Upregulation of Notch pathway molecules in oral squamous cell carcinoma

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Abstract. The constitutive activation of the Notch pathway has been demonstrated in various types of malignancies. However, it remains unclear how the Notch pathway is involved in the pathogenesis of oral squamous cell carcinoma (OSCC). We investigated the expression of Notch pathway molecules in OSCC cell lines and biopsy specimens and examined the effect of Notch pathway inhibition. Reverse transcription-polymerase chain reaction revealed upregulation of *Notch1*, *Notch2*, *Jagged1*, *HES1* and *HEY1* in both OSCC cell lines and biopsy specimens. Immunohistochemical examination showed that the Notch intracellular domain accumulates in the nucleus of cells in OSCC cell lines and biopsy specimens. In addition, Jagged1 is expressed in the cytoplasm of cells in OSCC cell lines and biopsy specimens. Furthermore, Notch pathway inhibition using a γ-secretase inhibitor prevented the growth of OSCC *in vitro*. These findings suggest that inhibition of the Notch pathway suppresses OSCC growth and may be a useful approach for the treatment of patients with OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common head and neck cancer, and the overall prognosis of patients with OSCC remains poor. Because of extensive recent research on its pathogenesis and management, the 5-year survival rate for patients with OSCC has improved slightly within the last 15 years, but it still remains at 60% (1). A number of etiologic factors have been implicated in the development of OSCC, such as the use of tobacco and alcohol and the presence of incompatible prosthetic materials

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(2). However, some patients develop OSCC without risk factors, which suggest that host susceptibility may also play a role in the development of OSCC. Molecular alterations in a number of oncogenes and tumor suppressor genes associated with the development of OSCC could be important for improving early detection and effective treatment of OSCC (3).

Members of the Notch family are highly conserved trans-membrane receptors that influence the proliferation and apoptosis of diverse types of cells in a variety of organisms (4). Activation of Notch signaling requires binding of its ligands, Jagged and Delta-like (DLL) (5,6), followed by proteolytic release of the Notch intracellular domain (NIC) and its translocation to the nucleus (7). NIC interacts with CSL, CBF1/RBP-Jκ, Su (H), Lag-1, transcription factors and converts them from repressors to activators, thereby promoting transcription of downstream genes involved in various differentiation programs (8).

Recent studies have demonstrated constitutive activation of the Notch pathway in various types of malignancies. The oncogenic potential of Notch has been reported in human T-cell acute lymphoblastic leukemia (9), non-small cell lung cancer (10), ovarian carcinomas (11), colon cancer (12), pancreatic cancer (13) and osteosarcoma (14). On the other hand, Notch signaling promotes differentiation in skin cells (15), and loss of function of Notch is permissive for tumor formation in a mouse model (16). Although 3 expression array studies reported the upregulation of some members of the Notch signaling pathway in head and neck SCC including OSCC (17-19), the function of the Notch pathway in oral squamous cell tumorigenesis has not been previously characterized.

To explore the involvement of aberrant Notch signaling in the pathogenesis of OSCC, we investigated the expression of the Notch pathway molecules in both OSCC cell lines and biopsy specimens of OSCC patients, and we examined the effects of Notch pathway inhibition by a γ-secretase inhibitor (GSI), a pharmacological agent known to effectively block Notch activation by inhibiting the proteolysis and translocation of NIC to the nucleus. We found that *Notch1*, *Notch2*, *Jagged1* and the Notch targets *HES1* and *HEY1* (20,21) were upregulated in cells in both OSCC cell lines and biopsy specimens. In addition, γ-secretase inhibition prevented the growth of OSCC cells *in vitro*.

