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Twist Expression in Pleomorphic Adenoma, Adenoid Cystic Carcinoma and Mucoepidermoid Carcinoma of Salivary Glands

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ABSTRACT

Objective: Twist is an important transcription factor that induces epithelial-mesenchymal transition and therefore plays an important role in tumor progression. There are a few studies on Twist expression in salivary gland carcinomas. The aim of this study was to evaluate and compare the expression of Twist in the most common benign and malignant salivary gland tumors and to determine whether its expression was correlated with any tumor characteristics.

Material and Method: In this retrospective cross-sectional study, 45 cases including 11 cases of normal salivary gland, 12 pleomorphic adenomas, 12 adenoid cystic carcinomas and 10 mucoepidermoid carcinomas were enrolled. The mean and intensity of Twist expression were evaluated immunohistochemically and were compared using statistical analysis.

Results: The expression of Twist was higher in malignant salivary gland tumors in comparison with normal glands and benign tumors (p= 0.03). It was also higher in pleomorphic adenomas in comparison with normal tissue. Adenoid cystic carcinomas and mucoepidermoid carcinomas showed no significant difference in Twist expression (p= 0.50). There was no correlation with the size, stage or grade of tumor.

Conclusion: The findings showed that Twist might play a role in the formation of salivary gland neoplasm and also may affect malignant transformation and tumoral invasion. The exact mechanism of this marker and the possibility of using it as a therapeutic target require further investigation.

Key Words: Twist transcription factor, Immunohistochemistry, Pleomorphic adenoma, Adenoid cystic carcinoma, Mucoepidermoid carcinoma

INTRODUCTION

Salivary gland tumors are one of the crucial neoplasms in maxillofacial pathology, and constitute 3-6% of all head and neck tumors (1). These tumors show a wide spectrum of clinical and pathologic variants that lead to difficult diagnosis and management. Pleomorphic adenoma (PA), mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (AdCC) are the most common benign and malignant tumors and have histopathologic similarities in many cases. Although, hematoxylin and eosin-stained (H& E) tissue sections are used routinely for diagnosis in many cases, the definite diagnosis is sometimes difficult. These tumors are managed differently and there is therefore a need to identify of diagnostic, prognostic, or therapeutic markers to explain their different invasiveness and biological behavior (2).

It has recently been recognized that epithelial-mesenchymal transition (EMT) play a key role in tumor invasiveness and progression and is a necessary step for metastasis (3,4). In this process, the epithelial cells lose their adhesion and polarity and acquire mesenchymal properties and increased motility (5). EMT is necessary for several developmental processes during embryogenesis (6). Twist is an important transcription factor that induces EMT by dysregulation of N-cadherin and E-cadherin expression (7).

In adults, this marker has been demonstrated in precursor cells such as the placenta, heart, and skeletal muscles (8). Twist proteins (Twist 1 and 2) play an important role in tumor progression by promoting cell invasion, metastasis, tumor growth, and angiogenesis (7). Moreover, Twist regulates matrix metalloproteinase (MMP) expression and inhibits apoptosis (9).

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Overexpression of Twist has been reported in several malignant tumors including head and neck squamous cell carcinoma, nasopharyngeal carcinoma, prostate, bladder, and breast cancer (3, 4, 10-12). In several types of cancer, Twist was an independent prognostic factor and a predictor of survival. The studies showed that Twist may be an oncogene that induced tumorigenesis as well as tumor progression in the malignant cells. Twist may be a contributing factor in anticancer drug resistance and Twist inactivation has increased chemodrug-induced apoptosis and suppressed invasion ability in prostate cancer cells (3). One study showed that Twist expression contributed to invasiveness of salivary AdCC (13). Twist may also be associated with perineural invasion of this tumor (14). Although a few studies explained Twist overexpression in AdCC, there is no data about MEC as a common malignant salivary gland tumor. It seems that Twist can be a novel target for enhancing the efficacy of cancer treatment; therefore, this study was designed to evaluate and compare the expression of Twist in the most common benign and malignant salivary gland tumors and to scrutinize whether its expression was correlated with any tumor characteristics such as tumor stage and grade.

MATERIAL and METHOD

The data of this cross-sectional study were collected from the archive of Oral Pathology department of Shiraz of Dental School, Iran. The selected cases included 12 PA, 12 AdCC, 10 MEC and 11 normal salivary glands (NSG). All cases had adequate tissue. Cases with uncertain diagnosis were excluded.

