Investigation of the proteins associated with epithelial-mesenchymal transition in skin tumors

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Abstract
Aim: While epithelial-mesenchymal transition (EMT) is associated with the complex morphogenetic events during embryogenesis, EMT has also been shown to play an important role in the progression of epithelial cancers. There have been few studies examining ZEB1 and SMAD protein expressions in skin tumor tissues in the literature, and there are no studies evaluating GIT1 protein expression.

Materials and Methods: Thirty-seven pieces of squamous cell carcinoma (SCC), 34 pieces of basal cell carcinoma (BCC), 11 pieces of actinic keratosis (AK), 9 pieces of in situ SCC (SCCIS) and 7 pieces of normal skin tissue were included in this study.

Results: A statistically significant difference was found between the SMAD1, AREB6 and GIT1 H-scores values of the individuals in the five groups. The levels of SMAD1, AREB6 and GIT1 were higher in SCC than in the control group. In binary comparisons, the SMAD1 H-score values of BCC, SCCIS groups were statistically significantly higher than normal skin. GIT1 H-score values of SCC and BCC groups were significantly higher than normal skin.

Conclusion: In skin tumors, EMT is an ever-active mechanism. It is thought that this mechanism is highly controlled in SCC, a more aggressive type of cancer, than in BCC. These results suggest that the investigation of genes related to SMAD1, AREB6 and GIT1 may be useful for research into new molecular targets for the treatment and prevention of metastasis in nonmelanotic skin tumors.

Keywords: AREB6; GIT1; non-melanoma skin tumors; SMAD1

INTRODUCTION
Epithelial–mesenchymal transition (EMT) is a process in which the cells undergo rearrangement of cytoskeleton, acquire mesenchymal characteristics, and their movements are increased with the loss of membrane-related specific connections in epithelial cells (1). While EMT is associated with the complex morphogenetic events during embryogenesis, EMT has also been shown to play an important role in the progression of epithelial cancers (1,2). Additionally, it has recently been revealed that EMT participates in tissue repair, cancer progression and organ fibrosis (3). Malignant skin tumors are classified into two types, melanomas and non-melanoma skin cancers (NMSC) (4). NMSCs are the most common form of malignancies in humans, accounting for 95% of all cutaneous neoplasms (5). In EMT, in response to the stimulation of cancer cells with soluble factors such as TGF-β or TGF-α in the tumor microenvironment, E-box binding factors (such as ZEB1 and ZEB2) and various transcription factors such as SNAI1, SNAI2, “basic helix-loop-helix (bHLH) factor Twist” are expressed (6). There have been few studies examining ZEB1 and SMAD protein expressions in skin tumor tissues in the literature, and there are no studies evaluating GIT1 protein expression (7-9).

Therefore, the aim of this study was to evaluate the expression levels of EMT-related proteins, namely SMAD1, AREB6 and GIT1, in non-melanoma skin tumors (squamous cell carcinoma (SCC), in situ SCC (SCCIS), basal cell carcinoma (BCC) and actinic keratosis (AK)) via immunohistochemistry, and to determine the relationships between these expression levels and clinicopathological characteristics.

MATERIALS and METHODS
Study Design
Thirty-seven pieces of SCC, 34 pieces of BCC, 11 pieces of AK, 9 pieces of SCCIS and 7 pieces of normal skin tissue were included in this study. All materials used in this study had been sent to the Department of Pathology between 2007 and 2014.
Ethics
This study was approved by the local Non-Interventional Clinical Research Ethical Advisory Board with the decision number 07/07 on 24.02.2014. The study was conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice. Informed consent was obtained from all individual participants included in the study.

Measurements
The most appropriate blocks for immunohistochemistry were selected by re-evaluating the H&E stained preparations of each case. Paraffin tissue blocks that met the histological criteria of each patient were taken for histopathological examination. Basal cell carcinomas were classified as non-infiltrative BCC (BCC-nonINF) when they had less than 50% of infiltrative component, and those over 50% were classified as infiltrative BCC (BCC-INF). Accordingly, 7 cases were grouped as BCC-INF and 27 cases were grouped as BCC-non-INF. Squamous cell carcinomas were classified as poorly-differentiated, moderately-differentiated and well-differentiated according to Broder’s grading system. Seventeen of SCC cases were well-differentiated (SCC-WD), and 20 of them were moderately differentiated (SCC-MD). The tumor diameter, perineural invasion, vascular invasion and the presence of inflammation were reevaluated. The primary antibody SMAD1 (AB25837, Abcam, USA), AREB6 (ZEB1) (AB87280, Abcam, USA) and GIT1 (AB153958, Abcam, USA) were used for immunohistochemistry. In preliminary studies, SMAD1 1/200, AREB6 1/250, GIT1 1/100 were determined as appropriate concentrations, and the primary antibodies were applied in these dilutions. Colon carcinoma for SMAD1, breast carcinoma for AREB6, and ovarian carcinoma for GIT1 were used as positive controls.

