellular domain induce proteolytic cleavage by tumor necrosis factor- α -converting enzyme (TACE) and γ -secretase complex, following the release of the intracellular domain, which enters the cell nucleus to modify gene expression.

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and colleagues (Capobianco et al., 1997) demonstrated that Notch1, as well as Notch2, could transform primary rodent epithelial cells by adenoviral E1A. Subsequently, the dysregulated expression of Notch receptors, ligands and targets is also observed in solid tumors, including lung, pancreatic, cervical and prostate carcinomas (Weijzen et al., 2002; Miyamoto et al., 2003; Zayzafoon et al., 2004; Westhoff et al., 2009). As well as influencing tumor initiation, it has been reported that Notch signaling also plays an important role in tumor progression, including angiogenesis, epithelial to mesenchymal transition (EMT)-driven metastatic tumor growth and the maintenance of cancer stem cells (Ranganathan et al., 2011). With the similar concept, we have reported the biological significance of Notch1 signaling in several types of tumors (Yoshida et al., 2013; Hassan et al., 2014; Wael et al., 2014; Hassan et al., 2016). In addition to these basic investigations, the clinical significance of Notch signaling related molecules has been elucidated. For example, high levels of Notch1 and JAG1 were noted in a subset of breast cancers with poor prognostic pathological features (Reedijk et al., 2005), and high-level of expression of JAG1 has been associated with cancer progression and metastasis in patients with prostate cancer (Santagata et al., 2004).

On the other hand, there is growing evidence that Notch signaling activation may have growth-suppressive functions in a variety of tumor types. In hematological tumor, Klinakis and colleagues (Klinakis et al., 2011) reported a novel inactivating mutation of Notch pathway molecules in patients with chronic myelomonocytic leukemia (CMML). They also showed that inactivation of Notch signaling in mouse hematopoietic stem cells (HSCs) results in an aberrant accumulation of granulocyte/monocyte progenitors, thereby inducing extramedullary hematopoiesis and CMML-like disease. In solid tumors, Hanlon et al. (2010) reported a tumor suppressive function of Notch1 in a mouse model of K-ras-induced pancreatic ductal adenocarcinoma. In hepatocellular carcinoma, activation of Notch pathway via E2F transcription factors serves as a negative feedback mechanism to suppress HCC growth, during tumor progression in mouse model, deleting the three

members of the Rb gene family: Rb1, p107, and p130 (Viatour et al., 2011). The tumor suppressor role of Notch signaling is generally thought to be a result of crosstalk with other signaling pathways, that govern decreased cell proliferation, increased apoptosis or promoting cell differentiation (Ranganathan et al., 2011). A tumor suppressive effect of Notch signaling has been elucidated in the skin. Nicolas and colleagues (Nicolas et al., 2003) described an increased incidence of skin cancers in conditional Notch1 knockout mice. Proweller et al. (2006) next demonstrated that the forced expression of pan-Notch inhibitor, dominant-negative Mastermind Like 1 (DNMAML1), in murine skin, led to the development of cutaneous SCC. It is postulated that the loss of Notch function contributes to skin carcinogenesis via both cell autonomous and non-cell autonomous manners. Dotto and colleagues (Rangarajan et al., 2001; Restivo et al., 2011) identified several Notch targets, that may mediate pro-differentiation and anti-growth effects, such as p21 and IRF6. On the other hand, Kopan and colleagues (Demehri et al., 2009) postulated that epidermal barrier defects caused by Notch loss-of-function produce a chronic cutaneous inflammatory state, that promotes skin tumorigenesis.

As described above, the bifacial roles of Notch signaling in solid tumor have been reported by many researchers (Table 1) (Capobianco et al., 1997; Sriuranpong et al., 2001; Weijzen et al., 2002; Miyamoto et al., 2003; Nicolas et al., 2003; Qi et al., 2003; Thorland et al., 2003; Parr et al., 2004; Veeraghavulu et al., 2004; Zayzafoon et al., 2004; Ishimura et al., 2005; Proweller et al., 2006; De La et al., 2008; Reedijk et al., 2008; Bin Hafeez et al., 2009; Demehri et al., 2009; Mittal et al., 2009; Miyaki et al., 2009; Mullendore et al., 2009; Westhoff et al., 2009; Whelan et al., 2009; Hanlon et al., 2010; Harrison et al., 2010; Ranganathan et al., 2011; Restivo et al., 2011; Viatour et al., 2011; Sun et al., 2014; Wael et al., 2014). The cellular reactions and outcomes of Notch1 signaling activity are highly dependent on contextual cues, such as interactions with the tumor microenvironment and crosstalk with other signaling pathways. In the following section we will discuss the oncogenic and tumor-suppressive role of Notch1 signaling in OSCC.

