ITRAQ-based Bioinformatics Analysis Reveals the Potential Anticancer Effects of ATP4B in Gastric Cancer

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Abstract

Background: Gastric cancer (GC) is one of the major malignancies of gastrointestinal tract. Hydrogen-potassium ATPase beta (ATP4B) gene is aberrantly downexpressed in gastric cancer that is associated with worse disease outcome. The objective of this study was to investigate the biological significance of ATP4B in GC carcinogenesis and development.

Methods: The expression level of ATP4B was analyzed via clinical tissues and TCGA database. Then, we overexpressed ATP4B in SGC7901 and utilized isobaric Tags for Relative and Absolute Quantitation (iTRAQ) technique to validate the ATP4B-regulated proteomics profile alterations. Bioinformatics analysis was performed to evaluate the biological processes of ATP4B in GC. Western blot was used for the verification of significant downstream proteins of ATP4B based on bioinformatics analysis data.

Results: We identified 293 differentially expressed proteins between the ATP4B overexpressing and control groups in SGC7901, including 145 upregulated proteins and 148 downregulated proteins. GO enrichment analysis indicated that ATP4B-modulating downstream proteins were primarily related to mitochondria function and metabolism. ATP4B-induced enrichments of biological functions were partly associated with suppressing tumor advancement, illustrating an inhibitory role for ATP4B in the progression of GC. Co-expression interaction network analysis exhibited the significant alterations in p53 and STAT3/NF- κ B signaling pathway. Consistently, KEGG pathway analysis showed that DEPs are enriched in cell metabolism and cancer-related signaling pathway. Western blot validated the activation of p53 pathway and the inhibition of NF- κ B /CD44 pathway after ATP4B overexpressing in GC cells.

Conclusion: ATP4B plays a critical anticancer effect by regulating p53/NF- κ B/mitochondrial pathway. Our data suggested a novel role and mechanism for ATP4B in GC progression.

Introduction

As one of the most deadly threats to humans due to the high morbidity and mortality, gastric cancer (GC) is the third most frequent cause of tumor-related mortality in both sexes worldwide [1]. There were greater than 1,000,000 new cases and an estimation of 783,000 gastric cancer-associated mortalities globally in 2018 [2], which brought great socio-economic burden on patients, their families, and the whole society. The advancement of diagnostic techniques and therapeutic strategies has been provided, but the prognosis of patients with this disease has not improved significantly. GC remains an important health burden worldwide owing to the lack of effective diagnostic biomarkers for early diagnosis [3]. Therefore, it is a critical need to discover the prospect novel diagnostic biomarker and new therapeutic targets for GC.

With the ongoing development of sequencing and proteomics mass spectrometry techniques, analyzing the alteration of gene expression profiles using high-throughput platforms provides more effective way to explore specific biomarkers and potential targets for a wide range of cancers, including GC [4]. In recent years, several genes significantly associated with GC have been identified via bioinformatics analysis based on dynamic transcriptomics, such as COL2A1, ATP4B, ATP4A, COL11A1, EGFR, and GIF [5–7].

Among them, we pay a great attention to the biological function of ATP4B gene in GC which is aberrantly downregulated expression in gastric tumor tissues [6].

ATPase H⁺/K⁺ transporting beta subunit (ATP4B) is mapped at human chromosome 13q34 [8], encoding a family of the P-type cation-transporting ATPases. ATP4B is mainly expressed in the parietal cells of the stomach and serves as a gastric function gene, which plays an essential role in the formation and secretion of hydrochloric acid [9, 10]. GC is a long-term progressive disease from inflammation to atrophy, intestinal metaplasia, dysplasia, and finally to gastric cancer, considered as an inflammation-driven tumor. It has been noted that the beta subunit of H⁺/K⁺ ATPase was a primary antigen recognized by sera from atrophic gastritis patients [11, 12]. In accordance with this, autoantibodies against ATP4B are regarded as serological diagnostic markers for patients with autoimmune atrophic gastritis, which are linked to an increased risk for gastric cancer [13]. Additionally, previous bioinformatics data have suggested that decreased ATP4B expressed in human GC tissue was substantially correlated with poor overall survival in patients with GC [14, 15]. Therefore, further investigation into biological function of ATP4B in GC may improve understanding of the molecular mechanisms responsible for providing novel therapeutic targets of GC.

In this study, we intended to reveal potential roles of ATP4B in GC progression. We overexpressed ATP4B in gastric cancer SGC7901 cell line and utilized iTRAQ proteomic technique to identify the proteins modulated by ATP4B. Integrate bioinformatics analyses and Western blot were subjected to validate some of the key effects and proteins regulated by ATP4B in GC cells. This study may provide a scientific basis for further research on the role of ATP4B in gastric cancer progression and introduce a promising therapeutic target in patients with GC.

Methods And Analysis

Clinical samples and immunohistochemistry (IHC) assay

Gastric carcinoma tissue and normal samples were collected from Seventh Medical University of Chinese PLA General Hospital and Beijing Cancer Hospital/Institute. Tissue sections (4 µm 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. Three independent pathologists scored the sections without the knowledge of patients' information.

Cell Culture

Human GC cell lines (SGC7901, AGS, BG823) were restored in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Grand Island, NY, USA) supplemented with

10% fetal bovine serum (FBS; Gibco, Life Technologies) at 37 °C with 5% CO₂. Cell growth was noted at regular intervals each day, with the culture medium changed according to the incubation condition.

