

TWIST expression in hypopharyngeal cancer and the mechanism of TWIST-induced promotion of metastasis

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Abstract. The transcription factor TWIST is an important factor in regulating epithelial-mesenchymal transition (EMT) and tumor metastasis. To explore the functions of TWIST in hypopharyngeal cancer, we investigated if overexpression of TWIST has an effect on FaDu cell morphology, and if alteration of TWIST has an effect on E-cadherin, N-cadherin, c-fos, MMP-9, as well as in cell migration, and the invasion ability of FaDu cells. Moreover, we also studied the relationship between TWIST overexpression and clinicopathological characteristics in hypopharyngeal cancer tissue samples by immunohistochemical assays. The results showed that overexpression of TWIST-induced morphological changes, such as occurrence of EMT. TWIST overexpression also increased cell migration and invasion ability, accompanied by an alteration of E-cadherin, N-cadherin, c-fos and MMP-9 expression. Furthermore, immunohistochemical assays showed that TWIST overexpression was related with tumor differentiation ($P=0.038$), tumor size ($P=0.048$) and lymph node metastasis ($P=0.044$). The data presented reveal that overexpression of TWIST plays a significant role in the metastasis of hypopharyngeal tumors, and alteration of TWIST has an effect on the EMT, c-fos and MMP-9 expression in FaDu cells. We conclude that TWIST promotes hypopharyngeal carcinoma metastasis, and the TWIST/c-fos/MMP-9 signaling pathway may play an important role in the metastasis of FaDu cells.

Introduction

Hypopharyngeal cancer is one of the most common head and neck malignancies. More than 75% of patients with hypopharyngeal cancer are at an advanced stage at the time of diagnosis (1). Lymph node metastasis is present in 60-80% of patients and it directly affects the prognosis of the disease (2). Although the locoregional control of this cancer has been significantly improved in the last decades because of the advent of new surgical techniques and approaches, this improvement does not significantly influence the overall survival rate, partly because of metastasis (3). Therefore, understanding the potential mechanism of hypopharyngeal cancer metastasis and investigating the related targeted factors of metastasis are of primary concern.

Recently, some studies have shown that TWIST, a basic helix-loop-helix (bHLH) transcription factor, is known to be essential for proper gastrulation, mesoderm formation, and neural crest migration (4). Interestingly, TWIST is required for epithelial-mesenchymal transition (EMT) (5). In addition, elevated TWIST expression was further related with tumor invasion and metastasis in esophageal squamous cell carcinomas (6). The role of TWIST in promoting EMT processes has also been reported in other solid cancers, such as prostate and uterine cancer (7,8). EMT and its accompanying reduction in E-cad expression have been shown to be essential for the extravasation of cancer cells into secondary organs (9). Previous research has confirmed that TWIST overexpression not only promotes the migration and invasion of cancer cells, but also decreases the sensitivity to chemotherapy (10,11). The overexpression of TWIST maybe the key for tumor metastasis and drug resistance, but the precise mechanism is still unclear (12). Recently, we have reported that TWIST may play a pivotal role in the paclitaxel-induced apoptosis of human laryngeal carcinoma Hep-2 cells (13). As yet, there are very few studies on the role of TWIST in head and neck tumors, especially in hypopharyngeal cancer. The concrete role of TWIST in the hypopharyngeal cancer and its detailed mechanism remain unclear.

Matrix metalloproteinases (MMPs), which comprise a large family of metalloendopeptidases with Zn^{2+} and Ca^{2+} and share a similar structure and function, can degrade the structural elements of the extracellular matrix (ECM) and cell-cell and cell-ECM adhesion molecules (14). Matrix

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Abbreviations: bHLH, basic helix-loop-helix; EMT, epithelial-mesenchymal transition; MMPs, matrix metalloproteinases; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcriptase polymerase chain reaction; FBS, fetal bovine serum

Key words: TWIST, c-fos, MMP-9, EMT, hypopharyngeal carcinoma FaDu cells, metastasis, immunohistochemistry

Table I. Forward and reverse primers used for reverse transcription PCR.

