ANP32E contributes to gastric cancer progression via NUF2 upregulation

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Abstract. Acidic nuclear phosphoprotein 32 family member E (ANP32E) is a histone chaperone that removes H2A.Z from chromatin. ANP32E is implicated in numerous cellular processes, including cell proliferation, apoptosis and cell differentiation. Increasing evidence suggests that dysregulation of ANP32E expression is strongly associated with carcinogenesis. However, the relationship between ANP32E in the development of gastric cancer (GC) is unknown. The present study aimed to explore the potential role of ANP32E in the development of GC using gain-of-function, loss-of-function, CCK-8, colony formation, apoptosis, reverse transcription-quantitative PCR, immunoblotting and luciferase reporter assay. The results of the present study demonstrated that ANP32E expression levels were significantly increased in GC tissues. ANP32E knockdown markedly inhibited GC cell proliferation and colony formation and significantly induced GC cell apoptosis, whereas overexpression of ANP32E significantly induced GC cell malignancy. Furthermore, the results demonstrated that there was a positive association between ANP32E and NUF2 component of NDC80 kinetochore complex (NUF2) expression levels. By assessing NUF2 expression levels, it was demonstrated that ANP32E promoted tumor cell proliferation and inhibited cell apoptosis by increasing NUF2 expression levels in GC cell lines. In conclusion, the present study indicated that ANP32E may function as an efficient oncogene, which promotes tumorigenesis of GC cells by inducing NUF2 expression.

Introduction

Gastric cancer (GC) is a type of gastrointestinal tumor and the fifth commonest type of cancer overall (1). GC also ranks second in cancer-related mortalities due to lethal malignancy and poor prognosis rates and therefore remains a major global public health problem (2,3). Early stage GC can be treated by endoscopic resection or alternative, less invasive, surgical approaches. However, in most cases GC is asymptomatic at the early stage and progresses to advanced stages prior to diagnosis. Therefore, it is important to identify novel biomarkers to predict disease relapse or distant metastasis.

Current molecular genetic analysis suggests that GC is a heterogeneous disease that is complicated by certain epidemiological and histopathological characteristics. The Cancer Genome Atlas Research Network (4) demonstrates that for 295 primary GC tissues, which divide GC into four molecular subtypes (tumors positive for the Epstein-Barr virus, genomically stable tumors, microsatellite unstable tumors and tumors with chromosomal instability), dysregulated signaling pathways and driver mutations contribute towards GC. These included genes related to carcinogenic or tumor suppressor signaling pathways, increased amplification rates of Janus kinase 2, programmed cell death-ligands 1 and 2 and chromosomal aberrations, in addition to differential gene expression and epigenetic alterations. A global gene expression profile identifies numerous driver mutations in 300 GC samples provided by the Asian Cancer Research Group, which identifies that tumor protein p53, N-α-acetyltransferase 10, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α, Kirsten rat sarcoma virus and phosphatase and tensin homolog mutations are present in GC tissues (5).

As a differentially expressed regulator, acidic nuclear phosphoprotein 32 family member E (ANP32E), has attracted significant interest as it acts as a member of the histone chaperones with leucine-rich repeats that removes histone variant H2A.Z from chromatin. These proteins serve important biological functions in multiple cellular process,
including cell adhesion and tumor progression (6,7). One study demonstrates that ANP32E upregulates E2F transcription factor 1 (E2F1) expression to promote tumor formation in triple-negative breast cancer (TNBC) cells (8). Other studies report that ANP32E is involved in DNA double-strand breakages, inducing nucleosome recombination and DNA repair (9-11). However, the biological role of ANP32E in GC remains to be elucidated.

NUF2, an important component of the NDC80 kinetochore complex, is critical for chromosome segregation and is involved in the cell cycle and proliferation of tumor cells (12). NUF2 is significantly dysregulated in numerous types of cancer and functions as a valuable prognostic biomarker in predicting tumors in their early stages (13,14). However, the precise function and specific mechanisms of NUF2 in GC remain to be clarified.

In the present study, ANP32E was demonstrated to be significantly highly expressed in GC tissues and three GC cell lines. ANP32E significantly induced the proliferation and colony formation of GC and suppressed apoptosis by upregulating the expression of NUF2. These results suggested that ANP32E may be used as a potential prognostic biomarker in the treatment of GC.