Cell Transfection

In order to generate recombinant ATP4B plasmid and condition of ATP4B transfection, PIREs-ATP4B and vector control pIREs (Clontech, US) were constructed in our study. The full length ATP4B cDNA in pIREs was sequenced to confirm the identity and orientation of the ATP4B gene in this construct. GC cells at the logarithmic growth phase were digested with trypsin and respectively inoculated in a six-well plate. Cells were transfected with PIREs-ATP4B or PIREs plasmid when the cell confluence was 80%. In *vitro* transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction.

In order to observe the live GC cells with ATP4B-EGFP overexpressing, based on the empty vector, pEGFP-N1, We designed the primers as follows to form the novel ATP4B-EGFP overexpressing plasmid, F-ATP4B-EGFP: 5'- CCGCTCGAgATGGCGGCTCTGCAGGAGAAG-3' and R-ATP4B-EGFP: 5'- CCCAAGCTTCTTCTCAATCTTGAGTTTGAAGTC-3' and the results of some plasmids were identified by commercial sequencing (Tianyi Huiyuan Ltd., Beijing, China). By plasmid transfection (pEGFP-N1 and ATP4B-EGFP vectors) and confocal analyses, the live cells with different green fluorescence distribution, the cells with pEGFP-N1 transfection, nuclear and cytoplasm green fluorescence, while the only cytoplasm green fluorescence location was detected in live cells with ATP4B-EGFP transfection. RT-PCR [16] and western blot were also used to examine the expression ATP4B plasmid in GC cells.

Itraq Proteomics

SGC7901 GC cells transfected with PIREs-ATP4B or vector for 48 h were harvested and wash twice with PBS. Washed cells were dissolved in RIPA lysis buffer to extract protein. Then, the protein samples were added dithiothreitol to a final concentration of 10 mM in a water bath for 1 h at 56 °C and then supplemented with iodoacetamide to a final concentration of 55 mM for an alkylation reaction at room temperature for 1 h in the dark. Every sample was precipitated with four volumes of cold acetone (–20 °C) were added to the for protein precipitation more than 3 h at –20 °C. After centrifugation (20000 g, 4 °C, 20 min), the supernatant was discarded and protein precipitation was re-suspended in lysis buffer. The Bradford method was used to determine the final protein concentration. 100 µg of protein from each of samples incubated with trypsin and TEAB overnight at 37 °C. Then, the iTRAQ Reagent – 8PLEX Multiplex Kit (Thermo Fisher Scientific) was used for peptide labeling according to the manufacturer's instructions. Protein samples of SGC7901 with overexpression ATP4B and controls were labeled with 119, 117 iTRAQ tags. The iTRAQ-labeled samples (mixed with buffer) were desalted by Strata X C18 SPE column and dried by vacuum centrifugation. HPLC-MS/MS analysis by using ESI-QUAD-TOF tandem mass spectrometer (Thermo Fisher Scientific) was applied to identify peptide signal data files. Mascot database (Version 2.3.01, Matrix Science, London, UK) combined with Non-Redundant protein database

of NCBI were used to extract proteins abundant profiles and quantification information. Differential expressed proteins (DEPs) were identified by two-tailed Student's *t*-tests with Fold Chang > 1.2 and *p* value < 0.05.

Bioinformatics Analysis

Functional Enrichment Analysis of Gene Ontology (GO)

In order to clarify the biological function significance correlated with ATP4B-modulated DEPs, we used plug-in ClueGO app [17] in the Cytoscape software (version 3.72, Cytoscape Consortium, New York, NY) to create GO functional categories (biological process, cellular component and molecular function). The GO terms that have a adjust *p* value < 0.05 were regarded as cut-off criterion and top 15 terms visualized by R software (R3.6.1, <https://cran.r-project.org/>).

Co-expression Network With Protein–protein Interaction Analysis

To further understand the molecular interactions between the identified DEPs, Co-expression network analyzed the protein-protein interactions ((Protein-Protein Interaction Databases. http://www.Geneinfinity.org/sp/sp_proteininteraction.html). In addition, DEPs were submitted onto STRING online database (<http://www.string-db.org/>) was to select interacting proteins. Cytoscape software (version 3.72, Cytoscape Consortium, New York, NY) was used to visualize the networks. Meanwhile, hub proteins in PPI network were demonstrated by Cytoscape MCODE and cytoHubba plug-in.

Kyoto Encyclopedia Of Genes And Genomes (kegg) Pathway Analysis

To identify the crucial signaling pathways and diseases related DEPs, KEGG pathway was performed on DEPs via R package clusterProfiler [18] (<http://www.bioconductor.org/packages/clusterProfiler/>) in R software. We used *p* value screening threshold of 0.05 to explore enriched pathways. In our study, we chose the top twenty KEGG pathways.

Ingenuity Pathway Analysis

DEPs were analyzed with QIAGEN's Ingenuity Pathway Analysis algorithm (<http://www.qiagen.com/Ingenuity>, IPA, QIAGEN Redwood City) to identify the hidden biological significance of ATP4B in GC cells. Based on the IPA's analysis, interactions network of DEPs associated with cell metabolism, biological function and disease as well as upstream regulatory network were

algorithmically generated based on published literature and observed protein expression from IPA experimental data set. Significant results were then ranked by z -score and p value for prediction the activation status of these processes. $|z| > 2$ is empirically considered statistical significance.

