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Snail controls the mesenchymal phenotype and drives erlotinib resistance in Oral epithelial and HNSCC cells

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Abstract

PURPOSE—The presence of regional metastases in HNSCC patients is a common and adverse event associated with poor prognosis. Understanding the molecular mechanisms that mediate HNSCC metastasis may enable identification of novel therapeutic targets. Our recent work on human HNSCC tissues underlies Snail's role as a molecular prognostic marker for HNSCC. Snail positivity is significantly predictive of poorly differentiated, lymphovascular invasive, as well as regionally metastatic tumors. We recently reported the role of Snail in the inflammation-induced promotion of EMT in HNSCC. However, other important Snail-dependent malignant phenotypes have not been fully explored. Here, we investigate the capacity of Snail to drive EMT in human oral epithelial cell lines, and its ability to confer drug resistance.

EXPERIMENTAL DESIGN—Snail was overexpressed HNSCC and oral epithelial cell lines. AIG assays, wound healing assays, invasion & migration assays, spheroid modeling, and cell survival assays were performed.

RESULTS—The overexpression of Snail in human HNSCC and oral epithelial cell lines drives EMT. The sole transfection of Snail confers the expression of a mesenchymal molecular signature including down-regulation of the epithelial adherens, such as E-cadherin and β -catenin, and

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induction of mesenchymal markers, Snail overexpressing cell lines demonstrate rapid growth in Anchorage-independent growth assays; a decreased capacity to form tight spheroids; increased resistance to erlotinib; and have an increased capacity for invasion.

CONCLUSION—Snail controls the mesenchymal phenotype and drives erlotinib resistance in HNSCC cells. Snail may prove to be a useful marker in predicting EGFR inhibitor responsiveness.

INTRODUCTION

Every year, 550,000 new cases and over 300,000 deaths are attributed to non-cutaneous head and neck cancer worldwide [1]. The majority of these cancers are head and neck squamous cell carcinomas (HNSCC) that cause significant mortality and morbidity. Despite surgical and pharmaceutical advancements, the 5-year survival for head and neck cancer has been steady at a dismal 62.7% from 1999–2006 [2]. Recently, there has been a concerted effort to study the molecular biology of HNSCC in hopes of improving the above statistics.

Epithelial-mesenchymal transition (EMT) is a physiologic model that explains embryologic processes like gastrulation, neural crest delamination, developmental morphogenesis, and tissue homeostasis [3]. Loss of cell-cell adhesion and gain of migratory function is a hallmark of cells undergoing EMT. This transition to a mesenchymal phenotype is mimicked by epithelial carcinomas. EMT has been proposed to explain a tumor cell invading the basement membrane and traversing the lymphovascular system. EMT causes cells to lose their epithelial phenotype and gain mesenchymal characteristics, increasing expression of markers like vimentin. By studying the EMT process, our group is focusing on elucidating the molecular pathways of how epithelial carcinomas metastasize. Mechanisms that block EMT are likely to decrease metastatic potential in epithelial carcinomas. Our previous work has established Snail as a key transcription factor in EMT in HNSCC [4].

Although metastasis is the most lethal consequence of tumor progression, comparatively little is known regarding the molecular machinery governing this process. In many carcinomas, there is a robust correlation between the expression of the transcription factor Snail and a poor prognosis, but the contribution of this protein to the metastatic process remains unresolved. Specifically, the question as to whether Snail is sufficient to drive EMT in HNSCC remained unexplored.

Herein, using Snail overexpressing HNSCC cell lines (OSC, Tu212, Tu686) and oral epithelial cell lines (OKF6, HOK-16B), we now identify Snail as a sufficient to drive EMT in HNSCC. Snail overexpression alone drives EMT in head and neck cancer cells by: inducing a fibroblastoid and invasive phenotype, down-regulating E-cadherin and β and γ -catenin, and increasing levels of vimentin and other mesenchymal markers. OKF-Snail and HOK-Snail lines demonstrate growth in Anchorage-independent growth assays; a decreased capacity to form tight spheroids; increased resistance to erlotinib; and they are highly invasive and migratory. Gene expression analysis also revealed Snail-associated differential gene expression with the potential to affect inflammatory cytokine regulation, migration, invasion and diverse aspects of HNSCC progression

