Effects of ADAM10 upregulation on progression, migration, and prognosis of nasopharyngeal carcinoma

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Asopharyngeal carcinoma (NPC) is the most common head and neck cancer in China, with 80,000 new cases and 50,000 deaths every year. The age of most NPC patients is approximately 30–50 years, which is younger than that found in other cancers. With the development of radiotherapy technology and the combined application of radiochemistry, the therapeutic efficacy is gradually improved, but it still fails to achieve the desired effect because of local recurrence and distant metastasis. Therefore, understanding the molecular events associated with NPC progression, invasion, and prognosis may improve earlier diagnosis, prognosis prediction, and development of novel therapeutic strategies.

A disintegrin and metalloprotease 10 (ADAM10) is a typical member of the ADAMs family, which has been reported to be upregulated in various types of cancers and contribute to cancer progression and metastasis. However, little is known about the role of ADAM10 in nasopharyngeal carcinoma (NPC). The purpose of this study is to explore ADAM10 expression status and its biological functions in NPC. We first examined the expression of ADAM10 in NPC tissues and cell lines by immunohistochemistry, Western blotting, PCR, and immunofluorescence analysis. We observed that ADAM10 was significantly elevated in NPC and its expression level was correlated with T classification (P = 0.044), distant metastasis (P = 0.016), TNM clinical stage (P = 0.013), and proliferation marker Ki-67 expression (P = 0.001). Patients with NPC with high expression of ADAM10 had shorter overall survival rates. In addition, knockdown of ADAM10 by RNAi was found to inhibit the CNE-2 cell proliferation and migration. Our findings hinted that overexpression of ADAM10 promotes the progression and migration of NPC, which makes it a potential therapeutic target for the treatment of tumor metastases in NPC.
The data revealed that ADAM10 is a prognostic marker of NPC and promotes NPC progression and migration.

Materials and Methods

Tissue specimens and ethics. All NPC tissues were obtained from histologically confirmed NPC patients who underwent biopsy at the Affiliated Hospital of Nantong University (Nantong, China). Twenty-four non-cancerous nasopharyngeal tissues were collected from patients with clinical symptoms suggestive of NPC, but ruled out by biopsy. None of the patients had received antitumor therapy prior to biopsy. The characteristics of 118 NPC patients are listed in Table 1. Furthermore, 20 paired of fresh NPC and non-cancerous nasopharyngeal tissues were snap-frozen in liquid nitrogen and stored at −80°C after biopsy. This study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University and conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Tokyo 2004). All participants gave informed consent.

Immunohistochemical staining and Western blot analysis. Immunohistochemistry and Western blot analysis were carried out according to the previous report. For assessment of ADAM10, the staining intensity and relative percentage of immunostained cells were analyzed and evaluated by two pathologists blinded to the data. The staining intensity was categorized as strong (2), weak (1), or negative (0) semiquantitatively using the following scale: 0, 0% of cells; 1, 1–25% of cells; 2, 26–50% of cells; 3, 51–75% of cells; and 4, 76–100% of cells. The scoring results of intensity and extent were multiplied as described previously. The samples with a final score <4 were regarded as ADAM10 negative or weak expression and 4–8 were determined as overexpression. When evaluating the Ki-67 expression, 51–100% of positively stained cell nuclei was classified as the high expression group and 0–50% was the low expression group.

For Western blot analysis, anti-ADAM10 (1:300; Sangon Biotech, Shanghai, China), anti-Ki-67 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-proliferating cell nuclear antigen (PCNA) (1:1000; Santa Cruz Biotechnology), anti-E-cadherin (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-N-cadherin (1:1000; Cell Signaling Technology), anti-vimentin (1:1000; Cell Signaling Technology), and anti-β-actin polyclonal antibody (1:2000; Santa Cruz Biotechnology) antibodies were used.

