Xenografted Tumorigenesis in the oral vestibule of nude mice by Snail transfection: Histological and immunohistochemical study

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Abstract (J. Kor. Oral Maxillofac. Surg. 2009;35:199-204)

Purpose: The purpose of this study is to investigate the epithelial-mesenchymal transition (EMT) induced by Snail transcription factor and Snail-transfected *in vivo* tumors with histopathological features.

Materials and methods: We induced *in vivo* xenografted tumorigenesis in the oral vestibules of nude mice by a Snail transfected HaCaT cell line and investigated morphological and immunohistochemical features in Snail expressive tumors.

Results: We identified tumor masses in 14 out of 15 nude mice in the HaCaT-Snail cell inoculation group, but no tumors were present in any of the HaCaT cell inoculation group. Induced tumors showed features of poorly differentiated carcinoma with invasion to neighboring muscles and bones. The HaCaT-Snail tumors showed decreased expressions of E-cadherin and cytokeratin, but showed increased expressions of vimentin and N-cadherin.

Discussion: The Snail transfected xenograft can improve productivity of malignant tumors, show various histopathological features including invasive growth, and aid in the investigation of tumor progression and the interaction with surrounding tissues.

Key words: E-cadherin, neoplastic cell transformation, Snail transcription factor, xenograft model

〔원고접수일 2009.6.2. / 1차수정일 2009.6.12. / 2차수정일 2009.6.27. / 게재확정일 2009.7.13.〕

Introduction

The epithelial-mesenchymal transition (EMT) has been observed during malignant transformation in some epithelial tumors[1]. E-cadherin, which is located in adherens junction and mediates homophilic cell-cell adhesion, is repressed during EMT[2]. E-cadherin is also involved in maintenance of the cytoskeleton through intracellular binding to actins via catenins[3]. Since the reduction of its expression has been reported to be associated with tumor invasiveness and prognosis[4,5], the E-cadherin gene is also called an invasion suppressor gene[6]. The E-cadherin loses its expression when an advanced epithelial tumor has its ability to invasion[7].

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Meanwhile, N-cadherin, which expressed in nerve cells and fibroblasts and is involved in heterophilic cell-cell adhesion[8], is called an invasion promoter[10] since there are some relationships between tumor progression and an increase of its expression[11]. N-cadherin is often up-regulated in situations where E-cadherin has been repressed[11,12].

The Snail transcription factor, first described by Bouley et al[13], induces EMT by down-regulation of E-cadherin. Snail has 4-6 C_2H_4 zinc finger domains and binds to CACCTG (or CAGGTG) specifically in promoter regions as a transcriptional repressor. More than 50 homologues have been reported, and Snail-transfected cells show repression of E-cadherin. Snail binds to the promoter of E-cadherin and represses its expression, resulting in loss of cell-cell adhesion and phenotypic changes, with additional effects on cell survival and migration.

The role of Snail in tumorigenesis as well as tumor invasion and metastasis has been studied in breast, gastric, and hepatic cancers. Yokoyama et al[14] reported that repression of E-cadherin was observed in Snail mRNA expressive oral squamous cell carcinoma culture cells and Takkunen et al[15] reported that

*This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-005-J00803).

EMT in oral squamous cell carcinoma cells could result from Snail, a E-cadherin repressor.

We developed a Snail transfected cell line derived from HaCaT cell line, which is a spontaneously immortalized keratinocyte cell line derived from normal adult human skin. It was called HaCaT because it had developed from human adult skin ketatinocytes during prolonged cultivation at a reduced Ca_{2+} concentration and elevated temperature[16]. We used this cell line for a xenografted tumor model in nude mice to investigate morphological and immunohistochemical changes in Snail expressive tumors.

Materials and methods

1. Plasmid

A human Snail cDNA was subcloned into the pBabePuro retroviral vector to generate pBabePuro-Snail-FLAG plasmid (Fig. 1). The amphotropic Phoenix cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand island, NY) supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FBS). The plasmid was transfected into the amphotropic Phoenix retrovirus packaging cell line by FuGENE™6 (Boehringer Mannheim, Roche Diagnostics, Laval, PQ) to generate a retroviral stock.