METHODS

Cell Culture

HNSCC cells utilized in this study included Tu686, Tu212 (generously provided by Dr. D. Shin; Emory University, Atlanta, GA), OSC (generously provided by Dr. M. Nagayama; Tokushima University, Japan). Cells were cultured in RPMI Medium 1640 (Invitrogen) with 10% FBS and 1% penicillin/streptomycin and kept at 37 degrees Celsius, 5% CO₂. The HNSCC cell lines Tu686, Tu212, and OSC are used for all of experiments presented unless otherwise specified. The oral epithelial cell lines HOK-16B and OKF-6 are used for all of experiments presented unless otherwise specified. All experiments were performed in triplicate.

Snail Overexpression

pcDNA3-Snail cDNA (a generous gift from Dr. E. Fearon, University of Michigan) was subcloned into the retrovirus vector pLHCX (Clontech). For virus production, 70%-confluent 293T cells were cotransfected with pLHCX-Snail or pLHCX (vector alone). Tumor cells were then transduced with high-titer supernatants producing either Snail, or pLHCX virus. Following transduction, the tumor cells were characterized by Western blot for expression of Snail.

Wound Healing

Cells were grown in RPMI-1640 medium (Invitrogen) mixed with 10% FBS and 1% penicillin/streptomycin. When cells reached 100% confluence, a scratch was made with a pipette tip (10 μ l). Light microscopy was used to monitor wound closure at 0-, 12-, and 24-hour time points.

Invasion/Migration

Experiments were carried out using the Cytoselect 24-well cell migration and invasion assay (8 μ m, colorimetric format) #CBA-100-C (Cell Biolabs, Inc.) 1×10^6 cells were cultured in the upper chamber overnight. After removal of all non migratory (for migration assay) or non-invasive cells (for invasion assay), cells that had passed through membrane were quantified using OD 560nm.

Soft agar Assay

Methods were adapted from previous reports [5] and the CytoSelect 96-well cell transformation assay (Cell Biolabs, Inc.).

Three-dimensional culture

Experiments were performed abiding to the protocol published by Bissell [6]. Light microscopy was used to image the morphology of cells.

Western Blot

Cells were washed with PBS and whole cell lysate was prepared with modified RIPA buffer. Proteins were resolved by SDS/PAGE and transferred using PVDF membranes (Millipore,

Bedford, CA). The membranes were probed with anti-E-cadherin, anti-vimentin, anti- β -catenin, anti-Snail, or anti- γ -catenin antibody (BD Biosciences Pharmingen/Transduction Laboratories, San Jose, CA) at 1:5000 dilution in TBST containing 1.0% nonfat dry milk. The membranes were developed by the ECL chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence Staining

Cells were cultured on coverslips, fixed in methanol, postfixed in cold methanol-acetone (1:1) for 5 min, and dried. The coverslips were blocked with Dako Protein Block (DAKO, Mississauga, Ontario, Canada) and incubated with antibody against E-cadherin (BD Biosciences) or Snail (Abcam) diluted in Dako Protein Block. Cells were examined using a Zeiss Axiophot epifluorescence microscope equipped with a digital camera (Q Imaging, Burnaby, British Columbia, Canada).

Erlotinib Assay

Cells were plated in a 96-well Nunclon plate (Cole-Palmer) at a concentration of 2000 cells/well. Cells were incubated with RPMI-1640 medium (Invitrogen) with 10% FBS overnight. Erlotinib was serially diluted with RPMI-1640 and 10% FBS medium. 100 μ l of the appropriate concentration of erlotinib was pipetted into each well, along with 100 μ l of culture medium. Plates were incubated for 72 hours. Proliferation of cell was measure with ATPlite luminescence assay (Perkin Elmer #6016731). Plates were read by the FLx800 fluorescence reader (Bio-Tek Instruments).

Statistics

Data are present as the mean $\pm$ s.d. The significance of the difference between groups was evaluated with the Student's t-test or χ^2 test. $P < 0.05$ was considered significant.

