AHNAK Downregulation Contributes to Nasopharyngeal Carcinoma Tumorigenesis and Metastasis

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Abstract

Purpose

Nasopharyngeal carcinoma (NPC) is an aggressive head and neck disease with a high incidence of distant metastases. Enlargeosomes are cytoplasmic organelles marked by, desmoyokin/AHNAK. The purpose of this study was to evaluate the expression of AHNAK in NPC and its effect on enlargeosomes, and to investigate the correlation between AHNAK expression levels and clinical NPC patient characteristics.

Methods

Primary nasopharyngeal carcinoma (NPC) and NPC specimens were evaluated by analyzing public data, immunohistochemistry. Systematic in vitro and in vivo experiments were performed using different NPC-derived cell lines and mouse models.

Results

In this study, we detected AHNAK and Annexin A2(ANXA2), a protein coating the surface of enlargeosomes, in NPC samples. We found that AHNAK was down-regulated, whereas Annexin A2 was upregulated in human NPC tissues. Down-regulation of AHNAK was associated with poor overall survival in NPC patients. Upregulation of Annexin A2 was associated with lymph node metastasis and distant metastasis in NPC patients. Functional studies confirmed that silencing of AHNAK enhanced the growth, invasion, and metastatic properties of NPC cells both in vitro and in vivo. In terms of mechanism, loss of AHNAK led to increase of annexin A2 protein level in NPC cells. Silencing ANXA2 restored the migrative and invasive ability of NPC cells upon loss of AHNAK. Moreover, transcription factor FOSL1-mediated transcriptional repression was responsible for the low-expression of AHNAK by recruiting EZH2.

Conclusion

Here, we report AHNAK as a tumor suppressor in NPC, which may act through annexin A2 oncogenic signaling in enlargeosome, with potential implications for novel approaches to NPC treatment.

Introduction

NPC originates from the nasopharyngeal mucosal epithelium. Nearly 70% of new cases occur in southern China and Southeast Asia(1, 2). The main pathogenic factors are Epstein-Barr virus (EBV) infection, genetic susceptibility and environmental factors(3). Because of the hidden anatomical position of the nasopharynx, the disease is not easy to detect early. The early detection of NPC is usually due to cervical lymph node metastasis(4), and its 5-year survival rate is 80%. Recurrence and distant metastasis are the main causes of death(5, 6).
AHNAK (Desmoyokin) is a macromolecular scaffold protein that regulates calcium channel homeostasis, cytoskeletal structure, muscle regeneration and signaling(7–9). AHNAK acts as a biomarker for enlargeosomes and is translocated from the cytoplasm to cell membrane when the cell is stimulated(10). An essential requirement for plasma membrane repair is calcium-regulated extracellular leakage, controlled by a selection of proteins that coordinate membrane resealing. One exocytotic involved in membrane fusion is the enlargeosomes, which features the large scaffolding protein AHNAK, which is transferred to the plasma membrane immediately after membrane damage. (11). Lourusso A et al. demonstrated that Annexin A2 is localized on the membrane surface of enlargeosomes, which is essential for the regulation of exocytosis action(12). The c-terminal domain of AHNAK is a binding target for the tetramer S100A10 – Annexin A2, and annexin A2 binds to acidic phospholipids at the plasma membrane in a Ca^{2+}-dependent manner, pulling the protein AHNAK toward the plasma membrane and initiating membrane repair(12–16). So, desmoyokin /annexin A2(d/A) plays a role in enlargeosome by regulating exocytosis and changing the cell membrane area, which is conducive to cell deformation and movement(12, 17). Previous study shows that the NGF-induced exocytosis of enlargeosomes sustained neurite outgrowth(18–20).