Materials and methods

Patient samples. Patients with GC (19 males and 11 females, aged 54-67) were enrolled from The First Affiliated Hospital of Jiamusi University (Jiamusi, China) between May 2017 and June 2021. The subsequent experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Jiamusi University (approval no. 202078). None of the patients, who were diagnosed as gastric cancer by three independent pathologists, received any interventions before surgery. Written informed consent was provided by each patient. GC and paired normal adjacent tissue samples 5 cm away from the tumor tissues were also obtained in addition to the cancerous tissue sample.

ANP32E and NUF2 expression analysis using the cancer genome atlas (TCGA) database. The mRNA levels of ANP32E and NUF2 expression, and the correlation between ANP32E and NUF2 expression in gastric cancer were analyzed using GEPIA (http://gepia.cancer-pku.cn/), which is based on TCGA genome atlas (TCGA) database. A total of 408 tumors and 211 normal tissues were analyzed. ANP32E and NUF2 expression in gastric cancer were analyzed using GEPIA database.

ANP32E overexpression. The pcDNA3.1 vector was used for the overexpression of ANP32E in HGC27 and MKN45 cells. Empty vectors were used as negative control for overexpression. The coding sequence of ANP32E was cloned into the pcDNA3.1 vector, then the empty vectors or ANP32E-overexpressing vectors (1 µg) were transfected into cells (2x10^5 cells/well) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and the cultured at 37°C for 10 h. At 48 h post-transfection, ANP32E overexpression was examined by western blotting.

Cell proliferation. Cell proliferation assay was assessed using the Cell Counting Kit-8 (CCK-8) assay in HGC27 and MKN45 cells. Briefly, transfected GC cells (2.5x10^5 cells/well) were seeded into a 96-well plate, supplemented with 100 µl culture medium and maintained for 5 days at 37°C. CCK-8 reagent (10%; Beyotime Institute of Biotechnology) was subsequently added to each well on days 1, 2, 3 or 4, following the initial culturing for 5 days. Cells were incubated at 37°C for 3 h. The optical density at 450 nm was analyzed with an absorbance microplate reader. All the experiments were repeated four times.

Colonies formation. Transfected GC cells (2x10^5 cells/well) were seeded into 6-well plates. After 14 days, colonies (>50 cells) that had formed were subsequently fixed in 4% paraformaldehyde for 10 min, stained with crystal violet for 20 min and dried at room temperature. Images were captured using a camera.

Cell apoptosis. Trypsin and PBS were used to digest and wash the cells. Subsequently, 5.0x10^5 cells (HGC27 or MKN45) were seeded in six well plates. When the cell confluence reached 60-80%, the cells were transfected with si control (Ctrl) or siANP32E1/2, respectively. After transfection for 48 h at 37°C, the cells were resuspended using 1X annexin-binding buffer and were stained using an Alexa Fluor 488, annexin V and PI cell apoptosis analysis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. PAC-1 (2 µM; Selleck Chemicals; cat. no. S2738) was used to induce cell apoptosis, then the cells were subjected to cell apoptosis assay. Subsequently, a cyt-FLEX flow cytomter (Beckman Coulter, Inc.) was used to detect cell apoptosis. The data were analyzed by CytExpert (version 2.4.0.28, Beckman Coulter, Inc.).
Reverse transcription-quantitative PCR (RT-qPCR). Total RNA derived from tissues (5-10 mg) or cell lines (5x10^5) was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.). The tumor tissues and normal adjacent tissues were placed on ice and cut into pieces and then homogenized with TRIzol® reagent according to the manufacturer's protocols. Subsequently, purified total RNA was subjected to reverse-transcription to obtain complementary DNA using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega corporation) according to the manufacturer's protocols. ANP32E mRNA expression levels were determined using SYBR green master mix (Takara Biotechnology co., ltd.) on a 7500-fast machine (Thermo Fisher Scientific, Inc.). The thermocycling conditions as follow: Pre denaturation: 95˚C, 10 min; thermal cycling: 45 cycles; denaturation: 95˚C, 20 sec; annealing: 60˚C, 20 sec; elongation: 72˚C, 30 sec. The qPCR primers used were: ANP32E forward (F), 5'-TGc cTG TGT GTc aaT GGG G-3' and reverse (r), 5'-Gca GaG cTT cTa C T G T A C T G A G A ‑ 3 '; and GAPDH F, 5'-TGA CTT CAACG CGACACCCA-3' and R, 5'-CACcCTGTGTGCTGTA GCC AAA-3'. Cyclin D forward (F), 5'-CCTcCGGTGTcCTACT TCA-3' and Reverse (R), 5'-CTCCTCAGcACTTGTcTTC-3'; Cyclin E forward (F), 5'-GTTATAAGGAGACGcGGGAG-3', Reverse (R), 5'-TGcCTGCTTcTTACCcGTC-3'. ANP32E mRNA expression levels were normalized to GAPDH, which was used as the internal reference gene. The mRNA expression was quantified using the 2^-∆∆Cq method (15). The experiments were conducted for three independent repeats.

