Abstract. The aim of this study was to determine the effect of A disintegrin and metalloprotease 10 (ADAM10) protein expression on the progression, migration and prognosis of hypopharyngeal squamous cell carcinoma (HSCC). Immunohistochemistry and western blot analysis were performed to detect ADAM10 expression in human HSCC specimens. Cell Counting Kit-8 (CCK-8) assay, flow cytometry analysis and wound-healing assay were employed to investigate the effects of ADAM10 knockdown (ADAM10-RNAi) on major oncogenic properties of FaDu cells. We detected that ADAM10 was overexpressed in HSCC specimens and its expression level was associated with differentiation (p<0.001), tumor size (p=0.019), lymph node metastasis (p=0.001), clinical stage (p<0.001), proliferation marker Ki-67 expression (P=0.001) and overall survival (p<0.046). ADAM10-RNAi in FaDu cells resulted in the inhibition of proliferation and the decrease in migration. Moreover, mechanistic experiments revealed that ADAM10-RNAi resulted in an increase in E-cadherin and a decrease in N-cadherin and vimentin expression. Our study implies that high expression of ADAM10 promotes the proliferation and migration of HSCC. These findings may help to provide a method for treatment of HSCC.

Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) is a common head and neck malignancy, accounting for 2-6% of all head and neck cancers (1,2). Due to the lack of evident clinical symptoms, easy lymph node metastasis and local infiltration in patients with early-stage HSCC (3,4), make treatment of HSCC one of the toughest challenges in human malignancies. Although the curative effect has progressively improved with the development of science and clinical technology in recent years, the overall survival has not improved due to the lymph node metastasis and distant metastasis of HSCC (5,6). Therefore, studying the molecular mechanisms of HSCC progression and migration, would improve the diagnosis and prognosis of HSCC, and help to develop a new therapeutic strategy.

The A disintegrin and metalloprotease (ADAMs) family which has been reported in more than 30 species, and contains common structural features, consists of multidomain transmembrane proteases (7). ADAMs plays an important role in the degradation of intercellular adhesion, cell-matrix adhesion and basement membrane. Aberrant expression of ADAMs is closely related to tumor proliferation, differentiation, adhesion, migration and invasion in tumors (7-11). ADAM10 is a crucial member of the ADAMs family (12,13). The chief function of ADAM10 is shedding certain protein molecules in the extracellular region, thereby activating molecules, such as Ephrins (14), N-cadherin (15), E-cadherin (16), Notch receptor and its ligand δ 1 (17). Previous research has revealed that ADAM10 is highly expressed in a variety of human types of cancer, including liver cancer (18), nasopharyngeal carcinoma (19), lung (20), gastric (21) and bladder cancer (22). Overexpression of ADAM10 promotes tumorigenesis and the progression, metastasis and invasion of tumors as well as the poor prognosis for cancer patients, by participating in a variety of signaling pathways (7-11,23-25). However, the expression of ADAM10 in HSCC and the role of ADAM10 on the progression, metastasis and poor prognosis of HSCC have yet to be elucidated.

In the present study, we first studied the expression of ADAM10 in HSCC, and the effect of ADAM10 expression on the proliferation and migration of HSCC. We found that ADAM10 overexpression in HSCC was associated with clinicopathological characteristics and the poor prognosis of patients with this disease. Moreover, inhibiting the expression level of ADAM10 decreased the proliferation and migration ability of the FaDu cell line. Our data indicated that ADAM10
is a potential molecular target involved in the progression and metastasis mechanism of HSCC.

Materials and methods

**Tissue specimens.** All of the HSCC samples were collected from the Affiliated Hospital of Nantong University and pathologically diagnosed as HSCC by tissue biopsy. The clinical features of 46 patients with HSCC are listed in Table I. Fifteen pairs of fresh HSCC tissues and adjacent tissues were stored in -80°C in a refrigerator. All selected patients had not undergone preoperative radiotherapy and chemotherapy. Informed consent was obtained from all individual participants included in the study. This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University and conformed to the provisions of the Declaration of Helsinki in 1995.