Rna Extraction And Real-time Quantitative Pcr

30 GCs and 30 matched normal adjacent tissues were used in the RT-qPCR validation assay. Total RNA was extracted from the tissue samples according to a standard Trizol protocol (Invitrogen, Carlsbad, CA, USA). 5 µg of total RNA were reverse transcribed to cDNA with 200 U MMLV reverse transcriptase (Promega, Madison, USA). RT reaction was set as following conditions: 37°C for 60 min, 72°C for 10 min. Quantitative real-time PCRs were performed in a total 20 µl reaction containing 2 µl of cDNA, 0.6 µl 20 × Eva Green (Capital Bio Corp., Beijing, China), 0.5 µl of each 10 µM forward and reverse primers, 0.5 µl of 2.5 mM dNTP, 1.5 U Cap Taq polymerase (Capital Bio Corp., Beijing, China), 10 µl 2 × PCR Buffer for Eva Green and 4.4 µl of H₂O. Using RT-Cycler™ 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 final Ct values were determined by the average Ct value of the three reaction. The melting curves for each PCR reaction were carefully analyzed to avoid nonspecific amplifications in PCR products. The expression of each gene was transformed using the $2^{-\Delta\Delta Ct}$ formula and normalized with β -actin expression. The primer pairs for ATP4B and β -actin were below:

ATP4B: 5'-TTCGCCCTGTGCCTCTATGT-3' (forward) and 5'-TGTGAGGTCTGCC CAGGTT-3'(reverse);

β -actin: 5'-TTAGTTGCGTTACACCCTTTC-3' (Forward) and 5'-ACCTTCACCGT TCC AGTTT-3' (Reverse).

Western Blot Analysis

Western blot analysis

Whole cell lysates were prepared from GC cells after 48 h transfecting, and standard western blotting was done according to the protocol from the manufacturer. Protein concentrations were quantified via BCA Protein Assay Kit (Thermo Scientific). 30 µg of extract subjected to polyacrylamide gel electrophoresis. Primary antibodies against ATP4B, p53, p21, p16 STAT3, p-STAT3, I κ B- α , p-I κ B- α and p65 (NF- κ B main subunit), Bcl-2, Bax, Bid, and CD44 were used based on the results of bioinformatics analysis. Protein sample loading was monitored by incubating the same membrane filter with an anti- β -actin (Sigma, US). Membranes were incubated with antibodies specific for these related proteins and Peroxide-conjugated secondary antibody. The blots were visualized by enhanced chemiluminescence immunoblotting detection system (Amersham, NJ, US).

Results

ATP4B expression level was reduced in paired GC tissues

To overview the expression levels of ATP4B in human gastric tissues, TCGA data from online analytic tools, including The Human Protein Atlas and GEPIA were used. The data indicated that ATP4B was high-expression in human gastric mucosa (Fig. 1a) and lower expression of ATP4B gene in gastric cancer tissues compared to paired normal tissues was also observed (Fig. 1b). To further validate this result, we analyzed the mRNA expression level of ATP4B in 30 fresh frozen GC samples compared with the paired adjacent normal appearance tissues from Seventh Medical Center of PLA Hospital, and found a decreased expression in GC tissues (Fig. 1c). Moreover, IHC tissue microarray analysis was performed in a cohort including 247 GC cases and 120 adjacent normal tissues from Beijing Cancer Hospital. The results showed that the positive rate of ATP4B expression in tumors was significantly less than that of normal tissues (Fig. 1d). From this cohort, only 118 GC patients were enrolled with complete follow-up for Kaplan-Meier analysis, evaluating the possible prognostic value of ATP4B. We found that GC patients with low expression of ATP4B was significantly associated with worse survival (Fig. 1e, $P < 0.001$).

Effects Of Atp4b On Protein Expression In Gc Cells

To investigate the roles of ATP4B in GC progression, we restored ATP4B expression in SGC7901 (adenocarcinoma cell line with lowly ATP4B expression [10]) and detected the alterations of ATP4B-mediated proteins expression in GC cells. Immunofluorescence exhibited the cytoplasmic expression of ATP4B after transfecting with ATP4B-EGFP in SGC7901 (Fig. 2a). RT-PCR and by Western blot validated the stable expression of ATP4B-EGFP in SGC7901 cells (Fig. 2b). ITRAQ proteomics technology is a powerful methodology in the search for disease-specific biomarkers and gene downstream targets. Therefore, we used iTRAQ proteomics to erect protein expression profiles of over-expression ATP4B in SGC7901 compared with the matched controls (Fig. 2c). All proteins with adjusted p values (Benjamini-Hochberg, FDR) < 0.05 and altered expression levels at $|\text{fold change}| > 1.2$ were identified as DEPs. A total of 293 proteins were differentially expressed in SGC7901 cells with ATP4B overexpression; 145 proteins were upregulated and 148 were downregulated (Fig. 2d).

Atp4b Regulated Mitochondria-related Functional Categories

Gene Ontology Functional Enrichment Analysis was performed on the DEPs in two groups to examine the biological significances. Significant GO terms were functionally classified by biological process (BP), cellular component (CC), and molecular function (MF). In the light of BP category, differential proteins were strongly associated with cell metabolic processes, including energy metabolism, amino acid metabolic processes and cellular lipid catabolism. Other significant BP terms were correlated with translation and viral gene expression process (Fig. 3a, Table 1). For the CC category, the differential proteins were predominantly located in mitochondrion including mitochondrial membrane, mitochondrial

envelope, mitochondrial matrix, mitochondrial protein complex as well as oxidoreductase complex (Fig. 3b). The prominent MF of differential proteins was involved in oxidoreductase activity and coenzyme binding, enoyl – CoA hydratase activity and fatty-acyl-CoA binding and NAD(P)H/NAD binding, which are associated with energy production and cellular metabolism (especially glucose and lipids metabolism) in the mitochondria (Fig. 3c). Collectively, these data suggest that ATP4B acts a prominent role in regulating the mitochondria bio-function and energy metabolism.

