Down-regulation of Wnt-4 and up-regulation of Wnt-5a expression by epithelial-mesenchymal transition in human squamous carcinoma cells

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Gene expression of Wnt-1, 2, 3, 4, 5a, 6 and 7a was analyzed by RT-PCR in eleven squamous cell carcinoma (SCC) cell lines and compared with that in two normal oral keratinocyte strains. There appeared to be an inverse relationship between Wnt-4 and Wnt-5a expressions, i.e., Wnt-4 was not expressed in HOC719-NE, HOC313 or TSU cells, while Wnt-5a was strongly expressed only in these cells. These cell lines showed decreased expression of E-cadherin and elevated expression of vimentin accompanied with strong expressions of Snail and δEF1, which have been reported to be transrepressors of E-cadherin and to trigger epithelial-mesenchymal transition (EMT), suggesting associations of Wnt-4 with epithelial phenotype and Wnt-5a with mesenchymal phenotype of SCC cells. To study whether the expressions of these *Wnt* genes are regulated by EMT, we transfected a Snail-expression vector into A431 and OM-1 cells, which express Wnt-4 but not Wnt-5a. The stably Snail-overexpressing clones showed spindle morphology, increased expression of vimentin and decreased expression of E-cadherin accompanied with augmented expression of δEF1. In these clones, down-regulation of Wnt-4 and up-regulation of Wnt-5a were clearly observed. These results indicated that Wnt-4 and Wnt-5a are oppositely affected by EMT, and down-regulation of Wnt-4 and up-regulation of Wnt-5a are possible markers of the malignant phenotype of human SCC. (Cancer Sci 2003; 94: 593–597)

Wnt genes encode a large family of secreted glycoproteins which are developmentally important signaling molecules.[1–3] Wnt proteins control cell fate specification, migration and polarity of cells through cell surface receptors encoded by frizzled genes and intracellular signaling pathways to modulate transcription of specific target genes. Recently, it has been found that Wnt proteins activate different intracellular signaling pathways. One is the Wnt/β-catenin pathway, also referred to as the canonical pathway, which stabilizes β-catenin and induces translocation of cytoplasmic β-catenin to the nucleus. The nuclear-translocated β-catenin forms complexes with members of the lymphoid enhancer factor/T cell factor (LEF/TCF) family of DNA binding factors and controls the transcription of the target genes. The other pathways, non-canonical pathways, do not require β-catenin. They include the Wnt/Ca^{2+} pathway mediated by protein kinase C (PKC) and calmodulin-dependent protein kinase II (CamKII), and the Wnt/c-Jun N-terminal kinase (JNK) pathway, which involves dishevelled (dsh), the small GTPase rhoA and JNK.[4–7]

The involvement of Wnts and their signaling in tumor formation has been suggested based on the following observations: (a) *Wnt-1* and *Wnt-3* were initially identified as the genes activated by MMTV insertions in murine mammary epithelial tumors.[8, 9] (b) Wnt-1, 2, 3a, 5b, 7a and 7b induced transformation of mouse mammary epithelial cells.[10, 11] (c) Several components of the Wnt-signaling pathway, including β-catenin and APC, are frequently mutated in human cancers. (d) *Wnt* genes are aberrantly expressed in many types of cancer.[12–15] In contrast, however, down-regulation of Wnt expression has been reported in endometrial carcinomas[16] and in invasive ductal breast carcinomas.[17] Further, members of the Wnt-signaling pathway have recently been reported to be associated with invasion, scattering and promotion of epithelial-mesenchymal transition (EMT) in cancer cells.[18–21]

In the present study, we analyzed the expression of seven *Wnt* genes in squamous cell carcinoma (SCC) cells, compared with that in normal oral keratinocytes. We found an inverse relationship between Wnt-4 and Wnt-5a expressions in highly invasive SCC cells with mesenchymal phenotype and increased expression of Snail, a transrepressor of E-cadherin that is implicated in EMT,[22–25] and in other cells with epithelial phenotype. Furthermore, a similar relationship was also observed between EMT-induced clones of SCC cells transfected with Snail and the control clones that retained the epithelial phenotype. Our results indicated that EMT alters the expression of Wnt-4 and Wnt-5a in human SCC cells and suggest that down-regulation of Wnt-4 and up-regulation of Wnt-5a are possible markers of highly invasive SCC.