**Immunohistochemical staining.** Tissue samples were cut into paraffin sections for immunohistochemical staining. After deparaffinization and rehydration, the tissue sections were heated with 1X sodium citrate solution at 100°C for 30 min and subsequently washed 3 times. Finally the tissue sections were incubated with the primary antibodies overnight at 4°C. A two-step incubation with a secondary antibody was performed using an immunohistochemistry universal kit (ZSGB-BIO, Beijing, China). DAB staining then followed and microscopic observation. Two pathologists assessed the staining intensity and percentage of stained cells in the tumor area, respectively. The expression level of ADAM10 was evaluated according to a semiquantitative scoring system named 'H-score approach' (26). The staining intensity was termed category A and assigned scores as strong (3); moderate (2); weak (1) or negative (0). The proportion of cells in the tumor area was termed category B and scored from 0 to 4: 0 (0%); 1 (1-25%); 2 (26-50%); 3 (51-75%) and 4 (76-100%). A final score was calculated by multiplying A by B (minimum 0, maximum 12). A final score of <6 was regarded as negative or weak expression, and a score of ≥6 was regarded as high expression.

**Cell lines, small interfering RNAs (siRNAs) and transfection.** The FaDu cell line from our laboratory was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, USA). Control non-targeted siRNAs and ADAM10-specific siRNAs were purchased from Ribobio (Guangzhou, China). Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, USA) following the manufacturer's protocol.

**Western blot analysis and antibodies.** Protein was extracted from tissues and FaDu cells for protein analysis. The samples were boiled at 100°C for 5 min. An equal amount of protein samples was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The membranes were incubated with primary antibodies overnight at 4°C. The antibodies used were as follows: anti-ADAM10 (1:300, polyclonal, rabbit anti-human; BBI Life Sciences Corporation, Shanghai, China), anti-Ki-67 (1:1,000; polyclonal, rabbit anti-human), anti-E-cadherin (1:1,000), anti-N-cadherin (1:1,000, monoclonal, mouse anti-human), anti-β-actin (1:1,000, monoclonal, mouse anti-human), and anti-β-actin polyclonal antibody (1:2,500, monoclonal, mouse anti-human) (all from Santa Cruz Biotechnology, USA). Immunoreactive protein bands were detected using an ECL detection system (Cell Signaling Technology, USA).

**Cell proliferation assay.** The CCK-8 assay was used to detect cell proliferation. The cells were seeded in 96-well plates (10,000 cells/well), incubated overnight, and then transfected with siRNA-ADAM10 or control non-targeted siRNAs and cultured for 12, 24, 36, 48, 60 and 72 h. The CCK-8 kit reagent (10 µl/well; BBI Life Sciences Corporation) was added and incubation followed in the dark in an incubator for 2 h. Subsequently the OD was assessed at 450 nm.

**Cell cycle analysis.** Flow cytometry was used to analyze the effect of ADAM10 on cell cycle progression. Briefly, FaDu cells were cultured in 6-well plates. The medium was replaced with 10% FBS at 6, 12, 24 and 36 h and then incubated with 0.1 µg/ml of siRNA-ADAM10 and cultured for 2 days. The adherent cells were collected by trypsin digestion and centrifuged at 95 x g for 5 min. The cells were then washed twice with phosphate-buffered saline (PBS), and fixed with

| Table I. Clinicopathological characteristics of HSCC patients and IHC staining score for ADAM10. |
|-----------------|-------|-----------------|------|
| Groups          | N     | ADAM10 IHC score | P-value |
| Age (years)     |       |                 |       |
| <60             | 20    | 5.80±3.736      | 0.741 |
| ≥60             | 26    | 6.15±3.461      |       |
| Sex             |       |                 |       |
| Male            | 43    | 6.05±3.605      | 0.740 |
| Female          | 3     | 5.33±3.055      |       |
| Differentiation |       |                 |       |
| Keratinizing    | 20    | 3.90±2.278      | <0.001 |
| Non-keratinizing| 26    | 7.62±3.238      |       |
| Tumor size (cm) |       |                 |       |
| ≤2              | 15    | 4.27±3.390      | 0.019 |
| >2              | 31    | 6.84±3.358      |       |
| Clinical stage  |       |                 |       |
| I-II            | 18    | 3.67±2.701      | <0.001 |
| III-IV          | 28    | 7.50±3.226      |       |
| Lymph node metastasis |     |                 |       |
| Negative        | 19    | 3.95±2.896      | 0.001 |
| Positive        | 27    | 7.44±3.274      |       |
| Ki-67 expression|       |                 |       |
| Low             | 31    | 4.45±3.064      | 0.001 |
| High            | 15    | 9.20±2.007      |       |