Immunofluorescence microscopy. Cells cultured on glass coverslips were fixed with 4% paraformaldehyde and blocked with 1% normal donkey serum. Cells were then incubated with primary antibodies overnight. Then, the cells were incubated with Alexa Fluor-conjugated secondary antibodies (1:1000; Invitrogen Life Technologies, Carlsbad, CA, USA), counterstained with Hoechst (Sigma-Aldrich Co., St. Louis, MO, USA) and observed with a fluorescence microscope.

Quantitative real-time PCR. Quantitative real-time PCR was carried out as previously described. The primers were purchased from Sangon Biotech and were as follows: ADAM9, 5′-GGTGCTGGTGAGTGCAGTG-3′ (forward) and 5′-CTCGG TGCCCTCGTGA-3′ (reverse); ADAM10, 5′-ATGGATTG TGGCTCAATTGG-3′ (forward) and 5′-TGCTGGAGGT GTGTTAGGA-3′ (reverse); ADAM12, 5′-CAGGAAAGCTT GGAGAC-3′ (forward) and 5′-AGCAGCGATCTTACATTTC-3′ (reverse); and ADAM17, 5′-GTATCTCGACACGACACC TG-3′ (forward) and 5′-CCTCTGGGACTTTCTTCTG-3′ (reverse). The expression levels of each gene were normalized by GAPDH.

Cell cultures. All four NPC cell lines (CNE-1, CNE-2, 5-8F, and 6-10B) maintained in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS (Gibco). The immortalized normal nasopharyngeal epithelial cell line NP69 was cultured in keratinocyte–serum-free medium (Invitrogen, Carlsbad, CA, USA).

Cell cycle analyses. Cells were fixed in 70% ethanol and then incubated with 1 mg/mL RNase A. Subsequently, cells were stained with propidium iodide (50 mg/mL; Becton Dickinson, San Jose, CA, USA) and analyzed using a Becton Dickinson flow cytometer (BD FACScan, San Jose, CA) and CellQuest acquisition and analysis programs. Gating was set to exclude cell debris, cell doublets, and cell clumps.

Small interfering RNAs and plasmids. The negative control siRNA and specific siRNAs for ADAM10 were designed and obtained from Guangzhou Ribobio (Guangzhou, China). The pcDNA-encoding ADAM10 reporter plasmid was purchased from GeneChem (Shanghai, China). The mock GV362 vector was used as a negative control. Cells were grown in 6-well plates and transfected with siRNAs and DNA plasmids using Lipofectamine 2000 (Invitrogen).

Cell proliferation assay. Cells were seeded in 96-well plates (20 000 cells/well) and grown overnight. The CCK-8 Kit reagents (10 μL/well, Sangon Biotech Co., Ltd, Shanghai, China) were added and incubated for 2 h at 37°C, and the absorbance was read at 450 nm in an automated plate reader.

Transwell assays. Cells (1 × 10⁵) were seeded into the upper chambers of cell culture inserts (24-well type, 8-µm pore size;
Corning, Corning, NY, USA) in the serum-free medium. Medium containing 10% FBS was added to the lower chambers. After 16 h of incubation, the cells that had migrated to the undersurface of the membrane were fixed and stained with crystal violet. Digital images were obtained from the membranes, and 10 random fields were counted.

**Wound-healing assay.** CNE-2 cells were plated in 6-well plates. When the cell confluence reached 80%, a scratch was made through the confluent cell using a 100-μL pipette tip. Photographs were taken at different time points within the scrape line.

**Calculation and statistical analysis.** Results from at least three independent experiments are reported as the mean ± SD. Statistical analyses were carried out using SPSS17.0 software (SPSS Inc., Chicago, IL, USA). Survival curves were estimated by Kaplan-Meier analysis and compared by the log-rank test. The χ²-test was used to determine the significance of differences in multiple comparisons. Statistical significance was assessed by two-tailed Student’s t-test for two groups and one-way ANOVA for more than two groups. \( P < 0.05 \) was considered statistically significant.

