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Deregulation of Snai2 is associated with metastasis and poor prognosis in tongue squamous cell carcinoma

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Abstract

The members of the Snail superfamily of zinc-finger transcription factors, including Snai1 and Snai2, are involved in essential biological processes, such as epithelial-mesenchymal transition (EMT). While Snai1 has been investigated in a number of cancers, our knowledge on Snai2 and its role(s) in squamous cell carcinoma of oral tongue (SCCOT) is limited. In this study, we confirmed the previous observation that over-expression of Snai2 is a frequent event in SCCOT. We further demonstrated that Snai2 over-expression is associated with lymph node metastasis in two independent SCCOT patient cohorts (total n = 129). Statistical analysis revealed that Snai2 over-expression was correlated with reduced overall survival. Furthermore, over-expression of Snai2 was correlated with reduced E-cadherin expression and enhanced Vimentin expression, suggesting a functional role of Snai2 in EMT. These observations were confirmed in vitro, in which knockdown of Snai2 induced a switch from a mesenchymal-like morphology to an epithelial-like morphology in SCCOT cell lines, and suppressed the cell invasion and migration. In contrast, ectopic transfection of Snai2 led to enhanced cell invasion and migration. Furthermore, Snai2 knockdown attenuated TGFβ1-induced EMT in SCCOT cell lines. Taken together, these data suggest that Snai2 plays major roles in EMT and the progression of SCCOT, and may serve as a therapeutic target for patients at risk of metastasis.

Keywords

epithelial menschymal transition; E-cadherin; Snai2; squamous cell carcinoma

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Introduction

Oral squamous cell carcinoma (OSCC) is a complex disease that arises at various sites. Tumors from these different sites have distinct clinical presentations and outcomes, and are associated with different genetic characteristics¹. In this study, we focused on squamous cell carcinoma of oral tongue (SCCOT), one of the most common sites for OSCCs. SCCOT is significantly more aggressive than other forms of OSCCs, with a propensity for rapid local invasion and spread². Despite continuous advancements in therapeutic strategies, the mortality rate of SCCOT patients has not changed significantly over the past 30 years, and patients continue to die from metastasis diseases^{3–7}. Recent studies have demonstrated that epithelial-mesenchymal transition (EMT) plays a major role in cancer cell invasion and the metastasis cascade^{8,9}. EMT is characterized by a spectrum of cellular and morphological changes that include loss of polarity and cell-cell adhesion, increased motility, and acquisition of the mesenchymal phenotype^{10,11}. These phenotypic changes are accompanied by alterations at the molecular level including the repression of E-cadherin gene expression (one of the hallmarks of EMT). E-cadherin is a major cell-to-cell adhesion molecule and plays a crucial role in the development and maintenance of epithelial cell polarity and tissue architecture¹². The loss of E-cadherin expression has been linked to enhanced SCCOT invasiveness, metastasis, and poor prognosis^{13,14}.

The members of the Snail superfamily of zinc-finger transcription factors, including Snai1 and Snai2 (human homologs of *Drosophila* snail and slug genes), are transcriptional repressors that have been implicated in EMT during embryonic development and tumor progression^{15,16}. While a number of studies have demonstrated the roles of Snai1 in the repression of E-cadherin expression and the induction of EMT in OSCC^{17–19}, only a few preliminary studies have reported on Snai2²⁰. The effect of Snai2 on SCCOT progression has not been defined. The aims of this study are to examine deregulation of Snai2 in SCCOT and to investigate its roles in EMT and SCCOT progression.

Materials and Methods

Patient

Two SCCOT patient cohorts were utilized in this study. The clinical characterization of the patients is summarized in Supplementary Table 1. The cohort #1 was used for evaluating the expression of Snail family members at the mRNA level. This group consists of 53 SCCOT cases and 22 matching adjacent non-cancerous samples with microarray data generated either from our previous study²¹ or downloaded from the GEO database^{22–24}. The cohort #2 was used for assessing gene expression at the protein level using immunohistochemical assay. This group includes 76 SCCOT patients who were diagnosed and underwent radical surgeries between 1996 and 2005 at the Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, Sun Yat-sen University. None of the patients received any form of adjuvant therapy prior to surgery. The tumor extent was classified according to the TNM system by UICC, and the tumor grade was classified according to the WHO classification of histological differentiation. Survival was calculated based on the date of surgery and the date of the last follow-up (or death). Median duration of follow-up was 65 months (range 3–120 months). This study was approved by the ethical committee of Sun Yat-Sen University.