Table I. PCR primer sequences.

| Target molecules		Primer sequence
Notch1	Forward	5'-GTGACTGCTCCCTCAACTTCAAT-3'
	Reverse	5'-CTGTCACAGTGGCCGTCACT-3'
Notch2	Forward	5'-AAAAATGGGGCCAACCGAGAC-3'
	Reverse	5'-TTCATCCAGAAGGCGCACAA-3'
Jagged1	Forward	5'-CGGGATTTGGTTAATGGTTATC-3'
	Reverse	5'-ATAGTCACTGGCACGGTTGTAGCAC-3'
HES1	Forward	5'-AGGCGGACATTCTGGAAATG-3'
	Reverse	5'-CGGTACTTCCCCAGCACACTT-3'
HEY1	Forward	5'-CGAGGTGGAGAAGGAGAGTG-3'
	Reverse	5'-CTGGGTACCAGCCTTCTCAG-3'
GAPDH	Forward	5'-GAAGGTGAAGGTCGGAGTC-3'
	Reverse	5'-GAAGATGGTGATGGGATTTC-3'
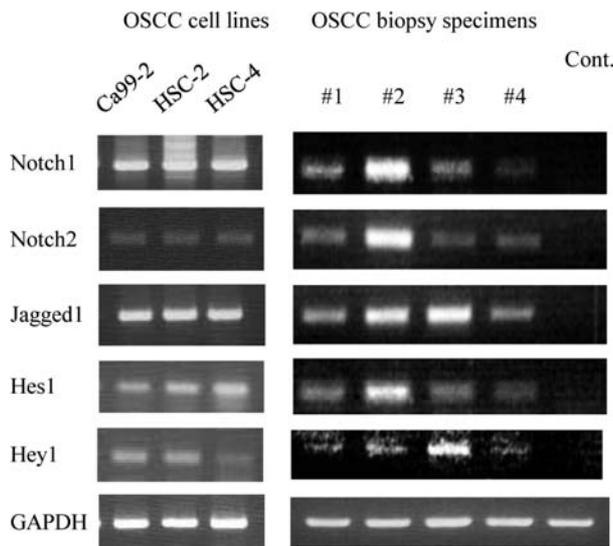
Figure 1. Expression of Notch signaling molecules in OSCC. RT-PCR revealed upregulation of Notch-related genes. *Jagged1* is a Notch ligand; *HES1* and *HEY1* are Notch target genes.

Materials and methods

Cell culture. The human OSCC cell lines Ca99-2, HSC-2 and HSC-4 were purchased from Health Science Research Resources Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were grown in a humidified atmosphere containing 5% CO_2 at 37˚C.

Biopsy specimens. All human OSCC biopsy specimens were obtained from primary lesions after informed consent according to a protocol that was approved by the institutional review board of Kagoshima University. Biopsies were performed before chemotherapy or radiotherapy to make the diagnosis. The respected tissues were divided into two parts: one was frozen immediately and stored at -80˚C until use, and the other was fixed in 10% buffered formaldehyde solution. Normal mucosa tissue used as a control was obtained from the tongue of a 76-year-old woman.

RT-PCR and real-time PCR. Total RNA was DNAse-treated and reverse-transcribed using oligo (dT) primers according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNAs were synthesized with 5 μg of total RNA as a template in a 20 μl reaction mixture by using Superscript II reverse transcriptase (Gibco-BRL). After reverse transcription, each reaction mixture was diluted 5-fold with H_2O, and 1 μl of each mixture was subjected to PCR using Taq polymerase (Takara, Tokyo, Japan). For real-time PCR assay, reactions were run using SYBR-Green (Bio-Rad, Hercules, CA, USA) on a MiniOpticon™ machine (Bio-Rad). The comparative Ct ($\Delta\Delta$Ct) method was used to determine fold change in expression using ßII-microglobulin. Each sample was run at three concentrations in triplicate. All primer sets amplified 100 to 200 bp fragments. The primers sequences used are shown in Table I. The cycling conditions were as follows: initial denaturation at 98˚C for 3 min; 30 cycles at 98˚C for 10 sec, 60˚C for 30 sec, and 72˚C for 1 min; and 72˚C for 7 min.