Table 1

Diseases and Bio Functions related to the ATP4B-modulated differentially expressed proteins in two groups.

Categories	diseases or Functions Annotation	p-Value	Activation z-score
Cancer, Cell Death and Survival, Organismal Injury and Abnormalities, Tumor Morphology	cell death of cancer cells	0.00000966	3.135
Cancer, Cell Death and Survival, Organismal Injury and Abnormalities	necrosis of epithelial tissue	0.000311	2.837
Cancer, Cell Death and Survival, Organismal Injury and Abnormalities	cell death of osteosarcoma cells	0.002	2.828
Lipid Metabolism, Small Molecule Biochemistry	metabolism of glycolipid	0.0000349	2.753
Cell Death and Survival, Organismal Injury and Abnormalities	cell death of epithelial cells	0.000165	2.694
Lipid Metabolism, Small Molecule Biochemistry	synthesis of glycolipid	0.000283	2.563
Lipid Metabolism, Small Molecule Biochemistry	Metabolism of glycosphingolipid	0.000233	2.558
Cell Death and Survival	cell death of hepatoma cell lines	0.000204	2.424
Cell Death and Survival	cell death	0.000000882	2.321
Cell Death and Survival	apoptosis of breast cell lines	0.00234	2.219
Cell Death and Survival	apoptosis of hepatoma cell lines	0.000306	2.213
Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	depolarization of mitochondria	0.000369	2.191
Cancer, Cell Death and Survival, Organismal Injury and Abnormalities, Tumor Morphology	apoptosis of tumor cells	0.00108	2.177
Cell Death and Survival	necrosis	0.0000108	2.172
Cell Death and Survival, Organismal Injury and Abnormalities	cell death of epithelial cell lines	0.000451	2.109

Z-score > 2 indicates Diseases or Bio Functions were activated;

Z-score < -2 indicates Diseases or Bio Functions are inhibited

Categories	diseases or Functions Annotation	p-Value	Activation z-score
Energy Production	consumption of oxygen	0.00272	2.002
Cancer, Organismal Injury and Abnormalities	malignant solid tumor	0.0000000916	-2.754
Cancer, Organismal Injury and Abnormalities, Respiratory Disease	pulmonary metastasis	0.00113	-2.621
Cancer, Organismal Injury and Abnormalities, Respiratory Disease	advanced lung cancer	0.00166	-2.621
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	accumulation of lipid	0.00000125	-2.528
Organismal Injury and Abnormalities, Reproductive System Disease	infertility condition	0.00211	-2.433
Cell Death and Survival	cell viability of colorectal cancer cell lines	0.00263	-2.414
Cancer, Organismal Injury and Abnormalities	abdominal neoplasm	0.0000027	-2.219
Cellular Movement	invasion of brain cancer cell lines	0.00000937	-2.203
Cancer, Organismal Injury and Abnormalities	metastasis	0.000861	-2.202
Cancer, Organismal Injury and Abnormalities	advanced malignant tumor	0.00276	-2.202
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	accumulation of glycosphingolipid	0.00000316	-2.197
Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	quantity of ceramide	0.000835	-2.173
Cancer, Organismal Injury and Abnormalities	growth of tumor	0.0018	-2.142
Cellular Movement	invasion of cells	0.000466	-2.033
Z-score > 2 indicates Diseases or Bio Functions were activated;			
Z-score < -2 indicates Diseases or Bio Functions are inhibited			

ATP4B-regulated DEPs were involved in activating cancer cell death and repressing cancer cell invasion, metastasis and advancement

To further predict which regulatory roles and biological functions/diseases might be activated or inactivated by the altered protein expression profiling after ATP4B overexpressing in GC cells, IPA upstream regulatory effect analysis and downstream function analysis were conducted based on IPA literature. Upstream regulatory effect analysis identified the potential upstream regulatory network and

downstream function in the DEPs network. PML, STAT4 and other molecules were predicted as upstream regulators. Top regulatory effect network identified FGF7, MAPK9, PML as upstream regulators that control the expression of BAX, CDKN2A, EGFR, FASN, HSPB1, NDRG1, S100A10, SCD, SQSTM1. Finally, related downstream anticancer effects functions were activated (Fig. 4a). Consistently, the results of disease/function analysis also indicated that ATP4B-induced protein expression alterations were remarkably associated with regulating cancer-related biological behaviors: repressing cell invasiveness and tumor metastasis, promoting cell death and cell apoptosis (Fig. 4b, Table 1). In line with the data of GO significant terms, the changed functions concluding metabolism of glycolipid and energy production were also observed after ATP4B overexpression in GC cells. As shown in Fig. 4b, we presented the regulation interaction between correlative DEPs and invasion of cells after ATP4B overexpression in GC cells.

Construction Of Molecular Networks

We further conducted biological protein–protein interaction network to predict the pivotal proteins regulated by ATP4B. According to the data of GO and IPA analysis, co-expression network related to cell apoptosis and metabolism were constructed. In total, the co-expression network contained 41 nodes. P53 and RELA were regarded as the central genes, exhibiting an important interaction between p53-related signaling and NF- κ B pathway (Fig. 5a). Following the construction of the PPI network, one hub-network included 119 nodes and 515 edges and the top ten core proteins (EGFR, UBC, PGK1, ALB, IDH1, NPM1, STAT3, EEF1G, CDKN2A, ALDH18A1) were presented (Fig. S1a). Additionally, we analyzed the interrelationships among the experimental proteins and the IPA database proteins via molecular network analysis. Intriguingly, the top-ranked network diagram indicated a major role of the DEPs in the regulation of mitochondrial metabolism (Fig.S1b).