Pooled-analysis to extract gene expression values from existing microarray dataset

The pooled-analysis was performed as described previously. In brief, the CEL files from all datasets (53 SCCOTs and 22 normal tongue samples) were imported into the statistical software R 2.4.1²⁵ using Bioconductor²⁶. The pooled-analysis was performed as

described²⁷, and the genome-wide expression pattern is presented in our previous study²¹. The Robust Multi-Array Average (RMA) expression measures²⁸ for each microarray dataset were computed after background correction and quantile normalization. Then, expression values of the overlapping probesets between U133A and U133 Plus 2.0 arrays were extracted. Probeset-level quantile normalization was performed across all samples to make the effect sizes similar among the four datasets²⁷. The expression values for probesets corresponding to Snai1 gene (219408_at) and for Snai2 gene (213139_at), Snai3 (1560228_at), E-cadherin (201130_s_at and 201131_s_at), and Vimentin (201420_s_at and 1555938_x_at) were then extracted from each dataset. Relative expression level for Snai1, Snai2, Snai3, E-cadherin, and Vimentin genes for each SCCOT samples were computed as previously described²⁹.

Immunohistochemical analysis

Immunohistochemical staining was performed on formalin-fixed, paraffin-embodied tissue sections. Representative sections were first stained with H&E and histologically evaluated by a pathologist. The selected sections were deparaffinized, quenched for endogenous peroxidase activity, and rehydrated as described previously³⁰. Sections were then blocked with 10% normal serum for 15 min at 37 °C followed by incubation with rabbit anti-Snai2 (Cell signaling, USA), mouse anti-E-cadherin (BD Bioscience, USA), or mouse anti-Vimentin (Cell signaling, USA) at a dilution of 1:100 for 16 h at 4 °C. After washing thrice in PBS, the sections were incubated with secondary antibody conjugated to biotin for 10 min at room temperature. After additional washing in PBS, the sections were incubated with streptavidin-conjugated horseradish peroxidase enzyme for 10 min at room temperature. Following final washes in PBS, antigen-antibody complexes were detected by incubation with a horseradish peroxidase substrate solution containing 3, 3'-diaminobenzidine tetrahydrochloride chromogen reagent, and counterstained with hematoxylin. Slides were rinsed in distilled water, cover-slipped using aqueous mounting medium, and allowed to dry at room temperature.

The relative intensities of the completed immunohistochemical reactions were evaluated using light microscopy by 3 independent trained observers who were unaware of the clinical data. All areas of tumor cells within each section were analyzed. The non-cancerous epithelium adjacent to the tumor cells were used as an internal control for the same cases. All tumor cells in ten random high power fields were counted. Immunoreactivity was semiquantitatively evaluated on the basis of staining intensity and distribution using the immunoreactive score, where Immunoreactive score = intensity score × proportion score. The intensity score was defined as 0: negative; 1: weak; 2: moderate; or 3: strong, and the proportion score was defined as 0: negative; 1: ≤ 10%; 2: 11–50% 3: 51–80% or 4: > 80% positive cells. The immunoreactive score ranged from 0 to 12. The immunoreactivity was divided into two groups on the basis of the immunoreactive score: low immunoreactivity was defined as a total score of 0–4, and high immunoreactivity was defined as a total score >4.

Cell Culture and the treatments of cell lines

The SCC9 cell line was acquired from American Type Culture Collection (ATCC), and the UM1 cell line was a gift from Dr. David Wong at UCLA School of Dentistry. These SCCOT cell lines were maintained in DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (GIBCO). For functional analysis, gene specific siRNA against Snai2 (On-TargetPlus SMARTpool, Dharmacon) or control siRNA was transfected into cells using DharmaFECT Transfection Reagent 1 as described previously^{31, 32}. The Snai2 expression vector (pCMV6-SNAI2, OriGene Technologies, Inc)

or empty vector was transfected into cells using Lipofectamine 2000 (Invitrogen). For the induction of EMT, cells were treated with 10ng/ml TGF β 1 as described previously^{33, 34}.