Materials and Methods

Cells and cell culture. A human vulval epidermal cell line A-431 was obtained from the Japanese Cancer Resources Bank. Eight human oral SCC cell lines, HSC-2, HSC-3, Ca9-22, HOC815, Mo-T, OM-1, TSU and HOC313, have been reported previously.[26–28] HOC719-PE and HOC719-NE cells were isolated from an SCC cell line, HOC719, which exhibits heterogeneous expression of E-cadherin.[29] Normal oral keratinocytes were isolated as previously described[30] and the two normal oral keratinocyte strains, HGE-2 and HGE-10, from gingival epithelium have been described previously.[31] All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. SCC cells were maintained with Dulbecco's modified essential medium (DMEM, Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS, Boehringer Mannheim, Tokyo). Oral keratinocyte strains were cultured with Keratinocytes-SFM (Gibco-BRL, Tokyo) including 25 μg/ml of bovine pituitary extract and 0.05 ng/ml of epidermal growth factor.

RT-PCR analysis. Total RNAs were isolated from the cells at 70 to 80% confluence using TRIZOL (Gibco-BRL). RT-PCR analysis was performed as described previously.[29] The RNA samples were first treated with deoxyribonuclease I (Gibco-BRL) and converted into cDNA using random hexamer primers and reverse transcriptase (Gibco-BRL). PCR consisting of 30 cycles of denaturing at 94°C for 30 s, annealing for 30 s and extension at 72°C for 1 min was carried out using PCR MASTER (Boehringer Mannheim). Amplified products were analyzed on 1.8% agarose gel. All of the primers were obtained from Hokkaido System Science Co., Ltd. (Sapporo). PCR product size,

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annealing temperature, and the primer sequence were: Wnt-1, 241 bp, 53°C, 5′-ctacttcgagaaatcgcccaac-3′ (forward), 5′-acagacactcgtgcagtacgc-3′ (reverse); Wnt-2, 289 bp, 55°C, 5′-gggaatctgccttttgtttatgcca-3′ (forward), 5′-gaaccgctttacagccttcctgcc-3′ (reverse); Wnt-3, 194 bp, 58°C, 5′-cagcagtacacatctctgggctca-3′ (forward), 5′-ctgtcatctatggtggtgcagttc-3′ (reverse); Wnt-4, 294 bp, 60°C, 5′-ctgaaggagaagtttgatggtgcc-3′ (forward), 5′-gtggaatttgcagctgcagcgttc-3′ (reverse); Wnt-5a, 273 bp, 58°C, 5′-ctcgcccaggttgtaattgaagc-3′ (forward), 5′-ctgccaaaaacagaggtgttatcc-3′ (reverse); Wnt-6, 300 bp, 51°C, 5′-cttggttatggaccctaccagcatc-3′ (forward), 5′-cactgcagcagctcgcccatagaa-3′ (reverse); Wnt-7a, 183 bp, 53°C, 5′-aatgcccggactctcatgaactg-3′ (forward), 5′-acggcctcgttgtacttgtccttga-3′ (reverse); E-cadherin, 653 bp, 60°C, 5′-agccatgggcccttggag-3′ (forward), 5′-ccagaggctctgtgcaccttc-3′ (reverse); vimentin, 750 bp, 55°C, 5′-tggcacgtcttgaccttgaa-3′ (forward), 5′-ggtcatcgtgatgctgagaa-3′ (reverse); δEF1, 317 bp, 60°C, 5′-gtggcccattacaggcaaccagt-3′ (forward), 5′-gctaggctgctcaagactgtagt-3′ (reverse); glyceraldehyde-3′-phosphate dehydrogenase (G3PDH), 452 bp, 52°C, 5′-accacagtccatgccatcac-3′ (forward), 5′-tccaccaccctgttgctgta-3′ (reverse).

