ATP4B suppresses cell proliferation and migration via mitochondrial/p53/NF- κ B pathway in gastric cancer

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Research

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Abstract

Background: Hydrogen/Potassium ATPase β (ATP4B) is a key protein in gastric mucosa barrier acting an essential role in gastric acid secretion. However, the exact role and precise mechanism of ATP4B in gastric cancer (GC) remain obscure. This study aimed to investigate the clinical significance of ATP4B in GC and its biological role in tumor progression.

Methods: We evaluated ATP4B expression in GC cell lines and patient specimens via qPCR and Immunofluorescence. The correlations between ATP4B expression level and clinicopathologic parameters, as well as the relevance of ATP4B expression with overall survival were assessed. The functional roles of ATP4B in GC were verified by gain- and loss-of-function cell models and xenograft tumor model. The possible downstream effects of ATP4B were analyzed by iTRAQ-based quantitative proteomics analysis.

Results: A dramatic decrease of ATP4B expression in GC cells and tissues compared with the adjacent normal tissues was observed; Downexpression of ATP4B was associated with the malignant transformation in gastric mucosa lesions and correlated with poor prognosis, high grade of TNM stage, and vessel carcinoma embolus in GC patients. Restoration of ATP4B expression in GC cells significantly inhibited cell proliferation, cell viability, migration, invasion, tumorigenicity and induced apoptosis, whereas ATP4B silencing exerted the opposite effects. Mechanistically, we found a constitutive activation of p53 and inactivation of NF- κ B signaling correlated with the mitochondrial pathway in ATP4B-overexpressing GC cells.

Conclusion: Our data suggest that ATP4B perform the promising tumor suppressor gene by regulating p53/NF- κ B/mitochondrial pathway in GC.

Background

Gastric cancer (GC) is a high-incidence-rate and high-mortality-rate malignancy neoplasm, and it is the second most common cause of cancer-associated death worldwide [1]. Tremendous improvements in the diagnosis and treatment of cancer, notwithstanding, current 5-year survival rate of individuals diagnosed with GC is still lowly with the reason that overwhelming majority of patients are first diagnosed at the advanced stage with malignant proliferation and metastasis [2]. As with other cancers, the challenge lies in the reality that the early symptoms tend to be relatively non-specific, and detection requires invasive physical procedures, such as gastrointestinal endoscopy, be carried out on a regular basis, which may not be practical for general screening [3]. Therefore, it is pivotal to identify novel effective therapeutic target for GC that can apply in the early diagnosis and prolonging the outcomes of GC patients.

High-throughput gene expression microarrays examined by machine learning algorithms are used to identify potential biomarkers with high sensitivity and specificity that recognize the biological characteristics of specific human diseases, including GC [4]. In recent years, several different algorithms are used to select feature genes based on differential expression profiling data of GC and a large number

of GC candidate biomarkers and related gene signatures have been identified [5]. It has been reported that ATP4B, COL1A2 and HADHSC are the promising biomarkers [6-8]. ATP4B is diagnostically significant with apparent fold-changes in our previous study [9]. While exhibiting the potential power, there is no research report so far about the role of ATP4B expression in human cancers yet.

ATP4B gene is located on human chromosome 13q34, encoding the member of the P-type cation-transporting ATPases, which is the main isoform of H⁺/K⁺-ATPase family. The H⁺/K⁺-ATPase is a membrane-associated enzyme that provides the driving force for HCl secretion into the stomach. H⁺/K⁺-ATPase couples the electroneutral exchange of extracellular K⁺ and intracellular H⁺ to the hydrolysis of ATP, thereby generating an extremely high transmembrane pH gradient. The enzyme consists of two subunits. α subunit contains the catalytic site for ATP hydrolysis (ATP4A). It has been reported that mice lacking the gastric ATP4A were observed with chronic loss of acid secretion, with progressive hyperplasia, mucocystic and incomplete intestinal metaplasia to cancer phenotype [10]. ATP4B is a glycosylated polypeptide and tightly associated with the stabilization and maturation of the catalytic α subunits [11]. Analysis of the H⁺/K⁺- β -deficient mice indicated that ATP4B involved in the differentiation and development of mature parietal cells [12]. It has been reported that decreased ATP4B expression is detected in human GC tissues closely associated with poor prognosis of GC patients [9, 13]. Our previous showed that ATP4B silence in GC may contribute to the intragenic DNA methylation and histone deacetylation [14].

We designed this study to illuminate the mechanism of ATP4B in GC initiation and progression as well as its potential clinical application in GC patients. Our findings suggest ATP4B could serve as a tumor suppressor in the tumorigenesis and progression of GC.

Materials And Methods

Human GC specimens and patients' information

We used human paired gastric cancer tissues preserved in the tissue bank of Beijing Cancer Hospital/Institute and obtained information about the patients from the comprehensive database of the bank. Primary gastric cancer in these patients was diagnosed and treated at Beijing Cancer Hospital/Institute from 2002.01 to 2010.12. The patients had a well-documented clinical history and follow-up information. None of them underwent preoperative chemotherapy and/or radiation therapy. We randomly selected 221 GC cases with 120 adjacent normal tissues (ANTs), in the cohort, 221 cases represent all of the stages and histopathologic types of GC. All the patients have undergone radical resection with lymph node dissection. 118 GC patients were enrolled with complete follow-up from this cohort. 45 patients had died, whereas 73 were still alive. We obtained 70 lymph node metastasis specimens and 48 with no lymph node metastasis. Details of the patients' characteristics and ATP4B expression are provided in tables.