Fluorescent immunocytochemical analysis

Cells were fixed on the slides and permeabilized as previously described³⁵, and then blocked with 5% BSA for 30 minutes, followed by incubation with primary antibodies (E-cadherin, BD Bioscience, USA and Vimentin, Cell signaling, USA) at 4°C for 16 h. The slides were then washed with PBS and incubated with Rhodamine-conjugated secondary antibody (for Vimentin) or FITC-conjugated secondary antibody (for E-cadherin) for 1 hour at 37°C. The slides were then washed with PBS and incubated with 5 μ g/ml DAPI for nuclear staining. The cells were visualized for immunofluorescence with a laser scanning Zeiss microscope system (Zeiss Axivert 400C, Germany).

Western blot analysis

Western blots were performed as described previously³² using antibodies specific to Snai2 (Cell Signaling, USA), E-cadherin (BD Biosciences, USA), Vimentin (BD Biosciences, USA), beta-actin (Sigma, USA), and a Immu-StarHRP Substrate Kit (BIO-RAD, USA).

In vitro cell migration and invasion assay

The in vitro cell migration and invasion were measured using the BD BioCoat system (BD Biosciences, USA) following the manufacturer's instructions. In brief, cells were seeded in the upper chambers, and culture medium with 10% FBS was added to the lower chambers. For invasion assay, transwell inserts coated with Matrigel were used. After 24 h incubation, cells that migrated to the reverse side of inserts were stained with Diff-Quik stain kit (Polyscience, USA) and quantified.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Science (SPSS, Chicago, IL), Version 17.0. Spearman Correlation Coefficient was used to assess correlations among the gene expression and clinical and histopathological parameters. One-way ANOVA was used to assess the association of Snai2 expression with pN. Kaplan-Meier plots were constructed to present survival outcomes. Cox regression was used for both univariate and multivariate analysis. For multivariate analysis, grade, tumor size, pathological T-stage (pT), pathological N-stage (pN), clinical stages and the expression of Snai2, E-cadherin and Vimentin were considered as co-variables. For all statistical analyses, $p < 0.05$ was considered statistically significant.

Results

The expression of snail family members at mRNA level in SCCOT

Pooled-analysis was performed on previously existing microarray datasets to determine the expression of Snail family members at the mRNA level in SCCOT (n=53) and normal control samples (n=22). As illustrated in Supplementary Figure 1, Snai2 is significantly over-expressed in SCCOT as compared to normal tissue. A statistically significant increase in Snai2 expression was also observed in the primary tumor tissues with lymph node metastasis (pN+) when compared to those with negative status (pN-). However, no difference in Snai1 expression was observed between normal and tumor samples, and no statistically significant difference in Snai1 expression was observed between primary tumor tissues with (pN+) or without lymph node metastasis (pN-). The expression of Snai3 was not detected in these cases.

The Snai2 protein level in SCCOT

To confirm our observation and further elucidate the role of Snai2, the expression of the Snai2 gene was examined by immunohistochemistry (IHC) in an additional SCCOT patient cohort (n = 76) (Supplementary Table 1). As illustrated in Figure 1A, in normal epithelium, Snai2 was detectable only in basal layers. In cancer cells, predominant nuclei staining of Snai2 were observed. Semi-quantitative analysis revealed that Snai2 is significantly over-expressed in SCCOTs when compared to normal tissue (Supplementary Table 2). Among 76 cases of SCCOT samples that we examined, 48 cases (63.16%) exhibited low nuclear staining for Snai2 (Figure 1B) and 28 cases (36.84%) exhibited high staining for Snai2 (Figure 1C). Furthermore, a dramatically increased nuclear staining of Snai2 was observed at the invasive front (Figure 1D and Supplementary Figure 2).

