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Original Article

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 immuostained 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 OSSC 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.[1] 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.[8] 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.[5] 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.[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.[6] Moreover, it is perceived as an important factor for metastasis in various types of cancer.[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.[19]

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

Silva *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 ß-catenin protein and interference in ß-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.[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[17] and entering of tumoral cells into the blood circulation and metastasis.[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."[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%).[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 OSSC (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].

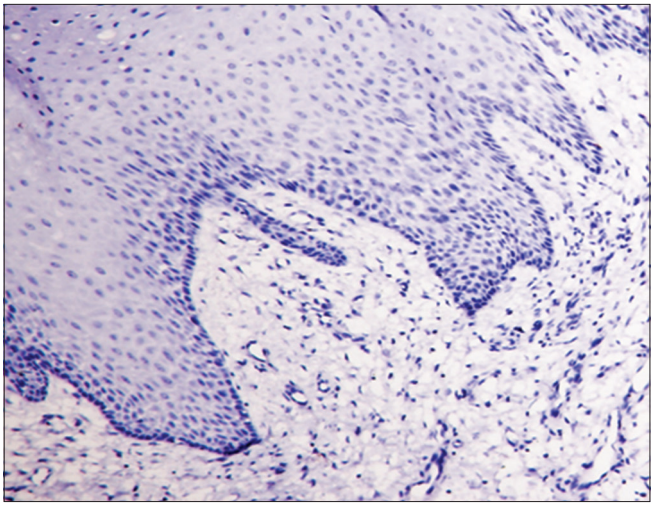

Figure 1: Negative expression of TWIST in normal mucosa (streptavidin-biotin ×200)

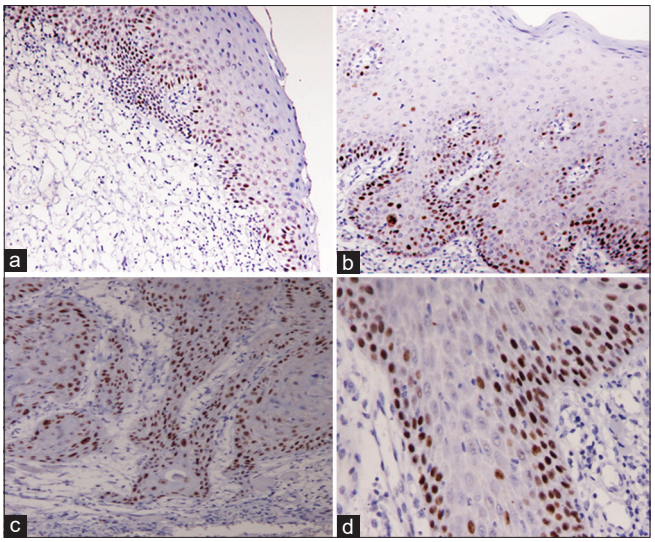

Figure 2: Expression of TWIST in epithelial dysplasia: (a) Mild; (b) moderate; (c and d) severe. Weak staining in the basal and parabasal layers in mild dysplasia (a). Nuclear expression of TWIST increase in moderate epithelial dysplasia in the basal and parabasal layers (b). Strong expression of TWIST in severe epithelial dysplasia, in the basal and parabasal layers (c and d) (streptavidin-biotin) (a and c, ×100; b, ×200; d, ×400)

Discussion

OSCC development is linked to the genetic alterations that lead to the lack of mechanisms that control the growth and differentiation of cells.[2] 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.[9] 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.

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	
Sever	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	

In one of the previous studies,[17] 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

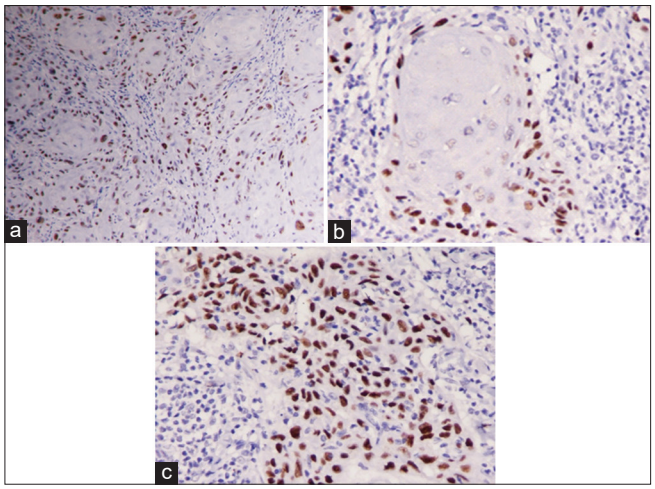

Figure 3: Expression of TWIST in oral squamous cell carcinoma (OSCC). Tumor cells with strong positive staining observed in the periphery of cancer nests of well-differentiated OSCC (a) and moderately differentiated OSCC (b). Diffuse localization of the positive cells in poorly differentiated OSCC (c) (streptavidin-biotin (a) ×400, (b) ×100, (c) ×400)