RESULTS

Increased Snail expression results in decreased epithelial markers and increased expression of mesenchymal markers

In order to define the role of Snail in EMT in HNSCC, we generated genetically modified Snail over-expressing (Snail-S) HNSCC cells, and oral epithelial cells (as well as vector controls). To substantiate Snail overexpression in human oral squamous cell carcinoma, western immunoblots were performed. (data not shown). We started by examining the effects of Snail expression in Tu686, Tu212, and OSC HNSCC cells on E-cadherin levels. (These HNSCC cell lines are used for all of the subsequent experiments unless otherwise specified). Snail overexpression significantly decreased E-cadherin levels (Figure 1A and 1B).

In addition to assessing the effects of Snail overexpression on E-cadherin levels, we went on to look at the effects of Snail overexpression on multiple EMT markers (E-cadherin, β and γ -catenin, and vimentin). We studied the effects of Snail overexpression in HNSCC cell lines as well as oral epithelial cells (OKF6, HOK-16B), and noted that Snail overexpression alone reproducibly increases the levels of mesenchymal markers and decreases the levels of

epithelial markers in both head and neck cancer and oral epithelial cell lines (Figure 2A, B, C, D).

Snail overexpression in normal oral keratinocytes and HNSCC cells is sufficient to alter three-dimensional morphology in spheroid culture

Three-dimensional (3D) cultures allows for observation of three-dimensional morphologies and organization. A more epithelial phenotype organizes into a round or mass-like spheroid configuration. When HOK or OKF6 cells are grown in 3D culture, a tight spheroid shape is observed. Even malignant cells (Tu212, Tu686) are somewhat organized in tight spheres in 3D cultures. However, when Snail is overexpressed in all of these cell lines, a botryoid to stellate organization is observed (Figure 3: A–H). This change in morphology corroborates the changes in gene expression documented above.

Overexpression of Snail in HNSCC augments wound healing, cell migration and basement membrane invasion

Cells undergoing EMT lose cell-cell adhesion and increase their motility. Wound-healing assays demonstrated significant differences in directional cell migration and motility between oral epithelial cell lines and HNSCC cell lines transfected with empty vector vs. Snail overexpression (data not shown).

HNSCC cells overexpressing Snail were assayed for migration through a porous membrane. Migration was significantly greater in cells with overexpression of Snail as compared with vector overexpressing controls (0.38 vs. 0.23, $p < 0.001$, Figure 4). A similar assay utilizing a nonporous membrane measured the invasive abilities of the different cells. Tu686-Snail showed a significantly increased average optical density (OD) of 0.31, while parental Tu686-V cells had an average (OD) of 0.25 ($p = 0.009$, Figure 4). These results parallel the results of the wound-healing assay, further supporting the hypothesis that in HNSCC Snail drives EMT and the mesenchymal phenotype.

Snail overexpression in HNSCC allows for anchorage-independent growth in soft agar

Normally, epithelial cell growth and proliferation are dependent on adhesion to neighboring cells and the basement membrane. Without anchorage, epithelial cells undergo anoikis, attachment regulated apoptosis [8]. However, overexpression of Snail results in increased anchorage-independent growth. Optical density ratios measured at excitation/emission wavelengths of 570/600 nm are significantly higher (more growth) in HNSCC cells overexpressing Snail as compared to controls (0.65 vs. 0.74, $p = 0.017$, Figure 5).

Snail overexpression in HNSCC confers resistance to erlotinib

Erlotinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) whose targeted therapy has been used to prolong survival in advanced non-small cell lung and pancreatic carcinomas. This drug targets epithelial cells; however, resistance is seen in tumors with mesenchymal phenotypes that have undergone EMT. Snail overexpression was found to enhance HNSCC cell survival when treated with increasing concentrations of erlotinib. The survival of Tu686 cells transfected with empty vector as compared to the

parental Tu686 (control) cells showed no difference, $p=0.26$. However, Tu686 -Snail cells had significantly higher resistance to erlotinib, $p<0.001$ (Figure 6).

DISCUSSION

A decisive factor in the multistage process of metastasis is the early step of local invasion of carcinoma cells. However, the mechanisms coordinating the increased motility of proliferating cancer cells remain elusive. Members of the Snail family of transcription factors have garnered widespread interest in this context, as they are expressed in a variety of carcinomas and are associated with recurring or metastasizing tumors. Snail proteins have a well-established role in embryogenesis during which they mediate a process known as an epithelial-mesenchymal transition (EMT) to facilitate tissue formation (3). The Snail-mediated EMT causes cells to lose their epithelial characteristics such as E-cadherin-mediated adhesion and polarity while adopting phenotypes of mesenchymal cells such as an increased migratory capacity. Given the similarities to its effects in development, it is widely extrapolated that Snail functions similarly in the metastasis of somatic cells during tumorigenesis.

