Evaluation of immunohistochemical expression of TWIST in oral epithelial dysplasia and squamous cell carcinoma

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ABSTRACT

Introduction: The major transcription factor, which modulates the epithelial-mesenchymal transition in different types of cancers, is known as TWIST oncogene. It binds to the promoter of E-cadherin and suppresses its transcription. The current study aims to assess the expression of TWIST protein in oral squamous cell carcinoma (OSCC), epithelial dysplasia (ED), and normal oral mucosa to verify whether such protein is useful as a marker in oral epithelium malignant transformation.

Methods: Thirty-five paraffin-embedded tissue samples of oral lesions with ED and OSCC and five samples of normal oral mucosa were immunostained with anti-TWIST antibody using the streptavidin peroxidase method.

Results: TWIST expression was negative in all cases of normal oral mucosa, whereas all cases of ED and OSCC showed positive immunoreactivity to TWIST varied from weak to strong expression. In ED, there was a significant difference between severe dysplasia and the other two types (P = 0.03). TWIST expression had no significant relationship with the clinical parameters of OSCC clinical stage and grade (degree of differentiation). Only two cases of OSCC with lymph node metastasis showed strong nuclear TWIST expression. Intergroups assessment indicated a significant increase of TWIST expression in OSCC compared to ED (P = 0.000).

Conclusion: A significant increase of TWIST expression in OSCC compared to ED may suggest its role in carcinogenesis, it may be a useful marker in malignant transformation of oral epithelium. Therefore, TWIST might be an important target for therapeutic approaches in patients with OSCC, which requires further investigations.

Keywords: Epithelial dysplasia, immunohistochemistry, squamous cell carcinoma, TWIST

Introduction

The most prevailing oral cavity malignancy is oral squamous cell carcinoma (OSCC), which usually has a relationship with a poor prognosis.[³] The majority of cases of OSCC are preceded by visible changes of the oral mucosa, the most prevalent one is idiopathic leukoplakia.[²] This term should be used to recognize white plaques of questionable risk having excluded other known diseases or disorders that carry no increased risk for cancer.[³,⁴] When the epithelial dysplasia (ED) exists, this will be considered an essential prognostic indicator signaling malignant transformation.[⁵] However, the degree to which the dysplasia grading is accurate depends on specimen quality and lesion location from which the biopsy was removed. Moreover, such system of grading is too subjective and there is a variability of inter- and intra-observers.[⁶,⁷]

As a result, to overcome this variability, relevant studies should focus on the assessment of molecular markers effectiveness in the prediction of premalignant lesions prognosis.[⁹] Recently, the malignant potential of potentially malignant oral lesions presenting with different degrees of ED was evaluated using various molecular markers as adjuvants.[⁹-¹¹]

The first in cascading events resulting in metastasis development is the invasion. There is still little understanding of invasion and metastasis’s basic biological regulation.[¹²] A key mechanism leading to the inducement of tumors’ invasion and metastasis is epithelial–mesenchymal transition (EMT), which is the process through which the polarity of epithelial cells is lost and thus their conversion to mesenchymal phenotypes takes place.[³,¹²] One of the key features of epithelial cells’ losing their adhesion is the decrease in E-cadherin expression.[¹³-¹⁵]

The major factors of transcription, including TWIST, slug, and snail, modulate the EMT in different types of cancer through binding the promoter of E-cadherin and repressing its transcription.[¹⁶,¹⁷] TWIST oncogene is considered an important
transcription factor with helix-loop-helix structure, which has that type of nature that integrates EMT and it is also a major regulator of embryonic morphogenesis.\textsuperscript{[18]}

In this current study, our focus was on TWIST as it is perceived as an EMT master regulator through E-cadherin indirect TWIST suppression.\textsuperscript{[6]} Moreover, it is perceived as an important factor for metastasis in various types of cancer.\textsuperscript{[7]} There is evidence that TWIST reduces cell-to-cell adhesion by direct suppression of E-cadherin. It promotes loss of cell-to-cell adhesion, causing an increase in cell motility through EMT and conditions to create metastasis.\textsuperscript{[19]}

TWIST has several properties that facilitate tumor progression, including the triggering of EMT, inhibition of apoptosis, and the enhancement angiogenesis.\textsuperscript{[10]}