ATP4B-modulated downstream effectors were significantly correlated with p53/ NF- κ B/ mitochondria signaling pathway

KEGG pathway enrichment analysis was performed to identify the potential mechanism of ATP4B-regulated bio-function associated with GC progression in our study. It revealed that ATP4B-modulated DEPs were primarily involved in pathways associated with cell metabolic pathways including: Biosynthesis and metabolism of amino acids, Carbon metabolism, Sulfur metabolism, Fatty acid metabolism and degradation, Citrate cycle (TCA cycle). In addition, p53 signaling pathway and PPAR signaling pathway (mainly regulated lipid metabolism) are also important pathways capable of regulating cancer initiation and development (Fig. 5b).

To our knowledge, the p53 pathway and NF- κ B are mutually repressed, involving in suppressing the tumorigenesis and development of cancer [19]. Besides, p53 gene and NF- κ B gene have been reported to regulate tumor cell metabolism, mitochondrial function [20, 21]. In order to prove the results of our bioinformatics analysis above, we utilized western blot analysis to validate selected significant DEPs. The results showed that the protein levels of p53, p21 and p16 increased following ATP4B overexpression

SGC7901 cells and the apoptosis related genes, such as Bax, Bid were increased while anti-apoptotic proteins Bcl-2 decreased (Fig. 5c). Moreover, our analysis also showed that the protein level of p65 and p-I κ B α , pSTAT3, CD44 were decreased after upon ATP4B overexpressed in SGC7901 indicating ATP4B inhibited NF- κ B pathway (Fig. 5c). In agreement with this, we found the same corresponding protein expression after overexpressing ATP4B in AGS and BGC823 (Fig.S2). Theoretically, we believed that restoration of ATP4B expression in GC cells regulated mitochondrial function and cell death through activation of p53 pathway along with the inhibition of NF- κ B/CD44 pathway, determining the inhibitory role of ATP4B in progression of GC (Fig. 5d).

Discussion

The initiation and development of GC is a multi-stage, heterogeneous and multifactorial pathology process involving numerous genetic, epigenetic and environmental factors alterations [22]. Recently, studies have reported that the ATP4B gene is downregulated in GC, which plays a negative role in the progression of GC [6, 15]. Restoring ATP4B expression in GC cells may heighten the inhibitory effects of chemotherapeutic drugs on GC cell growth [10]. Consistent with these studies, our current study verified the lower ATP4B expression in GC tissues than normal gastric mucosal via TCGA data, presenting a worse survival. ITRAQ proteomics and bioinformatics analysis were used to further investigate target profiles and the signaling pathway related to ATP4B regulation, unveiling the potential molecular roles of ATP4B in gastric cancer progression.

GO analyses in our study revealed a large proportion of ATP4B-DEPs were closely correlative to mitochondria-related GO terms and involved in energy production and cellular metabolism by affecting the mitochondrial enzymes. Mitochondria are complex organelles whose major functions are energy conversion and production ATP through OXPHOS system [23]. It is well-known that mitochondrial dysfunctions have broad impacts on suppression of tumor growth as they could cause the aberrant bioenergy metabolism of cancer cells [24, 25]. The most extensively studied metabolic alterations in cancer are glucose metabolic reprogramming. Besides altered glucose metabolic phenotype, biosynthesis and utilization of lipids abnormality as well as amino acid metabolism changes are also recognized as the one of the most common metabolic features of cancer cells. In the last few decades, increasing studies suggested that dysregulation of lipid metabolism, especially for the reactivation of de novo fatty acid products, were essential for tumor progression [26, 27]. High rate of glucose uptake and deregulated lipid metabolism have been recognized as a pivotal hallmark of cancer [28]. GC cells and normal cells exhibit metabolic differences in glucose metabolism as well as the metabolism of lipids and amino acid [29]. In the present study, the GO molecular function showed that the dominant biological roles of ATP4B-modulated DEPs regulated the enzymes activity of lipids and glucose metabolism, such as acyl-CoA hydrolase, NADH dehydrogenase (ubiquinone) activity, oxidoreductase activity. In agreement with the role of ATP4B in regulating cellular energy metabolism, GO cellular component categories for DEPs were enriched in mitochondrial protein complex and mitochondrial envelope and inner membrane, and it is the organelles where fatty acid β -oxidation, TCA cycle occur. Comprehensively, high ATP4B expression leads to mitochondrial function and energy metabolism alterations in gastric cancer.

Based on IPA analysis, our study theoretically showed that ATP4B gene exhibits an inhibitory role in GC cells, repressing cell invasion, metastasis and inducing apoptosis, preventing the advancement of gastric cancer. In addition, ATP4B-mediated functions were also associated with regulating cell metabolism, such as cell energy production and metabolism of glycolipid. The results of hub protein-protein interaction and IPA network analysis also indicated that a few highlighted hub proteins mainly took part in the energy metabolism process. Co-expression network analysis showed an important network related to p53 and NF- κ B pathway involved in regulating cancer progression. KEGG pathway analysis revealed that the top 20 pathways were relevant to cell metabolism pathways and p53 signaling pathway.