Table 1. Summary of the bifacial roles of Notch signaling in solid tumors.

Tumor type	Oncogenic function	Tumor-suppressive function	References
Breast	✓	✓	Capobianco et al., 1997; Parr et al. 2004; Mittal et al., 2009; Harrison et al., 2010
Colorectal	✓		Reedijk et al., 2008; Miyaki et al., 2009
Prostate	✓	✓	Whelan et al., 2009; Bin et al., 2009
Liver		✓	Qi et al., 2003; Ishimura et al., 2005; Viatour et al., 2011
Pancreatic	✓		Miyamoto et al., 2003; De La et al., 2008; Mullendore et al., 2009; Hanlon et al., 2010
Lung	✓	✓	Sriuranpong et al., 2001; Weijzen et al., 2002; Westhoff et al., 2009; Wael et al., 2014
Cervical	✓		Thorland et al., 2003; Zayzafoon et al., 2004; Veeraghavulu et al., 2004
Skin		✓	Rangarajan et al., 2001; Nicolas et al., 2003; Proweller et al., 2006; Demehri et al., 2009; Restivo et al., 2011

Oncogenic function of Notch1 in OSCC

Leethanakul and colleagues (Leethanakul et al., 2000) reported that Notch signaling pathway related molecules, such as Notch1, Notch2, JAG1, were overexpressed in four HNSCC cases including tongue squamous cell carcinoma, compared to matched normal tissues. Subsequently, several researchers reported an increased expression of Notch signaling-related molecules in OSCC. Hijioka et al. (2010) reported that mRNA levels of several Notch pathway-related genes; such as Notch1, Notch2, JAG1, Hes1 and Hey1, were upregulated in OSCC, compared to normal tongue tissues. Lee et al. (2012) reported that Hes1 expression was commonly upregulated in OSCC lesions, compared to precancerous dysplastic lesions. These early findings suggest the possible involvement of Notch1 signaling in OSCC progression. Recently, two studies have reported that the protein level of Notch1 and JAG1 were increased in oral dysplastic lesions, compared to normal mucosa (Yoshida et al., 2013; Gokulan and Halagowder, 2014). Especially in the rat animal model of tongue carcinogenesis, Notch1 expression exhibited the same tendency as that observed in the human tissue specimens (Yoshida et al., 2013). Sun et al. revealed that a larger subset exhibits other Notch1 signaling alterations, including increases in expression or gene copy number of the receptor or ligands, as well as downstream pathway activation by using large cohort of HNSCC samples including OSCC (Sun et al., 2014). This evidence strongly suggests that activated Notch1 signaling may contribute to oral carcinogenesis. In addition to this evidence which showed the possible involvement of Notch1 signaling in oral carcinogenesis, there were several reports that Notch1 signaling

participates in tumor progression of OSCC. Zeng et al. found that the level of Jagged1 was significantly correlated with tumor blood vessel content and associated with OSCC development (Zeng et al., 2005). Moreover, significant correlations were found between the expression of vascular endothelial growth factor and Notch1, and cervical lymph node metastasis, tumor depth, and microvessel density, in 51 patients with tongue cancer (Joo et al., 2009). Furthermore, Notch1 expression was found to be positively correlated with T-stage and clinical stage in OSCC patients, and Notch1 protein was localized to the invasive front of the tumor (Yoshida et al., 2013) (Fig. 2). These data suggest that Notch1 signaling plays an important role in tumor progression, by regulating proliferation, invasion and tumor microenvironment. A summary of the studies investigating the expression of Notch1 and clinical significance in OSCC is shown in Table 2 (Leethanakul et al., 2000; Joo et al., 2009; Gu et al., 2010; Hijioka et al., 2010; Zhang et al., 2010; Yoshida et al., 2013; Gokulan and Halagowder, 2014).

