

Notch1 signaling pathway participates in cancer invasion by regulating MMPs in lingual squamous cell carcinoma

BO YU^{1*}, JIANHUA WEI^{1*}, XINHONG QIAN², DELIN LEI¹, QIN MA¹ and YANPU LIU¹

¹Department of Oral and Maxillofacial Surgery, School of Stomatology and ²Department of Pediatrics, Xijing Hospital, The Fourth Military Medical University, Xi'an, Shannxi 710032, P.R. China

Received August 19, 2011; Accepted September 23, 2011

DOI: 10.3892/or.2011.1492

Abstract. Some studies show the Notch signaling pathway may participate in carcinoma invasion and metastasis. However, the mechanisms by which Notch1 mediates cell invasion and migration, especially in lingual squamous cell carcinoma, are not yet known. In the current study, we demonstrated for the first time the anti-invasion and anti-metastasis effect of down-regulation of Notch1 in lingual squamous cell carcinoma. Down-regulation of Notch1 could be an effective approach for inhibition of the expression of matrix metalloproteinase (MMP)-2 and MMP-9 resulting in the inhibition of invasion and metastasis, which could be useful for devising novel preventive and therapeutic strategies for lingual squamous cell carcinoma.

Introduction

Squamous cell carcinoma (SCC) of the tongue is a common oral malignancy, and despite advances in treatment, the worldwide trend in 5-year survival for SCC of the tongue since the early 1970s has remained relatively constant at about 60% (1). Lingual squamous cell carcinoma has a high incidence of nodal metastasis. Subclinical nodal metastasis is present in 30-50% of early T1 to T2 lingual carcinomas (2,3). Therefore, knowledge of the mechanism of invasion and metastasis is important to the advancement of therapies.

The metastatic process is complex. Cells move from the primary tumor, the basement membranes (BMS) and extracellular matrix (ECM) degrade and are invaded, and the tumor cells avoid the immune response and eventually colonize distant sites (4). Matrix metalloproteinases (MMPs), together

with cysteine proteinases, aspartic proteinases and serine proteinases are proteolytic enzymes involved in ECM and BMS degradation (5). This process is how cancer progresses and is frequent in oral squamous cell carcinoma, especially those in the tongue, which explains the poor survival rate of patients with this disease (6).

The Notch signaling pathway is an evolutionarily conserved signaling pathway that has been implicated in a wide variety of processes, including cell-fate determination, tissue patterning and morphogenesis, cell differentiation, proliferation and death. More recently, a number of studies have investigated the relationship between Notch signaling pathway and tumors in various anatomical sites. These studies have shown that the Notch signaling pathway is aberrantly activated in a variety of human cancers, including T-cell acute lymphoblastic leukemia, lung, colorectal, prostate and breast carcinomas (7-9). Some studies have shown that the Notch signaling pathway can regulate the MMPs (10-12). We thus hypothesized that the Notch signaling pathway may regulate the MMPs in the process of invasion and metastasis in lingual squamous cell carcinoma. To our knowledge, there have been no previous studies of the specific role of the Notch signaling pathway in lingual squamous cell carcinoma. The purpose of this paper was to assess the role of the Notch signaling pathway in invasion and metastasis of lingual squamous cell carcinoma.

Materials and methods

Cell culture and reagents. The UM1 and UM2 cells used in this study are paired cell lines with different metastatic potential that were generated from a single patient with lingual squamous cell carcinoma (13). These cell lines were maintained in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Normal oral epithelial cells were cultured in keratinocyte serum-free medium (K-SFM). The third or fourth passages of these cells were used in the study. The cells were maintained at 37°C in a sterile humid atmosphere under 5% CO₂. DMEM/F12, K-SFM, fetal bovine serum (FBS) and trypsin were obtained from Gibco (Grand Island, NY, USA). Streptomycin, penicillin and TRIzol were from Invitrogen (Carlsbad, CA, USA). Western blotting luminal reagent was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitrocellulose membranes were purchased from Promega

Correspondence to: Dr Yanpu Liu or Dr Qin Ma, Department of Oral and Maxillofacial Surgery, School of Stomatology, The Fourth Military Medical University, Xi'an, Shannxi 710032, P.R. China
E-mail: liuypfmmu886@gmail.com
E-mail: qinma@fmmu.edu.cn

*Contributed equally

Key words: Notch1, matrix metalloproteinase, invasion, lingual squamous cell carcinoma