Clinical samples of stomach precancerous lesions

121 precancerous lesions were obtained from endoscopic biopsy were collected in Aerospace Clinic Medical College of Peking University and 100 cases from the 7th Medical University of Chinese PLA General Hospital. The study protocol was approved by the Ethics Committee of the hospital, and conformed to the provisions of the Declaration of Helsinki 1995. These specimens were fixed immediately in 10% formalin and embedded, sectioned and stained with hematoxylin-eosin (HE).

Immunohistochemistry

Sections (4 μ m thickness) from formalin-fixed, paraffin-embedded specimens were prepared. The protein expression of ATP4B was detected with a mouse monoclonal antibody (Anti-hydrogen/potassium ATPase beta, 2G11, Thermo scientific, US. 1:300 dilution). A positive reaction was indicated by a reddish-brown precipitate in the nucleus and cytoplasm. The staining percentage and staining intensity of ATP4B were independently graded and calculated for the final staining score by three senior pathologists. The final results were obtained after evaluating the staining intensity of the positively stained cells within five areas at 200 $\times$ magnification. The proportion of positive cells was estimated from the following: 0, negative; 1, positive in $\leq 10\%$ of cells; 2, positive in $> 10\%$ and $\leq 50\%$ of cells; 3, positive in $> 50\%$ and $\leq 75\%$ of cells; and 4, positive in $> 75\%$ of cells. Then, staining intensity of this level was scored as 0, negative; 1, weak; 2, moderate; and 3, strong. The two scores were multiplied and expressed as graded: 0, negative; 1-4, weak expression; 5-8, moderate expression; and 9-12, strong expression. The median score was used as the expression cutoff point (ATP4B median score = 4). Patients were divided into high- or low-expression groups based on these values.

Cell lines and culture conditions

AGS, MNK45, N87, PHM82 were purchased from the American Type Culture Collection (Manassas, VA), and BGC823, MGC803, SGC7901, GES1 cell lines were obtained from Cell culture collection (Shanghai, China). All of the cell lines were maintained in plastic flasks as adherent monolayers in minimal essential medium supplemented with 10% fetal bovine serum in DMEM medium (GIBCO, US) at 37°C in a humidified atmosphere containing 5% CO₂.

Vector construction and transfection

To generate ATP4B overexpression plasmids, the full length ATP4B cDNA was sequenced and amplified by PCR and cloned into pIRES (Clontech, US) vector. ATP4B-pIRES and vector control pIRES were transfected into BGC823, SGC7901 and AGS cells, respectively using Lipofectamine 2000 (Invitrogen). Furthermore, GES1 cells and AGS cells were also transfected with siATP4B (Lifetech. Ltd, USA.) to knock down the expression of ATP4B.

In order to observe ATP4B expression in live GC cells, we designed novel ATP4B-EGFP overexpressing plasmid based on the empty vector, pEGFP-N1 (Tianyi Huiyuan Ltd., Beijing, China). Restoration and location of ATP4B expression were confirmed by RT-PCR, Western Blotting and Immunofluorescence.

MTT assay

Cells were seeded into 96-well culture plates, and MTT was added to the cells at 1-5 days. MTT was removed after 4 h incubation, and then dimethyl sulfoxide (DMSO) was added to solubilize the formazan product. The absorbency at 490 nm/570 nm was assayed by a microplate reader (Bio-rad680 ELISA).

Soft agar assay

Cells (1×10^4) were plated in complete culture medium containing 0.33% agar on top of 0.6% agar in the same medium after 48h transfection. Cells were then incubated for 4 weeks of incubation at 37°C with 5% CO₂, stained with vital tetrazolium dye INT (P-Iodonitrotetrazolium Violet, Sigma) to document the presence or absence of viable cell colonies. The soft agar was fixed with 100 µl methanol-acetic acid (3:1 v/v) and colonies were counted.

Apoptosis analysis

48h-transfection later, Cells were harvested and double-stained with FITC-conjugated annexin V and propidium iodide for 15 min at 4°C in a calcium-enriched binding buffer. All analysis was performed on a FACS Calibur Flow Cytometer with Cell Quest software (Becton Dickison, France).

Cell invasion and migration assay

The invasive capacity of cells was determined using the BD BioCoatMatrigel invasion chambers (8 µM; BD Biosciences) or Corning Incorporated Transwell (Corning, NY). Briefly, cells were seeded on the top chamber of each insert with complete medium added to the bottom chamber. After 24 hours, cells on the membrane were wiped off with a cotton swab. Fixed and stained with 1% toluidine blue, cells on the underside of the membrane were counted from 5 microscope fields (original magnification, 200×).

iTRAQ protein profiling

We constructed a quantitative proteomics analysis in GC cell lines (BGC823 and SGC7901) with overexpressing ATP4B using the multiplex capability of the iTRAQ approach as previously reported [15]. Based on commonly differential proteins (fold changes < 0.8 or > 1.2) after restoration of ATP4B in comparison with empty vector in GC cells, we used gene ontology (GO) analysis and co-expression network in analyzing the protein-protein interactions as well as downstream effectors of ATP4B. Furthermore, some proteins identified by the iTRAQ strategy as being regulated by ATP4B were validated independently by Western blotting.

