Notch signalling induces epithelial-mesenchymal transition to promote metastasis in oral squamous cell carcinoma

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Abstract. The activation of Notch signalling induces epithelial-mesenchymal transition (EMT), but this signalling pathway and its association with EMT in the context of cell motility in oral squamous cell carcinoma (OSCC) remains unclear. The present study aimed to investigate the role of the Notch signalling pathway and EMT in the metastatic potential of OSCC using 2 cell lines, Tca8113 and CAL27. The data demonstrated that zinc finger domain SNAI1 (Snail) knockdown by small interfering RNA decreased the expression of vimentin and increased the expression of epithelial cadherin (E-cadherin). In addition, silencing Snail also significantly inhibited cell migration in the 2 OSCC cell lines. It was also identified that blocking Notch signalling with the g-secretase inhibitor DAPT decreased the expression of the EMT markers Snail and vimentin and increased E-cadherin expression, accompanied by a significant inhibition of cell migration in the 2 OSCC cell lines. These data clearly indicate that Notch signalling mediates EMT to promote metastasis in OSCC cells. Therefore, targeting Notch signalling and its association with EMT may provide novel insights into the mechanism of invasion and metastasis in OSCC and potential therapeutic interventions.

Introduction

Oral cavity cancer is one of the most common types of cancer worldwide, and oral squamous cell carcinoma (OSCC) is the most prevalent type of tumour at this site, accounting for ~90% of all cases (1,2). Despite previous advancements in diagnostic techniques and therapeutic strategies, the prognosis of OSCC has not improved, with an overall 5-year survival rate of ~50% (2,3). This poor prognosis is primarily attributed to the high rates of invasion and metastasis (4). Therefore, it is important to understand the molecular events associated with tumour development and to identify biomarkers for prognosis assessment in OSCC.

Epithelial-mesenchymal transition (EMT) is a complex biological process that is important for embryogenesis, wound healing and malignant tumour metastasis (5). During EMT, epithelial tumour cells lose their polarity and adhesive phenotype, and they acquire mesenchymal traits, including an increase in cell motility potential and cytoskeletal remodeling (5). Alterations of adhesion molecules and extracellular proteins are key events in tumour cells during EMT. Epithelial cadherin (E-cadherin) and vimentin are two hallmarks of EMT; one is a predominant cell-surface protein for adherens junctions in epithelial cells, and the other is an intermediate filament in mesenchymal cells that controls cell motility, respectively (6). In primary head and neck squamous cell carcinoma cancer, the loss of E-cadherin and the gain of vimentin functions are associated with a higher incidence of distant metastasis (7). In addition, zinc finger protein SNAI1 (Snail) is one of the transcription factors involved in the EMT process and regulates various aspects of the EMT phenotype, including the upregulation of mesenchymal markers and the suppression of epithelial markers (6).

The Notch signalling pathway is evolutionarily conserved and serves important roles in numerous developmental processes, including cell fate determination, proliferation and differentiation (8). Notch signalling is initiated when a Notch ligand binds to an adjacent Notch receptor between two neighbouring cells. In mammals, the Notch family consists of 4 transmembrane receptors (Notch-1-4) and 5 ligands [Delta-like protein (Delta-like) 1, Delta-like 3, Delta-like 4, protein jagged (Jagged) 1 and Jagged-2] (9). Dysregulation of Notch signalling results in human developmental anomalies, including Alagille syndrome, cardiac disease and cancer development (10). Previous studies demonstrated that the expression levels of certain Notch receptors and ligands were dysregulated in human OSCC, which suggested that Notch signalling serves a vital role in tumour development in OSCC (11-13).

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Aberrant expression of EMT markers, including downregulated E-cadherin and β-catenin and upregulated vimentin and Neural cadherin, has been identified in OSCC tissues (14,15). The promotion of EMT in OSCC involved signalling, including the phosphoinositide 3-kinase/protein kinase B and Wnt/β-catenin signalling pathways (16). Notch signalling is also a key regulator in the induction of EMT, but the role of this signalling pathway and its association with EMT in cell motility in OSCC remains unclear. Therefore, the present study used a small-interfering RNA (siRNA) targeting Snail and a novel γ-secretase inhibitor, DAPT, to examine the role of the Notch signalling pathway and the EMT mechanism in the metastatic potential of OSCC in vitro. The Tca8113 and CAL27 human OSCC cell lines were adopted as cell models as they have been widely used to study the role of Notch signalling and EMT in cancer metastasis (11,17,18). The results of the present study demonstrated that Snail-induced EMT may promote the migration of OSCC cells, and that Notch signalling may mediate tumour metastasis in OSCC cells via its association with EMT progression.

