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Regulation of osteoprotegerin expression by Notch signaling in human oral squamous cell carcinoma cell line

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ABSTRACT

Objective: To investigate the influence of Notch signaling on osteoprotegerin (OPG) expression in a human oral squamous cell carcinoma cell line.

Methods: Activation of Notch signaling was performed by seeding cells on Jagged1 immobilized surfaces. In other experiments, a γ-secretase inhibitor was added to the culture medium to inhibit intracellular Notch signaling. OPG mRNA and protein were determined by real-time PCR and ELISA, respectively. Finally, publicly available microarray database analysis was performed using connection up- or down-regulation expression analysis of microarrays software.

Results: Jagged1-treatment of HSC-4 cells enhanced *HES1* and *HEY1* mRNA expression, confirming the intracellular activation of Notch signaling. OPG mRNA and protein levels were significantly suppressed upon Jagged1 treatment. Correspondingly, HSC-4 cells treated with a γ-secretase inhibitor resulted in a significant reduction of *HES1* and *HEY1* mRNA levels, and a marked increase in OPG protein expression was observed. These results implied that Notch signaling regulated OPG expression in HSC-4 cells. However, Jagged1 did not alter OPG expression in another human oral squamous cell carcinoma cell line (HSC-5) or a human head and neck squamous cell carcinoma cell line (HN22).

Conclusions: Notch signaling regulated OPG expression in an HSC-4 cell line and this mechanism could be cell line specific.

1. Introduction

Osteoprotegerin (OPG) is a decoy receptor regulating receptor and ligand interaction [1]. OPG is known to interact with receptor activator of nuclear factor kappa-B ligand (RANKL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), however, high-affinity binding was demonstrated for RANKL [1]. The role of OPG in osteoclastogenesis is well documented. OPG deficient mice exhibit a severe and early-onset osteoporosis phenotype as well as a high incidence of fractures [2]. Correspondingly, OPG transgenic mice that contained the human OPG promoter had significantly increased bone mass in cortical and trabecular bone [3]. In addition to the effect on bone phenotypes, OPG deficient mice demonstrate high aorta and renal artery calcification, implying the role of OPG in regulating pathological calcification [2]. Furthermore, OPG regulates other biological events, such as immune response, cell survival, and osteoblastic differentiation [1,4,5].

In cancer biology, OPG participates in bone invasion and apoptosis of cancer cells. OPG inhibits TRAIL-induced apoptosis in several types of cancers, including ovarian cancer cells, breast cancer cells, and colon cancer cells [6–8]. A

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strong negative relationship between endogenous OPG expression and TRAIL-induced cell apoptosis was observed in prostate cancer [9]. Exogenous OPG enhanced the proliferation of an osteosarcoma cell line partly via the nuclear factor kappa-B signaling pathway [10]. Further, a high RANKL/OPG ratio in human non-small cell lung cancer correlated with higher metastatic ability [11]. OPG also promotes endothelial cell survival and vessel formation [12]. Moreover, endothelial cells in malignant lesions express higher OPG levels than those in non-malignant tumors, implying the role of OPG in cancer angiogenesis [12]. Taken together, these data suggest a significant role of OPG in cancer cell behavior.

In oral cancer, OPG administration led to decreased mandibular bone invasion by transplanted human oral squamous cell carcinoma (OSCC) cells in nude mice [13]. In addition, OPG injected mice exhibit higher terminal deoxynucleotidyl transferase dUTP nick end labeling positive cancer cells compared with the control group, indicating that OPG promotes OSCC cell apoptosis [13]. Furthermore, OPG significantly inhibits RANKL-induced OSCC cell migration *in vitro* [13]. Another report indicated that OSCC promotes bone invasion via the suppression of OPG expression in the host stromal cells [14]. These results imply an important function of OPG in OSCC cell behavior.

Notch signaling is a highly conserved signaling pathway that regulates various cell functions in development, disease, and regenerative processes. It has been shown that Notch signaling regulates OSCC cell behavior [15,16]. In addition to the roles of Notch signaling in OSCC, it has been demonstrated that Notch signaling regulates OPG expression in osteocytes [17]. However, the relationship between Notch signaling and OPG expression in human OSCC cells remains unknown. Thus, the present study investigated the regulation of OPG expression by Notch signaling in a human OSCC cell line.