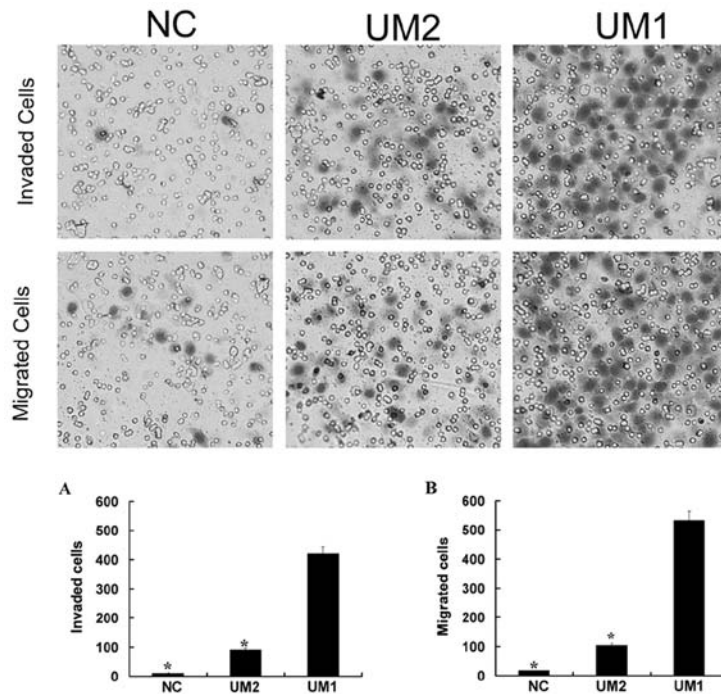


Figure 1. Invasion and migration abilities of UM1, UM2 and normal oral epithelial cells (NC). (A and B) Using Transwell cell culture chambers, we assessed the invasion and migration abilities of UM1, UM2 and normal oral epithelial cells (NC). The data represent mean \pm SD, * P <0.05 compared to the UM1 cells.

(Madison, WI, USA). The primary antibodies were from Santa Cruz Biotechnology. All secondary antibodies were obtained from Pierce (Rockford, IL, USA). Notch1 small interfering RNA (siRNA) and siRNA control were obtained from Santa Cruz Biotechnology. Lipofectamine 2000 was purchased from Invitrogen. All other chemicals and solutions were from Sigma-Aldrich, unless otherwise indicated.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from HepG2 cells by the TRIzol reagent (Invitrogen). Reverse transcription of total cellular RNA was performed using the one-step RT-PCR kit (MBI Fermentas, Lithuania) according to the manufacturer's instructions. The polymerase chain reaction (PCR) primers used were as follows: 5'-CCGTCATCTCCGACTTCATCT-3' (forward) and 5'-GTGTCTCCTCCCTGTTGTTCTG-3' (reverse) for Notch1; 5'-TCAACACGACACCGGATAAA-3' (forward) and 5'-CCGCGAGCTATCTTTCTTCA-3' (reverse) for Hes1; 5'-AGCGGGAAATCGTGCGTG-3' (forward) and 5'-CAGGGTACATGGTGGTGCC-3' (reverse) for β -actin. The PCR conditions were as follows: after initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 45 sec, annealing at each appropriate temperature as described for 30 sec and extension at 72°C for 45 sec. PCR products were separated by electrophoresis on 1% agarose gels and were visualized with ethidium bromide staining. Gene expression was presented by the relative yield of the PCR product from target sequences to that from the β -actin gene. Mean values from three independent experiments were calculated.

Small interfering RNA transfection. According to the protocol of Lipofectamine 2000, the UM1 cells were transfected with Notch1 siRNA and siRNA control, respectively. Cells were

allowed to further grow for 48 h and were harvested for further analysis.

Protein extraction and Western blotting. Cells were lysed in lysis buffer [50 mmol/l Tris (pH 7.5), 100 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP-40, 0.5% Triton X-100, 2.5 mmol/l sodium orthovanadate, 10 μ l/ml protease inhibitor cocktail and 1 mmol/l PMSF] by incubating for 20 min at 4°C. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% non-fat dried milk or bovine serum albumin in 1X TBS buffer containing 0.1% Tween-20 and then incubated with appropriate primary antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG was used as the secondary antibody and the protein bands were detected using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Quantification of Western blots was performed using laser densitometry and the results are presented as the mean of three independent experiments with error bars representing SD. For reprobing, membranes were incubated for 30 min at 50°C in buffer containing 2% SDS, 62.5 mmol/l Tris (pH 6.7) and 100 mmol/l 2-mercaptoethanol, washed and incubated with desired primary antibody.

