TFAP2A Regulates Nasopharyngeal Carcinoma Growth and Survival by Targeting HIF-1α Signaling Pathway

Dingbo Shi¹, Fangyun Xie¹, Yun Zhang¹, Yun Tian¹, Wangbing Chen¹, Lingyi Fu¹, Jingshu Wang¹, Wei Guo³, Tiebang Kang¹, Wenlin Huang¹,², and Wuguo Deng¹,²,³

Abstract

TFAP2A is a transcription factor that orchestrates a variety of cell processes, including cell growth and tissue differentiation. However, the regulation of TFAP2A in human nasopharyngeal carcinoma tumorigenesis and its precise mechanism of action remain largely unknown. In this study, we investigated the biologic role and clinical significance of TFAP2A in nasopharyngeal carcinoma growth and progression and identified the underlying molecular mechanisms. We found that TFAP2A was highly expressed in various nasopharyngeal carcinoma cell lines and tumor tissue specimens and was significantly correlated with hypoxia-inducible factor-1α (HIF-1α) expression. A positive correlation of TFAP2A overexpression with advanced tumor stage, local invasion, clinical progression, and poor prognosis of patients with nasopharyngeal carcinomas were also observed. Moreover, we found that knockdown of TFAP2A expression by siRNA significantly inhibited tumor cell growth in nasopharyngeal carcinoma cell lines and in a subcutaneous xenograft mouse model by targeting the HIF-1α–mediated VEGF/pigment epithelium–derived factor (PEDF) signaling pathway. Treatment of nasopharyngeal carcinoma cells with TFAP2A siRNA dramatically inhibited the expression and the release of VEGF protein but did not change the level of PEDF protein, resulting in a significant reduction of the ratio of VEGF/PEDF. Pretreatment with a HIF-1α siRNA did not significantly change the TFAP2A siRNA-mediated inhibition in cell viability. Our results indicate that TFAP2A regulates nasopharyngeal carcinoma growth and survival through the modulation of the HIF-1α–mediated VEGF/PEDF signaling pathway, and suggest that TFAP2A could be a potential prognostic biomarker and therapeutic target for nasopharyngeal carcinoma treatment. Cancer Prev Res; 7(2); 266–77. ©2013 AACR.

Introduction

Nasopharyngeal carcinoma is a squamous epithelial cancer arising from the lateral wall surface of nasopharynx (1). Unlike other head and neck cancers, nasopharyngeal carcinoma shows a clear regional and racial prevalence. The incidence rate of nasopharyngeal carcinoma is very high in China, Japan, and other Southeast Asian countries. But the southern Chinese have one of the highest incidences of nasopharyngeal carcinoma in the world (2). Etiology of nasopharyngeal carcinoma is considered to be related with a complex interaction of environmental and genetic factors as well as Epstein–Barr virus infection (3). At present, treatment of nasopharyngeal carcinoma is usually via radiotherapy. Nasopharyngeal carcinoma is more sensitive to ionizing radiation than other cancers (4). However, the treatment success mostly depends on the tumor, node, and metastasis (TNM) stage classification (5), which tend to be in the advanced stages at the point of diagnosis because the primary anatomical site of cancer growth is located in the silent painless area (6). Moreover, nasopharyngeal carcinoma has a poor prognosis because of late presentation of lesions, poor understanding of the molecular mechanisms, no suitable markers for early detection, and poor response to available therapies (7, 8). Therefore, there is an urgent need for further understanding the molecular mechanisms in nasopharyngeal carcinoma tumorigenesis and for identifying effective prognostic and diagnostic biomarkers and new therapeutic targets to improve the prognosis of patients.

TFAP2A, also called AP-2α, is a member of the AP-2 transcription factor family proteins, which includes 5...
different yet closely related 50 kDa proteins known as AP-2α, AP-2β, AP-2γ, and AP-2ε (9–12). AP-2 factors orchestrate a variety of cell processes, including cell growth, apoptosis, and tissue differentiation during embryogenesis (13). The AP-2 family is a DNA-binding protein (14) that presents a conserved helix–span–helix dimerization domain preceded by a DNA binding and a transactivation domain (15). AP-2 homodimers and heterodimers can activate transcription via GC-rich DNA sequences (16, 17). AP-2 has been shown to bind the palindromic consensus sequence 5'-GCCN3GCC-3' found in various cellular and viral enhancers. AP-2 family is known to exhibit both activating and repressing effects on target genes (18). A number of genes involved in cell growth, cell shape, cell movement, cell fate, and cell communication are regulated by AP-2 family (19–22). Reduced, or overexpressed TFAP2A expression is often detected in several types of cancers, such as melanoma, prostate, breast, ovary, neuroglioma, gastric, colon, and bladder cancers (23–32), indicating that loss of TFAP2A function, or TFAP2A overexpression may contribute to tumorigenesis and development of tumor malignancy. However, the biological role, prognostic value, and clinical significance of TFAP2, as well as its mechanism of action in nasopharyngeal carcinoma growth and progression remain unclear.