P53 gene, acted as a tumor suppressor, mainly controls cell cycle progression and cell death which play key roles in tumor suppression [30]. There are converging numbers of studies showing that p53 pathway also regulates cell metabolism-associated phenotype modifications, not only the control of glycolysis and mitochondrial respiration, but the effect on lipid and nucleotide metabolism [31]. P21, also known as cyclin-dependent kinase inhibitor 2A, is a well-known p53 target gene and its protein products link the p53 and cell cycle arrest [19]. Loss of p21 may predict poor outcome in gastric carcinoma which increases histologic grade or depth of invasion and lymph node and peritoneal metastases [32]. P16 is a cell cycle-associated protein, it has been reported that the activation of p16 led to potent antitumor efficacy in gastric cancer [33]. It has been universally acknowledged that the intrinsic pathway which may involve p53. P53 has a dual action of promoting anti-apoptotic members activities as well as down-regulation anti-apoptotic Bcl-2 family members, triggering mitochondria-mediated apoptosis pathway [34, 35]. Western blot verification in our study illustrated that ATP4B expression activated p53 pathway and promoted cell apoptosis.

Previous studies have verified that p53 and NF- κ B mutually repressed and overexpression of wildtype p53 inhibited NF- κ B activity, therefore induced apoptosis. Our co-expression network analysis presented a key interaction between p53-related signaling and NF- κ B pathway. Nuclear factor κ B (NF- κ B), a nuclear transcription factor, is composed of five family members: RelA (p65), p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), c-Rel, and RelB. NF- κ B is essential in various biological processes including cell survival, proliferation, apoptosis, adhesion, angiogenesis and inflammation [36]. Activation of NF- κ B mainly occurs via phosphorylation of I κ B proteins including I κ B α [37]. It has reported that NF- κ B is constitutively activated in gastric cancer tissues, inducing angiogenesis, cell proliferation, metastasis and evasion of apoptosis [38, 39]. CD44 is a target gene of NF- κ B pathway; overexpression CD44 in cancer cells can reroute NF- κ B pathway leading to cancer progression and malignancy [40, 41]. STAT3 serves a role as a signal transducing molecule between CD44 and NF- κ B. pSTAT3 an active form of STAT3 was positively associated with a poor prognosis for patients with gastric cancer [42]. In accordance with these findings, our study exhibited the reduced expression of NF- κ B, p65 and plkB α , CD44, pSTAT3 upon restoration of ATP4B, suggesting that ATP4B regulating the NF- κ B/CD44 pathway.

Limitations

There are some limitations in our current study. Although we have identified several ATP4B-mediated functions and pathways in gastric cancer cells by using bioinformatics analysis, there are no experiments to verify the relationship between ATP4B gene and these terms. Future experimental in vitro and in vivo studies are needed to better substantiate and validate the specific roles and regulatory mechanism of ATP4B in gastric cancer progression. Furthermore, the relevant detail by which ATP4B modulates the mitochondrial metabolic pathway remains unknown and should be addressed in future studies.

Conclusion

In summary, our results validate the lower ATP4B expression in GC tissues than normal gastric mucosal; patients with GC exhibits a worse overall survival. The comprehensive ATP4B proteomic changes in gastric cancer and its biological functions are closely with energy production and cell metabolism. Furthermore, ATP4B plays an anticancer effect in gastric cancer cells most likely by regulating p53/ NF- κ B/mitochondrial pathway. This study provides the useful resources for further understanding the potential mechanisms of ATP4B responsible for repressing the progression in gastric cancer.

Abbreviations

GC: gastric cancer; ATP4B: Hydrogen-potassium ATPase beta; iTRAQ: isobaric Tags for Relative and Absolute Quantitation; GO: Gene Ontology; KEGG: Kyoto encyclopedia of genes and genomes; IHC: immunohistochemistry; RT-PCR: Real-time quantitative polymerase chain reaction; DEPs: differentially expressed proteins; PPI: Protein-protein interaction; STRING: Search tool for the retrieval of interacting gene; IPA: Ingenuity Pathway Analysis; BP: biological process; CC: cellular component; MF: molecular function; OXPHOS: oxidative phosphorylation.

Declarations

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Authors' contributions: WJH and HYQ designed the study; LYJ and PYM performed the majority of the experiments; WJX and ZT helped collect and process data; WJX, ZT and ZLS assisted in writing the manuscript; FXT and LXJ analyzed data; SJQ, PYM, KQ conducted IHC assay. FXT and LYJ prepared tables and figures; LYJ drafted the manuscript; WJH and HYQ revised the manuscript. All authors read and approved the final manuscript.

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References

1. Venerito M, Vasapolli R, Rokkas T, Malfertheiner P: Gastric cancer: epidemiology, prevention, and therapy. *Helicobacter*. 2018, 23 Suppl 1:e12518.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*. 2018, 68(6):394-424.
3. Necula L, Matei L, Dragu D, Neagu AI, Mambet C, Nedeianu S, Bleotu C, Diaconu CC, Chivu-Economescu M: Recent advances in gastric cancer early diagnosis. *World journal of gastroenterology*. 2019, 25(17):2029-2044.
4. Zhang J, Huang JY, Chen YN, Yuan F, Zhang H, Yan FH, Wang MJ, Wang G, Su M, Lu G *et al*: Whole genome and transcriptome sequencing of matched primary and peritoneal metastatic gastric carcinoma. *Scientific reports*. 2015, 5:13750.
5. Fei HJ, Chen SC, Zhang JY, Li SY, Zhang LL, Chen YY, Chang CX, Xu CM: Identification of significant biomarkers and pathways associated with gastric carcinogenesis by whole genome-wide expression profiling analysis. *International journal of oncology*. 2018, 52(3):955-966.
6. Wang G, Hu N, Yang HH, Wang L, Su H, Wang C, Clifford R, Dawsey EM, Li JM, Ding T *et al*: Comparison of global gene expression of gastric cardia and noncardia cancers from a high-risk population in china. *PloS one*. 2013, 8(5):e63826.
7. Yan Z, Xu W, Xiong Y, Cheng Y, Xu H, Wang Z, Zheng G: Highly accurate two-gene signature for gastric cancer. *Medical oncology (Northwood, London, England)*. 2013, 30(2):584.
8. Song I, Brown DR, Yamada T, Trent JM: Mapping of the gene encoding the beta-subunit of H⁺,K⁺-ATPase to human chromosome 13q34 by fluorescence in situ hybridization. *Genomics*. 1992, 14(4):1114-1115.
9. Gooz M, Hammond CE, Larsen K, Mukhin YV, Smolka AJ: Inhibition of human gastric H⁺-K⁺-ATPase alpha-subunit gene expression by *Helicobacter pylori*. *American journal of physiology Gastrointestinal and liver physiology*. 2000, 278(6):G981-991.
10. Lin S, Lin B, Wang X, Pan Y, Xu Q, He JS, Gong W, Xing R, He Y, Guo L *et al*: Silencing of ATP4B of ATPase H⁺/K⁺ Transporting Beta Subunit by Intragenic Epigenetic Alteration in Human Gastric Cancer Cells. *Oncol Res*. 2017, 25(3):317-329.