Transfection with a Snail expression vector. Snail expression vector, pcDNA3-mm snail-HA[22] was a kind gift from Dr. de Herreros (Universitat Pompeu Fabra, Barcelona, Spain). OM-1 cells were transfected with 2 μg of pcDNA3-mm snail-HA or pcDNA3 (Invitrogen, Carlsbad, CA) using Tfx-20 (Promega, Madison, WI). Stable transfectants were selected with 600 μg/ml of G418 (Sigma) for 2 weeks, isolated using cloning rings (Iwaki, Tokyo) and maintained with 100 μg/ml of G418. A431-derived clones, A431SNA1, A431SNA2, A431pcD1 and A431pcD2 were similarly obtained by transfection with pcDNA3-mm snail-HA or pcDNA3 as described previously.[32]

Expression analysis of HA-tagged Snail protein in transfected cells by western blotting. Western blotting analysis was performed as previously reported.[32] Cell lysate was denatured at 100°C for 5 min, separated by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad, Richmond, CA). The membrane was incubated with a blocking buffer consisting of 5% skimmed milk and 1% bovine serum albumin in TBST (137 m*M* NaCl, 2.68 m*M* KCl, 0.1% Tween-20, 25 m*M* Tris-HCl, pH 7.5) for 3 h and with a rat monoclonal anti-HA, clone 3F10 (Roche, Mannheim, Germany) overnight, followed by horseradish peroxidase-conjugated goat anti-rat IgG (Sigma). The signals were detected by an enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK) and photographed with an ECL Mini-camera (Amersham).

Results

mRNA expression of Wnt-1, 2, 3, 4, 5a, 6 and 7a in normal oral keratinocytes and SCC cells. Gene expression levels of Wnt-1, 2, 3, 4, 5a, 6 and 7a in normal oral keratinocytes and 10 oral SCC cells and A431 cells were analyzed by RT-PCR (Fig. 1). Two strains of normal oral keratinocytes (HGE-2 and HGE-10) and HSC-3 cells showed similar levels of expression of Wnt-1 mRNA. Three cell lines (HOC815, OM-1 and Mo-T) showed elevated expression, but the other seven cell lines showed no expression of Wnt-1. Elevated expression of Wnt-2 was detected in five cell lines (A431, HSC-2, HOC815, HOC719-PE and HOC313) and similar expression was observed in two cell lines (HOC719-NE and TSU) compared with the oral keratinocytes, but no expression was seen in the other four cell lines. Expression of Wnt-3 was observed strongly in three cell lines (HOC-815, OM-1 and HOC719-PE), and weakly in A431, Ca9-22, and HOC719-NE cells, but was absent in the other five cell lines and the oral keratinocytes. Expression of Wnt-4 was observed similarly in the normal cells and Mo-T cells, and strongly in A431, HSC-2, HSC-3, HOC815, Ca9-22, OM-1, and HOC719-PE cells, but was absent in HOC719-NE, HOC313 and TSU cells. Expression of Wnt-5a was detected only in HOC719-NE, HOC313 and TSU cells. Wnt-6 was expressed strongly in OM-1 cells and weakly in HSC-2, HSC-3 and HOC313 cells, but not in keratinocytes or other cells. Wnt-7a was expressed similarly in Ca9-22, OM-1, HOC719-PE and the keratinocytes, and weakly in A431 and Mo-T cells, but was not detected in the other six cell lines.

These results did not reveal any clear relationships among the expression levels of Wnt-1, 2, 3, 6 and 7a mRNAs in normal oral keratinocytes and SCC cells. However, there was a distinct inverse relationship between Wnt-4 and 5a expressions. Three cell lines (HOC719-NE, TSU, HOC313) showed loss of Wnt-4 expression while Wnt-5a was expressed only in those cell lines.

Correlation of Wnt-4 and Wnt-5a expression with epithelial and mesenchymal phenotypes of SCC cells. HOC719-PE and HOC719-NE cells were isolated from an SCC cell line exhibiting heterogeneous expression of E-cadherin.[29] HOC719-PE cells with cuboidal morphology express E-cadherin protein, while HOC719-NE cells with a spindle morphology show loss of E-cadherin expression. Similarly, TSU and HOC313 cells are spindle-shaped and have no E-cadherin expression.[28, 29, 32] To study whether the loss of E-cadherin expression correlates with the increased expression of Wnt-5a and loss of Wnt-4 expression, we further studied the cellular characteristics of these cell lines. RT-PCR analysis demonstrated that these cells showed decreased gene expression of E-cadherin and elevated expression of vimentin and Snail compared to A431, OM-1 and HOC719-PE cells (Fig. 2). The inverse relationship between the expressions of Wnt-4 and Wnt-5a in these SCC cells was