Immunohistochemical examination. HSC-4 cells were fixed with 4% paraformaldehyde in PBS and subjected to immunofluorescent staining. Cells were blocked with PBS containing 10% FCS and 0.1% Triton X-100. The following primary rabbit polyclonal antibodies were used: anti-NIC (diluted 1:200; Calbiochem, Basel, Switzerland) and anti-Jagged1 (diluted 1:200; R&D Systems, Minneapolis, MN, USA). A fluorescent rhodamine-conjugated donkey anti-rabbit IgG antibody (diluted 1:200; Chemicon, Temecula, CA, USA) was used as the secondary antibody. The cells were counterstained with Hoechst 33258 (Molecular Probes, Carlsbad, CA, USA) to identify the nuclei. Immunohisto-chemistry with each secondary antibody alone without primary antibody was performed as a control.
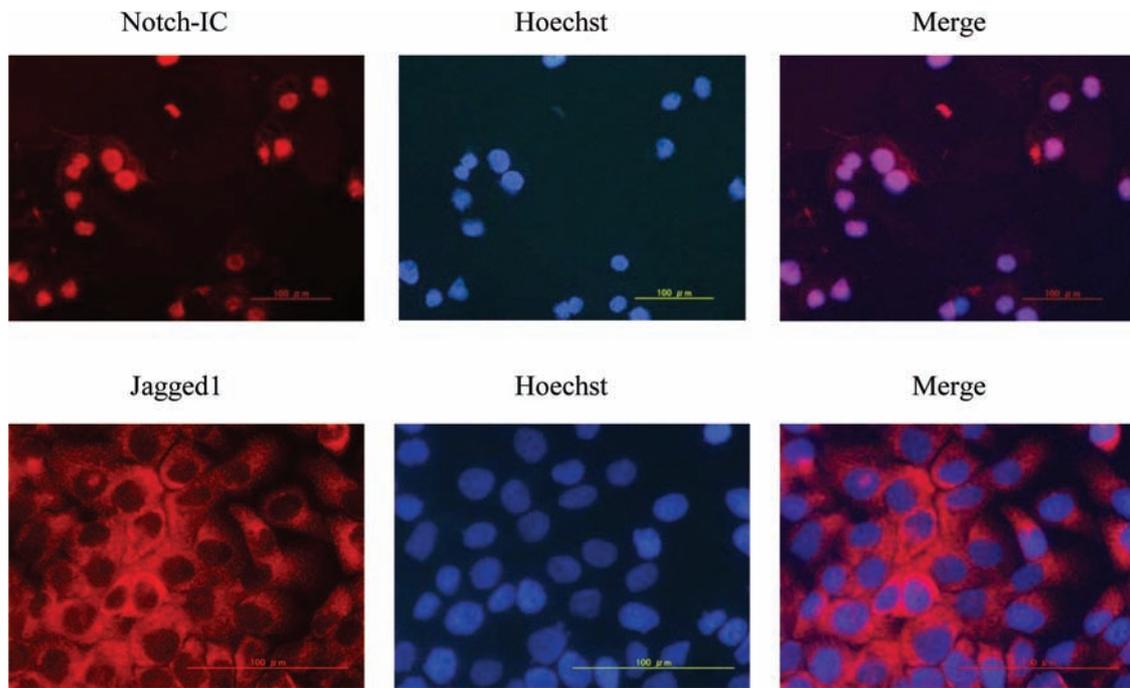
Figure 2. Expression of Notch intercellular domain (NIC) and Jagged1 in an OSCC cell line. Immunohistochemical examination revealed that NIC is accumulated in the nucleus of cells in OSCC cell lines, and Jagged1 is expressed in the cytoplasm of cells in OSCC cell lines.