Since overexpression of Snai2 has been linked to the induction of EMT, the levels of E-cadherin and Vimentin in these cases were also examined by IHC. As shown in Figure 1E, strong staining of E-cadherin was detected at the cytoplasmic membrane and the intercellular borders in the normal epithelium. Among 76 cases of SCCOT that we examined, high E-cadherin expression was observed in 34 cases (44.74%) (Figure 1F), and reduced E-cadherin expression was found in 32 cases (55.26%) (Figure 1G). SCCOT cells located at the invasive front were found to have no detectible E-cadherin staining (Figure 1H). As shown in Figure 1I, Vimentin staining was detected in the cytoplasm of the connective tissue mesenchymal cells, but not in normal epithelia cells. Of the 76 SCCOT cases, 38 (50.00%) showed negative or low cytoplasmic staining of Vimentin (Figure 1J), and 38 (50.00%) exhibited high Vimentin staining. Increased cytoplasmic staining of Vimentin was observed in the majority of SCCOT cells located in the invasive front and the de-differentiated cancer cells (Figure 1K and 1L).

Correlation among Snai2 expression, EMT markers and clinicopathological features in SCCOT

Correlations were tested among gene expression (e.g., Snai2, E-cadherin and Vimentin), clinical and pathological features for both SCCOT patient cohorts (Table 1). For patient cohort #1 (n = 53), the Snai2 mRNA level was positively correlated with pN ($P < 0.05$) and Vimentin expression ($P < 0.01$). For patient group #2 (n = 76), the Snai2 protein level was correlated with tumor size ($P < 0.01$), pT stage ($P < 0.05$), pN ($P < 0.05$) and clinical stage ($P < 0.05$). The expression of Snai2 significantly correlated with reduced E-cadherin expression ($P < 0.001$) and enhanced Vimentin expression ($P < 0.05$). It is important to note that of the 2 patient groups examined (total n = 129), correlation was consistently observed between Snai2 expression and pN. The Snai2 expression is significantly up-regulated in SCCOT cases with lymph node metastasis (pN+) as compared to those with negative status (pN-) (Supplementary Figure 1 and Supplementary Table 2).

The prognostic value of Snai2 deregulation for SCCOT patients

As illustrated in Figure 2, a striking difference in prognosis was observed between the high Snai2 expression group (5-year survival rate < 50%) and the low Snai2 expression group (5-year survival rate > 70%). The differences in survival were also observed when patients were grouped based on the expression of E-cadherin or Vimentin (Supplementary Figure 3). As illustrated in Table 2, univariate analysis indicated that grade, tumor size, pathological T-stage, lymph node metastasis, clinical stage, Snai2, Vimentin and E-cadherin were all significant prognostic factors for patients with SCCOT. Multivariate analysis showed that E-cadherin, and to a lesser extent Vimentin, were independent prognostic factors for SCCOT patients ($p = 0.01$, and $p = 0.062$, respectively). However, Snai2 appears not to be an independent prognostic factor.

The effects of Snai2 on EMT in SCCOT cell lines

UM1 is a SCCOT cell line that expresses high levels of Snai2 and exhibits the mesenchymal phenotype (low E-cadherin expression, high Vimentin expression, Supplementary Figure 4). When UM1 cells were treated with Snai2-specific siRNA, the cells were switched from a mesenchymal-like morphology to an epithelial-like morphology (Figure 3A and Supplementary Figure 5). This morphological change was accompanied by increased E-cadherin expression and reduced Vimentin expression (Figure 3A, 3B and Supplementary Figure 6), as well as reduced cell migration and invasion (Figure 3C and 3D). Similar results were observed in additional cell lines (Supplementary Figure 7A). When SCCOT cells were treated with Snai1-specific siRNA, enhanced E-cadherin expression and reduced Vimentin expression were also observed (Supplementary Figure 7B).

SCC 9 is a SCCOT cell line that exhibits the epithelial phenotype and can undergo EMT upon TGF β 1-treatment (Supplemental Figure 8). As illustrated in Figure 4A, when SCC9 cells were pre-treated with Snai2 specific siRNA, the TGF β 1-induced down-regulation of E-cadherin was blocked. The TGF β 1-induced up-regulation of Vimentin was also partially attenuated. Similar results were observed in additional cell lines (Supplementary Figure 7C). The knockdown of Snai2 also blocked the TGF β 1-induced cell migration and invasion activities in SCC9 cells (Figure 4B and 4C). Furthermore, ectopic transfection of Snai2 led to reduced E-cadherin expression and enhanced Vimentin expression (Figure 4D), and these expressional changes were accompanied by enhanced cell migration (Figure 4E) and invasion (Figure 4F).