Gene name	Forward and reverse primers	Amplified size
TWIST	5'-GGAGTCCGCAGTCTTACGAG-3' 5'-GTGGGGCGCCCCAGGCACCA-3'	200 bp
E-cadherin	5'-CCTTAGAGGTGGGTGACTACAA-3' 5'-TCAGACTAGCAGCTTCGGAAC-3'	567 bp
N-cadherin	5'-GCTTCTGGTGAAATCGCATTA-3' 5'-AGTCTCTCTTCTGCCTTTGTAG-3'	409 bp
c-fos	5'-GACTGATACTCCAAGCG-3' 5'-GCATAGAAGGACCCAGATAG-3'	417 bp
MMP-9	5'-TCTTCCCTGGAGCCTGAGAAC-3' 5'-GACACCAAAGTGGATGACGATG-3'	428 bp
Actin	5'-CTCCTTAATGTCACGCACGATTT-3' 5'-GTGGGGCGCCCCAGGCACCA-3'	550 bp

metalloproteinase-9 (MMP-9), which is one member of this family, has been associated with invasive and metastatic behavior in malignant tumors, and the high expression of MMP-9 in tumors is associated with distant metastases and poor prognosis (15). Furthermore, Hu *et al* (12) reported that c-fos is required for the expression of MMPs. In addition, c-fos overexpression has been identified as an independent predictor of survival in breast cancer (16). In contrast, some studies concluded that c-fos has tumor suppressor activity. The overexpression of c-fos was found to inhibit cell cycle progression and to stimulate murine hepatocyte cell death (17). Thus, c-fos was considered as a double-edged sword, which can promote or suppress tumorigenesis. This dual action was probably due to the variable protein compositions of cells and/or their microenvironment (18). However, few studies addressed the role of c-fos in metastasis of human hypopharyngeal cancer.

The experiment was designed to explore the alteration of TWIST expression and its effect on metastasis and the metastasis-related factors E-cadherin, N-cadherin, c-fos and MMP-9 in hypopharyngeal cancer and in the FaDu cell line, as well as to evaluate the significance of TWIST expression in human hypopharyngeal cancer tissue on the clinicopathological characteristics.

Materials and methods

Cells and reagents. The human hypopharyngeal carcinoma FaDu cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Primary antibodies against TWIST, E-cadherin, N-cadherin, c-fos, MMP-9 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All others agents were purchased from Sigma (St. Louis, MO, USA).

Plasmid constructions of pcDNA3.1-TWIST. Entire cDNA coding fragments of TWIST were amplified by RT-PCR and subcloned into the multicloning site of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) (pcDNA3.1-TWIST). The

primers sets of full length TWIST for amplification were forward 5'-GAGAGATGATGCAGGACGTGTC-3' and reverse 5'-CTAGTGGGACGCGGACATG-3'. The final constructs were confirmed by DNA sequencing.

Generation of miR-TWIST transfectants. The miR-TWIST vector was generated using the Block-iT™ *Po/II* miRNAi expression vector kit with EmGFP (Invitrogen) according to the manufacturer's instructions. The oligo sequences of the miR-TWIST were forward, 5'-TGCTGCTGCCGGTCTGGC TCTTCCTCGTTTTGGCCACTGACTGACGAGGAAGAC AGACCGGCAG-3' and reverse, 5'-CCTGCTGCCGTCTG TCTTCCTCGTCAGTCAGTGGCCAAAACGAGGAAGA GCCAGACCGGCAGC-3'. The oligo sequences of the negative control were forward, 5'-TGCTGAAATGTACTGCGC GTGGAGACGTTTTGGCCACTGACTGACGTCTC CACGCAGTACATTT-3' and oligo, reverse, 5'-CCTGAAAT GTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACG TCTCCACGCGCAGTACATTT-3'. The sequence targeting the TWIST gene-coding region was annealed and inserted into the pcDNA6.2-GM/EmGFPmiR vector to generate the miRNA interfering expression vector. The resulting vectors were then transfected into the FaDu cells.