Luciferase assay. To explore the interaction between ANP32E and NUF2, luciferase reporter assay was performed. Briefly, the PGL3 plasmids (Promega Corporation; cat. no. E1751) consisting of a firefly reporter were subjected to construct the plasmids containing the sequence of NUF2 promoter. Then, the plasmids with NUF2 promoter were co-transfected with Ctrl or ANP32E-overexpressed vectors into HGc27 cells using Lipofectamine 3000 (Thermo). After transfected for 48 h, the luciferase activity was evaluated using dual-luciferase reporter assay system (Promega Corporation).

Western blotting. RIPA lysis buffer (Beyotime Institute of Biotechnology) was used to lyse HGc27 and MKN45 cells transfected with siCtrl or siANP32E1/2, respectively. Protein concentration was determined using a BCA kit (Thermo Fisher Scientific, Inc.). A total of 30-50 µg protein was separated using SDS-PAGE on a 12% gel. Subsequently, separated protein was transferred to a PVDF membrane. Then, 5% non-fat milk was used to block the membrane for 1 h at
Membranes were incubated separately with specific primary antibodies against ANP32E (1:1,000; Abcam; cat. no. ab5993), GAPDH (1:10,000; Cell Signaling Technology, Inc.; cat. no. 5174) and NUF2 (1:1,000; Abcam; cat. no. ab122962), overnight at 4°C. Next, they were incubated with HRP-conjugated anti-rabbit secondary antibodies (1:4,000; ProteinTech Group, Inc.; cat. no. SA00001-2) at 25°C for 2 h, followed by visualization by using SuperSignal West Pico PLUS (Thermo Fisher Scientific, Inc.; cat. no. 34580). GAPDH was used as the loading control. Quantification of the western blotting results was performed using Image J (1.8.0.172; National Institutes of Health).

Statistical analysis. Data were analyzed using SPSS 25.0 (IBM Corp.) and GraphPad Prism 7.0 software (GraphPad Software, Inc.). One-way ANOVA followed by Bonferroni’s test and unpaired Student’s t-test were used to assess statistical differences among groups. P<0.05 was considered to indicate a statistically significant difference.

Results

ANP32E expression levels increase in GC tissues. The results demonstrated that ANP32E expression levels were significantly upregulated in GC tissues compared with normal adjacent tissues using RNA sequencing data downloaded from TCGA (P<0.05; Fig. 1A). ANP32E mRNA expression levels in the 30 GC tissues and paired normal adjacent samples were determined using RT-qPCR. The results demonstrated that ANP32E was significantly upregulated in GC tissues compared with healthy tissues (P<0.001; Fig. 1B), consistent with TCGA analysis. Regarding to the association between ANP32E expression and clinicopathological characteristics in gastric cancer patients, the results showed that ANP32E expression were
significantly associated with TNM stage (P=0.028, Table I) and tumor size (P=0.003, Table I). Subsequently, ANP32E mRNA and protein expression levels in several GC cell lines were determined using RT-qPCR and western blotting, respectively. The results demonstrated that ANP32E was highly expressed in MKN45, AGS and HGC27 cell lines, in contrast to GES-1, which displayed the lowest ANP32E levels among the cell lines tested (Fig. 1C and D).

**Figure 3.** ANP32E knockdown regulates gastric cancer apoptosis and cell cycle progression. PI/Annexin V-FITC staining and flow cytometry were performed to analyze apoptosis in siCtrl, siANP32E-1 and siANP32E-2 transfected (A) HGC27 and (B) MKN45 cells. PI staining and flow cytometry were performed to analyze the cell cycle in siCtrl, siANP32E-1 and siANP32E-2 transfected (C) HGC27 and (D) MKN45 cells. **P<0.01 and ***P<0.001. ANP32E, acidic nuclear phosphoprotein 32 family member E; si, small interfering RNA; Ctrl, control.