Western blot analysis

Whole cell lysates were prepared from tissue specimens or cell culture. 10 fresh gastric tumor tissue and paired normal gastric specimens were obtained from the patients with known levels of expression of ATP4B, as confirmed by immunostaining. Standard Western blotting was done with a monoclonal mouse

antibody against human ATP4B. Antibodies against p53, p21, p16, I κ B- α , p-I κ B- α (ser32/ser36) and p65 (NF- κ B main subunit), Bcl-2, Bax, Bid, and CD44 were used based on the iTRAQ analysis. Protein sample loading was monitored by incubating the same membrane filter with an anti- β -actin (Sigma, US). 30 μ g of extract subjected to polyacrylamide gel electrophoresis. Membranes were incubated with antibodies specific for these potential related proteins. Peroxide-conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham, NJ, US).

Reverse transcription-PCR analysis

AGS, BGC823, SGC7901 were seeded at 1.5×10^6 cells/dish in 60-mm cell culture dishes. 12 h later, the cells were incubated for 1 h at 37°C in serum-free medium alone or with pIRES-ATP4B or pIRES. 48 h after transfection, total RNA was extracted with the Trizol reagent (Invitrogen, US). The condition for RT-PCR was hot started at 95°C and then amplified for 25 cycles (95°C for 30 s, 58°C for 30s, and 72°C for 30s). PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. SAGE creation serve smartGel Image Analysis System was used for image acquisition and data analysis.

Real-time quantitative PCR

60 specimens including 30 GCs as well as 30 matched normal adjacent tissues were used in the qRT-PCR validation assay. Total RNA was extracted according to a standard Trizol protocol (Invitrogen, Carlsbad, CA, USA). 5 μ g of total RNA were reverse transcribed to cDNA with 200 U MMLV reverse transcriptase (Promega, Madison, USA). Quantitative real-time PCRs were performed in a total 20 μ l reaction containing 2 μ l of cDNA, 0.6 μ l 20 $\times$ Eva Green (Capital Bio Corp., Beijing, China), 0.5 μ l of each 10 μ M forward and reverse primers, 0.5 μ l of 2.5 mM dNTP, 1.5 U Cap Taq polymerase (Capital Bio Corp., Beijing, China), 10 μ l 2 $\times$ PCR Buffer for Eva Green and 4.4 μ l of H₂O. Using RT-CyclerTM 466 system (Capital Bio Corp., Beijing, China), PCRs were carried out with the following programmed parameters, heating at 95°C for 5 min followed by 40 cycles of a three-stage temperature profile of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. All reactions were performed in triplicates and the data were determined by the average Ct value. The melting curves for each PCR reaction were carefully analyzed to avoid nonspecific amplifications in PCR products. The expression of each sample was transformed using the $2^{-\Delta\Delta C_t}$ formula and normalized with β -actin expression [16]. The following primers were used: ATP4B: 5'-TTCGCCCTGTGCCTCTATGT-3' (forward) and 5'-TGTGAGGTCTGCCAGGTT-3' (reverse); β -actin: 5'-TTAGTTGCGTTACACCCTTTC-3' (Forward) and 5'-ACCTTCACCGTTCC AGTTT-3' (Reverse).

Animals and *in vivo* tumorigenicity

Female athymic BALB/c nude mice were purchased from the Vital River Laboratories (VRL) (Beijing, China). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 4 weeks old. BGC823 cells (5×10^5 cells/mouse) and SGC7901 cells (1×10^6 cells/mouse) stably transfected with ATP4B-pIRES vector or empty vector pIRES were injected subcutaneously into the dorsal flank of 4-week-old female BALB/c nude mice (ATP4B-overexpressed cells on the right flank and vector control clones on the left). Tumor diameter was measured and documented every 3 days until the

end of 3 weeks. Tumor volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumor and calculating as follows: $\text{Volume} = (\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$. The experiment was repeated for three times. Experimental mice were euthanized finally and the resected tumors were fixed with 10% formalin, embedded in paraffin, and sectioned ($4\ \mu\text{m}$). Sections were stained with hematoxylin and eosin (H&E) for immunohistochemical staining for light microscopy examination. The animal handling and all experimental procedures were approved by the Animal Ethics Committee of Peking University and according to NIH guidelines.

Transmission electron microscopy

After stably transfected with overexpressing ATP4B-pIRES plasmid, GC cells were digested with trypsin-EDTA solution. The cells were then washed in 3-4 changes of cacodylate buffer (pH 7.2) for 15 min in each change and post-fixed in cold osmium tetroxide for 2 h. Then, the samples were washed in three changes of cacodylate buffer for 15 min each. Dehydration was done using ascending grades of ethyl alcohol (30%-50%-70%) each for 2 h followed by 90% and 100% alcohol for 30 min each. Samples were then cleared in propylene oxide and embedded in Epon 812 using gelatin capsules. For polymerization, the embedded samples were kept in the incubator at 35°C for 1 day, then at 45°C for another day and in the 60°C during the third day. Ultrathin section ($50\text{-}80\ \text{nm}$) from selected areas were made and collected on copper grids. The ultrathin sections were contrasted with uranylacetate for 10 min and lead citrate for 5 min, and examined by Jeal 1009 transmission electron microscope and photographed at 80 kV (Assiut University Microscopy Unit).

Measurement of mitochondria membrane potential

After stable transfection of ATP4B-pIRES plasmid and the empty vector pIRES in GC cells, we further detected the variation of mitochondria membrane potential. Double fluorescence staining of mitochondria by JC-1, either as green fluorescent J-monomers (emission maximum of $\sim 529\ \text{nm}$) or as red fluorescent J-aggregates (with a specific red fluorescence emission maximum at $590\ \text{nm}$), was used for monitoring the mitochondrial membrane potential by flow cytometry.