Cell culture, transfection and generation of stable transfectants. FaDu cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin, and 100 mg streptomycin at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂. Cell transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were grown to 80-90% confluence without antibiotics. Vectors containing the different constructs (10 µg) were diluted in DMEM (100 µl) and then mixed with the transfection solution for 15 min. After washing, the cells were incubated with the transfection mixture at 37°C for 6 h and were allowed to grow in fresh media. For a transient expression of FaDu cells, the transfected cells were incubated at 37°C for 48 h and used for analysis. Stable FaDu cell transfectants were isolated by selection with 500 mg/ml of

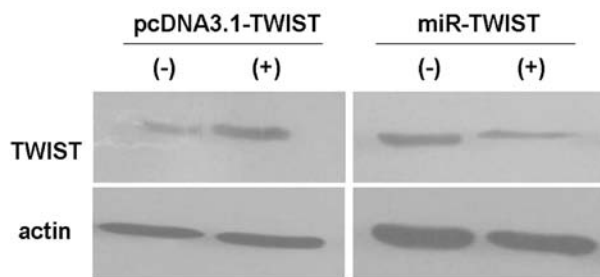


Figure 1. Western blot analysis of TWIST expression in the pcDNA3.1-TWIST and miR-TWIST FaDu cells. The expression of TWIST in the pcDNA3.1-TWIST and miR-TWIST FaDu cells was confirmed by Western blot analysis. Actin served as an internal control for normalization purposes. Compared with the control group, TWIST expression increased in the transfected pcDNA3.1-TWIST vector FaDu cells and decreased in the transfected miR-TWIST vector FaDu cells.

G418 (AMRESCO, Inc.) for two weeks. FaDu cells transfected with the pcDNA3.1 vectors were used as controls.

Observation of morphological changes. The morphological changes of the FaDu cells were observed using an inverted microscope. The photograph was taken using a Leica microscope image system (Leica, Mannheim, Germany).

Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis of gene expression. Detailed experimental procedures of RT-PCR and Western blot analysis of TWIST gene expression were described (13). RT-PCR primers are indicated in Table I. Western blot analysis was performed with antibodies against TWIST (1:200), E-cadherin (1:200), N-cadherin (1:200), c-fos (1:200), MMP-9 (1:200) and actin (1:3,000).

In vitro migration and invasion assays. Cell invasion assays were performed using Transwell™ chambers (Costar, MA, USA). After coating the filter with 80 µg of Matrigel (BD Biosciences, NY, USA) overnight at 4°C, cells (2×10^5 cells/well) were seeded on the top chamber of a 24-well plate in serum-free medium. The bottom chamber was filled with 0.6 ml DMEM with 10% FBS as a chemoattractant. After incubation for 24 h, non-invading cells were carefully removed with a cotton swab. The filters were fixed with 95% alcohol and stained with crystal violet for 15 min. The cells on the upper surface were gently removed with a cotton swab and the cells on the lower surface of the filters were quantified under a microscope at $\times 100$ magnification. All experiments were repeated in three replicates and were repeated three times. To assess migration, *in vitro* migration assays were conducted under the same conditions as the Transwell™ invasion assays, but in non-Matrigel-coated Transwell™ chambers.

Tissue samples. The study included 50 patients with primary hypopharyngeal tumors. The tumors tissues were obtained from patients who had undergone a partial or total resection between 2005 and 2008 at the Provincial Hospital affiliated to Shandong University. None of the patients had received pre-operative treatment. Each sample was used after written consent was obtained from the patients.