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that activates the expression of a number of genes involved in diverse aspects of cellular and physiologic processes (33, 34). Under hypoxic conditions, HIF-1α forms a heterodimer with HIF-1β, and binds to the hypoxia-responsive elements of the promoters to activate downstream hypoxia-responsive genes, including VEGF, to increase angiogenesis and tumor metastasis or to promote cancer cell proliferation and migration (35–37). VEGF has a crucial role in pathologic angiogenesis associated with tumors, intraocular neovascular disorders, and other conditions (36, 37). The potent angiogenic inhibitor pigment epithelium-derived factor (PEDF) counterbalances the effect of VEGF (38). The activity of HIF-1α is upregulated by a variety of nonhypoxic signals, including the activation by several oncogenic pathways such as Src, HER-2, Ha-Ras, and mitogen-activated protein kinase signaling pathways (39). HIF-1α is overexpressed in many human cancers, and several lines of evidence have indicated its essential role in tumorigenesis (40, 41). However, the detailed mechanism of action of HIF-1α and its relationship with TFAP2A in nasopharyngeal carcinoma tumorigenesis is still largely unknown.

In this study, we investigated the expression of TFAP2A in nasopharyngeal carcinoma cell lines and tumor tissues. The relationship between TFAP2A and HIF-1α expression and the clinicopathologic factors, as well as the functions and mechanisms by which TFAP2A regulated nasopharyngeal carcinoma growth and survival, were also evaluated. The regulation of TFAP2A in nasopharyngeal carcinoma tumor growth was further confirmed in a nasopharyngeal carcinoma xenograft mouse model in vivo. Our findings provide new insights into understanding the regulation of TFAP2A in nasopharyngeal carcinoma tumorigenesis and exploring the potential therapeutic targets for nasopharyngeal carcinoma therapy.

Materials and Methods

Cell lines and cell culture

The human nasopharyngeal carcinoma cell lines (CNE1, CNE2, HONE-1, and SUNE-1) and the immortalized normal nasopharyngeal cell line (NP69) were purchased from the American Type Culture Collection. Two CNE2 subclones (S18 and S26) and 2 SUNE-1 subclones (5-8F and 6-10B) were generously provided by Dr. M. Zeng (Sun Yat-sen University Cancer Center, Guangzhou, China). These 4 nasopharyngeal carcinoma cell lines and 4 subclones of nasopharyngeal carcinoma cells were cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The NP69 cells were cultured in Keratinocyte-SFM (Invitrogen). Each cell line was authenticated in March 2013 and maintained at 37°C in a humidified atmosphere and 5% CO₂ according the recommendations of the providers.

Induction of hypoxia

For hypoxic induction, the nasopharyngeal carcinoma cells were cultured in a modulator incubator chamber (autoflow gas mixing instruments incubator; Chang Jin Inc.) with 94% N₂, 1% O₂, and 5% CO₂ at 37°C at 37°C for 24 hours.

Patients and tissue specimens

This study was conducted on a total of 179 paraffin-embedded nasopharyngeal carcinoma samples, which were histologically and clinically diagnosed from the Cancer Center (Sun Yat-sen University) between 1999 and 2008. For the use of these clinical materials for research purposes, prior patient’s consent and approval from the Institute Research Ethics Committee were obtained. The disease stages of all the patients were classified or reclassified according to the 1992 nasopharyngeal carcinoma staging system of China. Clinical information of the samples was described in detail in Table 1. Patients included 139 males and 40 females, of ages ranging from 20 to 78 years (mean, 46.1 years). The figures on metastasis pertain to its presence at any time in follow-up. The median follow-up time for overall survival was 54.6 months for patients still alive at the time of analysis, and ranged from 12 to 128 months. A total of 44 (24.6%) patients died during follow up and 27 (15.1%) patients experienced metastasis.

RNA extraction and reverse transcription-PCR

Total RNA from cells was extracted using Trizol reagent (Life Technologies). The RNA was pretreated with DNase and used for cDNA synthesis with random hexamers. The full-length open reading frame of TFAP2A, VEGF, PEDF, and HIF-1α were PCR amplified from cDNA samples of the nasopharyngeal carcinoma cell lines. The primers were used.
for amplification of TFAP2A: sense primer, 5-GCGGCATG-AATCTATTGGAC-3, antisense primer, 5-CCCAGGAAGCCGTCTTTATT-3; VEGF: sense primer, 5-AGGAGGGCAGAATCATCACG-3, antisense primer 5-CAAGGCCCACAGGGATTTTCT-3; PEDF: sense primer, 5-TCCAATGCAGAGGAGTAGCA-3, antisense primer, 5-TGTGCAGGCTTAGAGGGA-3; HIF-1α: sense primer, 5-GCGGCATGAATCTATTGGAC-3, antisense primer, 5-CCCAGGAAGCCGTCTTTATT-3; GAPDH: sense primer, 5-CAAATTCCATGGCACCGTCA-3, antisense primer, 5-GGAGTGGGTGTCGCTGTTGA-3.