Silva \textit{et al}. stated that overexpression of TWIST in malignant tumors causes tumor progression by stopping the ability of differentiation through the Wnt pathway. Wnt pathway can have an important role in cancer for stabilization of β-catenin protein and interference in β-catenin and E-cadherin complex. Wnt pathway seems to be involved in the dysplastic changes that downregulate E-cadherin by TWIST and causes oral cancer.\textsuperscript{[20,21]}

TWIST has a key role in the development of an early tumor to the metastatic stage and its decreased expression prevents from metastasis to lymph node\textsuperscript{[17]} and entering of tumor cells into the blood circulation and metastasis.\textsuperscript{[1,12,14]} The current study aims to assess and compare the pattern of TWIST protein expression in OSCC and ED, and to examine the correlation between the expression of TWIST protein and the associated clinicopathological factors to verify whether such protein is useful as a marker in oral epithelium malignant transformation.

**Methods**

**Specimens**

This study included 35 paraffin-embedded tissue samples, 15 samples of oral leukoplakia which diagnosed histologically as ED, and 20 samples of OSCC were taken from the tongue, gingiva, buccal mucosa, and floor of the mouth of 10 females and 25 males. The mean range of their age was 58.8 years. All samples were chosen from the archives of the oral pathology department, University of Tanta.

Five samples of normal oral mucosa that was obtained from crown lengthening surgery in patients who were admitted for this purpose with minimal inflammation from the clinical and histopathologic aspects were chosen. Required information’s including age and sex of patients, tumor site, smoking habits, clinical tumor stage, grade of ED and histologic grade of OSCC, lymph node metastasis, and distant metastasis were collected from their medical records and pathological reports. To routinely stain with hematoxylin and eosin, samples were cut to 5 µm thick histological sections. Then, to confirm the diagnosis, they were analyzed using light microscopy. In accordance with the criteria of the World Health Organization (WHO), two independent oral pathologists examined the stained sections and determined histological grades of dysplasia (moderate dysplasia, severe dysplasia, and mild dysplasia). Based on the WHO criteria of histological typing of oral and oropharyngeal tumors, OSCC cases were also examined and graded histologically as “poorly differentiated, moderate differentiated, and well differentiated.”\textsuperscript{[18]}

**Immunohistochemical staining and evaluation**

From each paraffin-embedded block, 5 µm thick section was prepared. Through the use of streptavidin peroxidase method, the immunohistochemistry method was conducted. 98°C water bath with 0.01 L/mol citrate buffer solution (pH = 6) was used to heat the deparaffinized samples for 30 min, to block endogenous enzymes. Then, slides were incubated with anti-TWIST antibody (anti-rabbit polyclonal antibody, ART NO: Ab 50581, Abcam CO, UK) with the dilution of 1:100 in 4°C overnight. Then, the tissue surface was covered with a secondary antibody (Goat Anti-Rabbit IgG H and L [HRP] ART NO: ab97051-1 mg, Abcam CO, UK) for 30 min. DAB kit (Dako, Substrate Buffer-00046018, Denmark) was used to create the desired color intensity (for 10–15 min). Breast cancer was used as a positive control. The negative control was obtained by the omission of the primary antibody.

**Interpretation of immunohistochemical staining**

All the slides were evaluated with an optical microscope Olympus B × 41 (Olympus, Tokyo, Japan) by two independent pathologists who were unaware of the clinical characteristics of the samples. Evaluation of immunohistochemical TWIST expression was performed as follows: Five microscopic fields were randomly selected, and the percentage of stained cells (cytoplasmic or nuclear staining) was evaluated. The staining intensity was graded on microscopic examination using a three-grade scoring: 0 (no staining), 1 (weak staining = if the positive cells comprised <20%), and 2 (strong staining = positive cells >20%).\textsuperscript{[22]}

**Statistical analysis**

Through the use of SPSS software version 20, the statistical analysis was conducted. The difference in TWIST expression among the two groups was done using the Mann–Whitney test. Using Chi-square test, the intragroup comparisons were made when the value of $P < 0.05$, it was considered as statistically significant.