MTT assay. The transfected cells were seeded into 96-well plates at a density of 1×10^4 cells/well and grown for up to 48 h. Cell viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma) according to the manufacturer's protocol. Each experiment contained six replicates and was repeated three times. The data are expressed as the mean \pm SD.

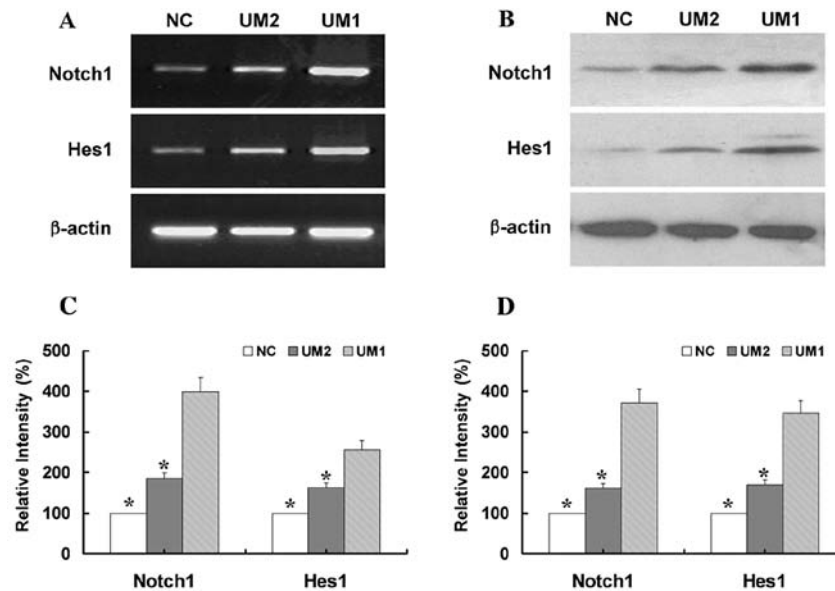


Figure 2. Expression of Notch1 and Hes1 in UM1, UM2 and normal oral epithelial cells (NC) at the mRNA and protein levels. (A and B) The expressions of mRNA and protein were determined by RT-PCR and Western blot analysis. (C and D) Relative intensity of of Notch1 and Hes1 expression against β -actin at 100%. * $P < 0.05$ compared to the UM1 cells.

Migration and invasion assays. Cell migration and invasion were determined with or without matrigel-coated Transwell cell culture chambers (8 μ m pore size) (Millipore, Billerica, MA, USA). Cells were seeded (5×10^4 cells/well) into the upper chamber with serum-free medium. The plates were incubated for 24 h. Cells on the upper side of the filters were mechanically removed and those that migrated onto the lower side were fixed with 4% formaldehyde, then stained with 0.5% crystal violet for 10 min. Finally, invasive cells were counted at x200 magnification from 10 different fields of each filter.

ELISA assay. Enzyme-linked immunosorbent assay (ELISA) technique (Amersham Pharmacia Biotech, Buckinghamshire, UK) was used to quantify the activity of MMP-2 and MMP-9. The samples were thawed on ice, and all reagents equilibrated to room temperature; assays were carried out according to the manufacturer's instructions.

Statistical analysis. Each experiment was repeated at least three times. All data are presented as mean \pm SD. The differences between the means were statistically analyzed using the t-test. All statistical analyses were performed using the SPSS 13.0 software (Chicago, IL). $P < 0.05$ was considered to indicate statistical significance.

Results

The invasion and metastasis abilities is high in UM1 cells. We used Transwell cell culture chambers to check the abilities of invasion and metastasis in the UM1, UM2 and normal oral epithelial cells. The invasion and metastasis abilities were highest in UM1 cells among the three cell lines.