*ADAM10 IHC score expressed as the mean ± SD. Student's t-test for two groups. &Statistically significant, P<0.05. ADAM10, A disintegrin and metalloprotease 10; HSCC, hypopharyngeal squamous cell carcinoma.
70% alcohol. The cells were then stored at -20°C for 24 h, washed with PBS 2 times and resuspended in 1 ml of 1x PBS containing 0.1% Triton X-100, 40 µg/ml RNase and 20 µg/ml propidium iodide (PI) followed by a 30-min incubation at room temperature. Finally, the samples were analyzed using the FACSCalibur flow cytometer (BD Biosciences, USA) and BD CellQuest software.

Migration assay. A wound healing assay was performed for cell migration. The cells were seeded in 6-well plates and incubated overnight, then transfected with siRNA-ADAM10 or control non-targeted siRNAs and a scratch wound was made using a 200-µl yellow tip when the cells reached ~90% density. Subsequently the cells were cultured with serum-free DMEM medium for 36 h. The percentage of migration was calculated based on the measured cell-free area. Similarly, Transwell migration assays were performed for cell migration. FaDu cells were transfected with siRNA-ADAM10 or control non-targeted siRNAs and seeded to the upper chamber containing serum-free DMEM with a non-coated membrane (24-well insert, 8-µm pore size; Millipore) and DMEM containing 10% serum was added to the lower chamber. After 24 h, the cells from the upper chamber were removed and the cells that had migrated to the lower chamber were fixed with formaldehyde, and then stained with 0.1% crystal violet. The cells were then counted using an IX70 inverted microscope.

Statistical analysis. Statistical software (IBM SPSS Statistics 20; IBM SPSS, Armonk, NY, USA) was used for statistical analysis. The Student's t-test was used to determine the statistical differences between groups. P<0.05 was considered statistically significant.

Results

ADAM10 protein overexpression in HSCC specimens. It was reported that ADAM10 is aberrant in tumors (18-22). Here, we first detected the expression of ADAM10 in HSCC. Immunohistochemical analysis of ADAM10 expression in hypopharyngeal normal tissues and tumor tissues revealed that ADAM10 was weakly expressed in normal tissues, moderately expressed in cancer in situ (CIS) and highly
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expressed in invasive cancer (IC) (Fig. 1A). The IHC score of ADAM10 was significantly decreased in normal tissues (1.93±0.88) compared with that in CIS (3.90±2.79, P<0.05) or IC (7.62±3.24, P<0.05) (Fig. 1B). In addition, there was a statistical difference in the IHC score of ADAM10 between CIS and IC tissues (P<0.05). Subsequently, we examined the expression of ADAM10 in 15 pairs of HSCC and precancerous tissues by western blot analysis, which revealed that the expression level of ADAM10 in cancer tissues was significantly higher than that in adjacent tissues (Fig. 1C and E). Finally, we also detected the expression of ADAM10 in the HSCC cell line FaDu (Fig. 1D). The results revealed that ADAM10 was overexpressed and may promote tumorigenesis and progression in HSCC.

Correlation of ADAM10 expression level with clinicopathological features. We further assessed the correlation of the expression level of ADAM10 with clinicopathological characteristics in HSCC. As shown in Table I and Fig. 2, high expression of ADAM10 in HSCC was significantly correlated with the degree of tumor differentiation (p<0.001). Squamous cell carcinoma is divided into keratinizing and non-keratinizing squamous cell carcinoma (27). In addition, overexpression of ADAM10 in HSCC was also associated with tumor size (p=0.019), lymph node metastasis (p=0.001) and clinical stage (p<0.001). There was no correlation with the sex and age of the patients and HSCC. Moreover, we also analyzed the correlation of the expression level of ADAM10 with Ki-67 expression (p=0.001). The result revealed that there was a significant positive correlation in the expression level of ADAM10 with the expression of Ki-67 (Fig. 3A). The data revealed that the expression of ADAM10 had a significant correlation with the clinicopathological characteristics of HSCC. Thus, high expression of ADAM10 may promote proliferation and migration.
High expression of ADAM10 is associated with the poor prognosis of HSCC patients. Similarly, we analyzed the relationship between the expression of ADAM10 and the prognosis of 46 patients with HSCC by statistical analyses. The result revealed that overall survival was significantly decreased in patients with high expression of ADAM10 than in those with low expression of ADAM10 (Fig. 3B) (p<0.046). It is therefore implied that overexpression of ADAM10 led to the poor prognosis of patients with HSCC.