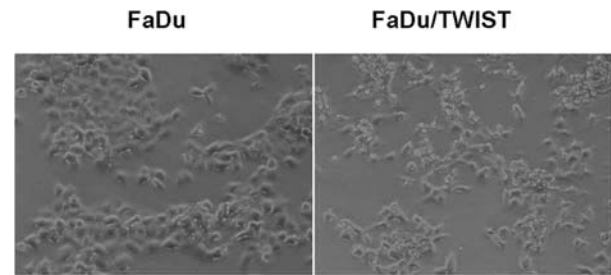


Figure 2. TWIST overexpression induces morphological changes. Changes in morphologies of FaDu cells from a well organized cell-cell adhesion and cell polarity in the control group to loss of cell-cell contacts and cell scattering in the pcDNA3.1-TWIST group. TWIST overexpression promoted the EMT in the transfected pcDNA3.1-TWIST vector FaDu cell.

Immunohistochemical staining. Immunohistochemistry for TWIST was performed on 4 µm sections from paraffin-embedded tumor tissue blocks with TWIST antibodies. The detailed experimental procedure was performed as previously described (19).

Statistical analysis. Data were presented as mean \pm standard deviation. One-way analysis of variance and least significance difference was applied to analyze the data. Expression of TWIST in human hypopharyngeal cancer tissue samples was analyzed using the χ^2 test and Fisher's exact test. Statistical calculations were performed using SPSS software package, version 13.0 (SPSS Inc., Chicago, IL, USA). P-values < 0.05 (two-tailed) were considered significant.

Results

TWIST expression increase in the transfected pcDNA3.1-TWIST vector FaDu cells and decrease in the transfected miR-TWIST vector FaDu cells. To investigate the function of TWIST in the hypopharyngeal cancer FaDu cells, FaDu cells were transfected with the pcDNA3.1-TWIST and miR-TWIST vectors, respectively. The expression of TWIST in different vectors was confirmed using Western blotting. Compared with the control group, the expression of TWIST increased in pcDNA3.1-TWIST group. Nevertheless, after transfecting the miR-TWIST vector into FaDu cells, TWIST expression decreased (Fig. 1).

Alteration in TWIST expression induced morphologic changes in FaDu cells. In order to explore whether TWIST overexpression has an effect on the morphology of FaDu cells, we performed the viviperception using an inverted microscope. The results showed that morphology of FaDu cells, after transfecting the pcDNA3.1-TWIST vector, was changed from well organized cell-cell adhesion and cell polarity to loss of cell-cell contacts and cell scattering. TWIST overexpression also promoted the EMT in the transfected pcDNA3.1-TWIST vector FaDu cells (Fig. 2).

Alteration in the TWIST expression affects the migration and invasion of FaDu cells. To explore whether TWIST overexpression has an effect on the migration and invasion of FaDu cells, we performed Transwell™ assays. In the migration assay, the number of invaded cells in the pcDNA3.1-TWIST