The expression of Notch1, Hes1, MMP-2 and MMP-9 was highest in UM1 cells among the three cell line. To explore the activity of Notch1 signaling pathway in the three cell lines,

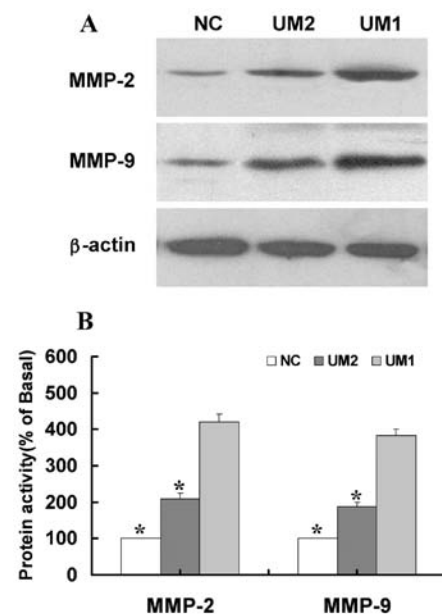


Figure 3. The protein expression and activity of MMP-2 and MMP-9 in UM1, UM2 and normal oral epithelial cells (NC). (A) The protein expressions of MMP-2 and MMP-9 were determined by Western blot analysis. (B) The protein activity of MMP-2 and MMP-9 was determined by an ELISA assay. * $P < 0.05$ compared to the UM1 cells.

RT-PCR and Western blotting were conducted to detect the alteration in the mRNA and protein expressions of Notch1 and Hes1. We found that at both the mRNA and protein levels, the expression of Notch1 and of the downstream target Hes1 were highest in UM1 cells (Fig. 2). This may suggest activated Notch1 signaling pathway may have something with invasion and metastasis. We also examined the protein expressions of MMP-2 and MMP-9 in the three cell lines (Fig. 3). We found that the protein expression and protein activity of MMP-2 and MMP-9 was also the highest in UM1 cells.

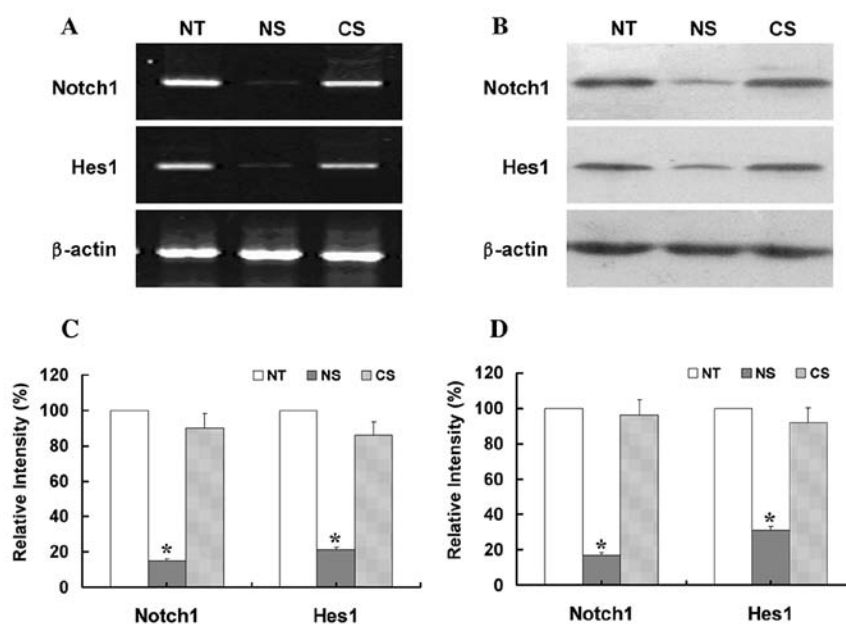


Figure 4. Inhibited effects of Notch1 signaling pathway by RNA interference in UMI cells. (A and B) Cells were treated with RNA interference. The mRNA and protein levels of Notch1 and Hes1 were determined by RT-PCR and Western blot analysis. (C and D) Relative intensity of expression against β-actin at 100%. *P<0.05 compared to non-transfected cells. NT, non-transfected cells; NS, Notch siRNA transfected cells; CS, control siRNA transfected cells.