2. Materials and methods

2.1. Cell culture and treatment

Two human OSCC cell lines (HSC-4 and HSC-5) and one HNSCC cell line (HN22) were used in the present study. The HSC-4 and HSC-5 cell lines were gifts from Professor Teuro Amagasa (Tokyo Medical and Dental University, Japan). The HN22 cell line was kindly provided by Professor J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, USA). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 unit/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B at 37 °C in a humidified 5% carbon dioxide atmosphere. All cell culture reagents were purchased from Gibco BRL (Carlsbad, USA).

For Notch signaling activation, Jagged1 immobilization on tissue culture surfaces was performed according to our previous publication [18]. Briefly, tissue culture plates were coated with recombinant protein G (50 μg/mL) for 16 h, followed by bovine serum albumin (10 mg/mL) for 2 h and recombinant human Jagged-1/Fc (R & D systems, USA) for 2 h. The surfaces were washed with sterile phosphate buffer solution between each step. Subsequently, 75 000 cells/well were seeded on the Jagged-1 modified surfaces in 24-well plates and maintained in culture medium for 48 h. For the inhibition experiment, the cells were seeded on 24-well plates as described above and treated with a γ-secretase inhibitor (DAPT 20 μmol/L, Sigma–Aldirch, USA) for 48 h.

2.2. ELISA

Culture medium was collected 48 h after treatment and used to determine the secreted OPG levels. OPG protein expression was measured using a human OPG/TNFRSF11B DuoSet kit R & D Systems, USA) according to the manufacturer's instructions. The absorbance was examined at 450 nm. The results were normalized to the control and presented as fold change.

2.3. Real-time PCR

Total cellular RNA was isolated using Isol-RNA Lysis reagent (5 Prime, USA). RNA (1 μg) was converted to cDNA using a reverse transcriptase kit (Promega, USA). FastStart® Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA) was employed for PCR in a Lightcycler Nano realtime PCR machine (Roche Applied Science, USA). The expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase expression and then normalized to the control results. The primer sequences are shown in Table 1.

Table 1
Primer sequences.

Gene	Primer sequences	Accession no.
GAPDH	F 5′-TCATGGGTGTGAACCATGAGAA-3′	NM_002046.3
	R 5′-GGCATGGACTGTGGTCATGAG-3′	
OPG	F 5′-AGCTGCAGTACGTCAAGCAGGA-3′	NM_002546.3
	R 5′-TTTGCAAACTGTATTTCGCTCTGG-3′	
HES1	F 5′-AGGCGGACATTCTGGAAATG-3′	NM_005524.2
	R 5′-CGGTACTTCCCCAGCACACTT-3′	
HEY1	F 5′-CTGCAGATGACCGTGGATCA-3′	NM_012258.3
	R 5′-CCAAACTCCGATAGTCCATAGCAA-3′	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; F: Forward; R: Reverse.

2.4. Microarray database analysis

Publicly available microarray expression database analysis was performed using connection up- or down-regulation expression analysis of microarrays software [19,20]. Briefly, the microarray dataset Gene Expression Omnibus (GEO) series were identified using the keywords “JAG1 or Jagged1”. The expression profile studies in human cancer cells containing three or more samples in each experimental group were included in our analysis. The GEO series and their platforms (GSE14995 and GSE36051) were downloaded from the GEO repository [21]. The differential expression of OPG (TNFRSF11B) mRNA was evaluated. Statistical analysis was performed using the two independent samples student's *t*-test, which is an extension of the connection up- or down-

regulation expression analysis of microarrays program. Differences at $P < 0.05$ were considered to be statistically significant.

2.5. Statistical analyses

The data were presented as box and whisker plots. The Mann–Whitney U test was employed to determine statistical differences. Differences at $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Attenuation of OPG expression in HSC-4 cells by Notch signaling activation

To activate intracellular Notch signaling, HSC-4 cells were seeded on Jagged1 immobilized surfaces. At 48 h, a significant increase in Notch target gene (*HES1* and *HEY1*) mRNA levels was observed (Figure 1A, B), implying successful Notch activation. Interestingly, Jagged1 treated HSC-4 cells exhibited a significant decrease in OPG expression at both the mRNA and protein levels (Figure 1C, D).