Biopsy specimens were fixed routinely in 10% formalin, embedded in paraffin, and sectioned into 4-μm thick slices. The immunostaining procedure was performed with the Evision kit/HRP (Dako, Glostrup, Denmark). In preparation for staining with anti-NIC and anti-Jagged1 antibodies, specimens were treated with 0.01 M citrate buffer (pH 6.0) for 10 min at 121˚C in an autoclave. The sections were then washed thrice with PBS and incubated with either rabbit polyclonal anti-NIC antibody (diluted 1:150; Cell Signaling Technology, Danvers, MA, USA) or rabbit polyclonal anti-Jagged1 antibody (diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS at 4˚C overnight. After incubation with primary antibodies, the sections were incubated with the appropriate secondary antibodies conjugated with peroxidase-labeled dextran polymers for 40 min. After rinsing in PBS, the immune complex was visualized by incubating the sections with diaminobenzine tetrahydrochroride. The sections were rinsed briefly in water, counterstained with Mayer's hematoxylin, dehydrated, and mounted. Negative controls for the immunostaining procedure were prepared by omitting the primary antibody.

MTT assay. Cells were incubated with the substrate MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 4 h, washed with PBS, and lysed to release formazan from cells. The cells were then analyzed in a Safire microplate reader (Bio-Rad) at 562 nm. GSI X (Calbiochem, Basel, Switzerland) was used for the MTT assay.

Results

Upregulation of Notch pathway molecules in human OSCC. RT-PCR revealed that all OSCC cell lines and biopsy specimens expressed *Notch1* and *Notch2* mRNA (Fig. 1). In contrast, neither *Notch1* nor *Notch2* were expressed in the normal tongue tissue (Fig. 1). To further examine the expression of Notch pathway molecules, we performed RT-PCR for Notch ligands and Notch target genes. *Jagged1* was expressed in all OSCC cell lines and biopsy specimens, but not expressed in the normal tongue tissue (Fig. 1). Both *HES1* and *HEY1* mRNA were expressed in all cell lines and biopsy specimens, but neither of them was observed in the normal tongue tissue (Fig. 1). Immunocytochemical examination revealed that NIC was accumulated in the nuclei of HSC-4 cells, and Jagged1 was expressed in the cytoplasm of these cells (Fig. 2). We further investigated the expression of NIC and Jagged1 protein in biopsy specimens from OSCC patients. Immunohistochemical examination revealed that NIC was accumulated in the nuclei and Jagged1 was expressed in the cytoplasm of tumor cells from patients' biopsy specimens as well as in cell lines (Fig. 3). These findings suggest that the Notch signaling pathway is activated in human OSCC.