ANP32E silencing effectively inhibits GC cell proliferation and induces cell apoptosis. As ANP32E mRNA and protein expression levels were significantly increased in HGC27 and MKN45 cells, these two cell lines were selected for assessment of the biological function of ANP32E. The results demonstrated that ANP32E expression levels were markedly suppressed by siANP32E-1 or siANP32E-2, in HGC27 and MKN45 cell lines, respectively (Fig. 2A). Subsequently, the
CCK-8 assay was performed to determine GC cell proliferation, which demonstrated that ANP32E silencing suppressed the proliferation of both the HGC27 and MKN45 cell lines (Fig. 2B and C). The effect of ANP32E expression on colony formation in the HGC27 and MKN45 cell lines was investigated. The results demonstrated that colony formation ability was significantly suppressed following ANP32E knockdown in GC cells (P<0.01; Fig. 2D and E). Moreover, qPCR analysis indicated that the expression of cyclin D and cyclin E were significantly decreased after ANP32E knockdown in GC cells (P<0.01; Fig. 2F). Taken together, these results indicated that ANP32E knockdown may inhibit GC cell proliferation.

ANP32E silencing regulates cell apoptosis and cell cycle progression. The downregulation of ANP32E significantly induced cell apoptosis in the HGC27 and MKN45 cell lines as indicated by the flow cytometry assay (P<0.05; Fig. 3A and B). The proportion of cells in the S and G2/M phases were decreased and the proportion of cells in the G0/G1 phase were increased following ANP32E knockdown in HGC27 and MKN45 cells (Fig. 3C and D). Collectively, these data suggested that ANP32E downregulation may promote cell apoptosis and cell cycle arrest.

ANP32E overexpression promotes GC cell proliferation. Based on the above results, ANP32E may act as an oncogene in GC development. In turn, ANP32E was overexpressed in GC cells to analyze its function. The results demonstrated that ANP32E protein expression levels were markedly upregulated in HGC27 and MKN45 cell lines (Fig. 4A). Overexpression of ANP32E significantly increased cell proliferation and colony formation in both HGC27 and MKN45 cell lines (P<0.01;
Figure 5. ANP32E promotes GC cell proliferation via NUF2 upregulation. (A) Spearman’s correlation between ANP32E and NUF2 expression levels was analyzed in tumor tissues using TCGA. r=0.6, P<0.05. (B) NUF2 expression levels in tumor (n=408) and adjacent normal (n=211) tissues were analyzed using TCGA. (C) Reverse transcription-quantitative PCR was used to determine NUF2 mRNA expression levels in Ctrl and ANP32E-overexpressing HGC27 cells. The NUF2 mRNA expression levels were normalized to GAPDH. (D) Luciferase reporter assay was performed to determine the interaction between ANP32E and NUF2. (E) Western blotting was used to determine NUF2 protein expression levels of NUF2 in Ctrl + sictrl, ANP32E-overexpressing + sictrl and ANP32E-overexpressing + siNUF2 HGC27 cells. (F) Cell proliferation and (G) colony formation was assessed in Ctrl + sictrl, ANP32E-overexpressing + sictrl and ANP32E-overexpressing + siNUF2 HGC27 cells. (H) PI/Annexin V-FITC staining and flow cytometry were performed to analyze cell apoptosis in Ctrl + sictrl, ANP32E-overexpressing + sictrl and ANP32E-overexpressing + siNUF2 HGC27 cells.
Fig. 4B–E). Furthermore, cell apoptosis of GC cells following ANP32E overexpression was investigated. The results demonstrated that ANP32E overexpression significantly decreased the percentage of apoptosis induced by PAC1 in HGC27 and MKN45 cell lines (P<0.001; Fig. 4F and G).