Materials and methods

Reagents. DAPT (GSI-IX) was purchased from Selleck Chemicals (Houston, TX, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, foetal bovine serum (FBS) and trypsin-EDTA were supplied by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Primary rabbit anti-human Notch1 intracellular domain (N1ICD; cat. no., ab83232) monoclonal antibodies were obtained from Abcam (Cambridge, MA, USA). The rabbit anti-human Snail (cat. no., 3879), E-cadherin (cat. no., 3195), and vimentin (cat. no., 5741) antibodies and the GAPDH (cat. no., 5174) control antibody were all acquired from Cell Signaling Technology (Danvers, MA, USA). The horseradish peroxidase-labelled secondary antibody (cat. no., EK020) was obtained from Xi'an Zhuangzhi Biotech Co., Ltd. (Xi'an, China).

Cell cultures. Established Tca8113 and CAL27 human OSCC cell lines were purchased from CHI Scientific, Ltd., (Wuxi, China). TCA8113 cells were maintained in RPMI-1640 medium containing 20% FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin, while CAL27 cells were maintained in DMEM containing 10% FBS, 100 U/ml penicillin G and 100 µg/ml streptomycin. The 2 cell lines were incubated in a humidified atmosphere of 5% CO2 at 37°C. Cells in the logarithmic growth phase were used in subsequent experiments.

Cell viability assay. 5x10^5 Tca8113 or CAL27 cells were seeded in 96-well plates for 24 h, then, after 4 h of incubation at 37°C, the medium was removed, and 150 µl DMSO was added to dissolve the formazan. Absorbance measurements were made with an automatic ELISA reader (Infinite M200; Tecan Group, Ltd., Männedorf, Switzerland) at 490 nm. Each experiment included 6 replicates and was repeated 3 times.

Transient transfection of Snail siRNA. Viral transfer vectors encoding green fluorescent protein and Snail siRNA, negative control (NC) siRNA, polybrene and Enhanced Infection Solution (ENi.S) were constructed by Shanghai Genechem Co., Ltd. (Shanghai, China). The target sequence for the Snail siRNA was as follows: 5'-AGGACTCTAATCCAGAGT-3'. Prior to viral infection, Tca8113 and CAL27 cells were seeded into 6-well plates at a density of 5x10^4 cells/well. Following 24 h of incubation at 37°C, the cells were transiently transfected with Snail siRNA or NC siRNA using viral transfer vectors when they reached 40% confluence. Cell transfection was performed according to the protocol of the manufacturer of the transfection kit. The CAL27 virus infection solution contained 20 µl virus at titer of 1E+8TU/ml, 880 µl ENi.S and 100 µl phycoerythrin (enriched with 50 µg/ml polybrene ENi.S solution), and the Tca8113 virus infection solution contained 120 µl virus at titer of 1E+8TU/ml, 780 µl ENi.S and 100 µl complete medium containing 50 µg/ml polybrene. After 12 h of incubation at 37°C, the virus infection solution was replaced with conventional culture medium to maintain cell growth for 96 h prior to the subsequent experiments.

Migration and wound-healing assays. Cellular Transwell migration was assayed by non-Matrigel-coated Transwell cell culture chambers (8-µm pore size; Corning Incorporated, Corning, NY, USA). Briefly, 1x10^5 Tca8113 or CAL27 cells were seeded into the upper chamber in 200 µl serum-free medium, and 600 µl complete culture medium with 10% FBS for each cell line was placed in the lower chamber as a chemoattractant. Cell migration assays following Snail knockdown were conducted at 37°C for 24 h, and subsequent to treatment with DAPT, the assays were conducted at 37°C for 24 and 48 h. Then, at room temperature, the cells were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 20 min. The unimmigrated cells were removed from the upper surface of the membrane using cotton-tipped swabs. Finally, the migrated cells were counted at magnification, x400 from 9 different randomly selected fields of each filter using an inverted light microscope.