Discussion

Cancer cells can de-differentiate through activation of specific biological pathways associated with epithelial-mesenchymal transition (EMT), thereby gaining the ability to migrate and invade. Recently, a number of EMT regulators have been described, such as microRNA-200 family³⁶, ZEB family³⁷ and Snail family^{15, 16}. The members of Snail family of transcription factors are zinc-finger transcriptional repressors. There are three known members of Snail family genes in the human genome: Snai1, Snai2 and Snai3. A number of in vitro studies suggested that Snai1 contributes to EMT by repressing E-cadherin expression^{17, 33, 38, 39}. However, several recent in vivo studies demonstrated that Snai1 expression is limited in OSCC, and its deregulation may not be directly associated with metastasis in OSCCs^{40, 41}. Based on our pooled-analysis of the existing mRNA microarray dataset on SCCOTs, no statistical significant difference in Snai1 expression was observed between SCCOT samples and the normal control samples, or between SCCOT samples with or without lymph node metastasis (Supplementary Figure 1A). This is in agreement with the previous in vivo observations on OSCCs^{40, 41}. Furthermore, among the cell lines we tested, only 1386Ln exhibits detectable Snai1 expression (Supplementary Figure 4B). In addition, Snai3 expression was not detectable in our samples.

Unlike Snai1, the role of Snai2 in OSCC has not been investigated extensively. Our pooled-analysis of the existing mRNA microarray dataset demonstrated that Snai2 is over-expressed in SCCOT when compared to the normal control samples. Furthermore, a statistically significant increase in Snai2 expression was observed in SCCOT cases with lymph node metastasis when compared to cases without metastasis. We further confirmed this association of Snai2 overexpression and lymph node metastasis in a second SCCOT patient cohort based on IHC analysis. In addition, we also found that the overexpression of Snai2 correlates with advanced clinical stage and reduced overall survival. Furthermore, we observed a high level of Snai2 expression in the invasion front of the primary cancer. More importantly, the over-expression of Snai2 is associated with reduced E-cadherin expression and increased Vimentin expression. These results indicate that Snai2 may suppress the E-

cadherin expression and induce EMT in SCCOT, which in turn contribute to invasion and metastasis.

To explore the functional relevance of Snai2 in SCCOT progression, we confirmed the roles of Snai2 in EMT in a set of in vitro experiments. Using the siRNA-based knockdown of the Snai2 gene, we were able to switch the SCCOT cells from a mesenchymal-like morphology to an epithelial-like morphology. This switch was accompanied by increased E-cadherin expression, decreased Vimentin expression, and reduced cell invasion and migration. In contrast, ectopic transfection of Snai2 led to reduced E-cadherin expression, enhanced Vimentin expression, and enhanced cell invasion and migration. We further demonstrated that knock-down of Snai2 can block the TGF β 1-induced EMT in SCCOT. Interestingly, Snai2 knock-down also leads to redistribution of E-cadherin from cytoplasmic to adherens junctions. These results suggest that Snai2 regulates EMT not only by repressing E-cadherin expression but also regulating the subcellular localization of E-cadherin in SCCOT.