To date, the biological significance of Notch1 signaling in OSCC has been mainly investigated by *in vitro* studies. Inhibition of Notch1 signaling by γ -secretase inhibitor significantly reduced cell proliferation in tongue carcinoma cells via down-regulation of Notch and Nuclear factor kappa B pathways (Yao et al., 2007). Similarly, the tumor suppressive effects of various γ -secretase inhibitors and the knockdown of *Notch1* gene were reported by several researchers (Hijioka et al., 2010; Yoshida et al., 2013). On the other hand, Zeng and colleagues (Zeng et al., 2005) showed indirect evidence that Jagged1 induced by growth factors, via mitogen-activating protein kinase (MAPK) in tongue squamous cell carcinoma cells,

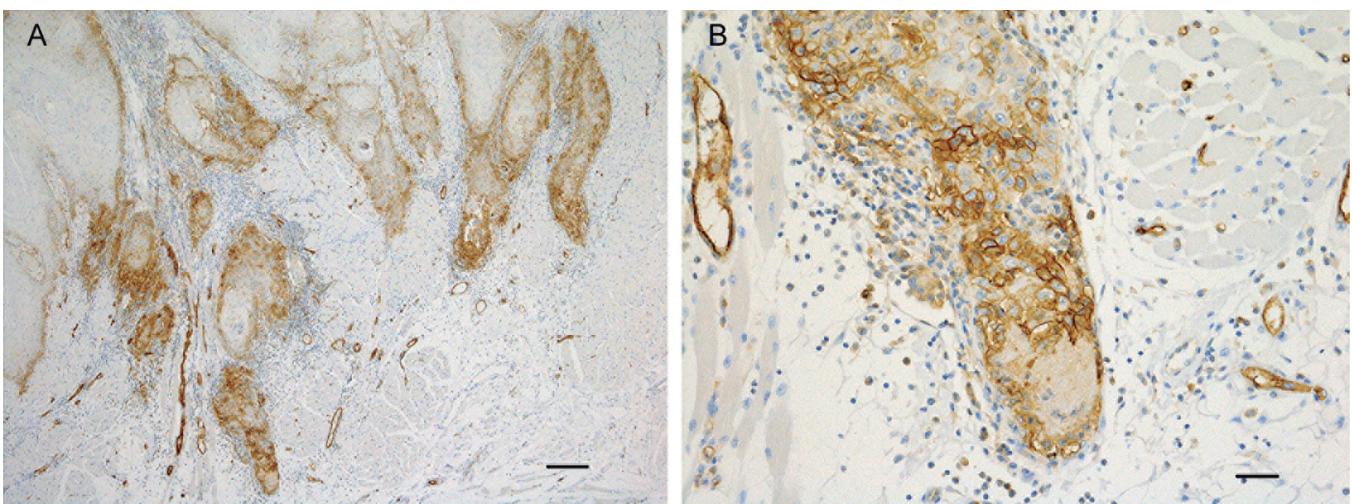


Fig. 2. Immunohistochemical staining of Notch1 in OSCC. **A.** Representative immunohistochemical stainings of Notch1 at OSCC in low power fields. Tissues were immunohistochemically stained using an antibody to Notch1. The membranous immunoreactions (brown) were regarded as positive for Notch1. **B.** Representative immunohistochemical stainings of Notch1 at OSCC in high power fields. Scale bars: A, 200 μ m; B, 100 μ m.

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triggered Notch activation in neighboring endothelial cells (ECs) and promoted capillary-like sprout formation. These authors also demonstrated that Jagged1-expressing tongue squamous cells significantly enhanced neovascularization and tumor growth *in vivo*. With regard to other malignant phenotypes, Yoshida et al. (2013) suggested that Notch1 may contribute in part to TNF- α -dependent OSCC cell invasion, via the transcriptional regulation of Slug and Twist, based on the findings that localizations of Notch1 and NICD were observed in invasive front of the tumors. Ishida et al. (2013) reported that hypoxia induces EMT in OSCC cell lines, via activation of Notch signaling. Accumulating evidence clearly indicated that the Notch signaling also plays a pivotal role in the maintenance of cancer stem cell (CSC) phenotype in many malignancies (Pannuti et al., 2010). In OSCC, the close relationship between Notch1 expression and cisplatin resistance was reported by Gu and colleagues (Gu et al., 2010). High expression levels of Notch1 were found to be closely associated with cisplatin resistance in cells obtained from patients with HNSCC (Gu et al., 2010). Further, Lee et al. (2016) reported that tumor necrosis factor alpha (TNF- α) -a major pro-inflammatory cytokine- enhances CSC characters of OSCC cells, such as an increase in tumor sphere-forming ability, stem cell-associated genes