11. Neumann WL, Coss E, Rugge M, Genta RM: Autoimmune atrophic gastritis–pathogenesis, pathology and management. *Nature reviews Gastroenterology & hepatology*. 2013, 10(9):529-541.
12. Rusak E, Chobot A, Krzywicka A, Wenzlau J: Anti-parietal cell antibodies - diagnostic significance. *Advances in medical sciences*. 2016, 61(2):175-179.
13. Lahner E, Marzinotto I, Brigatti C, Davidson H, Wenzlau J, Piemonti L, Annibale B, Lampasona V: Measurement of Autoantibodies to Gastric H⁺,K⁺-ATPase (ATP4A/B) Using a Luciferase Immunoprecipitation System (LIPS). *Methods in molecular biology (Clifton, NJ)*. 2019, 1901:113-131.
14. Rajkumar T, Vijayalakshmi N, Gopal G, Sabitha K, Shirley S, Raja UM, Ramakrishnan SA: Identification and validation of genes involved in gastric tumorigenesis. *Cancer cell international*. 2010, 10:45.
15. Yan Z, Luke BT, Tsang SX, Xing R, Pan Y, Liu Y, Wang J, Geng T, Li J, Lu Y: Identification of gene signatures used to recognize biological characteristics of gastric cancer upon gene expression data. *Biomarker insights*. 2014, 9:67-76.
16. Chen Z, Hu T, Zhu S, Mukaisho K, El-Rifai W, Peng DF: Glutathione peroxidase 7 suppresses cancer cell growth and is hypermethylated in gastric cancer. *Oncotarget*. 2017, 8(33):54345-54356.
17. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pagès F, Trajanoski Z, Galon J: ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics (Oxford, England)*. 2009, 25(8):1091-1093.
18. Yu G, Wang LG, Han Y, He QY: clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics : a journal of integrative biology*. 2012, 16(5):284-287.
19. Al Bitar S, Gali-Muhtasib H: The Role of the Cyclin Dependent Kinase Inhibitor p21(cip1/waf1) in Targeting Cancer: Molecular Mechanisms and Novel Therapeutics. *Cancers*. 2019, 11(10).
20. Johnson RF, Perkins ND: Nuclear factor- κ B, p53, and mitochondria: regulation of cellular metabolism and the Warburg effect. *Trends in biochemical sciences*. 2012, 37(8):317-324.
21. Zhou S, Kachhap S, Singh KK: Mitochondrial impairment in p53-deficient human cancer cells. *Mutagenesis*. 2003, 18(3):287-292.
22. Hsieh HL, Tsai MM: Tumor progression-dependent angiogenesis in gastric cancer and its potential application. *World journal of gastrointestinal oncology*. 2019, 11(9):686-704.
23. Vyas S, Zaganjor E, Haigis MC: Mitochondria and Cancer. *Cell*. 2016, 166(3):555-566.
24. Boison D, Lu WL, Xu QM, Yang H, Huang T, Chen QY, Gao J, Zhao Y: A mitochondria targeting Mn nanoassembly of BODIPY for LDH-A, mitochondria modulated therapy and bimodal imaging of cancer. *Colloids and surfaces B, Biointerfaces*. 2016, 147:387-396.
25. Książkowska-Łakoma K, Żyła M, Wilczyński JR: Mitochondrial dysfunction in cancer. *Przegląd menopauzalny = Menopause review*. 2014, 13(2):136-144.
26. Hao Y, Li D, Xu Y, Ouyang J, Wang Y, Zhang Y, Li B, Xie L, Qin G: Investigation of lipid metabolism dysregulation and the effects on immune microenvironments in pan-cancer using multiple omics