Determination of Intracellular ATP levels

The levels of intracellular ATP in BGC823 and SGC7901 which stably transfected with overexpressed ATP4B plasmid or control vector were determined using the ATP assay kit (Cat No. ab83355; Abcam) by following the instructions from the manufacturer.

ROS Assay

ROS concentrations were evaluated using the oxidant-sensitive probe 2,7-dichlorofluorescein diacetate (DCF-DA) according to the manufacturer's instructions. A fluorometric microplate reader (FilterMax F5, Molecular Devices, Sunnyvale, USA) with excitation and emission at 485 and $530\ \text{nm}$ was used to measure the fluorescence intensity.

LDH Release Assay

The amount of lactate dehydrogenase (LDH) released into the culture medium upon cell lysis was measured by the conversion of a tetrazolium salt into red formazan product according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI.; LDH kit No: 601170). The absorbance, proportional to the lysed cells was measured at 490 nm.

Immunofluorescence and confocal analysis

In order to observe the colocalization between ATP4B and mitochondria in live GC cells (transfected with ATP4B-EGFP or empty vector), MitoTracker (R) deep red (Life technologies, Eugene, USA) as the mitochondria probe was used to further analyze the co-localization between ATP4B protein and mitochondria under a confocal microscope.

Statistics

The two-tailed chi square test or student's t-test were done to determine the statistical significance. Survival durations were calculated with the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival durations in the patient groups. The Spearman rank test and Fisher's exact test were applied to demonstrate clinicopathological correlations. Univariate and multivariate risk ratios were computed, together with 95% confidence intervals. The SPSS software (Version 24.0, SPSS, Chicago) was used for the analysis. For *in vitro* and *in vivo* studies, all experiments were repeated at least triple. All the data were displayed as mean $\pm$ SD. Significantly statistical differences were defined as $P < 0.05$.

Results

Decreased expression of ATP4B in GC and precancerous lesions

To examine the biological activities of the ATP4B gene in gastric cancer cells, we evaluated its expression in various human gastric cancer cell lines at the mRNA level by RT-PCR and the protein level by western blot analysis. The results suggested that the expression of ATP4B was substantially decreased in all gastric cancer cell lines whereas the ATP4B was stable expression in GES1 cell line (Fig.1A-B). The mRNA expression of ATP4B was then detected in randomly selected 30 fresh frozen GC samples compared with the paired adjacent normal tissues and the levels of ATP4B mRNA expression in the tumor tissues was mostly down-regulation (26/30, 86.67%, $P=0.002$, Fig.1C). To validate our findings, Immunohistochemical staining and Immunofluorescence analysis were performed in a tissue microarray containing 221 formalin-fixed paraffin-embedded GCs (Table 1) and 120 adjacent normal appearance tissues. Most of normal tissues often exhibited cytoplasmic expression and levels of ATP4B expression were significantly decreased in gastric cancer tissues (Fig.1E) compared to the non-cancerous counterparts ($P < 0.001$, Table 2); the same results was also proved via Immunofluorescence analysis (Fig.1D).

ATP4B expression levels correlate with the with clinicopathologic outcome of gastric cancer

Univariate and multivariate analysis were used to investigate the correlation between ATP4B expression and prognostic factors in patients with GC. Univariate analysis indicated that downregulation ATP4B expression in GC was significantly correlated with poor survival (Table 1). This correlation was also observed in other prognostic marker including poor differentiation ($P<0.001$), vessel carcinoma embolus ($P=0.003$), lymph node metastasis ($P=0.002$), advanced TNM stage ($P<0.001$). Furthermore, by multivariate Cox proportional hazards regression analysis, only stage classification and ATP4B variation were independently associated with over-all survival ($P=0.046$ and $P=0.001$, respectively, Table 3). Patients with early stage (stage I, with higher ATP4B expression than other stages) was strongly associated with longer over-all survival ($P=0.001$, Table 3). Consistent with these results, we analyzed the association between ATP4B expression level and overall survival in GC patients *via* Kaplan-Meier analysis and found that GC patients with low expression of ATP4B in tumors displayed a worse overall survival in comparison with patients exhibiting high ATP4B expression (Fig.1F). Furthermore, a cohort of 118 GC patients with complete follow-up showed that a positive expression level of ATP4B was associated with differentiation ($P=0.044$), the status of vessel carcinoma embolus ($P=0.011$), the status of lymph node metastasis ($P=0.066$) and tumor-node-metastasis (TNM) stage ($P=0.001$). However, no correlation was found between the expression of ATP4B and other variables, including age and sex (Table 4).

To examine the clinical significance of ATP4B as a predictive biomarker for early diagnosis, histopathologic studies were conducted to identify a sequence of changes in gastric mucosa that apparently represent a continuum from superficial gastritis to precancerous lesions, with intermediate stages of chronic superficial gastritis, intestinal metaplasia, atrophy and dysplasia. We found that the expression of ATP4B was significantly reduced with the degree of gastric mucosal malignancy and negatively correlated with the early diagnosis of mucosa malignant transformation ($P<0.001$, Table 5, Fig.S2).

Taken together, these results revealed that ATP4B downexpression in GC is highly associated with tumorigenesis and an independent poor progression in patients with gastric cancer.