The baseline data including the patients' age and gender, tumor site and size as well as the grade and stage of the tumors were recorded according to the patients´ medical documents. Tumor stages were assessed based on the stages adapted by the American Joint Committee on Cancer (AJCC) TNM stage (15). Tumor grade in MEC was classified as grade I, if it demonstrated a well demarcated border, macrocystic spaces and a bland cyst lining; grade II, if it demonstrated a more solid growth with only few microcysts, and focal infiltration; and grade III, with no cystic spaces and a highly infiltrative growth pattern, and pronounced nuclear atypia. In ADCC, grade I is referred to as a tubular growth pattern, grade II as a cribriform growth pattern, and grade III as a solid growth pattern (16).

Immunohistochemical examinations (IHC) were performed on 4 µ-sections of formalin-fixed and paraffin-embedded specimens. Tissue sections were incubated in 60°c for 30 minutes, and then the sections were deparaffinized in xylene and rehydrated by alcohol. Antigen retrieval was performed by citrate solution in pH= 9 at 121°C for 20 minutes. To block the endogenous peroxidase activity, sections were immersed in 3% hydrogen peroxide for 30 minutes. The sections were then incubated with Twist1 polyclonal antibody (1:100, mouse, Abcam Corporation, ab49254, UK). After applying secondary antibody associated with the Envision system, sections were washed in PBS. 3.3´-Diaminobenzidine tetrahydrochloride was used as chromogen and slides were counterstained with Mayer's hematoxylin. A section of breast cancer was considered as the positive control. By omitting the primary antibody, sections were used as the negative control.

The cells with nuclear and/or cytoplasmic staining were considered as positive. The percentage of positive cells out of 500 tumoral cells was assessed in 10 microscopic fields at high magnification (x 400). Moreover, a similar evaluation was separately performed in 500 stromal fibroblasts. The intensity of staining in the parenchymal cells was marked as negative, weak, moderate, and intense in comparison with the control sample.

The statistical analysis was performed using the SPSS 15 software. Data were analyzed by Kruskal-Wallis and Dunn's tests at the 0.05 significance level.

RESULTS

In the present cross-sectional study, 45 cases including 11 NSG, 12 PA, 12 AdCC and 10 MEC were enrolled. Baseline data of all groups are summarized in Table I. Some cases of AdCC did not have any stage (2 cases) and grade (3 cases) data.

All cases were positive for Twist marker. Positive cells revealed brown nucleus and/or cytoplasmic staining. In all NSGs, ductal epithelium and some myoepithelial cells were cytoplasmic and/or nuclear positive for Twist (Figure 1A). In 5 cases (45.5%), acinar cells showed focal immunoreactions.

PAs showed Twist expression in ductal and some myoepithelial cells. The expression was 66.8% cytoplasmic, 16.6% nuclear and 16.6% both cytoplasmic and nuclear. Epithelial nests were also positive for this marker (Figure 1B). Cytoplasmic expression was predominant in PA stromal cells, which are between epithelial nests and sheets and includes myoepithelial, fibroblast, endothelial and inflammatory cells. In Table II, the mean± SD expression of Twist in tumoral parenchyma and stroma is shown.

Twist expression was positive in almost all cases of MEC. The only case that did not stain was a clear cell type

Table I: The base line data of all groups

Cases (number)	PA (n=12)	AdCC (n=12)	MEC (n=10)	NSG (n=11)	Total (n=45)
Age(Mean± SD)	37.2±15.3	50±14.4	52.6±22.1	39.4±20	44.6±18.6
Gender: Male: n (%)	2(16.7)	2(16.7)	5(50)	4(36.4)	13(29)
Female: n (%)	10(83.3)	10(83.3)	5(50)	7(63.3)	32(71.1)
Tumor site					
Major glands: n (%)	8(66)	5(41.6)	7(70)	9(81.8)	29(64.4)
Minor glands: n (%)	4(33)	7(58.3)	3(30)	2(18.1)	16(35.5)
Tumor size *					
T1: n (%)	3(25)	3(25)	4(40)		
T2: n (%)	9(75)	5(41)	3(30)		
T3: n (%)	0	2(16.6)	2(20)		
T4: n (%)	0	2(16.6)	1(10)		
Stage					
I + II: n (%)	---	6(60)	7(70)		
III + IV: n (%)	---	4(40)	3(30)		
Grade					
I: n (%)	---	2(22.2)	6(54.5)		
II: n (%)	---	7(77.8)	0		
III: n (%)	---	0	4(45.5)		

PA: Pleomorphic Adenoma, **AdCC:** Adenoid cystic carcinoma, **MEC**: Mucoepidermoid carcinoma**, NSG:** Normal salivary gland.
(T1: The greatest diameter of tumor (D) < 2cm, T2: D= 2-4 cm, T3: D > 4cm, T4: Tumor invades subjacent structures).