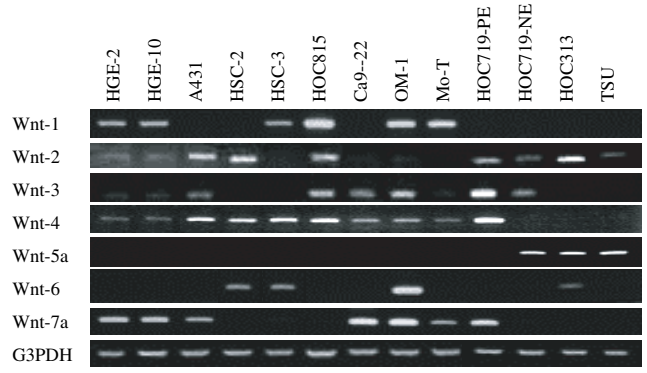

Fig. 1. Expression of mRNAs for Wnt-1, 2, 3, 4, 5a, 6, 7a and corresponding G3PDH in normal oral keratinocytes (HGE-2 and HGE-10) and SCC cells. Aliquots (2 μg) of total RNAs were analyzed by RT-PCR.

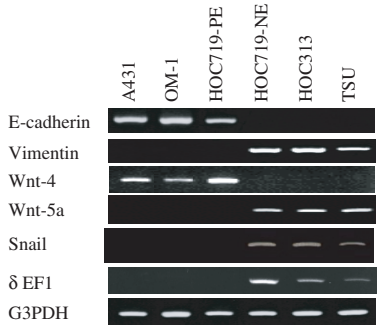

Fig. 2. Gene expressions in SCC cells with epithelial and mesenchymal phenotypes. Total RNAs were isolated from SCC cells which show E-cadherin expression (A431, OM-1 and HOC719-PE) and loss of E-cadherin (HOC719-NE, TSU and HOC313), and analyzed by RT-PCR.

again apparent, as in Fig. 1. Furthermore, we found strong expression of mRNA for δEF1, which has also been reported to transrepress E-cadherin expression,[33] in HOC719-NE, TSU and HOC313 cells, but not in A431, OM-1 and HOC719-PE cells.

Induction of EMT by Snail overexpression in SCC cells. To investigate the effect of EMT on Wnt-4 and Wnt-5a expression in SCC cells, OM-1 cells were transfected with an expression vector of Snail or a control pcDNA3 vector, and stable G418-resistant clones were isolated. The Snail-transfected clones showed spindle shapes (Fig. 3B), while the control vector-transfected clones retained their cuboidal morphology (Fig. 3A). The protein expression of transfected Snail was confirmed by western blotting analysis using anti-HA antibody, which recognizes the HA protein tag at the carboxyl terminus of Snail. The Snail-transfected clones (OM-1SNA1 and OM-1SNA2), but not the control clones (OM-1pcD1 and OM-1pcD2) showed immuno-reactive bands indicating protein expression from the transfected *Snail* gene (Fig. 3C). These results are consistent with our previous findings on A431-derived clones into which the Snail expression vector had been transfected.[32] Decreased gene expression of E-cadherin and increased expression of vimentin in Snail-overexpressing clones, but not in the control clones of both A431 and OM-1, were detected by RT-PCR analysis (Fig. 4). Furthermore, elevated expressions of *δEF1* gene was also observed in the Snail-overexpressing clones of both OM-1 and A431 cells. A similar association between Snail and δEF1 has been reported by Guaita *et al*.[34]

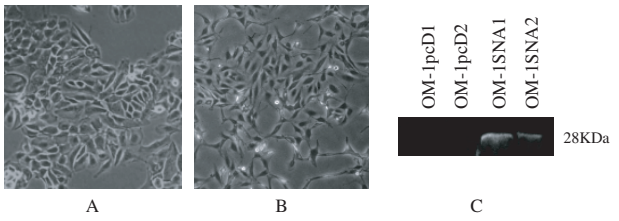

Fig. 3. Morphological change of OM-1 cells by overexpression of Snail. OM-1 cells were transfected with pcDNA3 (control) or pcDNA3-mm snail-HA and selected with 600 μg/ml of G418. Photographs of a control clone (A) and a Snail-transfected clone (B) were taken under a phase- contrast microscope at a magnification of ×100. (C) Expression of transfected Snail protein was analyzed by western blotting analysis using anti-HA antibody in control clones (OM-1pcD1 and OM-1pcD2) and Snail- transfected clones (OM-1SNA1 and OM-1SNA2).