The PCR products was confirmed by 1.2% agarose gel electrophoresis.

Western blot analysis

Equal amounts of whole cell lysates were resolved by SDS-PAGE and electrotransferred on a polyvinylidene difluoride (PVDF) membrane. The tissues were then incubated with primary rabbit monoclonal antibodies against β-actin, TFAP2A (Cell Signaling), VEGF (Merck Millipore), HIF-1α, and PEDF (Santa Cruz Biotechnology). The protein bands were detected by enhanced chemiluminescence.

Immunohistochemical staining

The 4-μm sections were cut from formalin-fixed paraffin-embedded tissue blocks and then deparaffinized in xylene and rehydrated in successive washes of ethanol. The sections were then heated in a microwave oven at medium power for 2 minutes in citrate buffer (pH 6.0) for heat-induced epitope retrieval. The sections were subjected to blockade of endogenous peroxidase activity and nonspecific binding of the primary antibody, and then target protein localization with the first antibody and visualization with the secondary antibody and color reaction as described above. Subsequently, the slides were incubated with rabbit monoclonal antibody anti-TFAP2A (Cell Signaling Technology; 1:100 dilution), anti-VEGF (Cell Signaling Technology; 1:50 dilution), anti-PEDF (Cell Signaling Technology; 1:50 dilution), anti-HIF-1α (Cell Signaling Technology; 1:25 dilution), and anti-CD34 (Life Technology; 1:100) for overnight at 4°C in a moist chamber. The slides were sequentially incubated with a secondary antibody and stained with 3,3′-diaminobenzidine. Finally, the sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted. A negative control was obtained by replacing the primary antibody with PBS. Known immunostaining positive slides were used as positive controls.

Stained tumor cells and paraffin sections were reviewed and scored using a light microscope by a pathologist blinded to the treatment group. Positivity of the stained tumor cells on cover slips and paraffin sections were defined by staining intensity and % of tumor cells; the staining intensity of protein expression was classified semiquantitatively into negative and weak, moderate, and strongly

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Table 1. Clinicopathologic characteristics and TFAP2A expression of patients with nasopharyngeal carcinoma

Immunostaining

The 4-μm sections were cut from formalin-fixed paraffin-embedded tissue blocks and then deparaffinized in xylene and rehydrated in successive washes of ethanol. The sections were then heated in a microwave oven at medium power for 2 minutes in citrate buffer (pH 6.0) for heat-induced epitope retrieval. The sections were subjected to blockade of endogenous peroxidase activity and nonspecific binding of the primary antibody, and then target protein localization with the first antibody and visualization with the secondary antibody and color reaction as described above. Subsequently, the slides were incubated with rabbit monoclonal antibody anti-TFAP2A (Cell Signaling Technology; 1:100 dilution), anti-VEGF (Cell Signaling Technology; 1:50 dilution), anti-PEDF (Cell Signaling Technology; 1:50 dilution), anti-HIF-1α (Cell Signaling Technology; 1:25 dilution), and anti-CD34 (Life Technology; 1:100) for overnight at 4°C in a moist chamber. The slides were sequentially incubated with a secondary antibody and stained with 3,3′-diaminobenzidine. Finally, the sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted. A negative control was obtained by replacing the primary antibody with PBS. Known immunostaining positive slides were used as positive controls.

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positive (0, +, ++, and ++++, respectively). In our study, gene expression was considered positive if the staining intensity was moderate or strong and the percentage of positive stained cells were >25%. However, the density of microvessels staining positive for CD34 was defined as positive at 400 power of a microscope field.

**Cell viability assay**

Cell viability was determined by a MTT assay (Roche Diagnosis). Briefly, cells plated in 96-well plates (2,000 cells/well) were treated with siRNA of TFAP2A and/or HIF-1α at the indicated doses. At 48 hours after treatment, cell viability was determined.

**Determination of VEGF and PEDF production by ELISA**

The nasopharyngeal carcinoma cells were seeded in 96-well plates and treated with siRNAs of TFAP2A and HIF-1α at 100 nmol/L for 48 hours. VEGF and PEDF levels in cell culture media were quantified using a VEGF Immunoassay Kit (968962; R&D Systems) and a Chemikine PEDF ELISA Kit (CYT420; Chemikine) according to the manufacturer’s protocols.

**Immunofluorescence and confocal microscopy**

Cells were fixed with 4% paraformaldehyde (w/v) for 20 minutes, quenched for 20 minutes with 50 mmol/L NH4Cl in PBS and permeabilized with 0.2% (w/v) saponin in PBS for 20 minutes. The saturation step was performed for 20 minutes in PBS containing 1% BSA and 0.2% saponin (w/v). Cells were then incubated overnight with the primary TFAP2A and HIF-1α antibodies diluted in PBS containing 1% BSA and 0.2% saponin. After PBS washings, cells were incubated for 2 hours with secondary fluorescein isothiocyanate or tetra-methyl rhodamine isothiocyanate–conjugated antibodies. TFAP2A and HIF-1α protein localization was assessed using a Leica confocal microscopy (Model TCS-NT). Files of microphotographs were processed with the Adobe Photoshop 5.0 software.