Expression of ADAM10 in proliferating HSCC cells. In order to further explore the possible biological roles of ADAM10 in HSCC we examined the expression level of ADAM10 in proliferating FaDu cells. First, we cultured FaDu cells in serum-free medium for 72 h to stop the cell cycle in the G1 phase. Then, we collected the cells after being cultured with 10% FBS at 0, 6, 12, 24, and 36 h. Flow cytometric analysis revealed that the percentage of cells in the S phase of the cell cycle increased from 6.99 to 45.66% (Fig. 4A and B). Similarly, the expression level
of ADAM10 in the cells was obviously increased as detected by western blot analysis (Fig. 4C and D). The result revealed that ADAM10 was involved in the process of cell proliferation.

The proliferation of FaDu cells was attenuated by knockdown of ADAM10. The above studies revealed that ADAM10 could promote the proliferation of HSCC. Therefore, FaDu cells were transfected with ADAM10 siRNAs to decrease the expression of ADAM10 in order to detect the function of ADAM10 in HSCC. First, we assessed the knockdown efficiency by western blotting and real-time PCR. The results revealed that the expression level of ADAM10 in the FaDu cell line was significantly decreased after knockdown, especially in ADAM10-siRNA3 (Fig. 5A-C). Subsequently, we examined cell proliferation by CCK-8 assay. The result revealed that the cell proliferation ability was significantly decreased after knockdown with ADAM10-siRNA3 (Fig. 5D).

The migration of FaDu cells was inhibited by knockdown of ADAM10. We then examined the migration ability of FaDu cells with ADAM10-siRNA3 knockdown by wound-healing and Transwell assay. The study revealed that wound healing was significantly decreased by ADAM10-siRNA3 compared with the control group after 36 h (Fig. 6A and B). Similarly, Transwell assay revealed that the number of migratory cells were significantly lower in the ADAM10-siRNA3 group than...
In conclusion, the migration ability of FaDu cells was significantly decreased after knockdown of ADAM10.

In the process of tumor migration, epithelial-mesenchymal transition (EMT) plays a decisive role (28). It could decrease cell adhesion, eliminate cell polarity, empower cell motility and invasive ability, thus leading to the metastasis of tumor cells from the original tumor location to adjacent organs and distant organs (29). The EMT process is usually accompanied with decreased expression of epithelial markers (E-cadherin, cytokeratin, and β-catenin) and increased expression of mesenchymal phenotypic markers (vimentin, N-cadherin and fibronectin), which play important roles in cell adhesion (29,30). Therefore, we detected the expression of N-cadherin, vimentin and E-cadherin in FaDu cells after treatment with ADAM10-siRNA3 (Fig. 6E). The result demonstrated that ADAM10 promoted tumor migration by affecting EMT.

Discussion

In our present study, we investigated whether ADAM10 had a potential effect on HSCC. To clarify the effect of ADAM10 on HSCC, we performed immunohistochemical and western blot analysis to detect the expression of ADAM10 and its relationship with clinicopathological characteristics. In addition, we observed the effect of the expression of ADAM10 on the proliferation and migration ability of the FaDu cell line. Collectively, our results revealed that ADAM10 was highly expressed and promoted tumor progression, migration and the poor prognosis of patients with HSCC.

ADAM10, a member of the ADAMs family, is a multinomial transmembrane protease, which is widely expressed in the body (31-34). The main function of ADAM10 is ectodomain shedding on a variety of transmembrane receptors, cytokines and adhesion molecules, such as TNF-α, EGF, E-cadherins, N-cadherins and Notch (15,16,31-35). High expression of ADAM10 has been reported in a variety of malignancies and is involved in the development and migration...
of tumors (7-11,16,23-25). However, the role of ADAM10 in HSCC has not been reported yet.