Recent interests focused on the role of AHNAK in cancer, but the evidence for its role in cancer is conflicting. On one hand, AHNAK acts as a tumor suppressor by inhibiting ERK phosphorylation and ROCK1 expression, thereby suppressing proliferation and migration of colorectal cancer (CRC) cells(21). AHNAK negatively regulates triple-negative breast cancer (TNBC) cells proliferation and metastasis through the Wnt/β-catenin pathway and AKT/MAPK signaling pathway(22). AHNAK could suppress the proliferation and invasion through inducing cell apoptosis or inhibiting epithelial-mesenchymal transition in gastric cancer(23, 24). AHNAK protein is lower expressed in human lung adenocarcinoma than in non-tumor lung tissues(25). Conversely, AHNAK is often overexpressed in many cancers, such as pancreatic ductal adenocarcinoma (PDCA) and melanoma, which could promote cancer cell metastasis(26). BRD4 promotes the regulation of AHNAK transcription, which promotes the migration and invasion of castration-resistant prostate cancer cells through its ability to form pseudopodia(27). AHNAK is overexpressed in human breast cancer and causes cells to produce extracellular vesicles, which are released to destroy the matrix through the surrounding fibroblasts and promote fibroblast migration(28). Interestingly, some studies showed that AHNAK promote the metastasis of liver cancer cells(29), while some studies showed that AHNAK inhibit the invasion and metastasis of liver cancer as a TGF-β signaling inhibitor(30). This discrepancy regarding the role of AHNAK in cancer may result from differences in the tumor microenvironment or in the strength or intensity of AHNAK signaling. However, the role and mechanism of AHNAK in NPC metastasis remain unclear.

The transcription factor FOSL1 is one of the molecules of the transcriptional complex AP1, which is closely associated with cell differentiation, metabolism, tumorigenesis, etc(31). According to the literature, FOSL1 can be detected in esophageal(32), bladder tumor(33), malignant glioma(34, 35) and breast carcinoma(36–38) samples, where high expression is associated with tumor grade, metastasis and prognosis. EGFR-PKM2 signaling induces the expression of FOSL1(39), long non-coding RNA
In our study, we investigated the role of the scaffold protein AHNAK in nasopharyngeal carcinogenesis. Our in vivo and in vitro studies confirmed that AHNAK mediated the growth and metastasis of NPC cells, possibly through the regulation of ANXA2 protein. And, FOSL1 inhibits the expression of AHNAK, in human NPC, loss of AHNAK is associated with poor survival.

**Materials And Methods**

**Data acquisition and analysis**

The mRNA sequencing data of 31 NPC and 10 normal nasopharyngeal tissue samples were downloaded from GEO database with clinicopathological data of patients.

**Tissue samples**

Expression levels of AHNAK and ANXA2 were measured using IHC in 150 human nasopharyngeal carcinogenesis tissues and 50 human nasopharyngeal inflammatory tissue that were collected after biopsy between 2005 and 2014 at the Second Xiangya Hospital of Central South University (Changsha, Hunan, China). The clinical profiles of all patients are listed in Supplementary Table S1. These cases were used to illustrate the correlation of AHNAK, ANXA2 expression and patients' survival outcomes. The study was authorized by Biomedical Research Ethics Committee of central south university, and all patients had provided informed consent.

**Immunohistochemistry and evaluation**

Antibodies for AHNAK (Santa, 390743) and Annexin A2 (Abcam, AB178677) were used in this study. After tissue sections were dewaxed and hydrated, specimens were repaired at high pressure in 1% EDTA buffer for 8 min to extract antigen and incubated at 3% H2O2 for 30 min at room temperature to block horseradish peroxidase. Then, tissue sections were incubated with anti-AHNAK and anti-Annexin A2 for 14h at 4°C. After washing with PBS 3 times, the tissue sections were incubated with enhancer for 15 min at room temperature and secondary antibody for 20 min, then washed 3 times with PBS before adding DAB solution. The cell nuclei of the sections were then counterstained with hematoxylin and sealed.

Total IHC score = staining area score × staining intensity score. Staining area: 0-10% (0 points), 11%-25% (1 point), 26%-50% (2 points), 51%-75% (3 points), 76%-100% (4 points). Staining intensity: no expression (0 points), weak expression (pale yellow, 1 point), moderate expression (yellow, 2 points), positive (brown, 3 points), strong expression (dark brown, 4 points). Therefore, the total theoretical score ranges from 0 to 16.
Cell lines and cell culture

The CNE1 cells, CNE2 cells, HK1 cells, HNE1 cells were maintained in our lab (6). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (44).

Generation of stable cell lines

CNE1 cells and HNE1 cells stably expressing control or AHNAK-specific shRNA. CNE1 cells and HNE1 cells were transfected with lentiviral supernatant, and selected using 5μg/ml puromycin 48h later for 7 days. Protein expression was observed by western blotting. The human AHNAK shRNA target sequences as follows: AHNAK shRNA #1: 5′- GCA CTT GAA GAT GCC CAA GAT T-3′; AHNAK shRNA #2: 5′- GCA GCT CTG AAG TGG TTC TGA -3′; AHNAK shRNA #3: CCC GTG AAG TCT TCA GCT.