Overexpression ATP4B shows anti-tumor activity *in vitro*

To determine the impacts of the overexpression of ATP4B on GC cell lines, multiply assays were performed on GC cells lines (BGC823, SGC7901 and AGS cell lines). The level of ATP4B expression was detected by western blotting and immunofluorescence after 48 h transfection, and the data showed that the protein level of ATP4B expression in the GC cell lines with ATP4B-pIRES was apparently higher than the control groups (Fig.2A-2B). Soft agar colony formation assay indicated that overexpression ATP4B significantly reduced colony formation abilities in BGC823, SGC7901 and AGS cells (Fig.2C, $P<0.0001$, $P=0.0003$, $P=0.002$, respectively), suggesting that ATP4B inhibited cell proliferation. MTT assay was used to examine the effect of overexpression ATP4B on GC cell viability, and demonstrated that ATP4B overexpression dramatically repressed cell viability in BGC823, SGC7901 and AGS cells (Fig.2D, $P=0.001$, $P=0.002$, $P=0.036$, respectively). The effects of ATP4B expression on cell cycle and apoptosis were detected by flow cytometric analysis. The results showed that the proportion of apoptotic cells in GC cell

lines with overexpression ATP was increasing compared to the empty vector groups ($P < 0.001$, Fig.2E). Cell invasion and cell migration assays presented that the invasion and migratory capabilities of GC cells were remarkably weakened by overexpressing ATP4B (Fig.2F). Conversely, knockdown ATP4B increased cell viability, migration and invasion and decreased the apoptosis ratio (Fig.S1). Collectively, these results suggest that ATP4B might play an inhibitory role in regulating the GC progression as a tumor suppressor gene.

The over-expression ATP4B suppress tumor growth *in vivo*

To validate the effect of ATP4B on growth of gastric cancer cells *in vivo*, BGC823 and SGC7901 cells carrying pIRES-ATP4B plasmid or pIRES were subcutaneous inoculation into the flank of nude mice to develop mouse xenograft model. It was shown that overexpressing ATP4B led to dramatically decreases of the average tumor volume in all the experimental groups, as compared with control groups ($*P < 0.001$). The overexpression ATP4B significantly suppressed tumor growth at the indicated time during the whole tumor growth period presenting tumorigenic ability was weakened after ATP4B overexpressing in GC cells (Fig.3A-B). Additionally, Immunohistochemical analysis showed that ATP4B and p16 were overexpressed in BGC823-ATP4B and SGC7901-ATP4B groups (Fig.3C). These data indicate that overexpressing ATP4B can suppress the growth of xenograft gastric tumors.

ATP4B regulates mitochondrial-related metabolism and functions

To clarify the regulatory mechanisms of ATP4B in gastric cancer, we performed iTRAQ proteomic analysis of ATP4B (Fig.4A). The downstream of differential proteins expression profiling were analyzed by iTRAQ analysis between BGC823 and SGC7901 cells. We found 245 intersected differential proteins of the two cell lines among (up-regulated 113, down-regulated 132, Fig.4B). Especially, 34 differential proteins (13.9%) were involved in mitochondria function and structure according to the cellular components (CCs) analysis in GO database (Fig.4C-D). The relationship between these mitochondrial related differential proteins indicated that p53 and RELA are central proteins (Fig.4E). In order to prove the iTRAQ data, we utilized western blot analysis to validate several genes related to p53 and NF- κ B pathway. The results showed that the protein levels of p53, p21 and p16 increased following ATP4B overexpression in AGS, BGC823 and SGC791 cells (Fig. 4F). In addition, the apoptosis related genes, such as Bax, Bid increased and anti-apoptotic proteins Bcl-2 decreased after overexpressing ATP4B (Fig. 4F). Moreover, our data also showed that the protein level of p65, p-I κ B α and CD44 were decreased while the I κ B α expression was upregulated upon ATP4B overexpressed in GC cells. In agreement with this, we found the reverse corresponding protein expression after ATP4B knockdown in AGS and GES-1 cells. Collectively, the overexpression ATP4B activates mitochondria mediated apoptosis through activation of p53 pathway along with the inhibition of NF- κ B/CD44 pathway (Fig.4G), determining the inhibitory role of ATP4B in progression of GC. Interestingly, nuclear factor kappa light-chain enhancer of activated B cells (NF- κ B) and p53 have been reported to regulate tumor cell metabolism, mitochondrial function, ROS generation and ATP production in cells [17].

ATP4B as a dominant regulator plays a role in mitochondrial pathway

To further confirmed that ATP4B were involved in mitochondrial apoptosis, we also examined the effect of ATP4B on ATP generation, reaction oxygen species (ROS) and mitochondria membrane potential and mitochondria mirco-structures analyses. Immunofluorescence was used to detect the localizations of ATP4B upon ATP4B overexpression in GC cells. We observed that ATP4B was coincided very closely with that of the mitoTracker, demonstrating an obvious co-location between the expression of ATP4B in live cells and mitochondria (Fig. 5A). ATP4B ectopic overexpression reduced the ATP generation in GC cells ($P<0.001$ Fig.5B) and increased intracellular ROS ($P<0.001$, Fig.5C) as well as LDH release (Fig. 5D, $P<0.001$) compared with cells with empty vectors. We also found that the mitochondria membrane potential of GC cells was reduced following ATP4B overexpression by JC-1 staining (Fig.5E $P<0.001$). Additionally, transmission electron microscopy (TEM) analysis indicated the decreasing number of mitochondria and the ultrastructural changes of mitochondria were observed upon ATP4B overexpression including the vacuolization and inner cristae disappearance in mitochondria (Fig.5F). In general, we confirm that ATP4B suppresses the progression of GC *via* regulating mitochondrial apoptotic pathway.