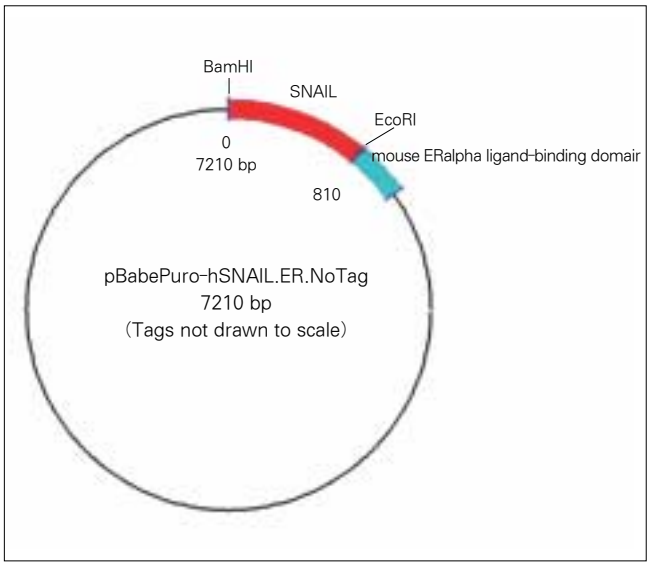

Fig. 1. Schematic diagram of pBabePuro-Snail with no tag ("Addgene plasmid 19292" which quoted from http://www.addgene.org/pgvec1?f=c&identifier=19292&atqx=snail&cmd=findpl).

2. Cell culture

The HaCaT cell line was cultured in a medium consisting of a 3:1 ratio of DMEM and Ham's nutrient mixture F12 (Gibco BRL, Grand island, NY), supplemented with 10% FBS, 1×10^{-10} M cholera toxin, 4 mg/ml hydrocortisone, 5 μg/ml transferring, and 2×10^{-11} M triiodothyronine (T3) under 5% CO_2 atmosphere at 37℃.

The virus-containing supernatant was obtained from the retroviral stock and applied to the cultured cell line.

3. Western blot analysis

A cell lysate was obtained from the transfected cell line and was extracted with a buffer solution of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS (sodium docecyl sulfate, Sigma, Saint Louis, MO), 1% NP_4O, and 1x Protease inhibitors (Roche, Indianapolis, IN). The proteins were resolved by 12% and 8% SDS-PAGE and transferred to nitrocellulose membranes. The membranes was first blocked by 5% skim milk for 2 hours, and then applied with the primary antibodies, anti-FLAG (monoclonal, Sigma-Aldrich, Saint Louis, MO), anti-E-cadherin (polyclonal, Santa Cruz, Santa Cruz, CA), and anti-β-actin (polyclonal, Sigma-Aldrich, Saint Louis, MO). The membranes were washed with phosphate buffered saline tween-20 (PBST) including 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20 for three times, and then the bound antibodies were applied with horseradish peroxidase conjugated secondary antibodies, respectively and visualized with the ECL system (Pierce).

4. Immunofluororescence

The cultured cells were transferred a chambered glass slide with 2×10^4 cells/chamber and fixed with 5% acetic acid in ethanol at -20℃ for 10 minutes. The cells were blocked by 2 mg/ml bovine serum albumin in phosphate-buffered saline (BSA-PBS) for 30 minutes and the primary antibodies for cytokeratin AE 1/3 (1:50, monoclonal, DAKO, Glostrup, Denmark), E-cadherin (1:100, monoclonal, BD Bioscience, San Diego, CA), and vimentin (1:100, monoclonal, DAKO, Glostrup, Denmark) were applied for 30 minutes at 4℃. After washing, they were stained with Alexa Fluor 594 dye (Molecular Probes Inc., Eugene, OR) conjugated secondary antibodies and fixed with ice-cold methanol for 10 minutes. They were then examined with a fluorescence microscope under FluoroGuard anti-fade mounting reagent (Bio-Rad, Hercules, CA).

5. *In vivo* tumorigenesis

The control HaCaT cells and transfected HaCaT-Snail cells were obtained at 5×10^6 cells/100 μl concentrations and 0.1 ml was injected into buccal vestibules of nude mice. The mice were sacrificed 8 weeks after inoculation to excise the resulting tumor mass, including surrounding tissues.