Protein extraction and western blotting

Cells were lysed in RIPA cell tissue lysate containing protease inhibitor cocktail (lysate: 100× Protease inhibitor cocktail=100:1) on ice for 30min, then centrifuged at 13000×g for 15min at 4°C. Collected the supernatants, protein expression was detected by primary (anti-AHNAK, anti- Annexin A2, anti-FOSL1, anti-EZH2) and secondary antibodies (45).

RNA extraction and qPCR analysis

Total RNA was extracted from the cells using Trizol (ambion, 15596018) following the manufacturer’s instruction. The cDNA was acquired using the reverse transcription kit (Thermo, K1622) was used as the template for PCR amplification. qPCR was performed on gene-specific primers for AHNAK, Annexin A2, FOSL1, EZH2 and GAPDH (as an internal control) with 2×Taq SYBR Green qPCR Premix (innovagene, SQ101) (46, 47). The primer sequences were as follows: AHNAK, forward5′ - ATG CGC CAG GGC TCA ACC T -3′ and reverse5′ - CGT GCC CCA ACG TTA AGC TT -3′; Annexin A2, forward5′ - TGA GCG GGA TGC TTT GAA C -3′ and reverse 5′ - ATC CTG TCT CGT TGC ATT GCT G-3′; FOSL1, forward5′ - CCG CCC TGT ACC TTG TAT CT-3′ and reverse 5′ - CTG CTG CTA CTC TTG CGA TG-3′; EZH2, forward5′ - GCC AGA CTG GGA AGA AAT CTG-3′ and reverse 5′ - TGT GTT GGA AAA TCC AAG TCA-3′.

Cell Counting Kit-8 (CCK-8)

Cells were accommodated in 96-well plates with a density at 1 × 10^3 cells/well and incubated 4 hours, followed by treating with 10 μl of CCK-8 (TargetMol, C0005) and incubating for 2 hours. The absorbance was detected by a microplate reader (Tecan Instruments, Switzerland) at 450 nm. Experiments were performed in five replicates (39).

Transwell migration and Matrigel invasion assays

In the transwell migration experiment, the upper chamber was inoculated with 200ul RMIP-1640 medium suspension containing 1×10^5 NPC cells, and the lower chamber was packed with 700μL RMIP-1640
medium comprising 15% FBS. After incubating for 18h at 37°C, the artificial basement membranes were
fixed with 4% methanol with 20 min, and then stained with 0.5% crystal violet and counted. For the matrix
invasion assay, the upper lumen was coated with Matrigel and incubated at 37°C for 1h, other steps were
performed as described previously(48).

**Confocal microscopy**

NPC cells seeded on coverslips in 6-well plates overnight were transfected for 48 hours, and fixed in 4%
paraformaldehyde. After 10 min of permeabilization with 0.1% Trion X-100, cells were blocked in 5
mg/mL BSA solution for 1 h. Then, cells were incubated with anti-AHNAK and anti- Annexin A2 at 4°C
overnight. After washing with PBS 3 times, the cells were incubated at 37°C in the dark with the
secondary antibody to 1 h, then sealed with anti-fluorescence quenching sealing liquid (with DAPI)(49).
The cells were photographed on the Operetta® High Content Imaging System as well as analyzed by
Harmony® High Content Image Analysis Software (USA).

**Xenografts**

4 - week - old BALB/c nude mice (Hunan SJA Laboratory Animal Co. Ltd) were injected with 5×10⁵ NPC
cells (n=10 mice per group) through tail vein for tumor metastasis. After 2 weeks, body weights were
measured every 3 days. 8 weeks later, lung and liver tissues were collected for standard histopathologic
study and metastatic assessment(50). All animal experiments were conformed to the Animal Care and
Use Committee's standard of Central South University (Changsha, Hunan, China).

**H&E staining**

Tissues were fixed with 4% paraformaldehyde and embedded with paraffin. Sections with a thickness of
4μm were attached to slides, dewaxed, hydrated, and stained with hematoxylin and eosin.

**Chromatin immunoprecipitation - qPCR (ChIP - qPCR)**

Cells were cross-linked with 1% formaldehyde and then burst with glycine. The cross-linked chromatin
was broken up with ultrasound and immunoprecipitated with antibodies specific for anti-FOSL1 (Abcam,
ab124722), EZH2 (Abcepta, AM1836A), H3K27me3 (Abclonal, A2363), H3K27ac (Abclonal, A7253). After
decrosslinking in a boiling water bath, purified short fragments of DNA were detected using AHNAK
promoter region primers and monitored by qPCR. The primer sequences of AHNAK-ChIP were as follows:
P1, forward5'-GCAGAGGAAGGCTGAGGTAG-3' and reverse5'- GAGGGAAGCCCAACTGTGA -3'; P2,
forward5' -CAGGCTGAGCAGGG AAGGA-3'and reverse 5' -TAAGGAGCGTGGAAGTGG-3'; P3, forward5' -
TCACTG ACTCACCCAAGT-3'and reverse 5' - AAGGACATGGGTGAAGAT-3'.