To further confirm the influence of Notch signaling on OPG expression by HSC-4 cells, a γ-secretase inhibitor (DAPT) was employed to inhibit the cleavage of the Notch receptor, leading to the attenuation of intracellular Notch signaling. The results demonstrated that DAPT inhibited *HES1* and *HEY1* mRNA expression (Figure 2A, B). Consistent with these findings, DAPT treatment promoted OPG expression (Figure 2C, D). This result may imply the role of endogenous Notch signaling in OPG expression in HSC-4 cells.

3.2. Effect of Jagged1 treatment on OPG expression in other human cancer cell line studies

To determine whether the role of Jagged1 attenuated OPG expression depended on cell type, a publicly available microarray database screening was evaluated. The investigations where human cancer cells overexpressed Jagged1 or were seeded on Jagged1 immobilized surfaces were included in our analysis (Table 2). In the database, there were only two studies that matched the inclusion criteria. These studies were performed using a human lung cancer cell line (CL1-0) or human breast cancer cell lines (MCF7 and MDA-MB-231). The results showed that there was no significant difference in OPG levels in the cell lines either overexpressing Jagged1 or exposed to Jagged1 immobilized surfaces, implying a cell type specific role of Jagged1 on OPG expression.

3.3. Specific influence of Jagged1 on OPG expression in OSCC cells

To determine the specific influence of Jagged1 on OPG expression in OSCC cells, an OSCC cell line (HSC-5), and an HNSCC cell line (HN22) were employed. The results showed that Jagged1 immobilized surfaces successfully activated Notch signaling in these cells as determined by the significant upregulation of Notch target genes (*HES1* and *HEY1*) (Figure 3). However, no alterations in OPG mRNA or protein levels were

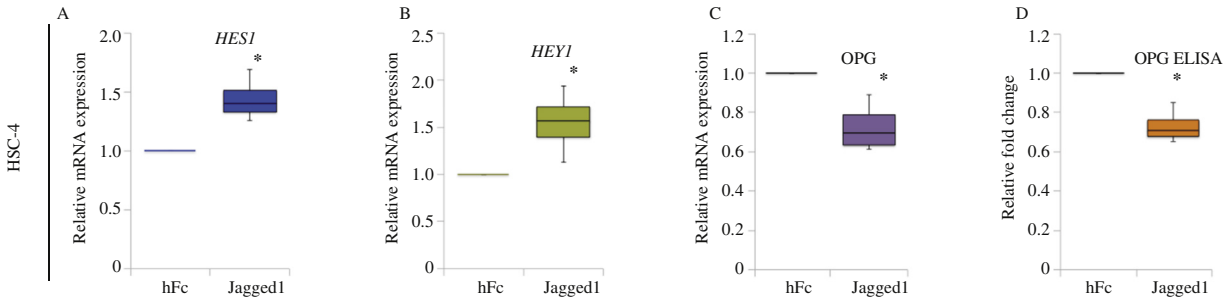

Figure 1. HSC-4 cells seeded on Jagged1 immobilized surfaces for 48 h.
The mRNA expression was determined using real-time PCR (A–C) and protein expression was measured using ELISA (D). Asterisks indicate a significant difference compared to the control.