**Co-immunoprecipitation assays**

The nuclei protein extracts were prepared and incubated with the antibodies for TFAP2A and HIF-1α for 24 hours at 4°C. Then, the sepharose-conjugated protein-A/G beads (Santa Cruz Biotech) were added and the mixture was incubated at 4°C for another 12 hours. After extensive washing with radioimmunoprecipitation assay buffer, the beads were boiled. The precipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes for Western blot analysis.

**Preparation of DOTAP cholesterol nanoparticles and encapsulation of siRNAs**

DOTAP-cholesterol was purchased from Avanti Polar-lipids Inc. High-performance liquid chromatography (HPLC)-grade chloroform was obtained from Sigma Chemical Co. The DOTAP-cholesterol nanoparticles were prepared by an EmulsiFlex-B3 high-pressure homogenizer (HPH; Avestin Inc.). In brief, the nanoparticles were prepared from a mixture of 2 lipids: cholesterol and DOTAP, at the molar ratio of 1:1. The lipids were dissolved in 15 mL HPLC-grade chloroform in a round bottom flask and then dried under nitrogen gas and overnight vacuum. The resulting films of the lipids were hydrated in deionized water to give a final concentration of 10 mmol/L. The lipid dispersions were warmed and mixed at 50°C for 45 minutes by rotation, followed by warming again at 35°C for another 10 minutes. The resultant dispersion was stored at room temperature for 3 hours before it was transferred into a scintillation vial and warmed again at 50°C for 10 minutes. The final lipid dispersion was homogenized using HPH at 20,000 psi for 5 cycles. Each time, 2.5 mL of lipid dispersion was subjected to homogenization and the resultant DOTAP-cholesterol nanoparticles were collected in another scintillation vial. The DOTAP-cholesterol nanoparticles were kept at room temperature for 1 hour before overnight storage at 4°C. TFAP2A or HIF-1α siRNAs duplexes were purchased from Santa Cruz Biotechnology and encapsulated using the DOTAP-cholesterol nanoparticles.

**Tumor growth inhibition by TFAP2A siRNA in a xenograft mouse model**

To determine the effect of TFAP2A siRNA on nasopharyngeal carcinoma cell growth in a xenograft model, CNE2 cells (2 × 10⁶) were inoculated subcutaneously into the flank of the nude mice. Once palpable tumors were observed, tumor volume measurements were taken every 3 days using calipers. The tumor volume was calculated using the following formula: \( V = \frac{(\text{width} \times \text{length})}{2} \). Body weights were also recorded. Two weeks after injection, the mice were randomized into 2 groups (7 mice/group). Group 1 received injection with In Vivo Ready nonspecific siRNA, and group 2 with In Vivo Ready TFAP2A siRNA. DOTAP-cholesterol nanoparticles encapsulated siRNA duplexes were injected into the tumors using insulin syringes at a concentration of 10 μg of siRNA/50 mm³ of tumor volume. All 2 groups were treated twice a week for 4 weeks. Upon termination, tumors were harvested and weighted. Animal experiments were approved by the Animal Research Committee of Sun Yat-sen University Cancer Center and were performed in accordance with established guidelines.

**Evaluation of TFAP2A knockdown and angiogenesis factors in xenograft tumor tissues**

Tumor tissues from the above treated animals were collected and placed in 10% formalin and embedded in paraffin for below analysis. The sections were stained with hematoxylin and eosin according to standard immunohistochemical procedures. Knockdown of TFAP2A by siRNA was determined by immunohistochemical staining with anti-TFAP2A antibody. To assess the effect of TFAP2A siRNA in vivo on angiogenesis factor VEGF, PEDF, and CD34, the embedded tissues were stained using anti-PEDF, VEGF, or CD34 antibody to determine the expression of angiogenic factors. A negative control was obtained by replacing the primary antibody with a normal rabbit or mouse immunoglobulin G. The immunoreactivity positive cells from each of the
differently treated tumor tissue sections were measured at × 200 magnification using a light microscope. The amount of proteins was analyzed by integral optical density using IPP (Image Plus Pro 6.0, Media Cybernetics, Inc., Rockville, MD).

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package (standard version 16.0; SPSS). Strong TFAP2A immunoreactivity was assessed for the association with clinicopathologic variables such as gender, age, and pathologic TNM stage by using the Pearson χ² test. Survival curves were calculated from the date of surgery to the time of death related to nasopharyngeal carcinoma or to the last follow-up observation. Kaplan–Meier curves were calculated for each relevant variable and for TFAP2A expression; differences in survival times among patient subgroups were analyzed by the log-rank test. Univariate and multivariate analyses were done with the Cox proportional hazard regression model to determine the associations between clinicopathologic variables and cancer-related mortality. First, we analyzed the associations between death and possible prognostic factors, including TFAP2A expression, gender, clinical stage, metastasis, T stage, and N stage, taking into consideration one factor at a time. Second, multivariate analysis was applied on forward (stepwise) procedures.