**Results**

**ADAM10 highly expressed in NPC.** It was reported that members of the ADAM family, including ADAM9, ADAM10, ADAM12, and ADAM17, are aberrant in cancers and critical during tumor progression in processes such as proliferation, migration, and invasion. Here, we first detected the expression of certain members of the ADAMs family such as ADAM9, ADAM10, ADAM12, and ADAM17 in NPC cells and tissues by PCR. We found that, compared with ADAM9, ADAM12, and ADAM17, ADAM10 had highest expression levels in NPC tissues as well as NPC cells lines (Fig. 1). This finding

![Fig. 1. Expression of types of a disintegrin and metalloprotease (ADAM) in nasopharyngeal carcinoma (NPC) tissues and cell lines. (a–d) mRNA levels of ADAM9, ADAM10, ADAM12, and ADAM17 in 20 paired NPC tissues by quantitative PCR. (e–h) mRNA levels of ADAM9, ADAM10, ADAM12, and ADAM17 in NPC cell lines by quantitative PCR. CNE-1, CNE-2, 5-8F, and 6-10B are human NPC cell lines; NP-69 is an immortalized normal nasopharyngeal epithelial cell line. The same experiment was repeated at least three times. * \( P < 0.05 \).](image-url)
suggests that ADAM10, but not other ADAMs, may act as a major sheddase for tumor progression in NPC.

We next detected the expression of ADAM10 and proliferation marker PCNA in 20 pairs of NPC and non-cancerous nasopharyngeal tissues by Western blot analysis. We found that the expression level of ADAM10 was similar to PCNA, both of which had higher expression in NPC than non-tumor tissues (Fig. 2a,b). Immunohistochemical analysis was further carried out to assess the overexpression of ADAM10 and proliferation marker Ki-67 (Fig. 2c). In agreement with the data above, ADAM10 and Ki-67 were highly expressed in NPC tissues (Fig. 2c). In contrast, there was no or little expression in non-tumor tissues (Fig. 2c). According to the statistical analysis, the positive expression rate of ADAM10 in the NPC was significantly higher than that in the non-tumor tissues ($P < 0.01$). The results of immunohistochemistry staining are summarized in Table 1.

Relationship between ADAM10 expression and clinicopathological characteristics. We further evaluated the association between ADAM10 expression and clinicopathologic variables in NPC. As shown in Table 1, high ADAM10 expression was significantly associated with T classification ($P = 0.044$), distant metastasis ($P = 0.016$), and clinical stage ($P = 0.013$). However, ADAM10 showed no statistical association with age, sex, smoking status, or N classification (all $P > 0.05$). Moreover, the high expression of ADAM10 was similar to Ki-67 in most specimens (Table 1). There was a positive correlation between ADAM10 expression and Ki-67-based proliferative activity ($P < 0.01$; Fig. 3).

ADAM10 expression significantly associated with survival of NPC patients. Next, the prognostic significance of ADAM10 was assessed using Kaplan–Meier analysis. In 118 NPC cases, patients with ADAM10 overexpression had worse prognoses than those with negative expression (Fig. 4) ($P < 0.001$).

Univariate analyses showed that distant metastasis ($P = 0.002$), clinical stage ($P = 0.001$), Ki-67 expression ($P < 0.001$), and ADAM10 expression ($P < 0.001$) were significantly related to poor survival in NPC (Table 2). Multivariate analysis showed that clinical stage ($P = 0.03$), Ki-67 expression ($P = 0.017$), and ADAM10 expression ($P = 0.032$) were independent prognostic factors in NPC patients (Table 3).

Expression of ADAM10 in proliferating NPC cells. To further investigate the potential biological roles of ADAM10 on NPC, cell experiments were carried out. We first investigated the expression of ADAM10 in four kinds of human NPC cell lines and the normal nasopharyngeal epithelial cell line. As shown in Figure 5, the expression of ADAM10 in four NPC cell lines

![Fig. 2. Expression of a disintegrin and metalloprotease 10 (ADAM10) in nasopharyngeal carcinoma (NPC) tissues. (a) Protein levels of ADAM10 in 8 of 20 paired NPC (T) and non-cancerous nasopharyngeal tissues (N) by Western blotting. (b) Quantitative results of Western blot analysis. (c) Immunohistochemistry analysis of ADAM10 and Ki-67 expression in NPC tissues (original magnification, ×400). β-actin was used as a loading control. Scale bar = 50 μm. The same experiment was repeated at least three times.](image-url)
increased dramatically, especially in CNE-2 and 5-8F cells, as compared with NP69 (Fig. 5).