ANP32E induces GC cell progression via upregulating NUF2 expression. To investigate whether ANP32E regulated GC cell growth by regulating another molecule, the correlation between ANP32E and NUF2 expression levels was investigated. The results demonstrated a strong positive relationship in GC tissues (Fig. 5A). Compared with healthy tissues, NUF2 was upregulated in GC tissues (Fig. 5B). Furthermore, the mRNA and protein expression levels of NUF2 were upregulated in ANP32E-overexpressing HGC27 cells, which was rescued after treated with siNUF2 (Fig. 5C and E). The luciferase assay results of the luciferase activity in NUF2-transfected HGC27 cells were significantly higher than that in cells transfected with pGL3-basic vectors (Fig. 5D). To explore whether NUF7 upregulation contributes to the oncogenic role of ANP32E in GC cells, the present study used siRNAs to silence NUF2 in ANP32E-overexpressing HGC27 cells. Firstly, it was shown that HGC27 cells transfected with siNUF2 had decreased mRNA compared with the cells transfected with siCtrl (Fig. S1). In addition, NUF2 was also markedly downregulated in ANP32E-overexpressing HGC27 cells transfected with siNUF2 (Fig. 5E). While overexpression of ANP32E promoted cell proliferation and colony formation, suppression of NUF2 combined with ANP32E overexpression abolished the oncogene roles mediated by ANP32E overexpression, including promoting cell growth as well as inhibiting cell apoptosis (Fig. 5F–H). Overall, these results suggested that ANP32E may promote GC cell proliferation and suppress cell apoptosis via increasing NUF2 expression levels.

Discussion

Surgery was previously considered as the only radical treatment for GC (16). Diagnostic and other therapeutic approaches have markedly improved in recent years. However, targeted therapy is especially effective in patients with GC (17). Therefore, an improved understanding of the molecular pathogenesis of GC development and progression is important to improve GC therapeutics. In the present study, an in vitro assay was used to determine the biological functions of ANP32E via its knockdown and overexpression in GC cell lines.

ANP32E is an oncogene in numerous types of solid tumors (18), including thyroid carcinoma, breast cancer (8) and lung cancer (19). ANP32E functions as an important gene that promotes cell proliferation and invasion of thyroid carcinoma via the activation of AKT/mTOR/hexokinase 2-mediated glycolysis (18). Other studies report that ANP32E induces TNBC cell proliferation and metastasis via the upregulation of E2F1 expression (8,20). In the present study, ANP32E was significantly upregulated in GC tissues, compared with adjacent normal tissues, which implied that ANP32E may serve a role in tumor progression. However, the present study could not obtain the expression of ANP32E at protein levels owing the lack of suitable antibody and the protein expression of ANP32E in gastric cancer tissues should be explored in the future. It was also observed that ANP32E expression markedly affected the proliferation, apoptosis and cell cycle in GC cells in vitro. The results indicated that ANP32E is an oncogene in GC, which was consistent with previous studies (20). Among the gastric cancer lines, GES-1 is a normal human gastric epithelial cell (21), AGS (poorly differentiated) and HGC27 (undifferentiated) are two well-characterized gastric cancer cell lines (22,23), whereas MKN45 (poorly differentiated) was established from the tumor of a 62-year-old patient with gastric cancer (24). The present study observed higher expression of ANP32E in the gastric cancer cell lines compared with normal gastric epithelial cell line. To the best of the authors' knowledge, the mechanism by which ANP32E acts as a GC oncogene was examined for the first time in the present study. The public microarray data also demonstrated a strong positive association between ANP32E and NUF2 expression. It can therefore be hypothesized that the upregulation of NUF2 may be an important mechanism by which ANP32E regulates GC progression. NUF2 is known to modulate tumor cell proliferation, invasion and apoptosis (25). NUF2 functions as a prognostic marker in breast cancer (26). Furthermore, NUF2 expression can be used to predict the early recurrence of hepatocellular carcinoma following surgical resection, serving as a promising prognostic biomarker (13). Other studies report that NUF2 knockdown suppresses proliferation and induces apoptosis of human osteosarcoma Saos-2 cells and hepatocellular carcinoma (14,27). As in a previous study (25), the present study indicated that ANP32E overexpression may be one of the causes of abnormal NUF2 expression in GC, which promotes GC progression.

In conclusion, the results of the present study demonstrated that ANP32E expression levels were significantly increased in GC and markedly promoted cell proliferation and inhibited cell apoptosis by upregulating NUF2 expression, thereby contributing to GC tumorigenesis. These findings have provided a novel biomarker for potential use in GC clinical practices.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors’ contributions

MZ and QW designed this study. XZ and YZ performed the experiments. TW, JN and QT aided with the experiments and performed the data analysis. MZ, XZ, YZ and QW confirm the authenticity of all the raw data. XZ, MZ and QW wrote and revised the manuscript. All authors read and approved the final manuscript.
Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Jiamusi University (approval no. 202078). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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