Table 3: Comparison of TWIST expression (positive cells %) among oral epithelial dysplasia and oral squamous cell carcinoma

Intensity of the stain	ED %	OSCC %	Mean rank	Sum rank	*P*-value
1 (week TWIST expression) %	40	15	5	45	0.000*
2 (strong TWIST expression) %	60	85	22.5	585	

*Significant at *P* level ≤0.05

et al. 2012,[21] it was concluded that the expression of TWIST has a relationship with the gaining of dysplastic phenotype of dysplastic epithelium in basal and parabasal layers. In their study, Seyedmajidi *et al*., 2018,[19] found that it is possible to detect TWIST in the carcinogenesis of head-and-neck region with expression and immunolocalization pattern which is correlated with the advancement of malignancy from normal epithelium to ED to carcinoma *in situ*, and finally SCC. As a matter of fact, in normal epithelium, TWIST is negative or weakly positive.

More importantly, TWIST expression in all the groups of ED in this study was highly immunolocalized in the parabasal and basal layers of the dysplastic epithelium. This is because there is an increase in stain intensity in the cytoplasm and nucleus associated with the severity of dysplasia. These results are consistent with those of the previous study where the expression of TWIST was revealed in the parabasal and basal layers in ED cases. Such areas are recognized for dysplastic changes initiation.[20] According to Silva *et al*. 2012,[21] staining was confined to the basal and parabasal cell layers in oral idiopathic leukoplakia. In addition, it was always limited to the nucleus. Further, they showed that in the dysplastic areas, strong nuclear staining was shown by cells while nuclear and cytoplasmic staining was shown in carcinoma *in situ*.[20,21] On the other hand, in invasive OSCC cases, it was revealed that there was strong positive nuclear and cytoplasmic staining of TWIST.[20,21]

In oral leukoplakia groups of the current study, parabasal and basal showed expression of TWIST may be due to the dysplastic phenotype at this area. In turn, this makes a suggestion that since early stages, TWIST can play an important role in the oral carcinogenesis. However, further study is required to explore the precise molecular mechanism behind the role of TWIST in the process of oral carcinogenesis and oral epithelium's malignant transformation process.

The progression and metastasis of malignant tumors include a series of multistep genetic changes, including abnormally activating tumor oncogenes and metastasis-related genes and abnormally inactivating tumor suppressor genes.[22,23] Evidence revealed that TWIST is the oncogene which causes a decrease in cell-to-cell adhesion through direct suppression of E-cadherin.[20,24] In turn, this results in the promotion of losing cell-to-cell adhesion, leading to increasing the motility of cells through the so-called "EMT process" and metastasis conditions.[25] There are several properties of TWIST which result in facilitating the progression of tumors including enhancement angiogenesis, apoptosis inhibition, and EMT triggering.[23-26] There is a possible relationship between OSCC progression and E-cadherin loss, which leads to a more invasive phenotype.[24] This may explain the strong TWIST expression in the two cases with lymph node metastasis in this study.

According to the study of Silva *et al*. 2012,[21] it was revealed that in OSCC, the expression of TWIST was increased significantly in comparison with normal epithelium and ED. Consequently, this makes a suggestion that the expression of TWIST represents an early incident in dysplastic lesions, which progress to oral cancer. These findings are consistent with the results of the current study in which there is a significant increase in expression of TWIST in OSCC compared to the ED and normal oral mucosa.

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 Seyedmajidi *et al*. 2018[19] and Silva *et al*., 2012.[21]

The results of Yuen *et al*. (2007)[26] showed that in prostatic tissue, the increase in the expression of TWIST has a positive relationship with the neoplastic transformation. Accordingly, TWIT 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

OSCC.[9] Recently, it has been shown to be associated with potentially malignant oral lesions transformed into OSCC.[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 nodes metastasis. These findings are similar to those of the previous studies[17,19] where there was a relationship between the expression of TWIST in OSCC and lymph node metastasis. According to Shibata *et al*. (2008),[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,[17,23] breast cancer,[28] gastric cancer,[29] prostatic cancer,[26] cervical cancer,[27] bladder cancer,[30,31] and pancreatic cancers.[32]

Silva *et al*. 2012[21] and Ou *et al*. 2008[33] reported the important role of TWIST in the prognosis of OSCC and also its correlation with the degree of tumor differentiation. However, in our study, and in the study of Seyedmajidi *et al*. 2018,[19] TWIST expression was not associated with the degree of tumor differentiation. Yang *et al*., 2009,[34] concluded that inhibition of TWIST expression in metastatic carcinoma leads to inhibition of metastasis to the lungs in breast cancer, and lack of TWIST reduced micrometastasis to the lung.

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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