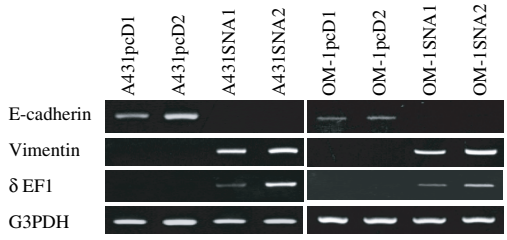

Fig. 4. Gene expressions in Snail-induced EMT clones and control clones of A431 and OM-1 cells.

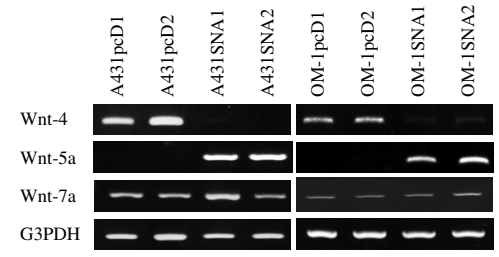

Fig. 5. Expression of mRNAs for Wnt-4, 5a, 7a and corresponding G3PDH in the clones of A431 and OM-1 cells.

Down-regulation of Wnt-4 and up-regulation of Wnt-5a by Snail-induced EMT in SCC cells. The Snail-expressing clones of both A431 and OM-1 showed clearly decreased expression of Wnt-4, but distinct expression of Wnt-5a compared to the control clones (Fig. 5). Although Wnt-7a, as well as Wnt-4, was expressed in A431, OM-1 and HOC719-PE cells but not in HOC719-NE, TSU and HOC313 cells (Fig. 1), the overexpression of Snail did not alter the expression of Wnt-7a (Fig. 5). Also, Wnt-1, 2, 3 and 6 were similarly expressed in the Snail-expressing and the control clones of A431 and OM-1 (data not shown). These results indicated that the expressions of Wnt-4 and Wnt-5a are inversely affected by EMT in human SCC cells.

Discussion

In this report, we found both increased and decreased expression of seven *Wnt* genes in SCC cells compared with two normal oral keratinocyte strains. Although there seemed to be no clear correlations among the expression levels of Wnt-1, 2, 3, 6 and 7a mRNAs in SCC cells, a distinct inverse relationship between Wnt-4 and Wnt-5a expressions was apparent; expression of Wnt-4 was not seen in three cell lines, HOC719-NE, TSU and HOC313, while strong expression of Wnt-5a was detected only in these cells.

We previously reported that HOC719-NE, TSU and HOC313 cells show a spindle morphology without E-cadherin expression; they are highly invasive and have elevated expression of Snail.[29, 32] Snail is a transrepressor of E-cadherin, which was originally identified as a transcription factor implicated in EMT during embryonic development.[24, 25] Increased expression of Snail in invasive cancer cells with the loss of E-cadherin expression has been reported in many types of human cancer, including SCC.[23, 24, 29, 35–38] To study the relationship between EMT and expression of these *Wnt* genes in more detail, we transfected a Snail-expression vector into SCC cells expressing Wnt-4, but not Wnt-5a. Overexpression of Snail in OM-1 cells resulted in the loss of E-cadherin expression, a change to spindle morphology, and the up-regulation of *vimentin* gene expression, indicating that EMT was induced in these cells, in accordance with our previous findings in A431 cells.[32] In these EMT-induced cells, down-regulation of Wnt-4 and up-regulation of Wnt-5a expression were clearly observed.