For immunohistochemistry, 0.4 μm-thick sections were taken from positive paraffin tissue blocks to positive charged slides. For the deparaffinization, the sections were kept in an oven at 70°C for 1 hour. The slides were placed on the Ventana BenchMark XT automated slide-staining system. After 1 hour and 45 minutes, the diluted antibodies were prepared according to the number of slides, and 150 μm antibodies were dropped on each slide. After the dyeing was complete, the tissues were removed from the device, immersed once in distilled water, and then immersed in 96% alcohol, and finally removed. After drying, they were kept in xylene and closed with entellan. Nuclear staining for SMAD1 and AREB6, and cytoplasmic staining for GIT1 were considered positive. Approximately 800 cells were counted with a microscope. A modified immunohistochemistry score (H-score) was used to evaluate the staining under the microscope. This scoring method was calculated using the formula $HS=\sum(Pixi/100)$; with $i$ as the density of staining and $Pi$ as the number of cells stained at this density. Nuclear staining for SMAD1 and AREB6, and cytoplasmic staining for GIT1 were evaluated separately using H-score.

Statistical Analysis
All statistical analyses were performed by using “PASW Statistics 17” program. The groups were compared with the Kruskal-Wallis test. The Bonferroni correction method was performed for post-hoc pairwise comparisons to reduce Type 1 errors. The relationship between quantitative variables was investigated with the Spearman correlation test. $p<0.05$ values were defined to demonstrate statistically significant results in all tests.

RESULTS
We have compared the baseline characteristics of the BCC and SCC group. There was no significant relationship between SCC and BCC groups in terms of perineural ($p=0.061$) and lymphovascular invasion ($p=0.051$). Tumor size ($p=0.023$) and tumor depth ($p=0.001$) were found to be superior in the SCC group compared to BCC group (Table 1).

Comparisons between the Main Groups
A statistically significant difference was found between the age values of the individuals in the five groups. In binary comparisons; the age values of SCC, BCC, SCCIS, AK groups were significantly higher than the age values of the control group. There was no statistically significant difference in other binary comparison results (Table 2).

SMAD1
A statistically significant difference was found between the SMAD1 H-score values of the individuals in the five groups. In binary comparisons, the SMAD1 H-score values of the SCC, BCC, and SCCIS groups were significantly higher compared to the SMAD1 H-score values of normal skin. The SMAD1 H-score value of the SCC group was also significantly higher than the AK group (Table 3, Figure 1).
Table 3. Comparison of SMAD1 H-score between groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>SMAD1 H-score</th>
<th>Median (Min-Max)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC</td>
<td>49.80</td>
<td>(7.12-200.0)</td>
<td></td>
</tr>
<tr>
<td>BCC</td>
<td>30.42</td>
<td>(0.44-104.80)</td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>23.86</td>
<td>(3.6-76.29)</td>
<td></td>
</tr>
<tr>
<td>SCCIS</td>
<td>43.95</td>
<td>(9.32-79.64)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control</td>
<td>0.80</td>
<td>(0.50-1.10)</td>
<td></td>
</tr>
</tbody>
</table>

* p<0.001, *p=0.004, *p=0.016

Figure 1. Distribution of SMAD1 H-score between groups

AREB6 (ZEB1)
A statistically significant difference was found between the AREB6 H-score values of the five groups. AREB6 H-score values of the SCC group were significantly higher than the BCC and SCCIS groups. The AREB6 H-score values of the SCC group were also significantly higher than the AREB6 H-score values of the AK group. When compared to normal skin, the AREB6 H-score values of SCC group were higher than normal skin (Table 4, Figure 2).