expression, chemo-radio resistance, and tumorigenicity. In line with this, Zhao et al. (2016) showed the elevated expression of Notch1 and Hes1 in human HNSCC, especially in tissues after chemotherapy and in cases of lymph node metastasis. Moreover, γ -secretase inhibitor treatment significantly reduces CSCs population and tumor self-renewal ability *in vitro* and *in vivo*. Furthermore, Lee et al. (2016) reported that the up/down regulation of NICD expression affects the CSC phenotypes of HNSCC cells and changes the expression of stem cell markers. Taken together, Notch1 may be a critical regulator of CSC phenotypes in OSCC cells, and inactivation of this pathway could be a potential target for the treatment of OSCC. A summary of studies investigating a functional role of Notch signaling in OSCC is shown in Table 3 (Hijioka et al., 2010; Lee et al., 2012; Yoshida et al., 2013; Sun et al., 2014; Zhao et al., 2016).

Tumor suppressive function of Notch1 in OSCC

In contrast to the oncogenic role of Notch signaling in OSCC, several reports described the tumor suppressive role of Notch1 in OSCC. In 2006, Duan et al. reported that overexpression of NICD in tongue squamous cell carcinoma cells resulted in growth

Table 2. Summary of the studies investigating the expression of Notch1 and clinical significance in OSCC.

Tumor samples	Control samples	Observation	Implication	References
HNSCC tissues including tongue tissue	Matched normal tissues	Overexpression of Notch1	Activated pathway	Leethanakul et al., 2000
Tongue tissues	NA	Notch1 expression was correlated with lymph node metastasis, tumor depth of invasion and microvessel	Metastasis	Joo et al., 2009
OSCC tissues	Normal tongue	Notch1 was upregulated in OSCCs	Activated pathway	Hijioka et al., 2010
HNSCC tissues including OSCC	NA	Notch1 expression positively correlated with cisplatin resistance	Chemoresistance	Gu et al., 2010
Tongue tissues	Adjacent non-neoplastic tissues	Notch1 overexpressed in cancerous tissue and high Notch1 expression positively correlated with lymph node metastasis	Activated pathway, Metastasis	Zhang et al., 2010
OSCC tissues	Normal and dysplastic tissues	Notch1 and NICD expression increased from normal, dysplastic to cancerous	Activated pathway	Yoshida et al., 2013
OSCC tissues	Normal and dysplastic tissues	NICD expression increased from normal, dysplastic to cancerous tissues	Activated pathway	Gokulan et al., 2014

Table 3. Summary of studies investigating a functional role of Notch signaling in OSCC.

Materials	Observation	Implication	References
OSCC cell line	Treatment with γ -secretase inhibitor evoked cell growth inhibition	Growth	Hijioka et al., 2010
OSCC cell line	Treatment with TNF- α enhanced cancer stem cell phenotypes via Notch1 signaling activation	Cancer stem cell phenotypes	Lee et al., 2012
OSCC cell line	Treatment with γ -secretase inhibitor and Notch1 knock-down reduced cell growth and invasion	Growth, Invasion	Yoshida et al., 2013
OSCC cell line	Treatment with γ -secretase inhibitor and Notch1 knock-down reduced cell growth	Growth	Sun et al., 2014
OSCC cell line	Notch1 inhibition enhanced the sensitivity of chemotherapy via suppression for cancer stem cell phenotypes	Chemoresistance, Cancer stem cell phenotypes	Zhao et al., 2016

Table 4. Summary of the studies investigating the tumor suppressive role for Notch signaling in OSCC.