- data. BMC bioinformatics. 2019, 20(Suppl 7):195.
27. Hu B, Lin JZ, Yang XB, Sang XT: Aberrant lipid metabolism in hepatocellular carcinoma cells as well as immune microenvironment: A review. *Cell proliferation*. 2020, 53(3):e12772.
 28. Brault C, Schulze A: The Role of Glucose and Lipid Metabolism in Growth and Survival of Cancer Cells. Recent results in cancer research Fortschritte der Krebsforschung Progres dans les recherches sur le cancer. 2016, 207:1-22.
 29. Liu Y, Zhang Z, Wang J, Chen C, Tang X, Zhu J, Liu J: Metabolic reprogramming results in abnormal glycolysis in gastric cancer: a review. *OncoTargets and therapy*. 2019, 12:1195-1204.
 30. Rivlin N, Brosh R, Oren M, Rotter V: Mutations in the p53 Tumor Suppressor Gene: Important Milestones at the Various Steps of Tumorigenesis. *Genes & cancer*. 2011, 2(4):466-474.
 31. Lacroix M, Riscal R, Arena G, Linares LK, Le Cam L: Metabolic functions of the tumor suppressor p53: Implications in normal physiology, metabolic disorders, and cancer. *Molecular metabolism*. 2020, 33:2-22.
 32. Tsihlias J, Kapusta L, Slingerland J: The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer. *Annual review of medicine*. 1999, 50:401-423.
 33. Ma JM, Shi ZJ, Chen L, Hu HZ, Wang WG, Su CQ, Yang CP: [Expression of multiple tumor suppressor gene p16 and its relationship with prognosis of gastric cancer patients]. *Zhonghua zhong liu za zhi [Chinese journal of oncology]*. 2012, 34(1):21-25.
 34. Bi L, Yan X, Chen W, Gao J, Qian L, Qiu S: Antihepatocellular Carcinoma Potential of Tetramethylpyrazine Induces Cell Cycle Modulation and Mitochondrial-Dependent Apoptosis: Regulation of p53 Signaling Pathway in HepG2 Cells In Vitro. *Integrative cancer therapies*. 2016, 15(2):226-236.
 35. Moll UM, Wolff S, Speidel D, Deppert W: Transcription-independent pro-apoptotic functions of p53. *Current opinion in cell biology*. 2005, 17(6):631-636.
 36. Hayden MS, Ghosh S: NF- κ B, the first quarter-century: remarkable progress and outstanding questions. *Genes & development*. 2012, 26(3):203-234.
 37. Schmitz ML, Bacher S, Kracht M: I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations. *Trends in biochemical sciences*. 2001, 26(3):186-190.
 38. Baldwin AS: Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. *The Journal of clinical investigation*. 2001, 107(3):241-246.
 39. Sasaki N, Morisaki T, Hashizume K, Yao T, Tsuneyoshi M, Noshiro H, Nakamura K, Yamanaka T, Uchiyama A, Tanaka M *et al*: Nuclear factor-kappaB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2001, 7(12):4136-4142.
 40. Fan F, Xiuwen Z, Yongyi L, Weiping C, Lu G, Yueqin L, Qi C, Huiling S, Xiaolan Z, Wenlin X: The CD44 variant induces K562 cell acquired with resistance to adriamycin via NF- κ B/Snail/Bcl-2 pathway. *Medical hypotheses*. 2018, 121:142-148.

41. Wei B, Sun X, Geng Z, Shi M, Chen Z, Chen L, Wang Y, Fu X: Isoproterenol regulates CD44 expression in gastric cancer cells through STAT3/MicroRNA373 cascade. *Biomaterials*. 2016, 105:89-101.
42. Woo S, Lee BL, Yoon J, Cho SJ, Baik TK, Chang MS, Lee HE, Park JW, Kim YH, Kim WH: Constitutive activation of signal transducers and activators of transcription 3 correlates with better prognosis, cell proliferation and hypoxia-inducible factor-1 α in human gastric cancer. *Pathobiology : journal of immunopathology, molecular and cellular biology*. 2011, 78(6):295-301.

Figures

Figure 1

ATP4B were downregulated in gastric cancer tissues. a. ATP4B mRNA expression levels in human different tissues. b. Box plot showing the ATP4B mRNA levels in stomach adenocarcinoma Based on GEPIA database. c. RT-qPCR results for relative mRNA expression of ATP4B in the 30 paired normal and Gastric cancer samples. d. Representative images of IHC staining for ATP4B in the primary GC and

adjacent normal tissues. The results of IHC assays analysis were shown. e. Kaplan-Meier analysis of survival of GC patients with ATP4B expression in the tumors.

Figure 2

Effects of ATP4B on protein expression in GC cells. (a) The transfection efficiency was determined by immunofluorescence. (b) The stable ATP4B expression in SGC7901 was validated by RT-PCR western blot. β -actin served as a loading control. (c) Workflow diagram of iTRAQ proteomics analysis in the

ATP4B-overexpression SGC7901 cells. (d) Bar graph displayed the number of up/down-regulation proteins from iTRAQ proteomics data.

Figure 3

Classification of DEPs based on their functional annotations via Gene Ontology (GO). GO terms related to (a) biological process, (b) molecular functions and (c) cellular components were represented.

A

B

Figure 4

IPA Upstream regulatory effectors and diseases/bio-functions analyses were performed. a. The heat map displayed potential upstream regulator in our study. Right panel: Regulator effect network analysis revealed the interaction relationship between upstream regulators, DEPs and functions. b. Diseases and bio functions heat map illustrated the cluster altered status of each disease or functions regulated by

DEPs. Right panel indicates the possible interaction and expression trends between the DEPs and top function based on literature in the Ingenuity knowledge base.

A

B

C

D

Figure 5

ATP4B-modulated downstream effectors were significantly correlated with p53/ NF-κB/ mitochondria signaling pathway. a. Co-expression network illustrates the predicted role of DEPs in our study. Up-regulated protein presented in red, moderate down-regulated protein in blue and apparent down-regulated

protein in purple. b. KEGG pathways Enrichment analysis of DEPs. Representative bubble plots are shown the top 20 KEGG pathway. c. Western blot detected of the activity of p53/NF- κ B signaling and Bcl-2, Bax, Bid expression after transfection with ATP4B-overexpression plasmid in SGC7901. d. Schematic representation of the functional mechanism of overexpression ATP4B in GC cells.

Supplementary Files

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