Discussion

Gastric cancer is among the most common cancers gastrointestinal malignancies with few effective treatment strategies. Previous studies have reported that ATP4B is downexpressed in GC tissues related to poor prognosis in patients with GC [9, 13, 14]. However, there are few researches involving in the role of ATP4B in tumorigenesis and progression of human gastric cancer. Therefore, our study presented here was designed to demonstrate the function of ATP4B in GC tissues and cells.

In the present study, we analyzed the expression of ATP4B and the correlation of ATP4B expression levels with GC clinical parameters in gastric cancer patients. We found the ATP4B expression levels was significantly downregulated in GC tissues and cells compared with normal controls; the low expression of ATP4B was strongly associated with some aggressive clinicopathological features of GC, including high TNM stage, lymph node metastasia, vessel carcinoma embolus and poor differentiation. IHC results suggested that ATP4B was mainly exhibited in cytoplasmic and negatively correlated with early malignant transformation of gastric. Kaplan-Meier analysis indicated low expression of ATP4B in GC was associated with shorter over-all survival. These data suggest that the expression of ATP4B negatively regulates the growth and progression of GC and relevant to patient OS as an independent prognostic factor of gastric cancer. Furthermore, restoration ATP4B expression in GC cells presented anti-tumor effects *in vitro* and *in vivo*, resulting in suppressing cell proliferation, migration, invasion, and inducing cell apoptosis via regulating p53/NF- κ B/mitochondrial pathway.

Mitochondria functionally control the production of energy, the electron transport chain, cell signaling, and apoptosis [18], playing crucial roles in tumor initiation, progression. Gastric parietal cells are rich in mitochondria [19], which generate ATP via oxidative phosphorylation by a proton-motive force ending in the joining of oxygen and protons to form water. Nevertheless, this process also results in the generation of superoxide with the release of reactive oxygen species (ROS) leading to mitochondrial dysfunction and cell death [20]. The severe accumulation of ROS in cancer cells results in increased vulnerability of

mitochondrial membrane permeability transition and impairs the membrane integrity [21]. Excessive ROS in mitochondria is shown to effectively kill cancer cells [22] and activates the intrinsic apoptotic pathway [23]. In the current study, a considerable increasing in ROS and apoptotic changes in mitochondrial morphology were observed. It has been shown that the accumulation of ROS and breakdown of mitochondrial membrane integrity could cause a decrease in mitochondrial membrane potential (MMP) [24]. The collapse of MMP contributes to the dissipation of the gradient with consequent loss of Cyt c interrupts electron flow between respiratory chain complexes III and IV, thereby reducing the production of ATP [25]. Our study illustrated that restoration of ATP4B in GC cells induced the decreasing mitochondria membrane potential (MMP) and ATP generation as well as the increasing of LDH. Tumor cells possess upregulated rates of aerobic glycolysis for energy production regardless of the oxygen concentration (Warburg effect) which can cause increased yields of lactic acid and more glucose uptake [26]. LDH is a glycolytic enzyme, which catalyzes the reversible conversion of lactate to pyruvic acid [27], and its release contributes to the induction of oxidative DNA damage [28].

It is widely accepted that p53, a tumor suppressor, leads to the endogenous apoptotic pathway (mitochondria-related apoptotic pathway) by regulating Bcl-2 family proteins [29, 30]. Inactivation of p53 gene leads to substantial impairment of the aerobic mitochondrial functions [31]. The mutation or suppression of p53, a frequent occurrence in cancer, results in promoting tumor initiation, metastasis, recurrence [32, 33]. To the best of our knowledge, p21 (CDKN1A), is a downstream gene of p53, functioning as a link between p53 and cell cycle arrest. Loss of p21 may predict poor outcome in gastric carcinoma which increases histologic grade or depth of invasion and lymph node, liver, and peritoneal metastasis [34]. p16, also known as cyclin-dependent kinase inhibitor 2A, is an activator of p53 protein [35]. Downregulation of p16 expression is associated with leading to cancer via the dysregulation of cell cycle progression [36]. In agreement with these findings, our study revealed that ATP4B expression activated the expression of p53, p21, and p16 as well as apoptotic related genes and inhibited anti-apoptotic proteins Bcl-2.

Intriguingly, p53 and NF- κ B mutually represses and overexpression of wildtype p53 inhibites NF- κ B activity, therefore induces apoptosis [37]. In our study, iTRAQ-based analysis and western blot validated the downexpression of NF- κ B, CD44, p65 and plkB α after restoration ATP4B expression. NF- κ B is composed of five family members: RelA (p65), p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), c-Rel, and RelB [38]. It is known that NF- κ B is persistently activated in numerous human cancers, including gastric cancer [39, 40]. Aberrant activation of NF- κ B supports tumor initiation, cell proliferation, metastasis and evasion of apoptosis, thus facilitates in cancer progression [41]. Activation of NF- κ B mainly occurs via phosphorylation of I κ B proteins including I κ B α [42]. In addition, there is also increasing evidence that the overexpression of CD44 in cancer cells can reroute NF- κ B pathway leading to cancer progression and malignancy [43-45].