Inhibition of the Notch pathway prevents OSCC growth in vitro. To determine whether Notch pathway activation is required for OSCC cell growth, we used GSI X. We performed real-time PCR to determine the concentration of GSI X that effectively inhibited Notch activity in OSCC cells, and then measured the expression of the Notch pathway target gene *HES1*. In HSC-2 and HSC-4 cells, GSI X at a concentration of 5 μM was found to reduce mRNA levels of *HES1*, particularly in HSC-4 cells in which *HES1* decreased by more than 90% (Fig. 4A). Because GSI X prevented osteosarcoma cell growth at 5 μM concentration (14), we decided that 5 μM was an appropriate concentration for
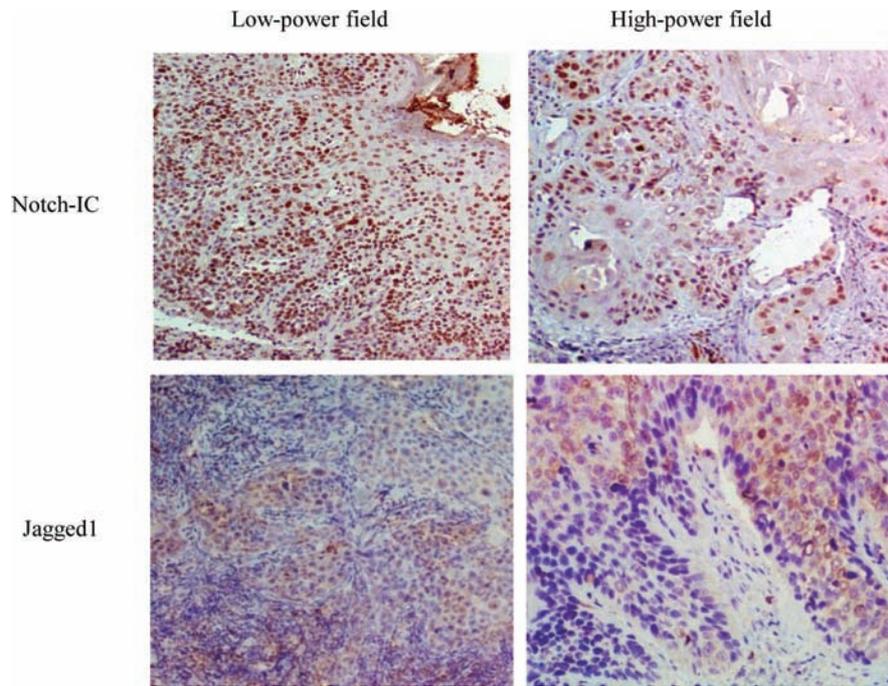
Figure 3. Expression of Notch intercellular domain (NIC) and Jagged1 in biopsy specimens from OSCC patients. Immunohistochemical examination revealed that NIC is accumulated in the nucleus of tumor cells in OSCC biopsy specimens, and Jagged1 is expressed in the cytoplasm of tumor cells in OSCC biopsy specimens. These results suggest that Notch signaling is activated in tumor cells in biopsy specimens from OSCC patients.
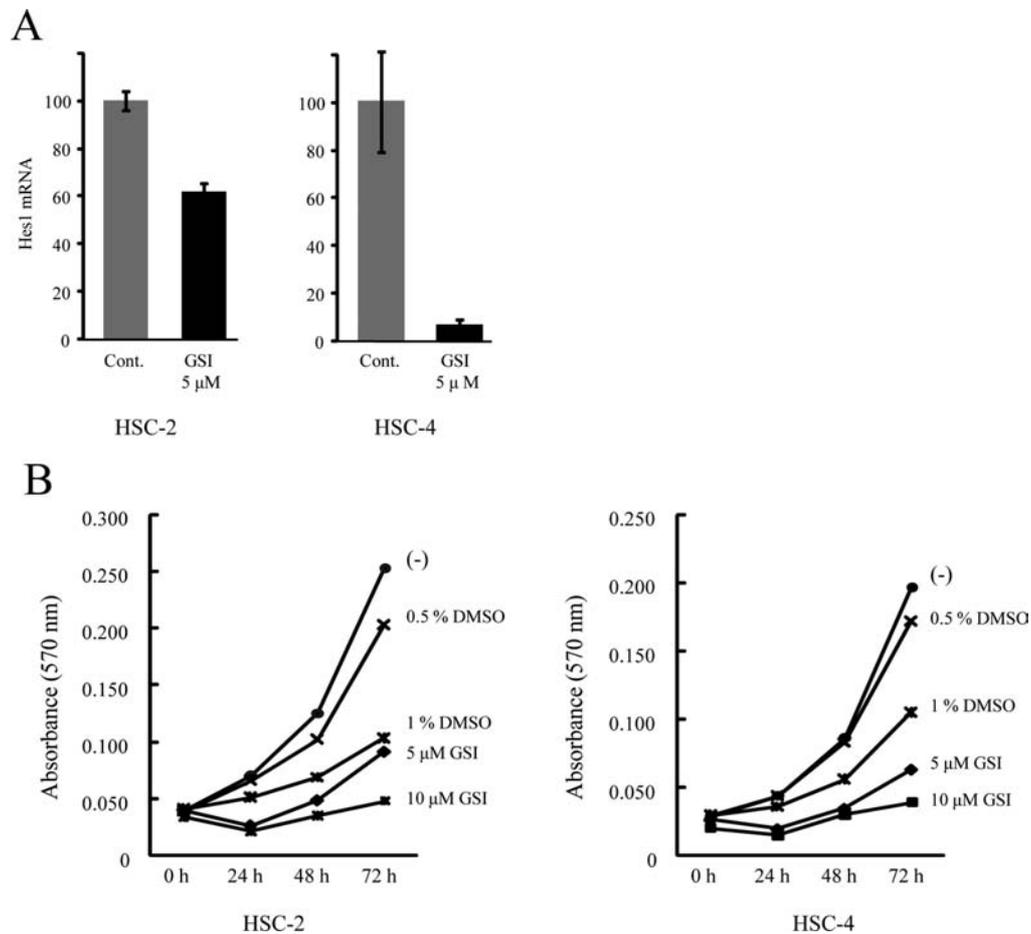