Table 4. Comparison of AREB6 H-score between groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>AREB6 H-score</th>
<th>Median (Min-Max)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC</td>
<td>27.17</td>
<td>(0.0-73.56)</td>
<td></td>
</tr>
<tr>
<td>BCC</td>
<td>0.78</td>
<td>(0.0-53.21)</td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>0.00</td>
<td>(0.0-53.51)</td>
<td></td>
</tr>
<tr>
<td>SCCIS</td>
<td>1.07</td>
<td>(0.0-13.62)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>(0.00-0.00)</td>
<td></td>
</tr>
</tbody>
</table>

* and *p<0.001, *p=0.005, *p=0.041

GIT1
A statistically significant difference was found between the GIT1 H-score values of the individuals in the five groups. GIT1 H-score values of SCC and BCC groups were significantly higher than normal skin. GIT1 H-score values of the SCC group were also significantly higher than the GIT1 H-score values of the SCCIS and AK groups. GIT1 H-score values of the BCC group were also significantly higher than the GIT1 H-score values of the AK group (Table 5, Figure 3).

Table 5. Comparison of GIT1 H-score between groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>GIT1 H-score</th>
<th>Median (Min-Max)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC</td>
<td>44.87</td>
<td>(1.32-194.48)</td>
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<tr>
<td>BCC</td>
<td>18.95</td>
<td>(0.0-188.47)</td>
<td></td>
</tr>
<tr>
<td>AK</td>
<td>3.45</td>
<td>(0.0-13.58)</td>
<td></td>
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<tr>
<td>SCCIS</td>
<td>5.17</td>
<td>(0.0-60.64)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Control</td>
<td>0.00</td>
<td>(0.00-0.00)</td>
<td></td>
</tr>
</tbody>
</table>

* and *p<0.001, *p=0.001, *p=0.022, *p=0.001

Subgroup Comparisons
The SMAD1 H-score values of the SCC-MD group were significantly higher than the SMAD1 H-score values of the SCC-WD group (p = 0.039). There was no significant difference between AREB6 and GIT1 H-score values of...
SCC-MD group compared to AREB6 and GIT1 H-score values of SCC-WD group.

There was no statistically significant difference between the SMAD1, AREB6 and GIT1 H-score values of the BCC-INF group, and the SMAD1, AREB6 and GIT1 H-score values of the BCC-nonINF group.

The SMAD1 H-score values of SCC stage 1 cases were significantly higher than those of stage 0 (noninvasive group AK + SCCIS). There was no significant difference between SMAD1 H-score values of SCC stage 1 and stage 2 groups (Table 6).

The ZEB1 (AREB6) H-score values of SCC stage 1 and stage 2 cases were significantly higher than those of stage 0 cases. There was no significant difference between the ZEB1 H-score values of SCC stage 1 and stage 2 groups (Table 6).

The GIT1 H-score values of SCC stage 1 and stage 2 cases were significantly higher than those of stage 0 cases. There was no significant difference between the GIT1 H-score values of SCC stage 1 and stage 2 groups (Table 6).

When the relationship between sociodemographics and clinical features and SMAD1, ZEB1 and GIT1 H-score values in SCC, BCC and SCCIS, AK cases were examined, it was determined that there was a weak negative relationship between tumor size and ZEB1 (AREB6) H-score values in SCC. There was a negative relationship between tumor size and GIT1 H-score values in SCCIS. There was a positive relationship between AREB6 and GIT1 H-score values in SCCIS. Other bilateral correlations were not significant (Table 7).
DISCUSSION

Plasticity associated with EMT morphology is a common theme in carcinogenesis, where cancer-initiating cells produce and invade their surroundings (10). EMT is a process that is likely largely controlled at the transcriptional level and is characterized by changes in the expression of at least 4000 genes (11). In our study, SMAD1 values were higher in all four patient groups compared to controls. The levels of AREB6 were higher in SCC and GIT1 were higher in SCC and BCC than in the control group.

SMAD1

In our study, we found that the SMAD1 H-score values in SCC were higher than those in healthy skin. This situation can be evaluated in favor of the EMT process in SCC. Moreover, in our study, SMAD1 H-score values in histopathological samples of stage II SCC cases were higher than those with stage 0 cancer. Although we found the SMAD1 expression in the samples of moderately-differentiated SCC cases to be higher than well-differentiated SCCs, it is surprising that we did not find any difference in terms of SMAD1 expression in SCC and SCCIS tumor tissues.