Conclusion

In conclusion, ATP4B expression is decreased in GC cell lines and specimens; lowly ATP4B expression is highly associated with the tumorigenesis and leads to a poor prognosis in GC patients. Furthermore, our study confirms that ATP4B plays a vital role in inhibiting gastric cancer growth as a tumor suppressor *via* mitochondrial/p53/NF- κ B signaling pathway *in vitro* and *in vivo*. The study of ATP4B will promote our understanding of the molecular mechanism of the tumorigenesis and progression of GC and is expected to be an independent prognostic biomarker and therapeutic target for the clinical application in GC.

Abbreviations

ANTs: Adjacent Normal Tissues;

AJCC: American Joint Committee on Cancer;

ATP4B: Hydrogen-potassium ATPase beta;

DCF-DA: 2,7-dichlorofluorescein diacetate;

DMEM: Dulbecco's Modified Eagle Medium;

GC: Gastric Cancer;

iTRAQ: Isobaric Tags for Relative and Absolute Quantification;

LDH: Lactate Dehydrogenase;

MMP: Mitochondrial Membrane Potential;

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

NF- κ B: Nuclear factor κ B

PAGE: Polyacrylamide gel electrophoresis;

PBS: Phosphate Buffer Saline;

qRT-PCR: Quantitative real-time PCR;

ROS: Reaction oxygen species;

TNM: Tumor, Node, Metastasis.

Declarations

Ethics approval and consent to participate: The study was reviewed and approved by the Faculty of Ethics Committee of Peking University Cancer Hospital/Institute. All participants provided written

informed consent prior to their treatments and study enrollment.

Consent for publication: I, as the corresponding author, give my consent for my photograph and other clinical data to be published in the public Library of Science (Cancer Communication) Journal. We have seen and read the material to be published.

Availability of data and materials: Not applicable.

Competing interests: The authors declare that there are no conflicts of interest related to this study.

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Authors' contributions: Yuanming Pan performed the majority of the experiments and drafted the manuscript; Youyong Lu and Jianjiu Sheng designed the research; Shuye Lin, Min Zhu, Yangjie Li, Jianxun Wang, Suo Miao, Jiheng Wang, Xianzong Ma, Junfeng Xu, Lang Yang, Peng Jin helped with data extraction and processing; Shuye Lin, Yangjie Li, Peng Jin, Guibin Yang and Lang Yang conducted the IHC assays and assisted in writing the manuscript; Min Zhu, Jiheng Wang, Suo Miao and Yuanming Pan collected and analyzed the data; Jiangqiang Huang, Youyong Lu, and Jianqiu Sheng provided critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Tables

Table 1. ATP4B expression associated with clinicopathologic parameters of GC (n=221)

Characteristics	No. Cases	ATP4B expression		P-value
		Positive (%)	Negative (%)	
Total	221			
Sex				
Male	189	28 (14.8)	161 (85.2)	0.541
Female	32	5 (15.6)	27 (84.4)	
Age at diagnosis				
≥60	130	22 (16.9)	108 (83.1)	0.323
<60	91	11 (12.1)	80 (87.9)	
Differentiation				
Well (0)	6	3 (50)	3 (50)	0.044
Moderate (1)	68	16 (23.5)	52 (76.5)	
Poor (2)	146	17 (11.6)	129 (88.4)	
Vessel carcinoma embolus				
Yes				0.011
No	84	6 (7.1)	78 (92.9)	
	137	27 (19.7)	110 (80.3)	
Lymph node metastasis				
Yes	151	18 (11.9)	133 (88.1)	0.066
No	70	15 (21.4)	55 (78.6)	
TNM stage				
I	17	8 (47.1)	9 (52.9)	0.001
II	51	9 (17.6)	42 (82.4)	
III	114	14 (12.3)	100 (87.7)	
IV	39	2 (5.1)	37 (94.9)	

Table 2. The protein level of ATP4B expression was reduced in paired GC tissues

Antobody	No.Cases	Positive	Negative	P-value
ATP4B	T = 221	36 (16.3%)	185 (83.7%)	
	N = 120	110 (91.7)	10 (8.3%)	< 0.001

Table 3. Logistic regression analysis of clinicopathologic features and prognosis in GC patients (n=118)

Features	Number (ratio, %)	Median time	<i>P</i> -Value	
Sex				
Male	95 (80.5)	62.9 ± 5.8		
Female	23 (19.5)	61.4 ± 14.8		0.646
Age				
≥60	71 (60.2)	54.2 ± 4.5		
<60	47 (39.8)	73.2 ± 8.1		0.347
Differentiation				
Well (0)	6 (5.1)	80.4 ± 19.8	0 vs. 2	0.08
Moderate (1)	40 (33.9)	63.6 ± 5.2	1 vs. 2	0.252
Poor (2)	72 (61.0)	59.7 ± 6.5		
Vessel carcinoma embolus				
Yes	46 (39.0)	39.6 ± 6.1		
No	72 (61.0)	77.8 ± 6.1		0.003
Lymph node metastasis				
Yes	70 (59.3)	46.0 ± 4.6		
No	48 (40.7)	85.9 ± 7.3		0.002
TNM stage				
I	11 (9.3)	77.0 ± 6.7		
II	31 (26.3)	66.8 ± 5.3	I vs. II	0.259
III	53 (44.9)	63.6 ± 7.6	II vs. III	0.033
IV	23 (19.5)	31.9 ± 6.8	III vs. IV	0.003
ATP4B expression				
High	27 (22.9)	79.5 ± 3.4		
Low	91 (77.1)	56.3 ± 6.2		< 0.001

Table 4. Cox regression analysis of clinicopathologic features and molecular signatures in GC patients (n=118)