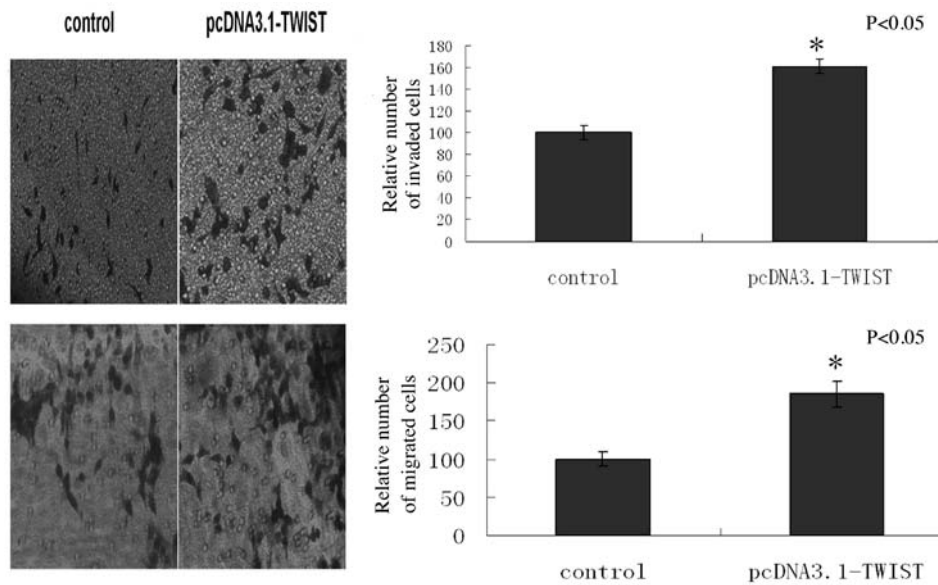


Figure 3. TWIST overexpression increases the migration and invasion of FaDu cells. Overexpression of TWIST increased cell migration and invasion in FaDu cells *in vitro*. Invasion (upper) and migration (bottom) were examined by the Transwell™ chambers assay in the control and pcDNA3.1-TWIST group, respectively (x100 magnification). Each data point represent the mean \pm standard deviation of 3 separate experiments.

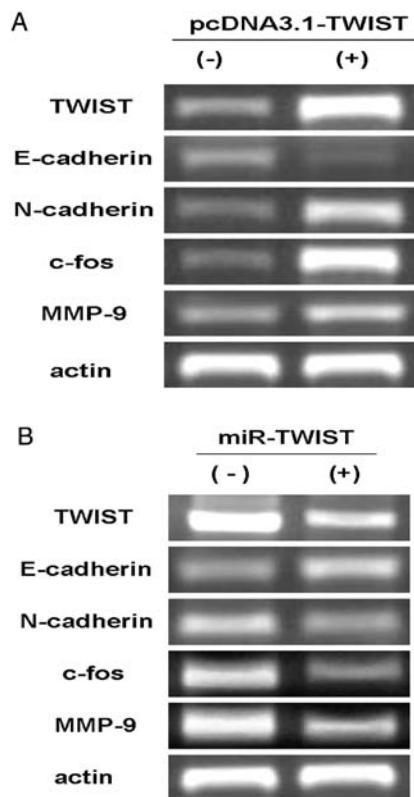


Figure 4. Alteration in the expression of TWIST in FaDu cells leads to an alteration of metastasis related factor expression by RT-PCR. Overexpression of TWIST decreased E-cadherin expression, and increased N-cadherin, c-fos and MMP-9 expression (Fig. 4A). Down-regulation of TWIST expression increased E-cadherin expression, and decreased N-cadherin, c-fos and MMP-9 expression (Fig. 4B).

group was 1.8-fold, compared with the number of invaded cells in the control group ($P < 0.05$) (Fig. 3). Similar results were obtained in the invasion assay. The number of invaded cells in

pcDNA3.1-TWIST group was 1.6-fold higher compared with the number of invaded cells in the control group ($P < 0.05$). Interestingly, after transfecting the miR-TWIST vector into FaDu cells, alteration in morphology, migration, and invasion of FaDu cells were completely reversed (data not shown).

Alteration in the TWIST expression leads to an alteration in E-cadherin and N-cadherin expression in the FaDu cell line. In order to elucidate the mechanism by which elevated expression of TWIST induced morphological change and increased the migration and invasion of FaDu cells, the expression of several proteins involved in cell metastasis was examined in the TWIST-overexpressing and control cells. We first examined E-cadherin, and found that TWIST overexpression had notably inhibited activation of E-cadherin in the FaDu-TWIST cell lines compared to the controls at the mRNA and protein levels, respectively (Figs. 4A and 5A). Examination of N-cadherin, revealed that the TWIST-overexpressing cell line increased the N-cadherin expression at the mRNA and protein levels, respectively (Figs. 4A and 5A). By contrast, use of the microRNA to knockdown TWIST expression, showed that down-regulation of TWIST expression strikingly increased the activation of E-cadherin and inhibited N-cadherin expression (Figs. 4B and 5B).