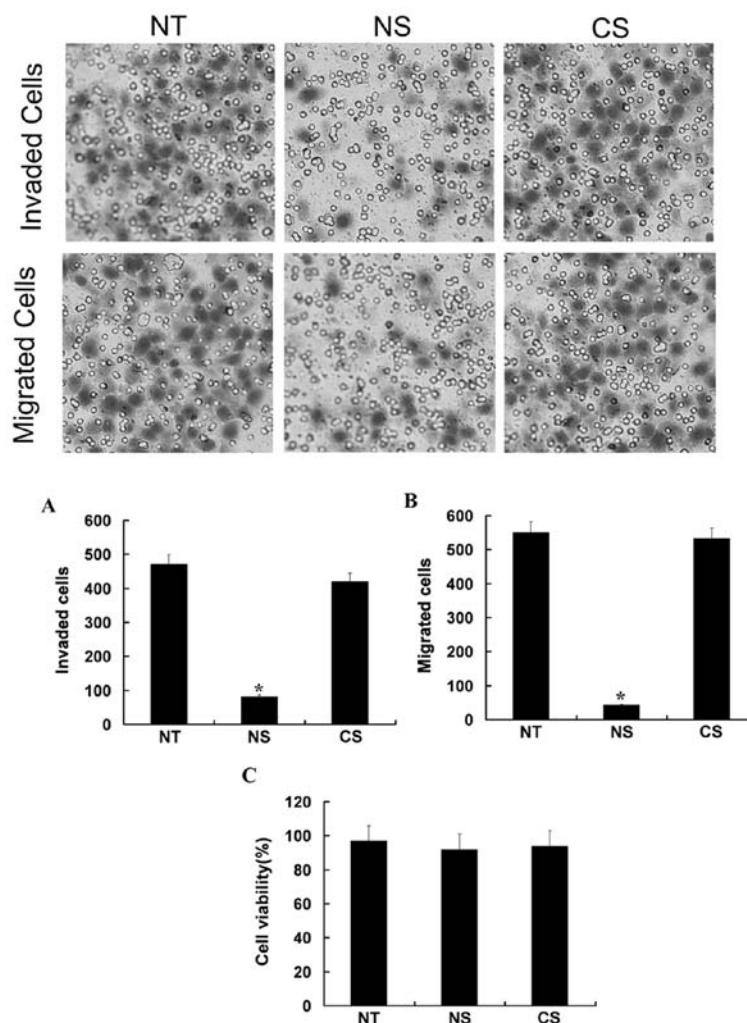


Figure 5. Effects of inhibition of Notch1 by RNA interference on invasion and migration of UMI cells. (A and B) Cell invasion and migration were measured by Transwell cell culture chambers. (C) UMI cell viability was examined. The data represent the mean ± SD, *P<0.05 compared to non-transfected cells. NT, non-transfected cells; NS, Notch siRNA transfected cells; CS, control siRNA transfected cells.

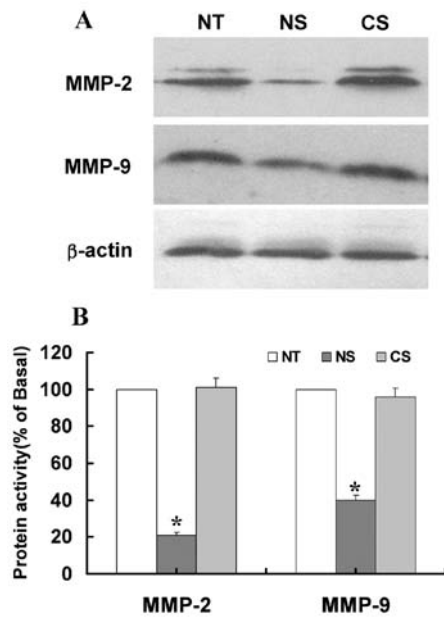


Figure 6. Effects of the inhibition of Notch1 by RNA interference on MMP-2 and MMP-9. (A) UM1 Cells were treated with RNA interference. The protein levels of MMP-2 and MMP-9 were determined by Western blot analysis. (B) The protein activity of MMP-2 and MMP-9 were determined by ELISA assay. * $P < 0.05$ compared to non-transfected cells. NT, non-transfected cells; NS, Notch siRNA transfected cells; CS, control siRNA transfected cells.

Down-regulation of Notch1 can suppress invasion and migration of UM1 cells but has no effect on cell viability. RNA interference can inhibit Notch1 and Hes1 effectively (Fig. 4). To further confirm the role of the Notch1 signaling pathway on the invasion and metastasis of UM1 cells, we used Transwell cell culture chambers with or without matrigel coating to examine the invasion and migration potentials of Notch1 siRNA transfected cells in culture. As shown in Fig. 5A and B, compared with non-transfected UM1 cells, the abilities of invasion and metastasis were strongly inhibited in Notch1 siRNA transfected cells. Next, we determined the cytotoxicity of Notch1 siRNA using the MTT assay. The use of Notch1 siRNA did not affect cell viability at the indicated concentrations (Fig. 5C).