**Statistical analysis**

Pearson's chi-square test was used to analyze the relationship between AHNAK expression and
clinicopathological factors. Spearman's rank Correlation test was used to calculate the correlations
between AHNAK and Annexin A2 expression levels. The prognostic relevance of AHNAK and Annexin A2 in univariate analysis were calculated using Kaplan-Meier survival analysis. Data are presented as the means ± standard deviation (SD). Student's t-test and one-way analysis of variance were used to compare experiment data and to obtain their \( p \) values. The following significance values were indicated by asterisks: \( p < 0.05 (*) \), \( p < 0.01 (**) \), \( p < 0.001(***). \)

**Results**

**AHNAK is downregulated and Annexin A2 is upregulated in human NPC and correlates with poor survival**

To investigate the role of AHNAK in the development of NPC, we first analysed GEO database data GSE12452 to examine AHNAK expression in human NPC tissues. These results showed that the mRNA levels of AHNAK were lowly expressed in NPC tissues. In comparison, AHNAK expression was higher in the normal nasopharyngeal tissue (Fig. 1A). Downregulation of AHNAK expression in tumor tissues was confirmed by immunohistochemistry, consistent with previous reports, Annexin A2 was highly expressed in NPC by our study, and exists on cell membranes. (Figs. 1B - D). Then we investigated the relationship between the levels of AHNAK and Annexin A2 and the clinicopathologic status of NPC patients. Pearson \( \chi^2 \) test analysis showed that AHNAK expression of NPC cells did not correlate with sex, age, TNM and clinical stage (Table 1), Annexin A2 expression in NPC cells associated with lymph node metastasis as well as distant metastasis (Table 2). However, Kaplan–Meier analysis showed low expression of AHNAK in NPC tissues was significantly associated with reduced survival (Fig. 1E). The Annexin A2 expression level in NPC tissues wasn't associated with survival rate (Fig. 1F). However, the survival rate of patients with low AHNAK expression and high Annexin A2 expression was significantly lower than that of patients with high AHNAK expression and low Annexin A2 expression (Fig. 1G). These data suggest that reduced AHNAK expression is correlated with the development of NPC.
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<th>High (%)</th>
<th>P-value</th>
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<td>13(38.2)</td>
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<td>T3-4</td>
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The Fisher's exact test was used to analyze the relationship between expression level of AHNAK and age and gender. Chi-square was performed to assess the relationship between expression level of AHNAK and clinicopathological features.
Table 2. Correlation between expression level of Annexin A2 and clinicopathological characteristics in NPC

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<th>characteristics</th>
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<th>High (%)</th>
<th>P-value</th>
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<td>26</td>
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The Fisher’s exact test was used to analyze the relationship between expression level of Annexin A2 and age and gender. Chi-square was performed to assess the relationship between expression level of Annexin A2 and clinicopathological features.

AHNAK deficiency promotes growth and metastasis of NPC cells in vitro

The results above suggested that the expression of AHNAK in NPC was decreased. Therefore, we wondered whether AHNAK has a causal role in modulating NPC cell phenotypes. We first transiently transfected siRNA/AHNAK to knock down AHNAK expression in CNE1 and HNE1 cell lines expressing high level of AHNAK protein. QPCR and Western blotting were used to verify the knockdown efficiency...
(Figs. 2A and B). The abilities of AHNAK to affect NPC cells proliferation, migration and invasion were first tested in vitro. CCK-8 assays showed that the ability of cellular proliferation was significantly enhanced after knocked down AHNAK in CNE1 and HNE1 cells (Figs. 2C and D). The wound-healing experiments showed that inhibition of AHNAK obviously promoted the migration of CNE1 cells and HNE1 cells at 24 h (Fig. 2E). In agreement with this result, in a transwell migration assay, AHNAK depleted CNE1 and HNE1 cells migrated approximately four times as much as the control cells (Fig. 2F). This is consistent with the results that knockdown of AHNAK can effectively enhances the invasiveness of NPC cells (Fig. 2G). These results suggested that AHNAK deficiency promotes the proliferation, migration and invasion of NPC cell in vitro.