Alteration in TWIST expression leads to an alteration in c-fos and MMP-9 expression in the FaDu cell line. Further analysis of the c-fos and MMP-9 protein expression in FaDu-TWIST cell lines and silent-TWIST FaDu cells, showed that overexpression of TWIST increased the protein expression of c-fos and MMP-9 at the mRNA and protein levels, respectively (Fig. 4A and 5A). In contrast, TWIST down-regulation expression inhibited the expression of c-fos and MMP-9 (Figs. 4B and 5B).

TWIST expression is related to tumor differentiation, tumor size, and lymph node metastasis in hypopharyngeal tumors.

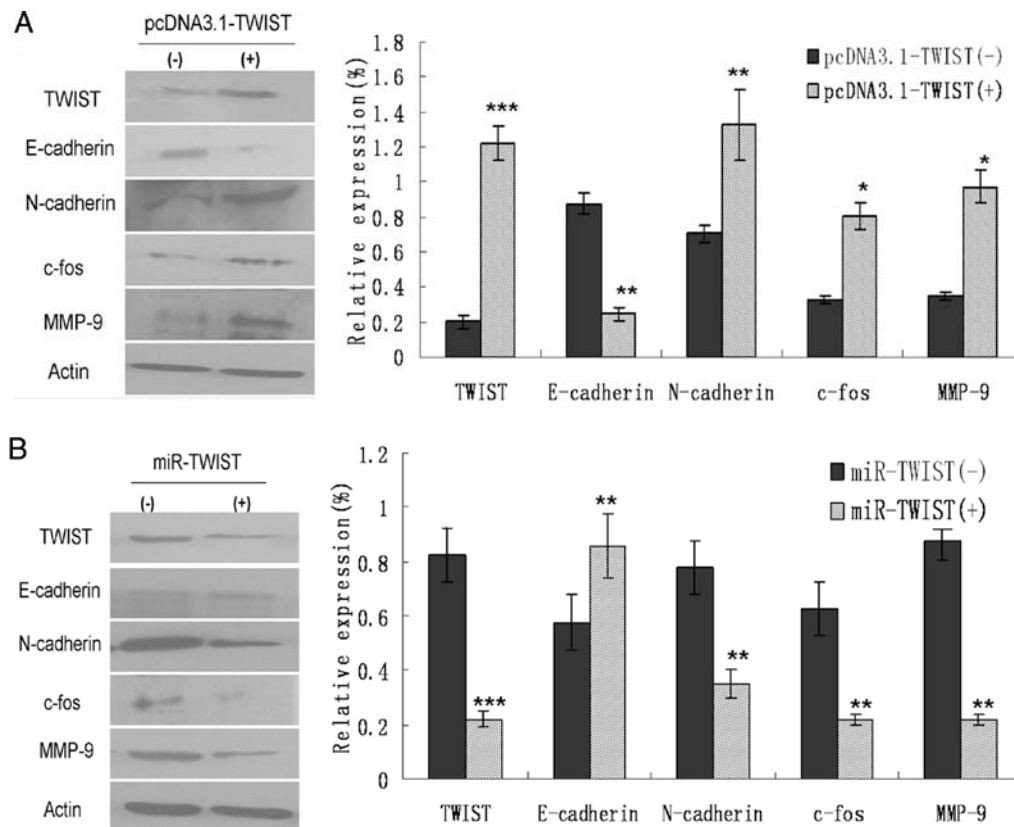


Figure 5. Alteration of TWIST expression in FaDu cells leads to an alteration of metastatic relation factor expression by Western blot analysis. (A) Overexpression of TWIST decreased the E-cadherin expression and increased the N-cadherin, c-fos, and MMP-9 expression. (B) Down-regulation of TWIST expression increased the E-cadherin expression, and decreased the N-cadherin, c-fos, and MMP-9 expression. Actin served as an internal control for normalization purposes. Bar graphs show densitometric analysis by the National Institutes of Health software Image J system of at least three separate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