Our findings above suggested that high ADAM10 expression might be related to the cell proliferation in NPC. So we detected the expression of ADAM10 during CNE-2 cell cycle progression. We found that CNE-2 cells were arrested in G1 phase after serum deprivation for 72 h. As cells re-entered S phase, S phase increased from 23.14% to 45.06% after serum addition (Fig. 6a). Western blot analysis showed that the expression of ADAM10 was upregulated (Fig. 6b,c). The expression of PCNA was upregulated (Fig. 6b,c). Thus, these results indicated that ADAM10 might play a crucial role in regulation of the NPC cell cycle progress and cell proliferation.

ADAM10 knockdown inhibits cellular proliferation and promotes cell cycle arrest. CNE-2 cells were transfected with
ADAM10 siRNAs to knock down ADAM10 expression for investigating the role of ADAM10 on NPC. ADAM10-si2 had the highest knockdown efficiency (Fig. 7a–c). To assess the effects of ADAM10 knocked down on CNE-2 cell proliferation, CCK-8 and flow cytometry assays were carried out. The CCK-8 assay showed that CNE-2 cells treated with ADAM10 siRNAs exhibited a significant deceleration of cell proliferation compared with the control siRNA (Fig. 7d). Flow cytometry analysis revealed that CNE-2 cells accumulated in the G0/G1 phase, whereas the S phase decreased after transfection of siRNAs (Fig. 7e), suggesting that ADAM10 could promote the accumulation of the percent of cells in S phase and thus the cell growth.

ADAM10 knockdown inhibits cellular migration. We next examined the impact of ADAM10 knocked down on the migration of CNE-2 cells by wound-healing and Transwell assays. As shown in Figure 8(a,b), the percentage of wound closure at 24 h was significantly shorter in ADAM10 siRNA-treated cells than in control cells (P < 0.05). The silenced ADAM10 measurably inhibited cell migration to the bottom chambers in Transwell assays (Fig. 8c,d). These data indicated that downregulation of ADAM10 reduced the migration of CNE-2 cells.

During the process of cancer metastasis, which is enabled by epithelial–mesenchymal transition (EMT), the disseminated cancer cells seem to acquire self-renewal abilities, similar to those shown by stem cells. To clear the mechanism of ADAM10 promoting migration, the expression levels of these EMT-associated markers in ADAM10 knocked down cells were compared with that of mock cells. As shown in Figure 8(e), the knockdown of ADAM10 in CNE-2 cells was accompanied by the upregulation of epithelial marker E-cadherin and downregulation of mesenchymal markers N-cadherin and vimentin (Fig. 8e). To confirm the role of ADAM10 in EMT, NP69 cells were transfected with GV362–ADAM10 to increase the expression of ADAM10. As shown in Figure 8(f), the increased expression of ADAM10 in NP69 cells was accompanied by the downregulation of E-cadherin and upregulation of N-cadherin and vimentin (Fig. 8f).
Discussion

The genesis and development of NPC are comprehensive pathologic processes involving complex alterations in oncogenes, which play roles in cell proliferation, cell cycle control, and migration through regulation of multiple signal transduction pathways. In the present study, we investigated the influences of ADAM10 on NPC. Our findings indicated that overexpression of ADAM10 promotes the progression and migration of NPC.