6. Histological Studies

The specimens were obtained and fixed in 10% neutral formalin for 24 hours, then embedded in paraffin blocks, from which 4 μm thick sections were prepared and transferred to glass slide.

7. Immunohistochemistry

The slides were deparaffinized in xylene for 30 minutes and rehydrated by sequential transfer to a 95%, 90%, 70% ethyl alcohol and distilled water. They were then immersed in 3% hydrogen peroxide for 20 minutes, to block the endogenous peroxidase activity. We used the Histostan-Plus kit (Zymed, South San Francisco, CA) to perform the immunohistochemical staining. Primary antibodies for this staining were E-cadherin (1:500 dilution, monoclonal, BD Bioscience, San Diego, CA), N-cadherin (1:100 dilution, monoclonal, Santa Cruz, Santa Cruz, CA), cytokeratin AE 1/3 (1:100, dilution, monoclonal, DAKO, Glostrup, Denmark), and vimentin (1:100 dilution, monoclonal, DAKO, Glostrup, Denmark). We washed the slides with phosphate-buffered saline (PBS) at every step. Finally, the slides were developed with DAB (3,3' -diaminobenzidine, Vector), and were counter-stained with Mayer' s hematoxylin (Fisher Scientific, Pittsburgh, PA). PBS solutions were used as negative controls and underlying connective tissues in the normal oral mucosa were used as positive controls.

Results

1. Western blot analysis

FLAG expressions were confirmed in Snail-transfected cell lines and HaCaT-Snail cells showed repression of E-cadherin (Fig. 2).

2. Phase contrast and fluorescence microscopy

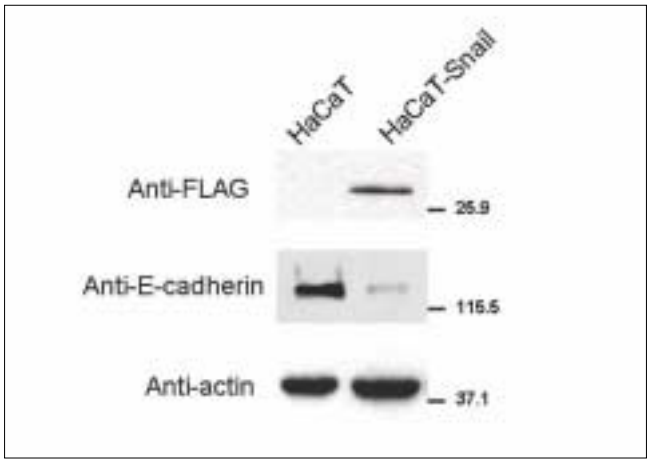

Fig. 2. The results of Western blotting after Snail transfection (left: HaCaT cell line; right: HaCaT-Snail cell line).

Table 1. Tumorigenesis after inoculation of HaCaT and HaCaT-Snail cell lines

Group	Mass formations
HaCaT	0 / 10
HaCaT-Snail	14 / 15

HaCaT-Snail cells showed loss of cell-cell adhesions in the phase contrast microscope and null expressions of cytokeratin and E-cadherin, and positive expressions of vimentin, in immunofluorescence microscopy (Fig. 3).

3. *In vivo* Tumorigenesis

We identified tumor masses in 14 out of 15 nude mice in the HaCaT-Snail cell inoculation group, but no tumors were present in any of the 10 nude mice in the control group (Table 1). Tumor masses in buccal vestibules extended to the lower face of nude mice (Fig. 4).

4. Histological Features

Tumors showed features of poorly differentiated carcinoma with central necrosis (Fig. 5), with invasion to neighboring muscles and bones (Fig. 6).

5. Immunohistochemistry

The HaCaT-Snail tumors showed decreased expressions of E-cadherin and cytokeratin, but showed increased expressions of vimentin and N-cadherin. E-cadherin was only slightly expressed while vimentin was substantially expressed. Cytokeratin showed negative expressions in almost all areas except some necrotic cells (Fig. 7).