**AHNAK deficiency promotes growth and metastasis of NPC cells in vivo**

We also evaluated the role of AHNAK in NPC cell growth and metastasis in vivo by establishing a tumor metastasis model with nude mice. We established cell lines with stable knockdown of AHNAK and verified the efficiency of knockdown by QPCR and Western blotting. (Figs. 3A and B). We first injected the same number of CNE1 cells that stably expressed AHNAK shRNA or control scrambled shRNA into nude mice through the tail vein. The mice were monitored daily and their body weights were measured every 3 days. The results showed that AHNAK-depleted mice were significantly emaciated as revealed by the weight (Fig. 3C). In this xenografted metastasis model, knocking down AHNAK expression significantly promoted tumor metastasis to lungs and livers. As shown in Figs. 3D-G, CNE1/shRNA/AHNAK cells formed more and larger micrometastases than the control cells. These statistical results of the number of micrometastases under the microscope were shown in Fig. 2H-I. The results indicated that AHNAK deficiency enhanced NPC cell growth and metastasis in vivo.

**AHNAK promotes invasiveness through upregulation of Annexin A2 protein in NPC cells**

Given the evidence linking aberrant Annexin A2 expression to tumor metastasis, to investigate the mechanisms by which AHNAK controls metastasis in NPC, we examined the possibility whether AHNAK interacts with Annexin A2. Western blotting assays exposed that knocking down AHNAK expression in CNE1 and HNE1 cells significantly enhanced Annexin A2 expression (Fig. 4B), but relative expression of Annexin A2 mRNA was unchanged (Fig. 4A). Compared with the control group, silencing AHNAK expression in CNE1 and HNE1 cells greatly increased the levels of Annexin A2 by immunofluorescent staining, and obvious colocalization was observed (Fig. 4C). Then we tested whether Annexin A2 is involved in AHNAK-derived migratory and invasive behaviors of NPC cells. Results from wound-healing assay showed that silencing AHNAK significantly enhanced the migratory ability of NPC cells. However, silencing AHNAK showed only a limited effect on NPC cell migration when Annexin A2 was depleted (Fig. 5A). Similar results were observed in the invasion assays (Fig. 5B). Together, these results suggest that the inhibitory effect of AHNAK on NPC cells metastasis is dependent on Annexin A2.

**FOSL1 inhibits the transcriptional activation of AHNAK in NPC cell**
We analyzed ENCODE database data GSM1010756 and found that the transcription factor FOSL1 had binding peaks in the AHNAK promoter region (Fig. 6A, top). To investigate the mechanism of FOSL1 regulate AHNAK expression in NPC, we further verified whether FOSL1 interacts with the AHNAK promoter region. As expected, ChIP-qPCR experiments revealed a constitutive interaction between endogenous FOSL1 and AHNAK promoter region in CNE1 cells (Fig. 6A, bottom). Given the evidence that FOSL1 as a transcription factor is associated with nasopharyngeal carcinoma metastasis, we sought to examine whether FOSL1 is associated with the expression of AHNAK. To exploit the underlying mechanisms of AHNAK low expression in NPC, we first used lentiviral vector-based shRNA technology to stably knock down FOSL1 expression in HK1 and CNE2 cell lines expressing high levels of FOSL1 protein or stably transfected with NC/PLV-FOSL1 in CNE1 and HNE1 cell lines expressing low levels of FOSL1 protein. The knockdown and overexpression efficiency of FOSL1, as well as expression of AHNAK were confirmed by RT-qPCR and western blotting (Fig. 6B, C). We found that the decrease in FOSL1 was accompanied by an increase in both mRNA and protein expression of AHNAK, and vice versa. Immunofluorescence analysis also showed that increasing FOSL1 decreased AHNAK expression in the cytoplasm of NPC cells (Fig. 6D). We speculate that FOSL1 may exert its pro-tumor function by suppressing AHNAK expression. To further support this idea, we tested whether FOSL1 was involved in the migration and invasive behavior of AHNAK-derived nasopharyngeal carcinoma cells. The results of wound-healing experiments and migration assays showed that silencing FOSL1 greatly reduced the migratory ability of nasopharyngeal carcinoma cells. However, when FOSL1 was depleted, silencing AHNAK had rescued effect on NPC cell migration (Figure 6E and 6F, top). Similar results were observed in the invasion assay (Figure 6F, bottom). These findings are summarized in Figure 6G. In addition, AHNAK siRNA significantly abolished the FOSL1 shRNA-induced decrease in NPC cell adhesion (Figure 6H). In summary, knockdown of AHNAK reversed the FOSL1 shRNA-induced downregulation of migration and invasion in NPC cells. Taken together, these results suggest that AHNAK expression is dependent on FOSL1.