For the wound-healing assays, 5x10^5 Tca8113 and CAL27 cells were seeded in 6-well plates in the aforementioned culture medium. When the cells reached 90% confluence, wounds were made by scratching a line across the bottom of the dish on a monolayer of confluent cells with a 10 µl pipette tip. Then, the cells were incubated in serum-free media at 37°C. The gaps between the cells at 0, 24 and 48 h were imaged on a microscope at a magnification, x100 and then quantified using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

Real time-quantitative polymerase chain reaction (RT-qPCR). The total RNA from all experimental and control cells was extracted using TRIzol® (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed using the PrimeScript RT Master Mix (Takara Bio, Inc., Otsu, Japan). The RT-qPCR was performed using a CFX96 real-time PCR detection system with SYBR® Premix Ex Taq II (Takara Bio, Inc.). Human GAPDH was used as an internal control to normalize the amount of mRNA in each sample using the 2-ΔΔCT method.
The conditions for PCR were as follows: 1 cycle of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. The primers used in the PCR reactions were as follows: Vimentin forward (F), 5'-AACCTGGCCGAG GACATCA-3'; vimentin reverse (R), 5'-TCAAGGTCAGA CGTGCCAGA-3'; Snail F, 5'-GACCATATGCCGCCTTCTC TT-3'; Snail R, 5'-TGCCTGTAGTTAGCCCTCCGATT-3'; Hes family bHLH transcription factor 1 (Hes1) F, 5'-GTG TAGA cGA cCA cGG GTA A-3'; Hes1 R, 5'-CAGATGACACC GGGACAC-3'; E-cadherin F, 5'-TGAGCCTGGCTCAAG TCAAG-3'; GAPDH F, 5'-GCCCGTCAGGGTCTAGA AC-3'; GAPDH R, 5'-TGGTGAAGACCAGCTGGA-3'.

Western blot analysis. The proteins were prepared using RIPA lysis buffer (Thermo Fisher Scientific, Inc.), and protein concentrations were determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). Each lane was loaded with 30 µg protein on 6-10% SDS-PAGE gels and then transferred onto polyvinyl difluoride membranes (EMD Millipore, Billerica, MA, USA), which were blocked with 5% non-fat dry milk/TBST (0.1% Tween-20) for 1 h at 25°C to prevent non-specific binding. The membranes were then incubated with primary antibodies (dilution, 1:1,000) in 5% w/v BSA, 1X TBS and 0.1% Tween-20 at 4°C with gentle shaking overnight. Following washing 3 times with TBS buffer (20 mM Tris-HCl, 137 mM NaCl and 0.1% Tween-20; pH 7.6) for 5 min/wash, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000) for 1 h at room temperature. Subsequently, the membrane was washed 3 times with TBS for 5 min/wash, and the protein bands were detected with the Millipore ECL luminous liquid (EMD Millipore). ImageJ 1.8.0 software was used to quantify the immunoblot bands.

Statistical analysis. Each experiment was repeated at least 3 times. All data are expressed as the mean ± standard deviation and calculated by SPSS 19.0 software (SPSS, Inc., IBM Corp., Armonk, NY, USA). The one-way analysis of variance followed by Fisher’s Least Significant Difference test were used to compare the differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.
Results

EMT participates in the migration of OSCC cells. To identify the role of EMT progression in the migration of OSCC cells, an siRNA method was used to knockdown Snail, a vital EMT transcriptional factor, in the OSCC Tca8113 and CAL27 cell lines. The mRNA level was first assessed using RT-qPCR, and the results demonstrated that the Snail siRNA model effectively suppressed Snail expression in OSCC cells. In addition, the expression level of the mesenchymal marker vimentin was decreased, while the level of the epithelial marker E-cadherin was increased in the Tca8113 and CAL27 cells (Fig. 1A and B). Then, changes in protein expression were examined with western blot analysis. In accordance with the mRNA results, Snail and vimentin levels were decreased, while E-cadherin was increased in the Tca8113 and CAL27 cells (Fig. 1C and D).

To assess the effect of Snail knockdown on cell migration in Tca8113 and CAL27 cells, a Transwell assay indicated that Snail-silenced cells exhibited a significant decrease in migration rate compared with the controls (Fig. 2A).