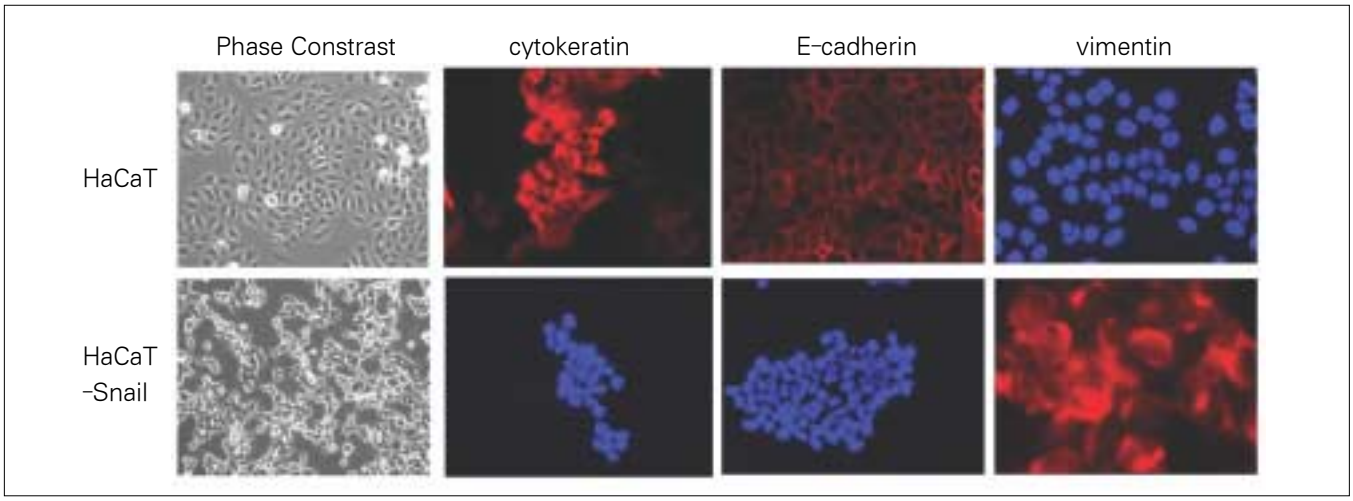

Fig. 3. Photographs of the phase contrast and fluorescence microscopy images, showing positive expressions as red colors.

Fig. 4. Photographs showing the tumor mass within the buccal cheek and vestibule of a nude mouse, extending to the lower face

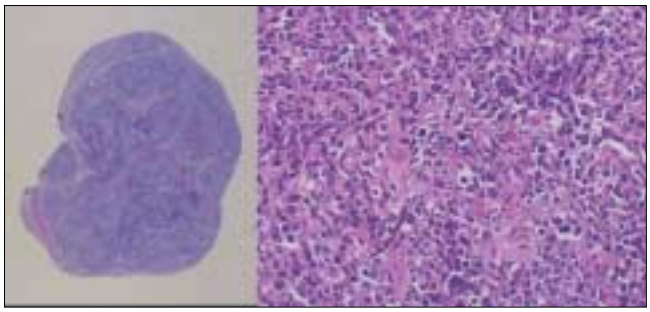

Fig. 5. Photographs showing the histological features of tumor mass (magnification, left: X 10 ; right: X 400). There are central necrotic areas in the tumor (left).

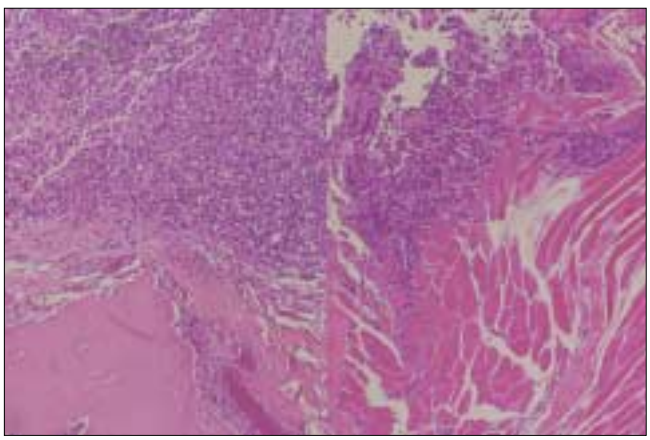

Fig. 6. Photographs showing invasions to neighboring muscles and bones (magnification, both: X 40).

