BAP-1 Inhibits Gastric Cancer Progression via PI3K/AKT Pathway by Suppressing FOXK1 Expression

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Abstract

BRCA1-Associated Protein-1 (BAP-1) gene functions as a vital mediator in tumor formation, progression, and metastasis. The involvement of BAP-1 in colon cancer has been widely reported, but the role of BAP-1 in gastric cancer (GC) is still unclear. In this study, we sought to investigate the contribution of BAP-1 in the pathogenesis of GC. qPCR and Western blot assay were used to detect the mRNA and protein expression of BAP-1 in GC cell lines. MTT and transwell assay were employed to determine the cell viability, migration, and invasion. Annexin V-PI double staining was used to evaluate cell apoptosis. We reported that BAP-1 was expressed at a low level in GC cell lines. Overexpression of BAP-1 inhibited cell viability, migration, invasion, and epithelial-mesenchymal transition (EMT) and promoted apoptosis in HGC-27 and AGS cells. BAP-1 inactivated the PI3K/AKT pathway by suppressing Forkhead-box K1 (FOXK1) expression. Moreover, overexpression of FOXK1 reversed the effect of BAP-1 on cell viability, apoptosis, migration, invasion, and EMT. Our data revealed that BAP-1 inhibits cell viability, migration, invasion, and EMT process and promoted apoptosis in HGC-27 and AGS cells through PI3K/AKT pathway via suppressing FOXK1 expression. Thus, BAP-1 can serve as a potential therapeutic target in GC treatment.

1. Introduction

Gastric cancer (GC) is a common digestive cancer with high mortality, and prevalent in many developing countries (Coburn et al., 2018). Early-stage gastric cancer is characterized by the pain and weight loss (Sitarz et al., 2018), and hepatic and renal metastases in the later stage will further results in cachexia (Kim et al., 2018). Although early diagnosis and new chemotherapy have achieved good results in the treatment of gastric cancer (Ahn et al., 2011), chemotherapy is not effective in patients with advanced disease due to drug resistance (Oba et al., 2013, Wu et al., 2015). Hence, the identification of targeted therapies and biomarkers has important clinical significance for the treatment of gastric cancer.

BRCA1-Associated Protein-1 (BAP-1) is a vital member of the ubiquitin c-terminal hydrolase (UCH) protein family (Ristic et al., 2014), which was recently identified as a crucial regulator of many cancers, including uveal melanoma, malignant mesothelioma, clear cell renal cell carcinoma, lung carcinoma, meningioma and breast cancer (Abdel-Rahman et al., 2011, Kang et al., 2018). Several studies have demonstrated that BAP-1 was aberrantly expressed in colorectal and renal cancer tissues and cells (Farid et al., 2019, Owen et al., 2017), and disturbing expression of BAP-1 was tightly linked to tumor formation and progression (Martorano et al., 2014). Moreover, BAP-1 also interacts with signal transfer molecules and inflammatory cytokines to affect tumor cell cycle and metastasis (Bishop et al., 2016, Kwan et al., 2015). A recent study reported that BAP-1 was downregulated in serum samples of GC patients (Wang et al., 2018). Nevertheless, there are no studies on the role and mechanisms of BAP-1 in GC.

Forkhead-box K1 (FOXK1) is a kind of transcription factor that has been found to be involved in cell differentiation, signal transduction, and metabolism (Li et al., 2019c, Ma et al., 2018). Upregulated FOXK1

was found in pancreatic cancer and esophageal cancer(Chen et al., 2017a), while FOXK1 expression was decreased in gastrointestinal tumors(Li, Qu, 2019c), FOXK1 also act a pivotal part in other types of cancer, such as liver cancer(Cui et al., 2018), prostate cancer(Chen et al., 2017b), and ovarian cancer(Li et al., 2017). A recent study demonstrated that FOXK1 promoted the GC cell proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) process, which may be a vital regulatory factor in the metastasis of malignant tumors(Peng et al., 2016). Furthermore, FOXK1 expression was regulated by kinase Gsk3 and participated in the mediation of glioma cell cycle(He et al., 2018, Ji et al., 2018). Mass Spectrometry analysis suggested that FOXK1 was coprecipitated with BAP1 in 293T cells(Machida et al., 2009). However, However, the mechanism of whether FOXK1 is involved in BAP-1-mediated GC processes is still unknown.

In this study, the aim was to investigate the contribution of BAP-1 in the GC cells cell migration, invasion, and EMT process. Further, we explored whether FOXK1 was involved in the BAP-1-mediated GC progression, so as to provide a new theoretical basis for the treatment of GC in clinical practice.

2. Materials And Methods

2.1 Cell culture

Human gastric cancer cell lines (HGC-27, AGS and MKN1) and epithelial cell strain GES-1 were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were incubated in RPMI-1640 (Sigma, St. Louis, MO, USA) medium containing 10% Fetal bovine serum (FBS, Gibco, Rockville, MD) in a humidified incubator (37°C, 5% CO₂). HGC-27 cells were treated with 100 ng/mL IGF-1 for 1 h.

2.2 Plasmid transfection

Overexpression plasmids of BAP-1 (BAP-1) and FOXK1 (FOXK1) were constructed and purchased from Bio-transduction Lab (Wuhan, China). Then, BAP-1, FOXK1, and empty overexpression plasmids negative control (NC) were transfected into GC cells using Lipofectamine®3000 (Life Technologies, USA), and its transfection concentration was 0.5 µg. Then transfected above plasmids and replenished culture medium after 48 h of post-transfection. The effectiveness of transfection was determined by qPCR and Western blot assay.