Results

TFAP2A is highly expressed in nasopharyngeal carcinoma cell lines and tumor tissues

We first examined the expression of TFAP2A at mRNA and protein levels in 8 human nasopharyngeal carcinoma cell lines and an immortalized normal human nasopharyngeal epithelial cell line (NP69) by reverse transcription-PCR (RT-PCR) and Western blot analysis, respectively. The TFAP2A mRNA levels were upregulated in 7 nasopharyngeal carcinoma tumor cell lines (CNE1, CNE2, S18, HONE1, SUNE1, 5-8F, 6-10B) by comparison with the expression of TFAP2A mRNA in normal cell line NP69 (Fig. 1A). Similarly, TFAP2A proteins were also highly expressed in 6 nasopharyngeal carcinoma cell lines (CNE1, CNE2, S18, S26, SUNE1, 5-8F). By contrast, TFAP2A protein was not detected in NP69 cells (Fig. 1B).

We also tested the expression of TFAP2A protein and its cellular localization in nasopharyngeal carcinoma cells by immunocytochemical staining. Consistent with the results from RT-PCR and Western blot analysis, the high levels of TFAP2A mRNA were observed in nasopharyngeal carcinoma cell lines. The expression of TFAP2A protein was detected in the nasopharyngeal carcinoma cell lines and the matched adjacent nasopharyngeal tissues. Magnification, ×200.
TFAP2A Regulates Nasopharyngeal Carcinoma Growth and Survival

TFAP2A proteins were detected in 6 nasopharyngeal carcinoma cell lines (CNE1, CNE2, SUNE1, S18, S26, S-8F). The HONE1 and 6-10B cells had weak staining of TFAP2A. Moreover, the results showed that the staining of TFAP2A proteins localized in the cell nuclei. By contrast, the normal nasopharyngeal cell line (NP69) had a relatively low expression of endogenous TFAP2A (Fig. 1C).

To further confirm the high expression of TFAP2A in nasopharyngeal carcinoma cells and to investigate the clinicopathologic significance of TFAP2A expression, we also analyzed the expression of TFAP2A in tumor tissues from patients with nasopharyngeal carcinoma by immunohistochemical staining assay. TFAP2A-positive staining was observed in the nucleus of nasopharyngeal carcinoma cells in tumor tissues, but staining was negative in any of the cells in adjacent nontumor tissue samples surrounding tumors (Fig. 1D). These results confirm the in vitro findings above and suggest that high levels TFAP2A may be a potential biomarker for nasopharyngeal carcinomas.

TFAP2A expression is associated with clinicopathologic characteristics

To gain further insight into the prognostic value of TFAP2A expression in patients with nasopharyngeal carcinoma, paraffin-embedded tissue sections (n = 179) with histopathologically confirmed nasopharyngeal carcinoma were examined using immunohistochemistry. The levels of TFAP2A immunoreactivity varied between the tumor tissue samples and the adjacent nontumor tissue samples. High positive TFAP2A expression was localized to the nuclei in 97 (54.2%) of the resected tumor tissue samples, whereas the remaining 82 cases (45.8%) displayed low levels nuclei TFAP2A expression (Table 1). Immunohistochemical determination of TFAP2A levels was also statistically analyzed to identify its association with the clinicopathologic features of nasopharyngeal carcinoma. TFAP2A expression was significantly correlated with gender (P < 0.001), T classification (P = 0.002), N stage (P = 0.001), clinical stage (P < 0.001), and distant metastasis (P < 0.001). However, there was no significant correlation between TFAP2A expression and age and recurrence (P = 0.925 and P = 0.516) (Supplementary Table S1). Spearman correlation analysis also revealed that TFAP2A expression levels were positively correlated with clinical stage (r = 0.286; P < 0.001), T classification (r = 0.277; P < 0.001), N classification (r = 0.248; P < 0.001), HIF-1α (r = 0.512; P < 0.001), and metastasis (r = 0.294; P < 0.001; Supplementary Table S2). Our data indicate that high TFAP2A expression significantly correlated with advanced tumor stage, local invasion, and clinical progression.

TFAP2A overexpression is associated with poor prognosis for patients with nasopharyngeal carcinoma

To investigate the biologic and clinicopathologic significance of TFAP2A in nasopharyngeal carcinoma carcinogenesis, we carried out immunohistochemical staining analysis in tumor tissues from the patients with nasopharyngeal carcinoma. The median survival time of the 179 patients with nasopharyngeal carcinoma was 54.6 months (range 12–128 months). The overall survival rate in the low TFAP2A expression group was significantly improved compared with the group with high TFAP2A expression (P < 0.001, Fig. 2A). In addition, the high HIF-1α expression group had significantly low rate of overall survival than the group with low HIF-1α expression (P = 0.04, Fig. 2B). Moreover, the group with low expression of both TFAP2A and HIF-1α had high overall survival rate than the group with high expression of both TFAP2A and HIF-1α (P = 0.008; Fig. 2C).