**Results**

Thirty-five samples were recruited into the study including 20 samples of OSCC and 15 ED (of 25 males [71.4%] and 10
[28.6%] female patients 17 patients ≤60 years and 18 patients over 60 years) with a mean age of 58.8 years and five normal tissue samples of five volunteers with a matching mean of age. The site of the ED and OSCC cases was tongue 15 cases (42.9%), buccal mucosa in ten cases (28.8%), gingiva and retromolar area in six (17.1%), and floor of mouth in four cases (11.4). In accordance with malignant tumors (UICCTNM) classification, OSCC clinical stage was determined including five (25%) with Stage I, seven (35%) with Stage II, two (10%) with Stage III, and six (30%) with Stage IV. From the recorded cases of OSSC two cases only showed lymph node metastasis.

The histological grades of ED were (mild dysplasia n = 6, moderate dysplasia n = 4, and severe dysplasia n = 5). OSCC was graded histologically into (well differentiated n = 8, moderate differentiated n = 7, and poorly differentiated n = 5). TWIST expression was negative in the five cases of the normal oral mucosa [Figure 1].

In all cases, it was observed that the expression of TWIST in the ED was nuclear and/or cytoplasmic staining. The localization of the majority of immunopositivity of TWIST was in confined to basal and parabasal layers of oral epithelium. There was a correlation of TWIST expression with dysplasia’s histological grade. In mild dysplasia cases, there was a weak cytoplasmic expression [Figure 2a]. In moderate ED cases, there was an increase in the nuclear staining [Figure 2b]. Moreover, in severe dysplasia cases, there was a strong cytoplasmic staining and nuclear expression [Figure 2c and d]. No significant difference was noticed between the expression of TWIST and the clinical parameters including patient sex and lesion site. On the contrary, significant difference was noticed between severe dysplasia and the other two types of dysplasia (P = 0.03) [Table 1].

As regard to OSCC, all cases showed an immune-positive reaction. TWIST strong nuclear and cytoplasmic staining were observed in cancer nests of moderately and well-differentiated OSCC mostly in the peripheral part. On the other hand, there was no particular localization of TWIST expression in poorly differentiated OSCC [Figure 3a-c].

TWIST expression in the nucleus and cytoplasm had no significant relationship with the clinical parameters of OSCC (site of the lesion and sex of the patients), clinical stage, and grade (degree of differentiation) of OSCC [Table 2].

Despite the fact that there was no correlation between the expression of TWIST and any of the clinicopathological parameters under evaluation, there was some observation of the relationship of lymph node metastasis with the stronger expression of TWIST. In addition, strong expression of TWIST was observed in two cases having lymph node metastasis.

Intergroups assessment indicated a significant increase of TWIST expression in the nucleus and cytoplasm of the epithelial cells in OSCC compared to ED (P = 0.000) [Table 3].

**Discussion**

OSCC development is linked to the genetic alterations that lead to the lack of mechanisms that control the growth and differentiation of cells. According to the previous studies, it was revealed that such genetic changes are existing in premalignant lesions as well, which indicates the potential role in the process of malignant transformation. According to the current study, there are significant differences in the expression of TWIST protein between OSCC, ED, and normal oral mucosa. This is in turn suggests that this type of protein is able to take part in oral carcinogenesis’s multi-step process.
In one of the previous studies, it was indicated that the expression of TWIST in normal oral mucosal cytoplasm was weak. This is on the contrary with the current study where negative stain for TWIST was observed in all cases of normal oral mucosa under study. This can be explained that normal mucosa is a non-dysplastic phenotype. According to Silva

### Table 1: Statistical analysis of the correlation between positive TWIST expressions in oral epithelial dysplasia with clinicopathologic factors

| Clinical and pathological factors | Expression of TWIST immunostain |  |  |  |
|----------------------------------|---------------------------------|---|---|---|
|                                  | 1 (week expression) % | 2 (strong expression) % | Total % | P-value |
| **Lesion sites**                 |                                 |                           |         |         |
| Buccal mucosa                    | 50                              | 22.2                      | 33.3     | 0.599   |
| Floor of the mouth               | 0                               | 11.1                      | 6.7      |         |
| Gingival tissue                  | 16.7                            | 33.3                      | 26.7     |         |
| Tongue                           | 33.3                            | 33.3                      | 33.3     |         |
| Total                            | 100                             | 100                       | 100      |         |
| **Gender**                       |                                 |                           |         |         |
| Male                             | 50                              | 11.1                      | 26.7     | 0.143   |
| Female                           | 50                              | 88.9                      | 73.3     |         |
| Total                            | 100                             | 100                       | 100      |         |
| **Grade of epithelial dysplasia**|                                 |                           |         |         |
| Mild                             | 66.7                            | 22.2                      | 40       | 0.03*   |
| Moderate                         | 33.3                            | 22.2                      | 26.7     |         |
| Severe                           | 0                               | 55.6                      | 33.3     |         |
| Total                            | 100                             | 100                       | 100      |         |