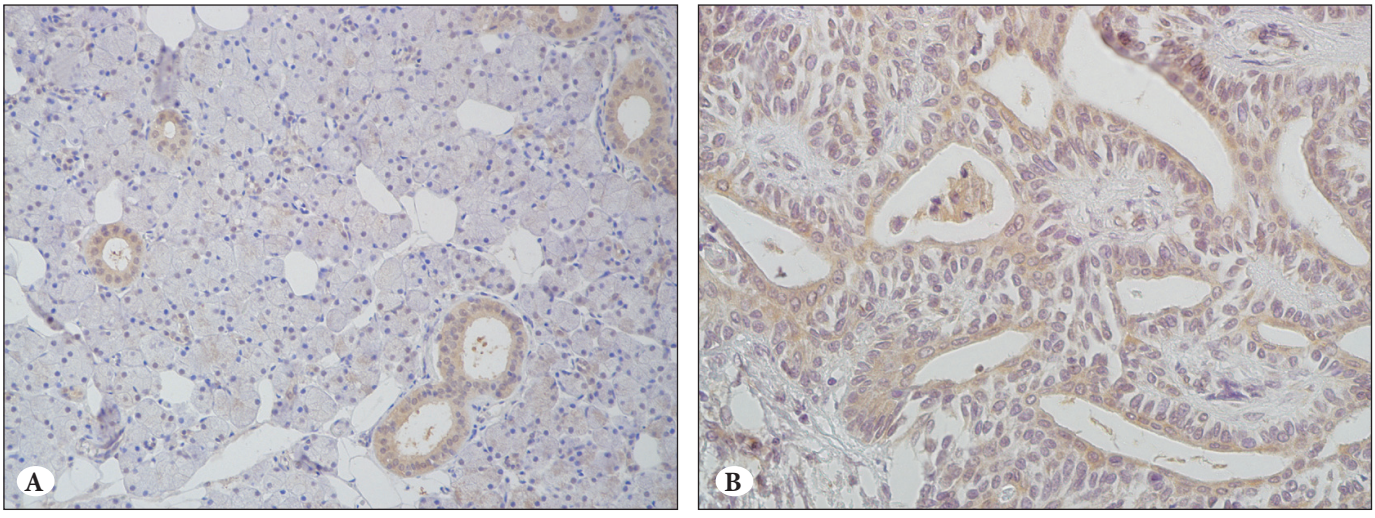

Figure 1: Moderate cytoplasmic Twist expression in **A)** ducts of normal salivary gland tissue (Twist; x200), **B)** ductal and myoepithelial cells of pleomorphic adenoma (Twist; x400).

MEC which was then excluded from statistical analysis. Epidermoid, mucous and intermediate cells showed 80% cytoplasmic, 10% nucleus and 10% both cytoplasmic and nucleus staining (Figure 2A). Clear cells were negative for Twist. Similar to the other tumors, stromal cells were stained with this marker. Table II shows mean±SD of Twist expression in the parenchymal cells and stroma.

AdCCs showed extensive nuclear expression in 67% of cases. Cytoplasmic expression was found in 16.5% and simultaneous cytoplasmic and nuclear staining was also found in 16.5% of the cases. All ductal cells were positive for Twist (Figure 2B). Stromal cells also showed immunoreaction. The percentage of expression mean is depicted in Table II.

Table II: Twist expression in benign and malignant salivary gland tumors

Cases (n)	Parenchyma (Mean ± SD) (Min-Max %)	Stroma (Mean ± SD)	p value (Parenchyma)
PA (n=12)	82.5± 19.6 (30-100)	35 ± 32.9	PA, AdCC:0.004
AdCC (n=12)	95.8 ± 9 (70-100)	45.8 ± 26.8	PA,MEC: 0.005
MEC (n=10)	97±9.5 (70-100)	46 ± 38.9	AdCC, MEC:0.5

PA: Pleomorphic Adenoma, **AdCC:** Adenoid cystic carcinoma, **MEC:** Mucoepidermoid carcinoma, **NSG:** Normal salivary gland.