**AHNAK is repressed by FOSL1 recruited EZH2-mediated promoter H3 methylation in NPC cell**

To elucidate the way FOSL1 binds to the AHNAK promoter region, we learned from the literature that FOSL1 acts as a transcription factor that may recruit the cofactor histone methyltransferase EZH2 to the promoter region of downstream target genes, forming a transcriptional complex that regulates gene transcription by regulating the DNA methylation and acetylation levels of target genes(51, 52). Therefore, we speculate that FOSL1 may regulate the expression of AHNAK by recruiting EZH2 to the promoter region and regulating the methylation and acetylation levels of AHNAK. Then, by using siRNA to knock down EZH2, we found that both mRNA and protein expressions of AHNAK were increased (Fig. 7A, B). To investigate whether FOSL1 recruitment of EZH2 is essential for AHNAK expression, we then knocked down EZH2 in NPC cells stably transfected with NC/PLV-FOSL1. Overexpression of FOSL1 induces loss of AHNAK, which is significantly reversed by depletion of EZH2 in NPC cells (Figure 7C, D). Moreover, immunofluorescence showed that FOSL1 co-localized with EZH2 in the nucleus (Figure 7E). We investigated the possibility of whether FOSL1 interacts with EZH2. Surprisingly, co-immunoprecipitation (co-IP) experiments revealed a constitutive interaction between exogenous EZH2 and FOSL1 in 293FT cells (Figure 7F). ChIP-qPCR experiments showed a significant enrichment of EZH2 and H3K27me3 in the
AHNAK promoter region after overexpression of FOSL1 (Fig. 7G, Fig. 7H, left), but H3K27ac was not significantly enriched (Fig. 7H, right). To further support this idea, we tested whether EZH2 is involved in FOSL1-induced migration and invasive behavior of NPC cells. The results of wound-healing and migration assays showed that overexpression of FOSL1 greatly enhanced the migratory ability of NPC cells. However, when EZH2 was depleted, overexpression of FOSL1 had only a limited effect on the migration of NPC cells (Figure 7I, top of 7J). Similar results were observed in the invasion assay (Figure 7J, bottom). In conclusion, these results suggest that the effect of FOSL1 on AHNAK is dependent on EZH2. These results confirm that FOSL1 suppresses AHNAK expression in NPC cells by recruiting EZH2-mediated methylation of H3K27.

**Discussion**

Recent evidence suggests that AHNAK gene aberrant expression plays a key role in tumorigenesis. But the expression profile and role of AHNAK in NPC remain unclear. In the present study, we observed that reduced expression of AHNAK in NPC was not associated with gender, age, TNM or clinical stage. However, NPC patients with high levels of AHNAK expression had longer survival time compared to those with low expression levels. The results of in vitro and in vivo experiments showed that silencing AHNAK expression significantly promoted the growth, migration and invasion of NPC cells. The differences between clinical and in vitro, in vivo results regarding metastasis should be mainly due to the small number of samples and the uneven number of groups. Therefore, these results strongly demonstrate that AHNAK plays an important role in the development of NPC, suggesting a novel tumor suppressor effect of AHNAK in NPC.