*Significant at P level ≤0.05

### Table 2: Statistical analysis of the relationship between the positive expressions of TWIST in oral squamous cell carcinoma and the clinicopathologic factors

| Clinical and pathological factors | Expression of TWIST |  |  |  |
|-----------------------------------|---------------------|---|---|---|
|                                  | 1 (week expression) % | 2 (strong expression) % | Total % | P-value |
| **Lesion sites**                 |                     |                           |         |         |
| Buccal mucosa                    | 33.3                | 23.5                      | 25       | 0.452   |
| Floor of the mouth               | 0                   | 17.6                      | 15       |         |
| Gingival tissue                  | 33.3                | 5.9                       | 10       |         |
| Tongue                           | 33.3                | 52.9                      | 50       |         |
| Total                            | 100                 | 100                       | 100      |         |
| **Gender**                       |                     |                           |         |         |
| Male                             | 66.7                | 70.6                      | 70       | 0.891   |
| Female                           | 33.3                | 29.4                      | 30       |         |
| Total                            | 100                 | 100                       | 100      |         |
| **Disease stages**               |                     |                           |         |         |
| Stage I                          | 66.7                | 17.6                      | 25       | 0.218   |
| Stage II                         | 33.3                | 35.3                      | 35       |         |
| Stage III                        | 0                   | 11.8                      | 10       |         |
| Stage IV                         | 0                   | 35.3                      | 30       |         |
| Total                            | 100                 | 100                       | 100      |         |
| **Histopathology of the disease**|                     |                           |         |         |
| Moderate differentiated           | 33.3                | 35.3                      | 35       | 0.338   |
| Poorly differentiated            | 0                   | 29.4                      | 25       |         |
| Well differentiated              | 66.7                | 35.3                      | 40       |         |
| Total                            | 100                 | 100                       | 100      |         |
There is a possible relationship between TWIST expression and malignancy in OSCC. A significant increase in TWIST expression was observed in OSCC compared to normal epithelium and ED. Consequently, this makes a suggestion that the expression of TWIST is a significant indicator of malignancy in OSCC.

In the current study, the immunolocalization of TWIST was detected in the cytoplasm and nucleus of tumor cells present in the periphery of neoplastic cell islands of moderate and well-differentiated OSCC types. In addition, it showed a diffusion in its distribution in the poorly differentiated SCC. These results are consistent with those of Silva et al. 2012 and Silva et al., 2012. The results of Yuen et al. (2007) showed that in prostatic tissue, the increase in the expression of TWIST has a positive relationship with the neoplastic transformation. Accordingly, TWIST higher nuclear expression can positively participate in metastasis promotion. In addition, TWIST is shown to be an independent prognostic factor in the case of patients having...
Recently, it has been shown to be associated with potentially malignant oral lesions transformed into OSCC.\(^\text{19}\)

According to this current study, in the nucleus and cytoplasm, the expression of TWIST had no significant correlation with OSCC’s clinical parameters, with the exception that in lymph node metastasis, there was a relationship between TWIST strong nuclear expression and lymph node metastasis. These findings are similar to those of the previous studies\(^\text{17,19}\) where there was a relationship between the expression of TWIST in OSCC and lymph node metastasis. According to Shibata et al. (2008),\(^\text{27}\) there is no relationship between clinicopathologic parameters and the expression of TWIST in cervical cancer. The role of TWIST in the metastasis and progression of cancer is revealed in a myriad of tumors including OSCC,\(^\text{17,23}\) breast cancer,\(^\text{28}\) gastric cancer,\(^\text{29}\) prostatic cancer,\(^\text{26}\) cervical cancer,\(^\text{27}\) bladder cancer,\(^\text{30,31}\) and pancreatic cancers.\(^\text{32}\)

**Conclusion**

According to the results of the present study, a significant increase of TWIST expression in the nucleus and cytoplasm of the tumor cells in OSCC compared to ED may suggest its role in carcinogenesis. TWIST protein may be a useful marker in the malignant transformation of oral epithelium and might be an important target for therapeutic approaches in patients with OSCC, which requires further investigations.

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