In this study, we found that ADAM10 was highly expressed in CIS and IC of HSCC compared with normal tissues, likewise, the expression level of IC was also higher than that of CIS (Fig. 1). Moreover, we found that the expression level of ADAM10 was correlated with tumor size, differentiation, clinical stage and lymph node metastasis in HSCC (Fig. 2). It is suggested that ADAM10 may be a potential oncogene and play an important role in the tumorigenesis and progression of HSCC. The aberrant expression of ADAM10 may be involved in tumor cell proliferation and migration-related signaling pathways, thus promoting tumor growth, lymph node metastasis and distant metastasis. These results were similar with those of You et al (19) that reported the effect of ADAM10 in nasopharyngeal carcinoma and Liu et al (36) that reported the effect of ADAM10 in hepatocellular carcinoma.

The studies revealed that the prognosis of HSCC was mainly related to the clinical stage, malignancy and lymph node metastasis and distant metastasis (5,6). Thus, we analyzed the effect of ADAM10 on the prognosis of HSCC. The results revealed that overexpression of ADAM10 significantly decreased the time of survival. Overall survival was significantly decreased in HSCC patients with high expression of ADAM10 compared to those with low expression (Fig. 3B). Combined with the above results (Figs. 1 and 2) it is implied that high expression of ADAM10 may promote migration and invasion, increase the difficulty of treatment and enhance the recurrence rate in HSCC, thus decreasing the overall survival rate of HSCC patients.

To further test our hypothesis, we used the HSCC cell line FaDu for validation. First, we detected the expression level of ADAM10 in proliferating FaDu cells, which revealed that the percentage of cells in the S phase increased with the increase of ADAM10 expression (Fig. 4). Subsequently, we knocked down the expression of ADAM10 in FaDu cells. The results revealed that the proliferation ability of FaDu cells (Fig. 5D) and the percentage of S phase cells (Fig. 5E and F) were significantly decreased after knockdown of ADAM10 expression. It was further demonstrated that the expression of ADAM10 promoted the abnormal proliferation of cells in HSCC. Furthermore, we investigated the effect of ADAM10 on the migration of FaDu cells. It was revealed that the ability of scratches to heal (Fig. 6A and B) and the number of cells that passed through the membrane (Fig. 6C and D) were markedly decreased after knockdown of ADAM10 expression in FaDu cells. These results indicated that downregulation of ADAM10 expression can significantly inhibit the movement and migration of FaDu cells. In other words, high expression of ADAM10 may promote the proliferation and migration of tumors in HSCC.

Tumor invasion and metastasis are very complex molecular regulation processes. The current study determined that EMT plays a decisive role in these processes (28). EMT is mainly characterized by the decrease of cell epithelial characteristics, the increase of interstitial characteristics, remodeling of the cytoskeleton and disappearance of cell matrix adhesion; and then a decrease of cell adhesion, elimination of cell polarity, empowerment of cell motility and invasive ability (29). This process is usually accompanied by decreased expression of epithelial phenotypic markers (e.g., E-cadherin and β-catenin) and increased expression of interstitial phenotypic markers (e.g., vimentin, and N-cadherin) (29,30). We employed proteomic analysis and found that the expression of E-cadherin was significantly increased, and the expression of vimentin and N-cadherin were significantly decreased after downregulation of ADAM10 expression in FaDu cells (Fig. 6E). The results suggested that high expression of ADAM10 may increase EMT regulation, and promote tumor cell migration and infiltration in HSCC.

In conclusion, complex biological processes are involved in the unrestricted tumor growth and metastasis of HSCC. Therefore, it is very important to understand the biological mechanism of the proliferation and metastasis of HSCC, which could detect the potential genes that promote the proliferation and metastasis of HSCC. In this study, our data clearly revealed that ADAM10 was highly expressed in HSCC and promoted the proliferation, migration and the poor prognosis of patients with HSCC. Thus, ADAM10 may be an important molecular target of HSCC proliferation and metastasis, with potentially important therapeutic implications.

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