In the literature, there are studies showing that the expression of some SMAD proteins in BCC tumor tissues were higher than the expression in the healthy skin of patients (7); further, several studies show that the expression of SMAD proteins in BCC tumor tissue is reduced (12,8). Our results showed that SMAD1 expression was elevated in BCC. This situation suggests that EMT also occurs in BCC. It can be expected that the EMT process in BCC-INF will be more intense than in BCC-nonINF. However, we did not find any significant difference between the SMAD1 expression levels of these two groups. We also found that the tumor size in BCC did not correlate with SMAD1 expression.

There are studies in AK showing that the expression of SMAD proteins varies depending on the path associated with the TGF-β1 pathway (8,13). In another study, it was found that some SMAD protein (Smad2, Smad7) expressions and phosphorylation in AK tissue were not different (14). To our knowledge, there are no studies in the literature evaluating SMAD1 expression in AK tissue. In the current study, no significant difference was found between the AK and the control group in terms of SMAD1 expression. From this point of view, SMAD1 expression in SCCIS is expected to be lower than in SCC. However, we did not find any significant difference between SMAD1 expression in SCC and SCCIS.

AREB6 (ZEB1)

ZEB proteins play an important role in human cancers (15). ZEB1 and ZEB2 transcription factors initiate the EMT process by reducing CDH1 expression (16). The expression of ZEB1 and ZEB2 has been associated with aggressive behavior of ovarian, stomach, pancreatic colorectal tumors, lung small cell cancer, oral SCC, osteosarcoma and uterine cancers (17-21). Our results showed that ZEB1 expression in SCC is higher than in BCC, AK, SCCIS and healthy skin. This is a finding that supports the functioning of the EMT process in SCC. The fact that we did not find any difference between SCCIS and the control group in terms of ZEB1 expression suggests that the EMT process is not evident (yet) in SCCIS. It was previously emphasized that, as the stage in SCC increases and differentiation deteriorates, the EMT process can be expected to become much more evident. In our study, the ZEB1 H-score values in histopathological samples of stage 1 and 2 SCC cases were higher than those of stage 0. There was no difference between stage 1 and 2 SCC in terms of ZEB1 expression. The expression of ZEB1 in the samples of moderately-differentiated SCC cases was not significantly different from the samples of cases with well-differentiated SCC. These results suggest that ZEB1 expression in SCC may increase to higher levels in patients with advanced disease.

We have not encountered any study evaluating the levels of ZEB1 in BCC tissues in the literature. However, inhibition of the “hedgehog” signal, which is associated with carcinogenesis and aggressive behavior of the tumor, induces the EMT process; thus manifesting as an increase in ZEB1 expression (22). The “Hedgehog pathway” is inhibited in advanced BCC (23) and therefore an increase in ZEB1 expression is expected. Our study showed that ZEB1 expression in BCC was not higher than controls. Furthermore, we could reliably expect the EMT process in BCC-INF to be more intense than in BCC-non-INF. However, there was no significant difference between the levels of ZEB1 expression in these two groups. We also found that the tumor size in BCC did not correlate with ZEB1 expression.

GIT1

It has been determined that GIT1 is an effective protein in the growth and metastasis of human liver cancer and GIT1 is high in breast cancer tissue (24,25). GIT1 expression in oral SCC tumor tissue has been found to be higher than normal oral tissue, and it has been reported that GIT1 can help predict metastasis and invasion as well as being a prognostic marker for oral SCC (26). In our study, we found that GIT1 expression in SCC tumor tissue was significantly higher than in controls, similar to the literature. Our review of the literature did not reveal any studies assessing GIT1 expression levels in BCC or AK. Our study showed that GIT1 expression in BCC was higher than in controls. In terms of GIT1 expression, no significant difference was found in the AK and SCCIS groups.

CONCLUSION

It is seen that the EMT process is an active mechanism in skin tumors. Our results support the hypothesis that, in SCC, which is a more aggressive cancer type, EMT is controlled at a higher degree than in BCC. These results suggest that the investigation of the genes related to SMAD1, AREB6 and GIT1 may be useful as new molecular
targets in non-melanotic skin tumors; thus, they may require further research to elucidate their possible effects in treatment of these cancers and also the prevention of metastasis. In this regard, we believe our results can shed light on the conduct and planning of future studies.

Competing Interests: The authors declare that they have no competing interest.

Financial Disclosure: There are no financial supports.

Ethical Approval: This study was approved by the local Non-Interventional Clinical Research Ethical Advisory Board with the decision number 07/07 on 24.02.2014.

REFERENCES