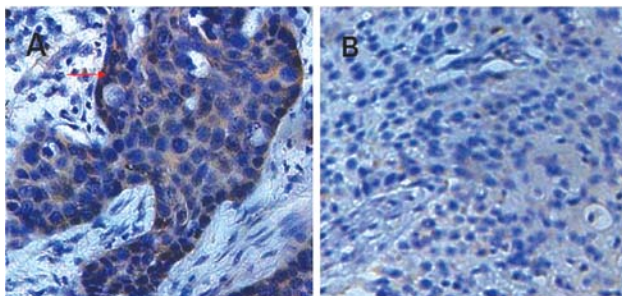


Figure 6. TWIST expression in the hypopharyngeal tumor tissue. Representative photomicrographs of the sections of hypopharyngeal tumor tissue stained with anti-TWIST antibody using immunohistochemistry (magnification, $\times 200$). Compared with adjacent non-tumor tissue (B), in the tumor tissue TWIST was expressed in both the nucleus and cytoplasm of tumors cells (A), but the expression of it in cytoplasm was more prominent than in the nucleus.

To explore whether TWIST expression has a function in hypopharyngeal cancer tissue, we examined the expression of TWIST protein in the adjacent non-tumor and hypopharyngeal cancer tissue samples by an immunohistochemical assay. The expression of TWIST in hypopharyngeal cancer tissues was higher than in the adjacent non-tumor tissue (Fig. 6). Immunohistochemical assays showed that TWIST was expressed in both the nucleus and cytoplasm of tumor cells, but the expression of TWIST in the cytoplasm was more prominent than in the nucleus (Fig. 6). TWIST expression was negative in the adjacent non-tumor tissue (Fig. 6). In order to

further explore the functions of TWIST expression in hypopharyngeal cancer, we investigated the relationship between TWIST expression and clinicopathological characteristics in the hypopharyngeal cancer tissue samples. The detailed data of the relationship between TWIST immunoreactivity and various clinicopathological variables are summarized in Table II. The results indicate that TWIST expression is related to tumor differentiation ($P = 0.038$), tumor size ($P = 0.048$), and lymph node metastasis ($P = 0.044$). The bigger the tumor size and the higher the grade in the lymph node metastasis, the higher the positive rate of TWIST expression. In contrast, no correlation was observed between TWIST expression and gender or age ($P > 0.050$).

Discussion

TWIST, as a tumor-related transcription factor, is overexpressed in the embryonic stage, and underexpressed in the adult stage. It has been reported that TWIST is overexpressed in solid cancers, such as prostate and uterine cancer (7,8), and elevated TWIST expression has been related to tumor invasion and metastasis in esophageal squamous cell carcinomas (6) as well as in head and neck carcinomas (20). The present study shows that alteration in TWIST expression is completely opposite between transfecting the pcDNA3.1-TWIST vector and transfecting the miR-TWIST vector in FaDu cells, indicating that transfected plasmid has an effect on TWIST expression. Next, we investigated if the alteration in TWIST expression

Table II. Correlation between TWIST expression and clinicopathological characteristics in hypopharyngeal cancer.