Figure 4. Inhibition of the Notch pathway prevents growth in OSCC cell lines. We performed real-time PCR to determine the concentration of GSI X that effectively inhibited Notch activity in OSCC cells, and then measured the expression of the Notch pathway target gene *HES1*. (A) GSI X at 5 μM reduced mRNA levels of *HES1* in HSC-2 and HSC-4 cells (error bar indicates standard deviation). (B) Growth of viable HSC-2 and HSC-4 cells over 3 days was slowed by GSI X in a dose-dependent fashion.

treating OSCC. We then assessed tumor growth *in vitro* under GSI treatment. The MTT assay revealed that GSI treatment slowed the growth of HSC-2 and HSC-4 cells in a dose-dependent fashion (Fig. 4B).

Discussion

Deregulation of Notch signaling is implicated in the development of various cancers, and Notch blockade appears to affect the survival and proliferation of multiple types of cancers (22-24). For example, *Notch* is activated by translocation or mutation in more than half of T-cell acute lymphoblastic leukemias, and anti-Notch treatments have been shown to slow the growth of acute lymphoblastic leukemia *in vitro* (25). In addition, combining GSIs, which inhibit Notch signaling, with anticancer drugs improves their anticancer effects (26,27).

We examined the expression of the Notch pathway genes in cell lines and tumors, and found that the expression levels of *Notch1*, *Notch2*, *Jagged1*, *HES1* and *HEY1* were upregulated compared to those of these genes in the normal oral tissue, suggesting that Notch signaling is active in OSCC. A number of previous studies support these findings and indicate a role for deregulation of Notch signaling in OSCC. Köse *et al* reported that Notch1 was absent in normal oral mucosa and even in oral pre-cancer lesions (28). Zeng *et al* revealed that Jagged1 was highly expressed in OSCC compared to that in the normal oral tissue, and Jagged1-expressing SCC cells were surrounded by microvessels (29). In recent expression array studies, two groups have independently reported significant upregulation of *Jagged1* and *Notch4* in OSCC compared to that in the normal oral tissue (17,18). In addition, Snijders *et al* have reported that DLL1 and RBPSUH are upregulated in OSCC (19).

GSIs prevent Notch receptor activation, and several forms of GSI have been tested for their antitumor effects (30). GSIs induce cell-cycle arrest and apoptosis in multiple cancer cell lines (26,31-33). We examined whether inhibition of the Notch pathway using GSI can block Notch signaling in OSCC and reduce tumor growth *in vitro*. Treatment of OSCC cells with GSI effectively reduced *HES1* mRNA and prevented growth in OSCC cell lines. These findings suggest that GSIs effectively inhibit the Notch pathway in OSCC cell lines and indicate an association between the activation of Notch signaling and pro-oncogenic effects in human OSCC. The role of GSI in inhibiting the growth of OSCC demonstrated in the present study is consistent with the previous findings in other malignancies (14,26,27,31,34-36).

In summary, our findings demonstrate that the Notch signaling pathway is functionally activated in human OSCC. This novel finding adds to the understanding of OSCC and may be important in understanding the proliferation of OSCC cells. Furthermore, the finding of growth inhibition by GSI, a Notch inhibitor, suggests that inactivation of Notch may be a useful approach for the treatment of patients with OSCC.

Acknowledgements

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