Features	95% CI				<i>P</i> -value
	B	HR	Lower	Upper	
Sex	-0.180	0.836	0.372	1.876	0.663
ATP4B	-1.811	0.163	0.056	0.478	0.001
Age	0.393	1.481	0.780	2.814	0.230
Differentiation	-0.327	0.721	0.428	1.216	0.220
Vessel carcinoma embolus	0.469	1.599	0.786	3.249	0.195
Lymph node metastasis	0.255	1.291	0.516	3.230	0.585
TNM stage	0.512	1.668	1.009	2.757	0.046

Table 5. ATP4B expression was reduced in gastric mucosa malignant transformation (n=221)

Precancerous lesions	No.Cases	ATP4B expression		<i>P</i> -Value
		Positive (%)	Negative (%)	
Chronic superficial gastritis	105	54 (51.4)	51 (48.6)	< 0.001
Intestinal metaplasia	44	7 (15.9)	36 (84.1)	
Atrophic gastritis	52	8 (15.4)	44 (84.6)	
Atypical hyperplasia	20	1 (5.0)	19 (95.0)	

Spearman correlation = - 0.388

Figures

Figure 1

ATP4B was downregulated in gastric cancer tissues and cell lines and correlated with poor survival. (A) The mRNA expression levels of ATP4B in GC cell lines and immortalized gastric cell line GES1 was detected by Semiquantitative RT-PCR. β -actin was used as a loading control. (B) Protein expression levels of ATP4B were analyzed by Western blotting. (C) RT-qPCR was used to quantify the amount of ATP4B mRNA expression in the primary GC and normal gastric samples. (D) Subcellular localization of ATP4B in gastric cancer cells was detected by immunofluorescence. Cells stained with anti-ATP4B antibody with green immunofluorescence in cytoplasm, and monitored DAPI as the nuclei staining (blue). Scale bars: 10 μ m. (E) Representative images of IHC staining for ATP4B in the primary GC and adjacent normal tissues. Brown staining indicates the cytoplasmic expression of ATP4B. (F) Kaplan-Meier analysis of survival of GC patients with ATP4B expression in the tumors. ATP4B+ means high expression of ATP4B in tumor tissues while ATP4B- means low expression of ATP4B in tumor tissues.

Figure 2

Restoration of ATP4B expression suppressed GC cell proliferation, viability, migration, invasion and induced apoptosis. (A) and (B) The transfection efficiency was determined by western blot and immunofluorescence. (C) Colony formation assay was conducted to detect the proliferation ability of GC cells after ectopic expression of ATP4B in BGC823, SGC7901 and AGS cells. Top panel: representative image; Bottom panel: quantitative graphs. (D) MTT assay performed to monitor the viability of GC cells

after transfection. (E) Flow cytometry assay detected apoptosis population of GC cells after ectopic expression of ATP4B in BGC823, SGC7901 and AGS cells. Data are the means $\pm$ SD. (F) and (G) Effects of ATP4B on GC cell migration and invasion were examined by Transwell assay in BGC823, SGC7901 and AGS cells after ectopic expression of ATP4B. Left panel: representative image; Right panel: quantitative analyses.

Figure 3

The effects of ATP4B on tumor growth in vivo. Comparison of the tumor size of xenograft tumors induced by ATP4B overexpression or empty vector transfected in BGC823 (A) and SGC7901 cells (B). Right panel: the corresponding growth curve analysis. Results shown represent the mean $\pm$ standard deviation (SD), *P<0.001, significant differences from the control. (C) Immunohistochemical staining of isolated xenograft tumors. Left panel: Brown staining indicates the expression of ATP4B. Magnifications: $\times 200$. Right panel: Brown staining indicates the expression of p16, respectively. Magnifications: $\times 200$.

Figure 4

ATP4B overexpression in GC cells leads to mitochondrial-related metabolism and function changes. (A) The workflow and protocol of iTRAQ for investigate the downstream of overexpressing ATP4B in BGC823 and SGC7901. (B) Differential proteins expression profiling by iTRAQ analysis of BGC823 and SGC7901 cells. (C-D) Differential proteins were cataloged according to cellular components (CCs) according to the GO database. (E) Protein-protein interactions between the mitochondrial related differential proteins. Up-

regulated proteins presented in red, moderate down-regulated protein in blue and apparent down-regulated protein in purple. (F-G) Western blot analyzed the activity of p53/NF- κ B signaling and Bcl-2, Bax, Bid expression after overexpressing (F) or knockdown (G) ATP4B in GC cells.

Figure 5

ATP4B overexpression regulated mitochondrial pathway. (A) Immunofluorescence staining of ATP4B and mitochondria in GC cells after ectopic expression of ATP4B-EGFP in BGC823 and SGC7901 cells to detect

the localization of ATP4B expression. Cells were labeled with mitoTracker, and monitored by DAPI (blue). Scale bars: 10 μ m. (B-D) Comparison of the alterations in ATP generation, ROS production and LDH after ATP4B transfection in BGC823 and SGC7901 cells. **, indicated $P<0.001$. (E) FACS analysis of mitochondria membrane potential after restoration of ATP4B. (F) Transmission electron microscopy (TEM) analysis indicated the mitochondrial morphology.

Figure 6

Schematic representation of the functional mechanism of overexpression ATP4B in GC cells. ATP4B activated the p53 signaling and suppressed NF-κB pathway regulated the Bax, Bid expression and decreased Bcl-2, CD44 expression, leading to the apoptosis and repressing proliferation and metastasis of GC.

Supplementary Files

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