Figure 2. Snail silencing decreases the motility of oral squamous cell carcinoma cells. The migration of Snail-silenced Tca8113 and CAL27 cells was measured by a (A) Transwell assay for 24 h and a wound-healing assay for (B) 24 and (C) 48 h. All data are presented as the mean ± standard deviation from triplicate experiments. *P<0.05 vs. control and NC group. Snail, zinc finger protein SNAI1; siRNA, small interfering RNA; NC siRNA, negative control siRNA.

Figure 3. Effects of DAPT on the viability of oral squamous cell carcinoma cells. (A) Tca8113 and (B) CAL27 cells were treated with DAPT at different doses for 3 days. The viability of Tca8113 and CAL27 cells was measured by an MTT assay. The basal group (without any treatment) was used as control. All data are presented as the mean ± standard deviation from triplicate experiments. *P<0.05 vs. basal group.
Furthermore, it was observed that Snail knockdown in OSCC cells significantly decreased the rate of cell migration from the edge of the wound following scratching (Fig. 2B and C).

**Inhibition of Notch signalling with DAPT inhibits EMT progression.** To confirm the association between Notch signalling and EMT progression, the γ-secretase DAPT was employed to block Notch signalling and detect changes in the EMT mechanism in OSCC cells. Firstly, an MTT assay was used to examine the effects of DAPT at different doses (1, 2, 5, 10, 20 and 40 µmol/l) on the growth and viability of Tca8113 and CAL27 cells, and untreated cells or cells treated with DMSO were used as controls. The results demonstrated that the viabilities of Tca8113 and CAL27 cells were markedly inhibited by DAPT in a time- and concentration-dependent manner when the concentrations of DAPT were ≥10 µmol/l (Fig. 3). Therefore, treatment with DAPT at concentrations of 1 and 5 µmol/l for 24 and 48 h was used in subsequent studies, which had no marked effect on the viability of Tca8113 and CAL27 cells.

Then, the role of DAPT in the expression of the Notch signalling pathway was examined in Tca8113 and CAL27 cells. The cells were treated with DAPT for 24 and 48 h, and the mRNA expression levels of Hes1 and the protein expression levels of N1Icd were measured. The results indicated that the mRNA expression levels of Hes1 and the protein expression levels of N1Icd were decreased in the 2 cell lines following treatment with DAPT in a time- and concentration-dependent manner (Fig. 4). Whether inhibition of the Notch signalling pathway with DAPT affected the expression of EMT markers in Tca8113 and CAL27 cells was also explored. The expression of Snail, vimentin and E-cadherin were detected at the mRNA and protein levels in the 2 cell lines. The results demonstrated that the inhibition of the Notch signalling pathway with DAPT downregulated the expression of Snail and vimentin and upregulated the expression of E-cadherin at the mRNA (Fig. 5A and B) and protein levels (Fig. 5C and D) in a time- and concentration-dependent manner.
Inhibition of Notch signalling with DAPT decreases OSCC cell migration. To determine whether Notch inhibition affects the migration potential of OSCC cells, Transwell chamber migration and wound-healing assays were performed. As indicated in Fig. 6A-D, the inhibition of Notch signalling with DAPT markedly decreased the migratory capabilities of Tca8113 and CAL27 cells in a time- and concentration-dependent manner, and the number of migrating cells was significantly decreased in the DAPT treatment groups compared with the controls.
Discussion

The high rate of distant metastasis is a major obstacle in improving the survival rates of patients with OSCC (20). Therefore, an understanding of the molecular mechanisms that regulate metastasis may provide novel insights for the development of effective treatments for OSCC, which in turn may decrease the rate of metastasis and improve survival. The present study aimed to understand the role of Notch signalling and its association with EMT in metastasis during OSCC development using 2 cell lines, Tca8113 and CAL27. Although STR detection identified that the Tca8113 cell line was contaminated by HeLa cells (21), previous studies revealed that contamination is unlikely to affect the outcomes of the present study (13,17,22). Firstly, the results verified that Snail regulates EMT and promotes tumour migration in OSCC cells, and then it was demonstrated that the inhibition of Notch signalling by DAPT decreased the migration of OSCC cells.
by inhibiting the EMT mechanism. The present study suggests that Notch signalling promotes EMT and is involved in tumour metastasis in OSCC cells.