The relative contributions of Snai1 and Snai2 to the induction of EMT are still unclear. Complementary roles of Snai1 and Snai2 have been previously proposed. While Snai1 is believed to initiate EMT by regulating the expression of basement membrane proteins, E-cadherin, and matrix metalloproteinase activities^{33, 42, 43}, Snai2 maintains the mesenchymal phenotype by sustaining the repression of E-cadherin⁴⁴. However, in some cancer types (e.g., bladder cancer), Snai2 appears to trigger EMT, whereas Snai1 acts later to complete the process by enhancing cell motility and initiating the switch from cytokeratin to Vimentin expression⁴⁵. Collaborative roles of Snai1 and Snai2 have also been suggested in skin cancer cell lines⁴⁶. In OSCC, both Snai1 and Snai2 have been showed to mediate TGF β 1-induced induction of MMP9 expression, suppression of E-cadherin expression, and enhancement of cell invasion^{20, 33}. Our knockdown experiment also confirmed that both Snai1 and Snai2 regulate E-cadherin expression in vitro (Supplementary Figure 7A and 7B). However, no apparent change in Snai1 protein level and slight down-regulation of Snai1 mRNA was observed in SCCOT cell lines treated with TGF β 1, whereas Snai2 was significantly up-regulated (data not shown). The TGF β 1-induced up-regulation of Snai2 was accompanied by dramatic down-regulation of E-cadherin expression (data not shown). Similar observations were reported by other SCCOT cell lines⁴³. These observations further illustrate the different roles of Snail family members in different cancer types, and Snai2 may serve as a novel therapeutic target for metastatic SCCOT. It is possible that in SCCOT, Snai2 is responsible for both initiating and maintaining EMT. Furthermore, the observed differences in in vitro studies may be cell line specific. It is possible that the cell lines we tested here (or the cell line used in the previous study) may have specific mutation(s) that dictates the effects of Snai1 and Snai2 in EMT. More in-depth functional analysis will be needed to fully evaluate the potential differential effects of Snai1 and Snai2 in EMT, as well as to assess the feasibility of utilizing Snai2 as biomarkers or therapeutic target for SCCOT patients at risk of metastasis. Nevertheless, our results suggest that Snai2 plays an important role in EMT and the progression of SCCOT.

While our retrospective analysis revealed a striking difference in the 5-year survival rate between the high Snai2 expressing patient group (< 50%) and the low Snai2 expressing patient group (> 70%), Snai2 appears not to be an independent prognostic factor. This may be due to the apparent correlation between enhanced Snai2 expression and EMT (characterized by reduced E-cadherin expression and enhanced Vimentin expression), which is associated with metastasis and disease progression. This is in agreement with the observed statistically significant increases in Snai2 expression in SCCOT cases with lymph node metastasis. These observations suggest that to achieve optimized prognostic assessment, it is important to integrate Snai2 with EMT-related markers (e.g., E-cadherin and Vimentin), as

well as clinical and pathohistological parameters. Additional studies with larger sample sizes will be needed to fully explore the prognostic values of these markers.

In summary, our results demonstrate that Snai2 deregulation is a frequent event in SCCOT, and is associated with disease progression. One of its major roles in SCCOT progression is promoting EMT which will enhance cancer cell migration, invasion and metastasis. Additional studies will be needed to fully explore the molecular mechanisms that contribute to the deregulation of Snai2 in SCCOT, and its potential as a biomarker and a novel therapeutic target for SCCOT patients at risk of metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

EMT	epithelial-mesenchymal transition
IHC	immunohistochemistry
OSCC	oral squamous cell carcinomas
pN	pathological N-stage
pT	pathological T-stage
SCCOT	squamous cell carcinoma of oral tongue

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Figure 1. Immunohistochemistry analyses of Snai2, E-cadherin and Vimentin expression in SCCOT.

Immunohistopathological analyses were performed as described in Material and Methods to examine the Snai2, E-cadherin, and Vimentin expression respectively on **A, E, and I**: adjacent normal epithelium (n = 10); **B, F, and J**: well differentiated primary (n = 52); **C, G, and K**: moderate or poor differentiated SCCOT (n = 24), **D, H, L**: invasive fronts of SCCOT (n = 76). Representative images of x200 magnification were presented. Scale bar = 200 μ m.

Figure 2. The effects of Snai2 on the prognosis SCCOT patients

Kaplan-Meier plots of overall survival in patient groups defined by the expression levels of Snai2. The differences in survival rates are statistically significant ($p < 0.05$).

Figure 3. Knock-down of Snai2 restores the epithelial phenotype in UM1 cells

UM1 cells were transiently transfected with Snai2 specific siRNA or control siRNA.

Fluorescent immunocytochemistry analyses were performed to detect the expression of E-cadherin (green) and Vimentin (red) in the intact cells (A). The expression of Snai2, E-cadherin and Vimentin were also measured by Western blots (B). Cell migration (C) and

invasion (D) were significantly reduced when cells were treated with Snai2 specific siRNA. Data represents at least 3 independent experiments with similar results. * indicates $p < 0.05$.