Materials	Observation	Implication	References
Tongue carcinoma cell line	Overexpression of NICD reduced cell growth	Growth	Duan et al., 2006
Oral precancerous and cancerous tissues	Reduced Notch1 expression in both precancerous and cancerous lesions	Inactive pathway	Sakamoto et al., 2012
OSCC cell line	Notch1 knock-down disrupted terminal differentiation of OSCC	Differentiation	Sakamoto et al., 2012

suppression *in vitro* and *in vivo*, accompanied by cell cycle arrest and apoptosis (Duan et al., 2006). To date, there is only one report describing reduced levels of Notch1 protein in primary OSCCs, compared to normal epithelium. In that report, the tumor suppressive role of Notch1 in oral carcinogenesis was explained based on its role of maintaining epithelium differentiation *in vitro* (Sakamoto et al., 2012). A summary of studies investigating the tumor suppressive role for Notch signaling in OSCC is shown in Table 4 (Duan et al., 2006; Sakamoto et al., 2012).

With the advent of next generation sequencing platform, a tumor suppressive role of Notch pathway in HNSCC was revealed by several research groups, using exome sequencing. Agrawal and colleagues showed that Notch1 was one of the most frequently mutated genes, with mutations being detected in 15% of HNSCCs, by using whole-exome sequencing and gene copy number analyses. In that report, a subset of mutations identified in Notch1 were predicted to produce truncated form protein. Therefore, the authors speculated that *Notch1* may behave as a tumor suppressor gene, rather than an oncogene in HNSCC (Agrawal et al., 2011). At the same time, it was also reported that about 30% of cases harboring mutations in genes that regulate squamous differentiation, have mutations in *Notch1*, implicating its dysregulation as a major driver of HNSCC carcinogenesis (Stransky et al., 2011). Pickering et al. revealed more somatic events than previously reported, by using comprehensive genomic analysis of gene expression, copy number, methylation, and point mutations in OSCC. The authors identified four major driver pathways (mitogenic signaling, Notch, cell cycle, and TP53) and two additional key genes (*FAT1*, *CASP8*) in integrated analysis. In that cohort, Notch pathway was defective in 66% of cases and in follow-up studies showed functional Notch1 signaling inhibited proliferation of OSCC cell lines (Pickering et al., 2013).

In contrast to the previous studies, Son and colleagues (Song et al., 2014) recently revealed that six mutations in Notch1 coding regions were detected in 4 (31%) of the 13 HNSCC cell lines. Moreover, forty-two somatic *Notch1* mutations within the domain, commonly harboring potential activating mutations in acute lymphoblastic leukemia, were detected in 43% of Chinese OSCC tumors. Furthermore, patients harboring Notch1 mutation had significantly poor survival rate, compared with those whose tumors harbored no Notch1 mutations. According to Izumchenko et al. (2015), *Notch1* mutations were found in 60% of premalignant

lesions and 54% of primary OSCC, and almost 60% of patients with leukoplakia carry Notch1 mutations that are also identified in OSCC. Interestingly, although there were overlaps in critical regulatory Notch1 domains alterations, when compared all known *Notch1* mutations identified in Chinese OSCC patients with those reported in Caucasian population, possible gain-of-function mutations of *Notch1* were predominantly seen in Chinese population. The authors concluded that gain of function mutation of *Notch1* was a main event in OSCC progression. It has been regarded that the discrepancy in the potential role of *Notch1* mutations may rely on the different mutation spectra, in different cohort studies or population. Collectively, further investigation of *Notch1* mutation signature in tumors, from distinct ethnic and geographic areas will be needed, to clarify the precise role of Notch signaling in development and progression of OSCC.

Conclusion

In this review, we have demonstrated that a number of studies have indicated that up-regulation of Notch signaling may contribute to the malignant phenotype in head and neck squamous cell carcinomas (HNSCCs), including those of the oral cavity (Yoshida et al., 2013; Song et al., 2014; Sun et al., 2014; Izumchenko et al., 2015). On the other hand, several researchers have shown a tumor suppressive function of Notch signaling (Duan et al., 2006; Agrawal et al., 2011; Sakamoto et al., 2012).

As OSCC is a very heterogeneous disease, thereby the functional role of Notch signaling is dependent on the genetic background and cross talking with other pathways of individual tumors. Therefore, investigating the factors which mediate the oncogenic and tumor suppressive activities of Notch signaling in OSCC will be required, to elucidate the uncertain mechanisms of development and progression of OSCC.

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