We further evaluated the potential correlation between expression of TFAP2A and HIF-1α in our nasopharyngeal carcinoma cohort. The results from immunohistochemical analysis showed a positive correlation between expression of TFAP2A and HIF-1α in nasopharyngeal carcinoma (Fig. 2D and E and Table S2). For the 97 nasopharyngeal carcinoma cases with high expression of TFAP2A, an average of 69.7% of the carcinoma cells stained positive with HIF-1α protein; the percentage was significantly higher than that (30.3%) in the remaining 82 nasopharyngeal carcinomas with low expression of TFAP2A (P < 0.001; Fig. 2D).

TFAP2A knockdown inhibits nasopharyngeal carcinoma cell growth by targeting HIF-1α–mediated VEGF/PEDF signaling

To determine the role TFAP2A in regulating the growth of nasopharyngeal carcinoma cells, we examined the effect of TFAP2A knockdown by DOTAP–cholesterol-based TFAP2A siRNA nanoparticles (si-TFAP2A) on cell viability in various kinds of human nasopharyngeal carcinoma cell lines, including high differentiated cell line CNE1, low differentiated cell line CNE2, and its clones S18 and S26, by a MTT analysis. The results showed that knockdown of TFAP2A expression by TFAP2A siRNA (si-TFAP2A, 100 nmol/L) for 72 hours significantly inhibited cell viability as compared with the transfection with the nonspecific scramble siRNA (si-NS; 100 nmol/L) and mock control groups, resulting in a 17% to 55% inhibition in cell viability in the 4 kinds of nasopharyngeal carcinoma cell lines (Fig. 3A).

Because TFAP2A expression positively correlates with HIF-1α levels in nasopharyngeal carcinomas and the upregulated HIF-1α can promote the expression of VEGF genes, we next determined the effect of TFAP2A knockdown on the expression of VEGF and PEDF at mRNA (Fig. 3B) and protein levels (Fig. 3C) in nasopharyngeal carcinoma cells by RT-PCR and Western blot analysis, and on the release of VEGF and PEDF proteins in cell culture media by ELISA in CNE2 cells (Fig. 3D). Transfection with TFAP2A siRNA (100 nmol/L) dramatically inhibited the expression of VEGF proteins, but did not significantly decrease the levels of PEDF protein expression (Fig. 3C). Similarly, treatment of nasopharyngeal carcinoma cells with TFAP2A siRNA (100 nmol/L) also significantly inhibited the release of VEGF protein, but did not affect the levels of PEDF in cell culture media, resulting in a significant reduction of the ratio of VEGF/PEDF (Fig. 3D). Knockdown of TFAP2A expression...
by si-TFAP2A (100 nmol/L) also suppressed VEGF expression at mRNA levels (Fig. 3B), but did not affect the levels of PEDF and HIF-1α. These results indicate that TFAP2A might also regulate tumor cell growth through the regulation of VEGF expression and the ratio of VEGF/PEDF in nasopharyngeal carcinoma cells.

**TFAP2A interacts with HIF-1α in nasopharyngeal carcinoma cells**

Because human VEGF promoter contains the binding sites for TFAP2A and HIF-1α, we next determined whether there was an association between these 2 proteins in nasopharyngeal carcinoma cells. We first analyzed the colocalization of TFAP2A with HIF-1α in CNE2 cells by immunofluorescence analysis. The cells were treated with CoCl₂ (200 μmol/L) or cultured in a modulator incubator chamber with 1% O2 for 24 hours to induce hypoxia status, and then incubated with an anti-HIF-1α or anti-TFAP2A antibody and followed by secondary antibodies conjugated to rhodamine and fluorescein isothiocyanate, respectively. As shown in Fig. 4A, both HIF-1α (red) and TFAP2A (green) staining were detected in cell nucleus and cytoplasm, but most staining were observed in cell nucleus. The colocalization analysis also showed that HIF-1α and TFAP2A had the same subcellular localization in nasopharyngeal carcinoma cells.

We also analyzed the interaction between the TFAP2A and HIF-1α proteins by immunoprecipitation assay. The nucleus proteins from CNE2 cells treated with 200 μmol/L CoCl₂ or cultured in a modulator incubator chamber with 1% O2 were incubated with anti-HIF-1α or anti-TFAP2A antibody, respectively, and then immunoprecipitated with protein A/G-agarose beads. The immune complexes were analyzed by Western blotting assay. The results showed that HIF-1α protein was presented in the immune complexes precipitated by the antibodies against TFAP2A, and also TFAP2A protein was detected in the immune complexes precipitated by the antibodies against HIF-1α (Fig. 4B).