Figure 4. Enhanced expression of Snai2 induces EMT in SCC 9 cells

SCC 9 cells were treated with siRNA specific to Snai2 or control siRNA. The cells were then treated with TGFβ1 for the induction of EMT (as illustrated in Supplementary Figure 5). The expression of EMT markers (E-cadherin and Vimentin) (A) and the cell migration (B) and invasion (C) were measured. SCC9 cells were transiently transfected with a Snai2 expression vector (Snai2 pcDNA), or an empty vector. The expression of E-cadherin and Vimentin (D), and the cell migration (E) and invasion (F) were measured. * indicates $p < 0.05$.

Table 1

Correlations among clinical and histopathological features of primary SCCOT[#]

	sex	age	tumor size	grade	pT	pN	C stage	E-cad	Vim	Snai2
Patient cohort #1 (n = 53)										
sex		0.06	na	na	0.08	-0.24	-0.08	0.23	-0.17	-0.23
age			na	na	0.00	0.07	0.03	0.17	-0.09	-0.05
tumor size				na	na	na	na	na	na	na
grade					na	na	na	na	na	na
pT						0.05	0.59*	0.03	-0.55*	-0.71*
pN							0.50*	0.03	-0.03	0.28*
C stage								0.02	-0.30	-0.37*
E-cad									0.02	0.06
Vim										0.67*
Snai2										
Patient cohort #2 (n = 76)										
sex		-0.10	0.19	-0.05	0.33*	0.03	0.22	0.12	-0.36*	-0.05
age			-0.09	-0.01	-0.04	0.01	-0.06	0.11	-0.05	-0.20
tumor size				0.03	0.71*	0.09	0.44*	-0.13	-0.06	0.28*
grade					0.10	0.17	0.10	-0.26*	0.23	0.18
pT						0.28*	0.65*	-0.15	0.06	0.31*
pN							0.77*	-0.39*	0.21	0.23*
clinical stage								-0.26*	0.11	0.25*
E-cad									-0.53*	-0.30*
Vim										0.27*
Snai2										

[#] Spearman correlation coefficients were presented.

The expression levels of Snai2, E-cadherin and Vimentin for Patient cohort #1 were based on the relative mRNA level. The expression levels of Snai2, E-cadherin and Vimentin for Patient cohort #2 were generated from semi-quantitative immunohistochemical analysis.

pT: pathological T-stage; pN: pathological N-stage; C stage: clinical stage.

* p < 0.05. The p values were computed using Fisher's transformed z-score test.

na: data not available.

Table 2

The effects of clinical and pathohistological parameters on prognosis*

		Univariate analysis			Multivariate analysis		
		HR	95%CI	p value	HR	95%CI	p value
Gender	Female	1					
	Male	1.117	0.516 – 2.421	0.779			
Age	≤ 55	1					
	> 55	0.948	0.452 – 1.987	0.887			
Grade	Well	1					
	Mediate	2.342	1.074 – 5.128	0.033	1.715	0.524 – 3.289	0.254
Tumor Size	Poor	3.278	0.767 – 14.925	0.108	1.852	0.320 – 10.753	0.492
	≤ 4 cm	1					
pT	> 4cm	3.257	1.517 – 6.993	0.002	2.262	0.507 – 10.101	0.285
	pT1-2	1					
pN	pT3-4	3.509	1.658 – 7.407	0.001	1.066	0.248 – 4.587	0.932
	Negative	1					
Clinical stage	Positive	5.848	2.469 – 13.889	<0.001	1.525	0.283 – 8.227	0.624
	I-II	1					
Snai2	III-IV	6.894	2.381 – 20.000	<0.001	5.952	0.697 – 50.000	0.103
	Low	1					
Vimentin	High	2.506	1.190 – 5.291	0.015	1.256	0.450 – 3.509	0.664
	Low	1					
E-cadherin	High	4.695	1.901 – 11.628	0.001	3.145	0.944 – 10.526	0.062
	High	1					
	Low	15.152	3.597 – 62.500	<0.001	9.709	1.718 – 55.556	0.010

* Analysis was done with Cox proportional hazard regression. HR: hazard ratio; 95% CI: 95% confidence interval.