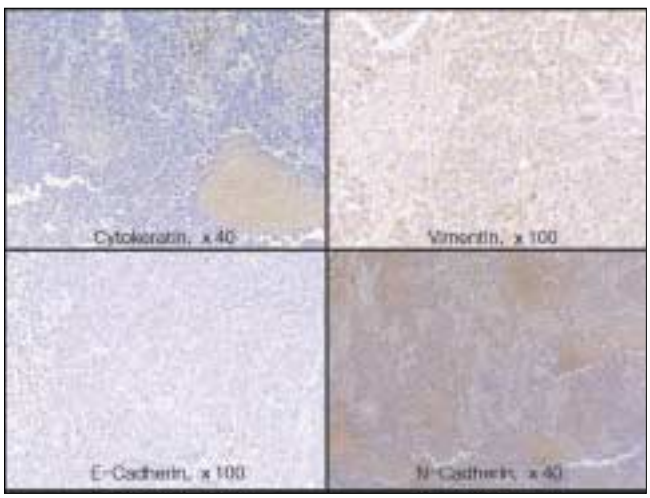

Fig. 7. Photographs of immunohistochemical staining showing decreased expressions of E-cadherin and cytokeratin and increased expressions of vimentin and N-cadherin (magnification, cytokeratin and N-cadherin: X 40; E-cadherin and vimentin: X 100).

Discussions

Tumorigenesis is now known to be a sequential multistep processes of genetic alterations[17,18]. As a result, subclones which have some advantages in survival can proceed to proliferation and reach potentials of growth, invasion and metastasis[19]. During these processes, epithelial tumor cells lose their cell-cell adhesions, have increased mobilities, and degrade their underlying connective tissue[20]. For these biochemical properties, they have to undergo epithelial-mesenchymal transition at first and reduce their E-cadherin expressions.

E-cadherins are bound to E-cadherins of neighboring cells to maintain extracellular cell-cell adhesions and are also bound to actin, via catenins, to maintain intracellular cytoskeletons.

β-catenins are known to have some important roles in tumorigenesis[21]. They act as transcription factors that control the Lef/Tcf promoter, stabilize intracellular cytoskeletons, and bind to E-cadherins[22,23].

Snail acts on the transcriptional control region of E-cadherin to repress its expression and increases the concentration of free β-catenins in the cytosol to activate a variety of tumor related genes[24].

Cano et al.[25] and Peinado et al.[26] reported subcutaneous formations in nude mice by Snail transfected cell lines. We also confirmed tumorigenesis in HaCaT cells, which used to be non-tumorigenic immortal keratinocytes, by Snail transfection. Snail is known to be degraded through ubiquitination via GSK-3β mediated phosphorylation and is so unstable that it has a short half-life of about 25 minutes[27]. We made a stabilized Snail transfectant that could induce E-cadherin repression.

We also observed increased expressions of N-cadherin. Hazan et al. reported increased expressions of N-cadherin in E-cadherin repressed tumor cells with high invasiveness[28,29]. N-cadherin is expressed in some tumors and is involved in cell mobility and invasion. It involves heterophilic cell-cell adhesion as well as homophilic cell-cell adhesion and its level could be increased by Snail in this study.

We investigated the *in vivo* state of Snail induced tumors in nude mice. The *in vivo* tumor model can have various phenomena, including central necrosis, invasion to surrounding tissues, and neoangiogenesis, as seen in naturally occurring tumors. In this study, we histologically observed partial necrotic regions and invasions to the underlying bones and muscles, and while we immunohistochemically saw uneven staining patterns of E-cadherin and viementin within the tumor mass. This may be a result of formations of certain different intraclonal variants or subclones in accordance with their environments.

We also observed that the tumors had the ability to infiltrate into their surrounding tissues, but we failed to find any distant metastatic lesions in any other organs. Certain other factors should be considered as inducing distant metastasis.

In conclusion, improved productivity of tumors can be obtained by Snail-transfected xenografts and various histopathological features, including invasive growth, can be observed with these *in vivo* tumors. This animal model can aid in the study of tumor progression and the interaction with surrounding tissues.

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