Down-regulation of Notch1 can decrease the expression and protein activity of MMP-2 and MMP-9. MMP-2 and MMP-9 are involved in cancer cell invasion processes. Therefore, we used Western blot analysis and ELISA assay to confirm the expression and protein activity of MMP-2 and MMP-9 in Notch1 siRNA transfected cells. As shown in Fig. 6, after Notch1 inhibition, the expression and protein activity of MMP-2 and MMP-9 decreased. These results indicate that underexpression of Notch1 decreased the protein expression and activity of MMP-2 and MMP-9.

Discussion

Carcinoma of the tongue is a common head and neck cancer. Nodal metastasis often occurs in the early stage (2,3). Improved therapy for lingual squamous cell carcinoma aiming at reducing its metastatic potential and aggressiveness is neces-

sary to increase the survival rate of patients with this disease. This study sought to identify and validate if Notch signaling pathway contributes to metastasis in lingual squamous cell carcinoma. In this research, we used the UM1 and UM2 cells. These cells are paired cell lines from a single lingual squamous cell carcinoma patient with different metastatic potentials (13). Specifically, UM1 is more aggressive than UM2 in terms of cell invasion (14). As a control, we chose to use normal oral epithelial cells. Our results confirmed that the invasion and metastasis abilities are highest in UM1 cells among the three cell lines.

The Notch signaling pathway has been known for decades to developmental biologists as a key player in cell fate determination (15,16) and tissue homeostasis by maintaining the self-renewal potential of some tissues and inducing differentiation of others (17), including formation of the prostate gland (18). Aberrant expression of Notch1 has been detected in various types of human cancers including T-cell acute lymphoblast leukemia (19), breast carcinoma (20) and brain tumor (21). The data in our studies showed that compared to normal cells, the expressions of Notch1 and Hes1 at the mRNA and protein levels were higher in carcinoma cells. It is interesting that the more aggressive UM1 cells had higher Notch1 and Hes1 in mRNA and protein levels. Inhibition of Notch1 by RNA interference in UM1 cells, resulted in decreases in the abilities of invasion and metastasis. These results suggest that the Notch1 signaling pathway may be involved in the invasion and metastasis of UM1 cells. However, the specific mechanism is unclear. We thus focused on the role of MMP-2 and MMP-9, which contribute to invasion and metastasis.

Metastasis, the spread of cells from the primary neoplasm to distant sites and their growth at that location, is the most fearsome aspect of cancer. An understanding of the progression of tumors to the metastatic state, and the biological changes in the tumor cells, is important in the development of new approaches to diagnosis, assessment, and treatment of malignant disease. Invasion of cancer cells involves degradation of the extracellular matrix and the basement membrane. The initial steps bring about the degradation of the extracellular matrix surrounding the endothelial cells by matrix metalloproteinases (MMPs). These are a group of over 20 enzymes which play an important role in extracellular matrix degradation (22).

Many studies have shown that gelatinases (MMP-2 and -9) are expressed in oral cancer and may have roles in tumor progression (23-25). The ability of MMP-2 and MMP-9 to initiate basement membrane destruction and further degrade the collagenous and non-collagenous components of the ECM suggests that they are important in this process. MMP-2 has been detected in oral carcinoma *in vivo* where it localised to small nests of cells at the advancing tumor front (24). MMP-9 is considered a key MMP in the invasion and metastases, over-expressed by cancer cells and induced by several cytokines, growth factors and oncogene products (26), its inhibition resulting in loss of metastatic phenotype (27).

Recent studies have shown that the expression and protein activity of MMP-2 and MMP-9 was highest in UM1 cells which had a high metastatic potential. It is thus suggested that MMP-2 and MMP-9 may participate in invasion and metastasis in lingual squamous cell carcinoma. Next, we examined the

functional activity of MMP-2 and MMP-9 in Notch1 knock-down UM1 cells and observed a decrease in their expression and activity, indicating that Notch1 may regulate MMP-2 and MMP-9.

Collectively the data indicate that the disruption of Notch1 in UM1 cells leads to a decrease in their invasiveness accompanied by a decrease in MMP-2 and MMP-9 expression and suggest the versatile role of Notch1 in UM1 cell invasion. Notch1 could be a target for intervention of human lingual squamous cell carcinoma.

Acknowledgements

We are grateful to Yang Xue for providing technical assistance.

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