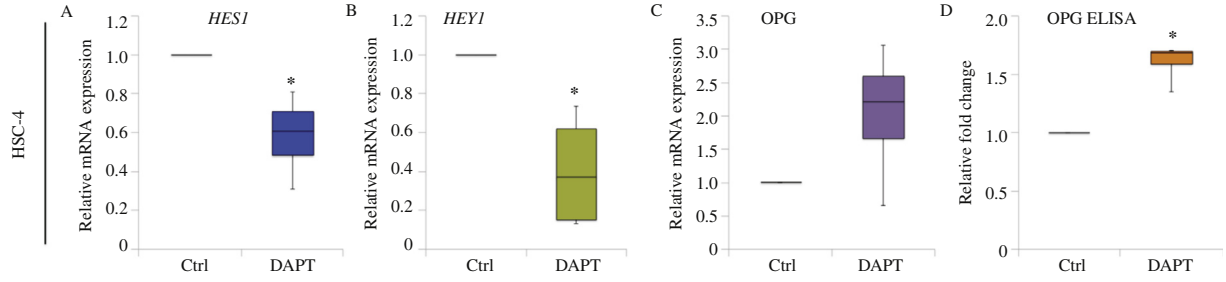

Figure 2. HSC-4 cells treated with a γ-secretase inhibitor (DAPT) for 48 h.
The mRNA expression was determined using real-time PCR (A–C) and protein expression was measured using ELISA (D). Asterisks indicate a significant difference compared to the control.

Table 2
Expression of OPG (TNFRSF11B) in the control and the overexpressing Jagged1 or immobilized Jagged1/Fc exposed groups.

GEO series	Probe ID	Conditions	Expression value (Mean ± SD)	*P*-value
GSE14995 Human lung adenocarcinoma cell line (CL1-0) Control group: CL1-0 cells (n = 3) Experimental group: Jagged1 overexpressing cells (n = 3)	204932_at	Control group Experimental group	0.880 ± 0.067 0.830 ± 0.046	0.334
	204933_s_at	Control group Experimental group	0.510 ± 0.003 0.510 ± 0.011	0.817
GSE36051 Human breast cancer cell line (MCF7, ER^+, wild-type p53, luminal type B breast cancer) Control group: Cells on Fc coated surfaces with dimethyl sulfoxide vehicle control (n = 3) Experimental group: Cells on Jagged1/Fc coated surfaces (n = 3)	ILMN_1676663	Control group Experimental group	20.140 ± 0.235 20.000 ± 0.000	0.423
GSE36051 Human breast cancer cell line (MDA-MB-231, ER^-, mutated p53, basal breast cancer) Control group: Cells on Fc coated surfaces with dimethyl sulfoxide vehicle control (n = 3) Experimental group: Cells on Jagged1/Fc coated surfaces (n = 3)	ILMN_1676663	Control group Experimental group Experimental group	20.020 ± 0.041 20.460 ± 0.790 56.530 ± 22.880	0.443