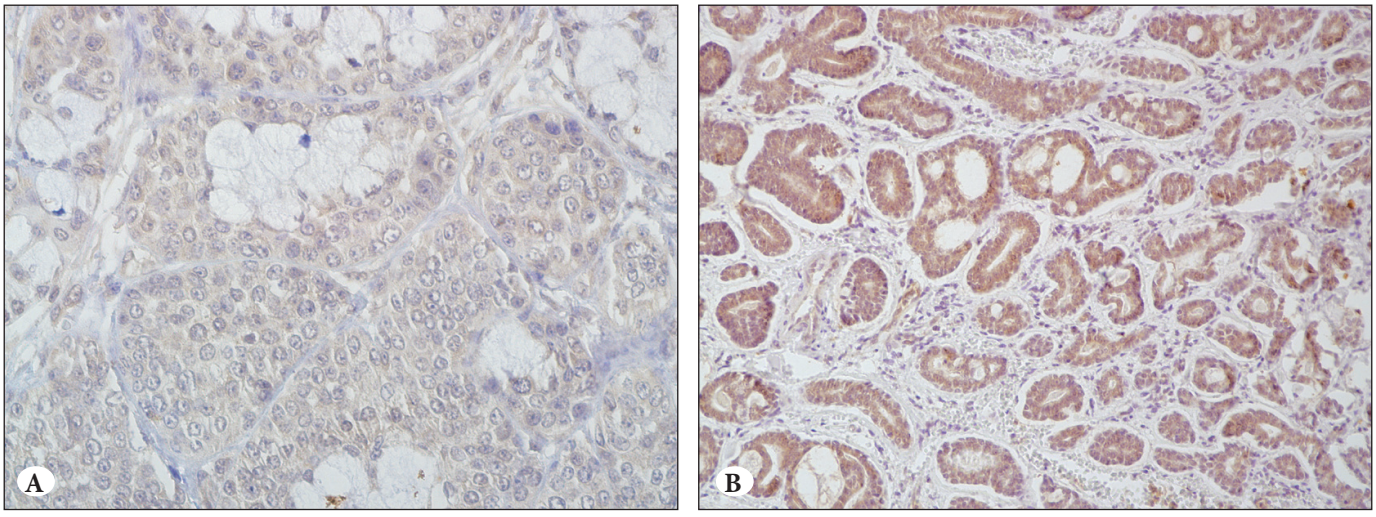

Figure 2: A) Weak cytoplasmic Twist expression in mucoepidermoid carcinoma (Twist; x400), **B)** Strong nuclear and cytoplasmic expression in adenoid cytic carcinoma (Twist; x200).

PAs and MECs showed weak or moderate staining, whereas, AdCCs revealed moderate or strong staining in most of the cases; all NSGs showed moderate staining. Table III illustrates the intensity of Twist expression in benign and malignant salivary gland tumors as well as NSG tissues.

The Kruskal-Wallis test showed a significant difference between the mean expression of Twist in the parenchymal component of the tumoral groups (p=0.03). Dunn's test revealed that PAs were significantly different from both AdCCs (p= 0.004) and MECs (p= 0.005) in Twist expression, but AdCC and MEC did not show any statistical difference in its immunoreaction (p= 0.50).

Using the Kruskal-Wallis test, the intensity of staining was significantly different among the groups (p=0.018) and by Dunn's test, the intensity of Twist was higher in AdCCs than those of MECs (p= 0.001) and PA (p= 0.003) but no significant difference was seen between MEC (P= 0.4) and PA.

Table III: Intensity of Twist expression in all groups

Cases (number)	Weak (%)	Moderate (%)	Strong (%)
PA (n=12)	40.6	59.4	0
AdCC(n=12)	8.3	58.3	33.3
MEC (n=10)	40	60	0
NSG (n=11)	------	100	------
Total (n=45)	22.2	68.9	8.9

PA: Pleomorphic Adenoma, **AdCC:** Adenoid cystic carcinoma, **MEC:** Mucoepidermoid carcinoma, **NSG:** Normal salivary gland.

Stromal cells in all tumors showed expression of Twist and there was no statistical difference among groups in stromal staining (Kruskal-Wallis test, p= 0.756).

The percentage and intensity of Twist expression in tumoral parenchyma were not statistically different among the groups as regards tumor size, stage, and grade (Kruskal-Wallis and Dunn's test, all p> 0.05)

DISCUSSION

EMT is one of the essential steps in metastasis of malignant tumors and is regulated by several genetic pathways (17). Twist is one of the important proteins that play a crucial role in EMT by reduction in E-cadherin and upregulation of mesenchymal markers (18). Twist includes two proteins: Twist1 and Twist2, which have shown similar expression in tumors (8). Twist expression was previously shown in some normal tissues such as prostate and breast (11, 19). It was also demonstrated that this protein showed higher expression in malignant tumors compared to benign tumors such as prostate, parathyroid and lung (19-21). In the salivary gland tumors, there has been some investigation on Twist expression in AdCCs and PA. These studies demonstrated expression of Twist in PA and overexpression of Twist in AdCC in relationship with perineural invasion and tumoral invasiveness (13); however, this protein was not studied in MECs as a common malignant salivary gland tumor. In the present study, we found Twist overexpression in the most common benign and malignant SGTs (MECs and AdCCs) and its possible role in tumor formation and malignant transformation of SGs.