Annexin A2 had an actin filament binding site and therefore contributed to the rearrangement of the actin cytoskeleton and regulates membrane transport processes such as endocytosis and cytokinesis(12, 53). Annexin A2 was overexpressed in a variety of malignancies and functions as a tumor-promoting factor that promotes angiogenesis, cell proliferation, migration and invasion(54–56), for instance, pancreatic cancer, ovarian cancer, hepatocellular carcinoma, glioma, etc(57–61). Annexin A2 was highly expressed in NPC and correlated with lymph node metastasis and distant metastasis, consistent with previous reports, but wasn't associated with survival rate in our study(62), this could be caused by the small sample size or the different scoring rules. We then correlated AHNAK expression on Annexin A2, and there was a direct negative correlation between the two groups (Pearson correlation coefficient of -0.172*). Patients with low AHNAK expression and high expression of Annexin A2 in NPC had shorter OS, exceeding the prognostic stratification of NPC patients based on high AHNAK expression alone. This striking association prompted us to develop models to test whether AHNAK could influence the biological behavior of NPC by regulating Annexin A2. We found that AHNAK affects the protein expression level of Annexin A2, but not the transcription. Thus, deletion of AHNAK may affect the synthesis or degradation pathway, or the transport of ANXA2 protein in NPC. Silencing of AHNAK expression in NPC cells was followed by immunofluorescence staining showing a significant increase for Annexin A2 levels, interestingly, a clear d/A-labeled apparent exocytosis was also visible (Fig. 4C).
AHNAK is well characterized in the regulation of various biological functions, such as participating in sarcolemma repair, structural rearrangement of cells, and migration(63, 64). The enlargosomes are involved in the process of plasma membrane expansion. Thus, in addition to regulating the assembly of microfilaments into stress fibers, AHNAK regulates the cytoplasmic membrane area by forming enlargeosomes, thus regulating cytoskeletal reorganization(10, 17, 65). A potential multiprotein repair complex includes S100A10, annexin A2, AHNAK, and actin found in enlargosomes. Annexin A2 is a centerpiece protein that participates in membrane transport events, such as intra/extra cellular interactions. The S100A10 dimer can orchestrate a pair of annexin A2 proteins that may bridge adjacent phospholipid membranes together in close proximity in a membrane fusion event. AHNAK is recruited to the plasma membrane by annexin A2 in complex with S100A10 to maintain the normal cellular architecture of epithelial cells. Previous study shows that AHNAK promotes the migration and invasion of castration-resistant prostate cancer cells through its ability to form pseudopodia(27). Therefore, we speculate whether AHNAK can alter exocytosis of the enlargeosome to change the area of the cell membrane and promote the invasion and metastasis of cancer cells by affecting the annexin A2 in NPC. More research is warranted to establish this conjecture.

Meanwhile, we investigated the mechanism of AHNAK low expression in NPC. Recently, the aberrant expression of AHNAK was shown to be regulated through transcription factors(27). FOSL1 has been reported to be a transcription factor that regulates the expression of proteins(31). In metastatic breast cancer, during IL-6 gene transcription, the methylase Ezh2, histone acetylases CBP, chromatin remodeling factor Brg1 and the kinase MSK1 were recruited by NF-κB p65/RelB and AP-1 Fra − 1/c-Jun members in the IL-6 gene promoter region(51). In colon cancer, EZH2 was highly expressed and could affect EMT by regulating ITGa2 transcription through H3K27me(52). In the present study, FOSL1 inhibited the transcriptional activity of AHNAK by recruiting EZH2 to methylate the AHNAK promoter region H3K27 in NPC. Blocking FOSL1-mediated malignant progression of AHNAK may offer a potential drug target for personalized treatment of patients with NPC.

Declarations

Ethical Approval

This study was endorsed by the institutional review board/ethics committee of central south university.

Competing interests

No potential conflicts of interest have been disclosed.

Authors' Contributions

Conception and design: B. Xiang, M. Yi

Development of methodology: X.-X. Lu, Y. Mei, B. Xiang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X.-X. Lu, Y. Mei, C.-M. Fan, P. Chen, X.-Y. Li, Z.-Y. Zeng, G.-Y. Li, W. Xiong, B. Xiang, M. Yi

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X.-X. Lu, Y. Mei, B. Xiang

Writing, review, and/or revision of the manuscript: X.-X. Lu, B. Xiang

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**Availability of data and materials**

The datasets used and/or analyzed in the current study are available upon reasonable request from the corresponding author.

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**Figures**
Figure 1

AHNAK downregulated and Annexin A2 upregulated in NPC suggest a poor clinical outcome. A, GEO data showed the expression of AHNAK mRNA was reduced in NPC (n=31) compared with normal (n=10). B, Representative images of IHC analysis for AHNAK and Annexin A2 in 106 cases of human NPC tissues and 30 cases of human nasopharyngeal inflammatory tissue. C, D, violin diagram analysis of AHNAK and Annexin A2 levels in 106 NPC tissue and 30 nasopharyngeal inflammatory tissue samples. E, Kaplan–Meier survival curve of NPC patients with low (n=51) and high (n=52) AHNAK expression. F, Kaplan–Meier survival curve of NPC patients with low (n=59) and high (n=47) Annexin A2 expression. G, Kaplan–Meier survival curve of NPC patients with low AHNAK & high Annexin A2 (n=21) and high AHNAK & low Annexin A2 (n=25), *p<0.05, **p<0.01, ***p<0.001.
Knockdown of AHNAK promotes NPC growth and metastasis in vitro. A, B, Representative images of RT-PCR and WB showed the expression of siAHNAK was reduced in CNE1 cells and HNE1 cells compared with control. C, D, CCK-8 assay showed that the proliferation of cells with knockdown AHNAK was significantly reduced. E, the representative photographs of wound-healing assay are presented (left; magnification, ×20), and the relative distance of healing was counted (right). F, G, transwell experimental
representative photographs are presented (F and G, left; magnification, ×20) and the relative number of migratory cells (F, right) and invasive cells (G, right) were counted.