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**Figure 2.** Overexpression of TFAP2A and HIF-1α is associated with poor prognosis of patients with nasopharyngeal carcinoma. A–C, Kaplan–Meier analysis of survival of patients with nasopharyngeal carcinoma expressing TFAP2AA, HIF-1α (B), and both proteins (C) (P < 0.001, log-rank test). D, the association between TFAP2A overexpression and HIF-1α protein levels in the same tumor tissues from patients with nasopharyngeal carcinoma. E, representative images of TFAP2A and HIF-1α protein expression in nasopharyngeal carcinoma tumor tissues by immunohistochemistry. Magnification, ×200.
These results suggest a physical interaction of HIF-1α with TFAP2A proteins in nasopharyngeal carcinoma cells.

To further confirm the role of TFAP2A in regulating the HIF-1α–mediated cell growth in nasopharyngeal carcinoma cells, we next inhibited the expression of HIF-1α protein in CNE2 cells by pretreating cells with the DOTAP-cholesterol–based TFAP2A siRNA nanoparticles (si-TFAP2A). At 72 hours after transfection, the effect of TFAP2A knockdown on cell viability (A) and the expression of TFAP2A, VEGF, PEDF, and HIF-1α mRNAs (B), and proteins (C) were detected by MTT, RT-PCR, and Western blot analysis, respectively. The amounts of VEGF and PEDF secretion in cell culture media were determined by ELISA, and the ratio of VEGF-to-PEDF was analyzed D. The cells treated with nonspecific scramble siRNA (si-NS) were used as the control groups. The data are presented as mean ± SD of 3 tests. *P < 0.05, significant differences between the si-TFAP2A or si-HIF-1-treated groups and the control siRNA groups.

TFAP2A knockdown inhibits tumor growth and reduces VEGF expression in a xenograft mouse model

The significant association of TFAP2A expression with nasopharyngeal carcinoma cell survival and clinical outcome led us to further verify the essential role of TFAP2A in regulating nasopharyngeal carcinoma growth in vivo. The CNE2 cells were injected subcutaneously into the flank of nude mice. After 2 weeks, visible tumors developed at injection sites (mean tumor volume = 150 mm³). The DOTAP-cholesterol nanoparticles encapsulated TFAP2A siRNA (si-TFAP2A) were then intratumorally injected 6 times at a regular interval of 4 days for up to 27 days. Treatment mice with DOTAP-cholesterol–based TFAP2A siRNA nanoparticles (si-TFAP2A) significantly inhibited the tumor volume as compared with the treatment with nonspecific control siRNA (si-NS; Fig. 5A). Xenografts were harvested and the weights of the tumors were also analyzed at 27 days after treatment. As shown in Fig. 5B and C, TFAP2A siRNA (si-TFAP2A) treatment
also significantly inhibited tumor growth and the weights of tumors. We also analyzed the levels of TFAP2A expression in tumors by immunochemistry and showed a significant inhibition of TFAP2A expression by TFAP2A siRNA (si-TFAP2A; Fig. 5D). To elucidate the potential mechanisms involved in tumor growth inhibition by TFAP2A siRNA in vivo, we carried out a systematic analysis to test the effect of TFAP2A siRNA on angiogenesis factors. We analyzed the expression of VEGF and PEDF in tumors by immunohistochemical staining analysis (Fig. 5D). Consistent with the in vitro data, TFAP2A knockdown by the DOTAP-cholesterol-based siRNA nanoparticles (si-TFAP2A) significantly inhibited VEGF expression, but did not change the PEDF protein levels by comparison with those treated with nonspecific scrambled siRNA (si-NC; Fig. 5D). We also examined the effect of TFAP2A knockdown on CD34 expression and microvessel density (MVD), an important indicator for angiogenesis, which was important for the tumor growth. Knockdown of TFAP2A expression by si-TFAP2A in the nasopharyngeal carcinoma nude mice significantly reduced MVD values of the tumors as compared with the control group (Fig. 5E). These in vivo results were consistent with those observed in vitro and confirmed the regulation of TFAP2A in nasopharyngeal carcinoma tumor growth and angiogenesis.

**Discussion**

In this study, we detected the high expression of TFAP2A in nasopharyngeal carcinoma tumor tissues and cell lines, which is correlated with nasopharyngeal carcinoma growth, survival, and HIF-1α expression. Silence of TFAP2A expression inhibited cell viability in nasopharyngeal carcinoma cell lines and suppressed tumor growth and microvessel density in vivo. We also found that TFAP2A interacted with HIF-1α to regulate cell growth and VEGF expression. We conclude that TFAP2A plays a potential oncogenic role in nasopharyngeal carcinomas through the mechanism by which TFAP2A regulate the HIF-1α-mediated VEGF expression and secretion, thereby promotes nasopharyngeal carcinoma cell proliferation, tumor growth, and angiogenesis. Our findings therefore provide new insights into...
understanding the mechanisms of nasopharyngeal carcinoma tumorigenesis and exploring the potential therapeutic targets for nasopharyngeal carcinomas.