The expression of Twist in this study was both nuclear and/or cytoplasmic. This pattern of expression was also found in cancers of breast, oral cavity, and pancreas and in ameloblastomas. (10, 22-25).

The present study showed that Twist was expressed in all NSGs in the ductal epithelium and some myoepithelial cells, but not in acinar cells. Zhao et al. demonstrated the expression of E-cadherin in normal salivary gland acinar cells with no expression of N-cadherin (26). Regarding the mechanism of Twist in downregulation of E-cadherin, our finding is indirectly in the same line with that study (18). Lee et al. stated that Twist had a role during the organogenesis of the parathyroids (27). As Twist has been shown to be a crucial protein in gastrulation and organogenesis of several organs (20), its expression in NSGs might be related to its role in the organogenesis of salivary glands.

The current study showed Twist expression in all benign tumors (PAs), predominantly with a cytoplasmic pattern and moderate intensity. Yuen et al. also explained that cytoplasmic expression of Twist was related to neoplastic transformation in prostate tissues (19). It seems that overexpression of Twist in our study was also related to the neoplasm formation in salivary glands. In agreement with our findings, Shen et al. reported overexpression of Twist in 30% of PAs (13). Overexpression of Twist has been previously demonstrated in some benign and malignant tumors. Benign tumors of prostate, parathyroid, lungs and precancerous lesions of the oral cavity have shown this protein in their parenchymal cells (19-21, 28).

All malignant tumors (cases of MEC and AdCCs) also revealed immunoreaction for Twist. MEC exhibited Twist expression in squamous and mucous cells. Cytoplasmic expression of Twist protein was dominant in this tumor. Most cases of MEC in this study had low histopathologic grade, and their cytoplasmic pattern of staining was similar to the samples included in the study of Lee at al. They reported the cytoplasmic expression of this marker in the well-differentiated squamous cell carcinomas (27).

In AdCCs, staining was predominantly nuclear with moderate and strong intensity. AdCC is a high-grade tumor with a high ability of metastasis (29). It was demonstrated that nuclear expression of Twist was related to tumor metastasis (19). Previous studies have demonstrated higher expression of Twist in high grade AdCC and its relationship with perineural invasion (13,14). Another study also showed nuclear immunoreaction with Twist in high-grade squamous cell carcinoma (12). According to invasive behavior of AdCC, our results support previous studies that have shown a relationship between nuclear Twist expression and increasing tumor invasiveness (10, 19).

The findings showed overexpression of Twist in malignant tumors in comparison with NSGs and benign tumors. Previous studies have demonstrated a reduction in E-cadherin expression in the majority of AdCC cases in comparison with PA, and also overexpression of this protein in benign salivary glands tumors (26,30). According to the explained mechanism of Twist in downregulation of E-cadherin, these findings indirectly supported the switching mechanism of Twist in salivary glands tumors (18). Our findings might indicate the role of Twist protein in malignant transformation of SGTs. Kwok et al. demonstrated Twist overexpression in malignant prostate cancer cells and concluded that Twist may be a novel oncogene resulting in malignant transformation of the cells (3). That result was a noticeable finding in our study. Several studies have demonstrated that Twist expression might be an independent prognostic factor which is useful for predicting patients' survival in breast cancer and melanoma (31,32). AdCCs and high-grade MEC are chemo-and radiotherapy-resistant malignant tumors (33). It was reported that inactivation of Twist increased the drug-induced apoptosis and therefore, it could promote the efficacy of anticancer treatments in cases of prostate cancer (3). Due to the overexpression of Twist in MEC and

AdCCs, Twist might be a novel target of treatment that needs further investigation.

In the present study, we observed no statistically significant difference between Twist expression and tumor grade, stage, or size. According to the different roles of Twist in tumor progression, it is possible that the limited number of our cases with complete clinical data have resulted in these findings. Also, future studies are suggested to evaluate the relationship between Twist overexpression with clinical behavior of salivary gland tumors.

In conclusion, the overexpression of Twist in salivary gland tumors in comparison with normal glands and also in carcinomas in comparison with benign tumors may indicate the role of this protein in the tumorigenesis of salivary glands and also in the malignant transformation and tumor invasion. In this regard, we suggest further investigations to evaluate the exact mechanism of Twist protein and its possible use as a therapeutic target.

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