![Graph A and B](image)

**Figure 3**

Knockdown of AHNAK promotes NPC growth metastasis in vivo. A, B, representative images of RT-PCR and WB showed AHNAK expressing in the cells stably expressing the shRNA vector was reduced.
compared with control. C, CNE1 cells stably expressing AHNAK shRNA (shRNA/AHNAK) or shRNA (shRNA/Control) were injected into nude mice. Macroscopic and body weight changes of mice after 8 weeks. D, F, H, macroscopic and microscopic representative images of tumors in lung tissue and the average number of metastases per group are shown. E, G, H, macroscopic and microscopic representative images of tumors in liver tissue and the average number of metastases per group are shown.

**Figure 4**

AHNAK regulates Annexin A2 protein levels. A, qPCR analysis of the Annexin A2 expression in CNE1 cells and HNE1 cells stably expressing AHNAK shRNA (shRNA/AHNAK) or shRNA (shRNA/Control). \( P>0.05 \) (ns). B, WB analysis the Annexin A2 expression with stable knockdown of AHNAK versus control. C, immunofluorescent staining of AHNAK (red) and Annexin A2 (green) in NPC cells stably expressing
AHNAK shRNA (shRNA/AHNAK) or shRNA (shRNA/Control). Cell nuclei stained with DAPI (blue). Representative images are shown (magnification, ×1000).

Figure 5

AHNAK regulates Annexin A2-dependent metastasis. A, wound-healing assay was performed in AHNAK shRNA transduced CNE1 cells and HNE1 cells with or without Annexin A2 siRNA. The representative photographs are presented (top; magnification, ×4) and statistical analysis (bottom) are shown. B, invasion assay of the representative photographs are presented (left; magnification, ×20) and statistical analysis (right) are shown.
Figure 6

FOSL1 is responsible for AHNAK-mediated NPC progression. A, ChIP-PCR assay FOSL1 binding to the AHNAK promoter region. B, C, Q-PCR, Western blot assay to detect FOSL1 and AHNAK expression in HK1, CNE1, CNE2 and HNE1 cells stably transfected with NC/Si-FOSL1 or transfected with NC/PLV-FOSL1. D, immunofluorescent staining of AHNAK (red) in CNE1, HNE1 cells transfected with NC/PLV-FOSL1. Cell nuclei stained with DAPI (blue). E, wound-healing assay was performed in FOSL1 shRNA transduced HK1
cells and CNE2 cells with or without AHNAK siRNA. The representative photographs are presented (left; magnification, ×4) and statistical analysis (right) are shown. F, G, migration and invasion assay of the representative photographs are presented and statistical analysis are shown. H, adhesion experiment relative adhesion analysis.

Figure 7
FOSL1 recruits EZH2 to silence AHNAK transcription. A, B, Q-PCR and Western blot assay to detect AHNAK expression in HK1, CNE2 cells transfected with NC/Si-EZH2. C, D, Q-PCR, Western blot assay to detect AHNAK expression in CNE1 and HNE1 cells stably transfected with NC/PLV-FOSL1 with or without transfected with NC/si-EZH2. E, immunofluorescent staining of EZH2 (green) and FOSL1 (red) in HK1, CNE2 cells transfected with PLV-FOSL1. Cell nuclei stained with DAPI (blue). F, Immunoblotting analysis of lysates from immunoprecipitated 293FT cells transfected with FLAG-EZH2 and FOSL1. G, H, ChIP-PCR detects the occupancy of EZH2, H3K27me and H3K27ac after FOSL1 overexpression. I, wound-healing assay was performed in NC/PLV-FOSL1 transduced CNE1 cells and HNE1 cells with or without EZH2 siRNA. The representative photographs are presented (top; magnification, ×4) and statistical analysis (bottom) are shown. J, migration and invasion assay of the representative photographs are presented (left; magnification, ×20) and statistical analysis (right) are shown.

**Supplementary Files**

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- ClinicalinformationofpatientswithNPC.xlsx