In connection with tumorigenesis, Wnt proteins can be divided into three groups; strong, weak and non-transformers of C57MG mammary epithelial cells. Both Wnt-4 and Wnt-5a belong to the non-transforming Wnts and do not induce accumulation of cytosolic β-catenin, which is induced by the transforming Wnts.[11, 39] In *Xenopus* development, Wnt-1, 3a, 8 and 8b belong to a class of molecules that promote duplication of the embryonic axis, whereas Wnt-4, 5a, and 11 belong to another class that alters morphogenetic movements and antagonizes the duplication of the embryonic axis induced by ectopic expression of Wnt-1.[40, 41] Wnt-5a directs the Wnt/Ca^{2+} pathway mediated by PKC and CamKII in a G-protein-dependent manner. Recently, calcineurin and nuclear factor of activated T cells (NF-AT) have also been identified as targets of this pathway.[42] In contrast, Wnt-4 directs the Wnt/focal adhesion kinase (FAK) pathway. Wnt-4 promotes cell movement by regulating focal adhesion formation through the activation of FAK during ovarian morphogenesis in *Drosophila*.[43]

The associations of Wnt-4 with epithelial cells and Wnt-5a with mesenchymal cells have been suggested in several previous reports. Saitoh *et al*.[44] reported that normal murine keratinocytes, but not dermal fibroblasts expressed Wnt-4 mRNA.

They also reported that the loss of *Wnt-4* gene expression was associated with a less differentiated, more malignant phenotype in keratinocyte cell lines. Stark *et al*.[45] reported that Wnt-4 is required for the mesenchyme to undergo epithelial transition during kidney development. In odontogenesis, Wnt-4 is expressed in the facial, oral and dental epithelium, but Wnt-5a is expressed in the dental papilla mesenchyme.[46] Similarly, Wnt-4 is expressed in epidermal cells and Wnt-5a is expressed in dermis throughout embryogenesis in skin development. The expression of Wnt-5a is up-regulated in dermal papilla of the hair follicle during hair follicle formation.[47]

Rhee *et al*.[48] studied the expression of five *Wnt* (*Wnt-1*, *5a*, *7a*, *10b* and *13*) genes in 10 SCC cell lines and reported that all the SCC cells showed markedly increased expression of Wnt-1, 7a, 10b and 13, although they did not examine SCC cells with mesenchymal phenotype. They also demonstrated that treatment with anti-Wnt-1 antibodies resulted in reduced activity of LEF/TCF, decreased expressions of cyclin D1 and β-catenin, and induction of apoptosis in an SCC cell line. In the present study, Wnt-1 and Wnt-7a were also frequently expressed in SCC cells with epithelial phenotype. Furthermore, all the SCC cells with epithelial phenotype showed increased expression of at least one of the transforming *Wnt* genes. These results indicate a strong correlation between Wnt/β-catenin signaling and tumorigenesis and proliferation of SCC cells. However, Snail-induced EMT did not affect the expressions of these *Wnt* genes, but altered the expressions of Wnt-4 and Wnt-5a, which belong to the non-transforming Wnts and direct the non-canonical pathways, in A431 and OM-1 cells. These results suggest that the loss of Wnt-4 expression and up-regulation of Wnt-5a expression do not directly correlate to the early steps of carcinogenesis. In SCC, these Wnts may alter cell characters such as mobility, adhesion and invasion accompanied with EMT at the later steps of cancer progression.

The Yamamoto-Kohama (Y-K) mode of invasion[49, 50] has been useful in predicting the prognosis of patients with an oral SCC. A significantly poor prognosis has been reported in the patients with SCC showing Y-K grade 4D (diffuse type) mode of invasion, the most invasive type in the Y-K classification, which is seen in about 10% of oral SCCs. Tumor cells of the 4D type are poorly differentiated and show strong invasion without cancer nests or cell-cell contact. We previously suggested that SCC cells with the 4D mode of invasion have acquired EMT, because of their fibroblastic morphology, highly invasive activity, loss of E-cadherin expression and increased expression of vimentin, MMP-2 and Snail.[32]

In contrast to mammary epithelial cells,[51] breast cancers[52] and uroepithelial carcinomas,[53] in which Wnt-5a functions as a tumor suppressor, Wnt-5a was identified as a molecular marker of highly invasive phenotype in melanomas.[54] Furthermore, increased invasion induced by overexpression of Wnt-5a in melanoma cells and a direct correlation between Wnt-5a expression and increased tumor grade in melanoma biopsies have been reported.[21] Our present results, taken together with these reports, suggest that the loss of Wnt-4 and up-regulation of Wnt-5a are possible molecular markers of a more malignant and invasive phenotype of SCC. Further immunohistological and biochemical analyses are needed to clarify the expression and function of Wnt-4 and Wnt-5a in human SCC.

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