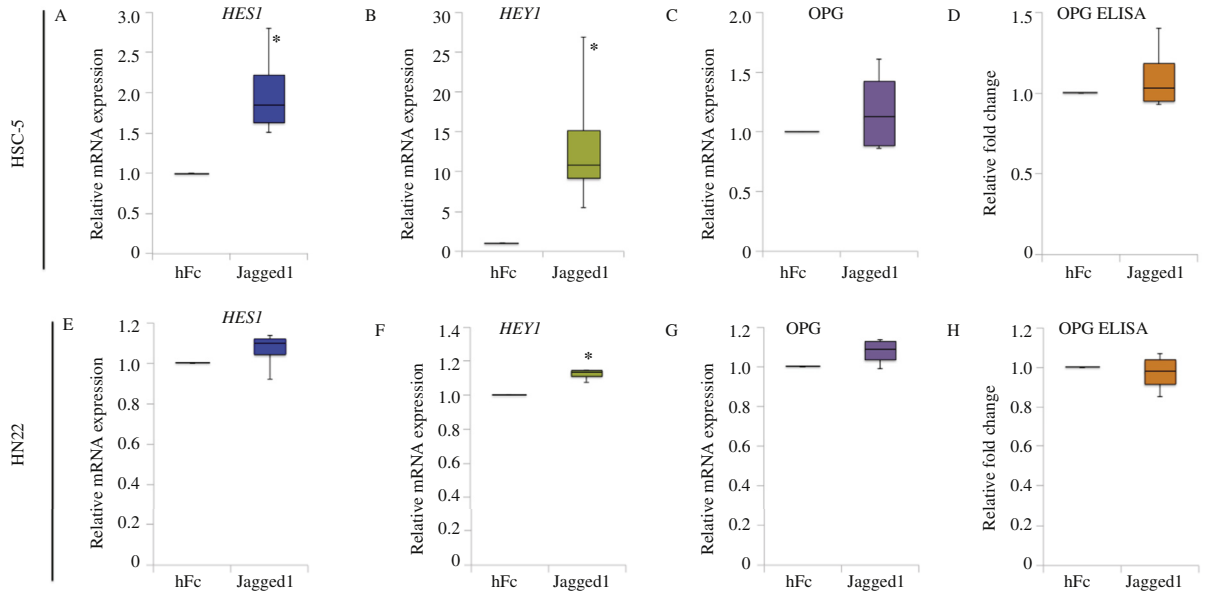

Figure 3. The human primary oral squamous cell carcinoma cell line (HSC-5) and human head and neck squamous cell carcinoma cell line (HN22) seeded on Jagged1 immobilized surfaces for 48 h.
The mRNA expression was determined using real-time PCR (A–C and E–G) and protein expression was measured using ELISA (D and H). Asterisks indicate a significant difference compared to the control.

observed. Together, these data implied that the Notch signaling mechanism regulating OPG expression was cell type specific.

4. Discussion

Several studies have demonstrated the role of OPG in OSCC. Immunohistochemistry analysis revealed that OSCC expressed OPG, while OPG staining was not observed in normal buccal mucosa [22]. However, there was no significant difference in OPG expression between OSCC with or without bone invasion [22]. Another study indicated a relationship between OPG expression and OSCC aggressiveness. It was observed that tumor samples from patients with malignant bone invasion expressed higher OPG levels than those of patients without bone invasion [23]. Correspondingly, an animal study showed that OPG administration decreased mandibular bone invasion by transplanted human OSCC cells [13]. Together, these studies may imply the influence of OPG in regulating OSCC behavior.

The present study demonstrated that the activation of Notch signaling attenuated OPG expression and Notch signaling inhibition resulted in increased OPG mRNA levels in an HSC-4 cell line. There are several hypotheses that may explain the role of Notch-regulated OPG in OSCC. First, Notch and OPG may

regulate the bone invasion by OSCC similar to their modulation of osteoclastogenesis. OPG treatment of mice transplanted with human OSCC cells in resulted in reduced mandibular bone invasion [13]. Decreased TRAP positive multinucleated cells were demonstrated in the OPG treated groups [13]. Jagged1, a Notch ligand, was shown to promote osteoclast formation and function [24]. Thus, Notch activation in HSC-4 cells led to the reduction of OPG expression and may participate in the enhancement of bone invasion by these cells. Second, Notch regulation of OPG expression may be involved in the control of cancer cell migration. It was shown that Notch signaling was involved in hypoxia-induced OSCC cell migration and invasion [15]. However, OPG inhibited RANKL-induced OSCC cell migration [13]. Together, these results imply that Notch attenuated OPG expression in HSC-4 cells promotes OSCC cell migration. Third, Notch modulation of OPG expression may be involved in the control of cancer cell apoptosis. It was demonstrated that Notch signaling inhibition by DAPT treatment in a human tongue carcinoma cell line resulted in the inhibition of Notch target gene (*HES1*) expression and the promotion of cell apoptosis [25]. In contrast, OPG administration led to increased cell apoptosis in an OSCC invaded bone model *in vivo*, but the *in vitro* portion of this study revealed no influence of OPG on OSCC cell apoptosis [13]. Therefore, the effect of OPG on cell apoptosis may be limited to the bone microenvironment *in vivo*. However, it could be hypothesized that Notch signaling activation leads to decreased OPG expression, resulting in decreased cell apoptosis. Further investigation is needed to confirm these hypotheses.

In the present study, the influence of Notch signaling on OPG expression was demonstrated in HSC-4 cells, but not in other cell lines (HSC-5 and HN-22). These results may imply that the role of Notch signaling on OPG expression may occur only in specific cell types. We focused on an OSCC derived cell line (HSC-4) that was derived from a lymph node metastasis that originated from the tongue. The HSC-5 cells were derived from a primary lesion at the maxillary sinus area. Previously, it has been shown that increased OPG expression in OSCC correlated with local bone invasion, poor response to neoadjuvant chemoradiotherapy, and decreased patient survival [23]. Thus, Notch regulated OPG expression in HSC-4 cells but not in HSC-5 cells may be due to different regulation of cell behavior between invasive and primary lesion derived cell lines.

Conflict of interest statement

We declare that we have no conflict of interest.

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