This differentiation of epithelial to mesenchymal cells (epithelial-mesenchymal transition) has been proposed to explain how cells lose polarity and cell-cell junctions thereby allowing them to invade the lymphovascular system and establish metastases in cancer. Our prior work has clearly demonstrated that Snail overexpression is significantly associated with regional metastasis and poor histopathologic findings in HNSCC tissues.. Snail overexpression was also associated with lymphovascular invasion, poor tumor differentiation, and basaloid classification. Snail(+) tumors also demonstrate significant association with nodal metastasis, which corresponds to the aggressive histopathologic features of Snail(+) tumors. We have also corroborated Snail's inverse correlation with E-cadherin, while demonstrating Snail's improved clinical utility over E-cadherin. The independence of Snail expression from HNSCC tumor subsites or from other common tumor markers (HPV, p16) supports a clinical role for Snail staining. Snail staining would allow head and neck surgeons to aggressively treat those patients most at risk for cervical metastasis.

We have also previously studied the molecular mechanisms underlying our above clinical observations. We found Snail to be an important transcription factor in EMT [10]. By binding to an E-box in the E-cadherin promoter, Snail represses the expression of E-cadherin and decrease cell-cell adhesion [11]. Our previous work has established that inflammatory mediators like IL-1 β and COX-2 upregulate Snail and repress E-cadherin [4]. The question as to whether Snail is sufficient to drive EMT in HNSCC remained unexplored.

In this study, we provide ample evidence that in human head and neck squamous cell carcinoma cells as well as oral epithelial cells, Snail contributes to tumor progression by acting as a crucial mediator of EMT. This conclusion is supported by several lines of evidence. First, the sole transfection of Snail confers the expression of a mesenchymal molecular signature including down-regulation of the epithelial adherens, such as E-cadherin

and β -catenin, and induction of mesenchymal markers, such vimentin (Figures 1 and 2). Second, Snail expression in oral epithelial cells confers a botryoid invasive phenotype in 3D culture (Figure 3). Third, Snail overexpression augments wound healing, cell migration and invasion, and allows for growth in soft agar (Figures 4 and 5). Fourth, Snail overexpression in HNSCC confers resistance to erlotinib (Figure 6). Loss of E-cadherin and gain of the expression of Snail are associated with resistance to EGFR TK inhibitors (4). This evidence supports a key role for Snail as an inducer of tumor invasion as well as a potential contributor to tumor growth and/or chemoresistance mechanisms. Thus, there is a dual purpose in studying pathways that regulate E-cadherin expression in HNSCC: Maintenance of E-cadherin expression may promote sensitivity to targeted therapy and prevent invasion and metastases.

The presence of regional metastases in HNSCC patients is a common and adverse event associated with poor prognosis. Understanding the molecular mechanisms that mediate HNSCC invasion and metastasis may enable identification of novel therapeutic targets for the prevention and management of metastasis. Although further studies are required to examine the complex connection network that regulates EMT in tumor metastasis, herein we provide the first report indicating that solely overexpressing Snail has the capacity to drive EMT in oral epithelial cell lines. Snail signaling orchestrates transcriptional programs that regulate E-cadherin down-regulation, EMT, and progression to invasive and metastatic head and neck carcinoma.

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Figure 1A:

Figure 1B:

Figure 1.

Figure 1A: The generation of Snail over expressing HNSCC lines diminishes E-cadherin
 Figure 1B: The generation of Snail over expressing HNSCC lines diminishes E-cadherin

Figure 2.
A: Snail Overexpression drives EMT

Figure 3A, B

HOK

HOK-SNAIL

Figure 3C, D

OKF

OKF-Snail

Figure 3E, F

Tu686-V

Tu686-Snail

Figure 3G, H

Tu212

Tu212-Snail

Figure 3.

Figure 4.
Invasion and migration

Figure 5.
AIG assay

Figure 6.
Overexpresion of snail resistance to Tarceva