TFAP2A plays a key role in regulating cell differentiation, proliferation, and apoptosis. A number of genes involved in cell growth, cell shape, cell movement, cell fate, and cell communication are regulated by AP-2. Therefore, the loss or upregulation of TFAP2A expression may result in differentiation, proliferation, and eventually, tumorigenesis, tumor progression, invasion, or metastasis. Previously, low or lost expression of TFAP2A has been reported in breast cancer, colon cancer, pancreatic cancer, bladder cancer, ovarian cancer, prostate cancer, gastric cancer, melanoma, glioma, and some other malignant tumors. And recently, some reports suggested AP-2 may be a tumor suppressor gene. However, in other malignant tumor, such as the head and neck squamous cell carcinoma, the expression of TFAP2A was upregulation. But to date, the biologic roles and clinical significance of TFAP2A as well as its precise mechanisms of actions in nasopharyngeal carcinoma tumorigenesis and progression have not yet been reported. Our results in this study have demonstrated a potential oncogenic role of TFAP2A in human nasopharyngeal carcinoma. We showed high expression of TFAP2A protein in both nasopharyngeal carcinoma cells and nasopharyngeal carcinoma tumor samples. Interestingly, clinicopathologic data from our study have also showed that patients with nasopharyngeal carcinoma, which highly expressed TFAP2A proteins, had shorter survival periods than those with TFAP2A lowly expressed tumors. In addition, multivariate analysis showed that the strong positivity of TFAP2A is a poorly independent prognostic factor. Moreover, we demonstrated that silence of TFAP2A expression inhibited the proliferation of nasopharyngeal carcinoma cells in vitro and in vivo.

Therefore, it seems that TFAP2A may be a potential oncogene involved in nasopharyngeal carcinoma tumorigenesis. These observations do not support a previous hypothesis that TFAP2A might be a tumor suppressor. To the best of our knowledge, it is the first time to report that TFAP2A plays an oncogenic role in nasopharyngeal carcinomas.

We also elucidated the molecular mechanisms by which TFAP2A regulates nasopharyngeal carcinoma cell growth and found that TFAP2A functions by targeting the HIF-1 and VEGF/PEDF pathway in nasopharyngeal carcinoma cells. The HIF-1α pathway exists as a critical step in carcinogenesis, which is linked to several oncogenic and tumor
suppressor gene pathways in cancers. As a transcription factor, HIF-1α heterodimerizes with the constitutively expressed HIF-1β subunit, and they activate the expression of a number of genes, including VEGF, to take part in tumor angiogenesis and tumor cell proliferation and invasion. By binding to the hypoxia-responsive elements on VEGF promoter, HIF-1 leads to the transcriptional activation of the VEGF gene (42). VEGF plays an important role in tumor angiogenesis, tumor cell growth, and survival. PEDF counterbalances the effect of VEGF. PEDF and VEGF have an inverse relationship with each other, especially in cancer in which low levels of PEDF are associated with the increased incidence of metastasis and poor malignancy prognosis (43). PEDF suppressed proliferation and induced apoptosis of the tumor cell (44). The increased ratio of VEGF/PEDF is required for angiogenesis and tumor growth. In this study, we found that HIF-1α interacted with TFAP2A to regulate VEGF expression in nasopharyngeal carcinoma cells, and knockdown of TFAP2A by siRNA inhibited the ratio of the VEGF/PEDF. Thus, our results show that HIF-1α signaling contributes, at least in part, to TFAP2A-mediated nasopharyngeal carcinoma carcinogenesis.

Moreover, we demonstrated that knockdown of TFAP2A by DOTAP-cholesterol–based TFAP2A siRNA nanoparticles markedly inhibited tumor growth in a nasopharyngeal carcinoma xenograft mouse model at least partially through the modulation of VEGF signaling. Immunohistochemistry analysis for the xenograft tumors showed that TFAP2A knockdown inhibited the angiogenesis-related protein factor VEGF. Thus, these in vivo studies confirmed the tumor inhibition effects by TFAP2A knockdown in vitro and provide a rationale for pharmacologic investigation of TFAP2A as a novel therapeutic target in nasopharyngeal carcinoma. The detailed mechanisms by which TFAP2A regulates VEGF remain to be elucidated.

In conclusion, our study demonstrate that high TFAP2A expression independently predicts worse overall survival in patients with nasopharyngeal carcinoma and TFAP2A plays a critical role in regulating human nasopharyngeal carcinoma carcinogenesis by targeting HIF-1α and VEGF/PEDF-dependent pathways. These results indicate that TFAP2A overexpression in patients with nasopharyngeal carcinoma is associated with a poor prognosis and could be a potential prognostic biomarker and therapeutic target for nasopharyngeal carcinoma treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: D. Shi, W. Deng
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Shi, L. Fu, W. Deng
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