EMT is a dynamic cellular process during which epithelial cells adopt characteristics of mesenchymal cells. EMT itself is essential for tissue construction during normal development and the development of metastatic disease (5). A number of in vitro and in vivo studies have demonstrated that the cancer-associated EMT process promotes cancer cell migration and invasion into the stroma (23). A loss of epithelial markers and cell-cell adhesion, and a gain of mesenchymal marker expression, are key events in EMT for tumour cell metastasis and invasion (24). In previous studies, the downregulation of E-cadherin and matrix metalloproteinase (MMP)-9 and the upregulation of vimentin and MMP-2 were observed in OSCC cell lines (16), and a trend towards decreased E-cadherin expression but increased vimentin expression in OS cc cell lines (14), and a trend towards decreased and the upregulation of vimentin and MMP-2 were observed in metastasis and invasion (24). In previous studies, the downregulation of E-cadherin and matrix metalloproteinase (MMP)-9 and the upregulation of vimentin and MMP-2 were observed in OSCC cell lines (16), and a trend towards decreased E-cadherin expression but increased vimentin expression was associated with increased disease severity of OSCC (14).

In addition, EMT markers, including E-cadherin, β-catenin, adenomatous polyposis coli and vimentin, have predictive value for the progression of multiple primary OSCC, and the simultaneous downregulation of E-cadherin and β-catenin exerted a significant prognostic effect in these patients (25). These data, combined with those from the present study, suggest that the EMT process serves a critical role in the progression of OSCC.

Snail is known as a pivotal mediator of EMT and contributes to the repression of the transcription of E-cadherin gene by binding to E-boxes during tumour progression (26). A number of studies have demonstrated that Snail promotes tumour development and metastasis through regulating EMT in a variety of types of cancer (27). A previous study demonstrated that Snail knockdown in OSCC cells significantly inhibited cell migration and invasion (28). An additional study suggested that the overexpression of Snail induces EMT and promotes cancer stem cell-like traits in OSCC SCC-9 cells (29). The results of the present study additionally confirmed that the knockdown of Snail upregulated E-cadherin expression, downregulated vimentin expression and decreased cell motility in Tca8113 and CAL27 cells. All of these data suggest that Snail-induced EMT promotes tumour metastasis in OSCC cells.

It is well-established that the EMT process is stimulated and regulated by a number of signal transduction pathways, including Transforming growth factor β, Wnt, Hedgehog and Notch signalling (30). Emerging evidence suggests that the Notch signalling pathway serves a vital role in the regulation of EMT, resulting in tumour invasion and metastasis (31,32), and the suppression of Notch signalling with the γ-secretase inhibitor DAPT restricts the growth, invasion and metastasis of gastric cancer by inhibiting EMT (33). Previous immunohistochemical examination demonstrated increased expression levels of Notch1, Notch2, Jagged1, Hes1 and Hairy enhancer-of-split related with YRPW motif protein 1 in oral tissues of OSCC compared with those in the normal controls, suggesting that Notch signalling is active in OSCC (13). However, the roles of the Notch signalling pathway and the EMT process in the metastatic potential of OSCC remains unclear. In the present study, DAPT was used to inhibit the Notch signalling pathway in Tca8113 and CAL27 cells. The results indicated that the mRNA expression level of Hes1, a downstream target of Notch signalling, and the protein expression level of the Notch1 intracellular domain (N1Icd) were decreased following treatment with DAPT. These data suggest that DAPT effectively inhibits the Notch pathway in OSCC cell lines. In addition, the results suggested that the inhibition of Notch signalling notably decreased Snail and vimentin and increased E-cadherin at the mRNA and protein levels during the EMT process. Furthermore, DAPT inhibition may markedly decrease the migration ability of OSCC cells. Together, these observations indicate that Notch signalling may be involved in the EMT-induced metastasis of OSCC cells.

In summary, the results from the present study confirmed that Snail-induced EMT promotes cell migration in OSCC cells, and additionally demonstrated that Notch signalling mediates tumour metastasis in OSCC cells through its association with EMT progression. Therefore, targeting Notch signalling and its association with EMT may provide novel insights into the mechanism of invasion and metastasis in OSCC, and the inactivation of Notch signalling or the inhibition of the EMT upstream molecule Snail may be useful approaches for the development of therapeutic strategies for OSCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JZ, GZ, QS and MY contributed to the study concept and experimental design. JZ, LZ, QS, XW, PL and TW performed the experiments. JZ, PL and TW contributed to the data acquisition and analysis. JZ and MY drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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