Characteristics	n	TWIST expression		P-value
		Negative (%)	Positive (%)	
Gender				
Male	47	13 (27.7)	34 (72.3)	0.211
Female	3	2 (66.7)	1 (33.3)	
Age (years)				
≤60	20	11 (55.0)	9 (45.0)	0.773
>60	30	14 (46.7)	16 (53.3)	
Differentiation				
I and II	32	8 (25.0)	24 (75.0)	0.038
III	18	10 (55.6)	8 (44.4)	
Tumor size				
T1 and T2	10	6 (60.0)	4 (40.0)	0.048
T3 and T4	40	9 (22.5)	31 (77.5)	
Lymph node metastasis				
N0 and N1	24	14 (58.3)	10 (41.7)	0.044
N2 and N3	26	7 (26.9)	19 (73.1)	

affects the character of FaDu cells. We observed that elevated expression of TWIST changed cell morphology from a well-organized cell-cell adhesion and cell polarity morphology to loss of cell-cell contact and cell scattering. The phenomenon is called epithelial-mesenchymal transition (EMT) (21). Some studies have shown that the epithelial cell polarity was lost, and the cell morphology changed from epithelial to mesenchymal morphology, epithelial markers were lost, and the mesenchymal markers were acquired. The process contributes to the progress of tumor metastasis (22). Simultaneously, we found that elevated expression of TWIST increased the migration and invasion of FaDu cells. The results also indicated that TWIST overexpression promoted metastasis in FaDu cells.

In order to elucidate the mechanism by which TWIST promoted the metastasis of FaDu cells, we further analyzed several proteins that were related with tumor metastasis, and found that elevated TWIST expression up-regulated N-cadherin and down-regulated E-cadherin, and *vice versa*. Results also showed that elevated expression of TWIST down-regulated E-cadherin and decreased the cell adhesion ability to increase the invasion ability (5). In addition, gain of N-cadherin promoted metastasis of breast and prostate cancer (23,24). Recently, EMT and its accompanying reduction in E-cad expression were shown to be essential for the extravasation of cancer cells into secondary organs (9). We confirmed that elevated expression of TWIST may have promoted metastasis by inducing EMT and decreasing the cell-cell adhesion ability in FaDu cells. Interestingly, we verified that both c-fos and MMP-9 expression were positively correlated with the alteration of TWIST in FaDu cells. After the silent TWIST expression, both c-fos and MMP-9 dramatically decreased, suggesting TWIST has a role in regulating c-fos and MMP-9 expression. The results agreed with the study that the depletion of TWIST results in decreased expression of the c-fos in

gastric cancer cells (25). It has been reported that c-fos has oncogenic activity, is frequently overexpressed in tumor cells, and is thought to enhance motility and invasiveness of cancer cells (26). MMP-9 and c-fos were related to tumor metastasis, and c-fos induced loss of cell polarity and EMT (27). MMP-9 has been implicated in the facilitation of cancer cell invasion and metastasis through degradation of surrounding ECM proteins (28). Moreover, the overexpression of c-fos, increased MMP-9 expression (29). Thus, we hypothesized that the TWIST mechanism which promotes the metastasis of FaDu cells *in vitro*, may change the morphology, and degrade the structural elements of the ECM, and the cell-cell and cell-ECM adhesion molecules by regulating c-fos, MMP-9 expression, and promoting the development of EMT.

In addition, elevated TWIST expression was further related with tumor invasion and metastasis in esophageal squamous cell carcinomas (6), as well as in head and neck carcinomas (20). The present results are consistent with those reports and show that TWIST expression was elevated in hypopharyngeal cancer tissue, and it was positively related with the tumor size, lymph node metastasis, and tumor differentiation. Results demonstrate that TWIST has a similar function that promotes the metastasis of hypopharyngeal cancer *in vivo*.

In summary, this study documented that overexpression of TWIST played a role in the metastasis of hypopharyngeal cancer *in vivo* and *in vitro*. Furthermore, the alteration of TWIST has an effect on the N-cadherin, E-cadherin, c-fos and MMP-9 in FaDu cells. We concluded that TWIST promoted hypopharyngeal cancer metastasis by regulating c-fos and MMP-9 expression, and by inducing EMT. However, the mechanism of TWIST activation and its downstream signaling pathway during tumor metastasis are still vague and the related experiments are currently underway to address these important questions.

Acknowledgements

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