The ADAMs family of proteins include a sequence that has similarities to the reprolysin family of snake venom metalloproteinases, and they share the metalloproteinase domain with MMPs. Functional ADAMs are involved in cleavage of diverse growth factors, membrane-bound cytokines, surface receptors, and adhesion molecules, thus increasing tumor proliferation, migration, and invasion. Certain members of the ADAMs family, including ADAM9, ADAM10, and ADAM17, are critical during genesis, development, and metastasis of cancers. ADAM10 has been reported to be overexpressed in various malignancies and to be involved in cancer progression. For example, Liu et al. established that downregulation of ADAM10 in HepG2 cells using RNA silencing significantly suppressed cell proliferation, migration, and invasion, and tumor growth in vivo. Further, silencing ADAM10 decreased constitutive phosphorylation of phosphoinositide 3-kinase (PI3K) and Akt, which indicates that ADAM10 is involved in the activation of the PI3K/Akt signaling pathway. In addition, Endres et al. showed that ADAM10 can cleave collagen type IV in the basement membrane, which is relevant to tumor metastasis and proliferation. Moreover, ADAM10 mediated shedding of the extracellular domain of E-cadherin, which in turn resulted in translocation of β-catenin to the nucleus and led to enhanced proliferation.

However, the contribution of ADAM10 to NPC carcinogenesis remained largely unidentified and needs to be determined.

This study is the first to show that expression of ADAM10 was increased in NPC and that overexpressed ADAM10 was significantly associated with T classification, distant metastasis, and clinical stage. A positive correlation between ADAM10 and Ki-67 (a useful marker of tumor proliferative activity) was also observed in NPC. Those results showed that the overexpressed ADAM10 functioned as a potential oncogene and played an important role in the malignant progression and migration of NPC.

Furthermore, survival analysis confirmed that patients with ADAM10 overexpression had a shorter survival time. In addition, univariate and multivariate analysis revealed that overexpression of ADAM10 was a remarkable independent predictor of poor prognosis for NPC.

The biological functions of ADAM10 in NPC are still unclear. Our data revealed that the expression level of ADAM10 was related to that of PCNA and Ki-67. In addition, ADAM10 expression was upregulated during G1 to S phase in NPC. It is known that the G1/S phase transition is a major checkpoint for cell cycle progression. These results revealed that ADAM10 might contribute to carcinogenesis by regulating cell proliferation. Therefore, to further determine the role of ADAM10 in the proliferation of NPC, we knocked down ADAM10 using siRNA. After ADAM10 was silenced, the proliferation of CNE-2 cells was significantly inhibited. Cell cycle analysis showed that downregulation of ADAM10 decreased the cell population in the S phase as well as augmented G1 cycle arrest, leading to the inability of CNE-2 cells to complete cell division. Taken together, these findings indicated that ADAM10 might contribute to tumor cell proliferation through the regulation of cell cycle distribution.
It was reported that ADAM10 depletion could inhibit melanoma growth, as well as metastasis.\(^{(13)}\) In addition, ADAM10 correlates with metastasis of lung, hepatocellular carcinoma, and uveal melanoma.\(^{(14,15,32)}\) These data were in accord with our results, confirming the involvement of ADAM10 in NPC metastasis. Wound-healing assays and Transwell migration assays showed that silencing of ADAM10 inhibited migration by Transwell assays. (d) Number of cells that invaded through the membrane was counted in 10 fields under \( \times 20 \) objective lens. Bars, SD. \( n = 3; P < 0.05 \). (e) Western blot analysis of E-cadherin, N-cadherin, and vimentin in CNE-2 cells transfected with ADAM10 siRNAs and non-specific siRNA. (f) Western blot analysis of E-cadherin, N-cadherin, and vimentin in NP69 cells transfected with GV362-ADAM10 and mock GV362 vector. The same experiment was repeated at least three times.

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In summary, our data offer convincing evidence that ADAM10 is overexpressed in NPC and the level of ADAM10 is associated with clinical progression and poor prognosis. Furthermore, we show that ADAM10 may be involved in cell proliferation, migration, and invading abilities of NPC cells, which makes it a novel therapeutic target for NPC treatment.

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Disclosure Statement

The authors have no conflict of interest.