2.3 RNA extraction and RT-qPCR

The total RNA was extracted from cells by RNA Extraction Kit (Takara, China), followed by synthesizing the first-strand cDNA using Revert Aid cDNA Synthesis Kit (Transgen Biotech; Beijing). The expression of BAP-1 was quantified by RT-qPCR in a DNA Engine Opticon™ system (MJ Research, MA). β-actin was used as the internal control. Gene-specific primers were designed as follows: BAP-1 forward, 5'-GACCCAGGCCTCTTCACC-3'; BAP-1 reverse, 5'-AGTCCTTCATGCGACTCAGG-3'; FOXK1 forward, 5'-GCTCGGTGGACTTGAGCAT-3'; FOXK1 reverse, 5'-AAACCGGAAGGTACACTGCT-3'; β-actin forward, 5'-CTGTACGCCAACACAGTGCTGTCT-3'; β-actin reverse, 5'-ATCCACATCTGCTGGAAGGTGGAC-3'.

2.4 Western blot analysis

The gastric cancer cells were lysed with RIPA lysis buffer. Protein concentration was determined by Protein Determination Kit (Cayman, USA). The total protein was separated by 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF (polyvinylidene fluoride) membranes (Millipore, Billerica, MA, USA). Then hybridizations with primary antibodies (BAP-1, FOXK1, N-cadherin, vimentin, E-cadherin, PI3K, p-PI3K, AKT, p-AKT and β -actin, Sangon Biotech; Shanghai, China) were carried out for 1 h at room temperature. Then, the membranes were incubated with secondary antibodies (Sigma, St. Louis, MO, USA). The protein complexes were detected by the enhanced chemiluminescence reagents (Abcam, Cambridge, UK).

2.5 MTT assay

Cell proliferation was measured using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Dojindo Laboratories, Kumamoto, Japan). The cell viability was detected using microplate reader (Thermo Fisher Scientific, Massachusetts, USA) by spectrophotometry at 570 nm.

2.6 Transwell assay

Cell migration and invasion were evaluated using Transwell migration chambers (8- μ m pore size; Millipore, Boston, MA, USA) precoated with or without a layer of Matrigel (excreta cellular matrix gel; Sigma, St. Louis, MO, USA). Cells were seeded into the upper compartment of the invasion chamber filled with FBS-free culture medium, the lower chamber contained 700 μ L RPMI-1640 medium with 10% FBS, which was served as a chemoattractant. After incubation at 37°C for 48 h, the cells remained in the upper compartment were removed by cotton swabs. The migrated and invaded cells were washed with PBS, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted by light microscopy.

2.7 Apoptosis test

AGS cells were inoculated in 6-well plates, and after different treatments, the cells were rinsed twice with cold phosphate buffer (PBS) followed by suspension with Annexin V-FITC/PI (Invitrogen, Carlsbad, CA, USA) apoptosis detection buffer. Detection of apoptotic cells by flow cytometry (Beckman Coulter, Fullerton, CA, USA)

2.8 Animals and treatment

Twelve male BALB/C nude mice aged 4–6 weeks were purchased from Laboratory Animal center of Shanghai and were randomize into four groups (n = 6). AGS cells (1×10^6 cells in 100 μ L PBS) transfected were overexpression plasmids of FOXK1, BAP-1 and/or treated with IGF-1 were injected into the mice. Tumor growth was determined by measuring the length (cm) and width (cm) of tumors every weeks starting the second week after inoculation. Tumor volume was calculated using the formula $V = \text{width} \times \text{length} \times 0.5$. Five weeks later, animals were sacrificed. All procedures were approved by the Committee of the first affiliated hospital of xi'an jiaotong university.

2.9 Statistical analysis

The data were represented as means $\pm$ SEM, and each experiment was performed in triplicate in this study. Two-way ANOVA and Student's unpaired *t*-test were used to analyze statistical significance. $P < 0.05$ were considered as statistically significant.

3. Results

3.1 BAP-1 was downregulated in GC cell lines

In our study, we detected BAP-1 expression in GES-1, MKN-1, AGS, and HGC-27 cell lines. RT-qPCR assay evidenced that BAP-1 mRNA was lower in GC cell lines than that in human epithelial cell strain GES-1 ($P < 0.05$, Fig. 1A). What's more, BAP-1 protein expression was also downregulated in in GES-1, MKN-1, AGS, and HGC-27 cell lines ($P < 0.05$, Fig. 1B and C).

3.2 Overexpression of BAP-1 inhibited cell viability and promoted apoptosis

To investigate the function of BAP-1 in GC cells, we investigated cell viability and apoptosis in HGC-27 and AGS cells. The results showed that the mRNA and protein levels of BAP-1 in HGC-27 and AGS cells transfected with BAP-1 were significantly increased ($P < 0.05$, Fig. 2A-C). As expected, transfection of BAP-1 dramatically inhibited cell viability ($P < 0.05$, Fig. 2D and E) and promoted apoptosis ($P < 0.05$, Fig. 2F) in HGC-27 and AGS cells. Meanwhile, the results demonstrated that BAP-1 significantly enhanced C-caspase-3 and BAX expression and reduced Bcl-2 expression ($P < 0.05$, Fig. 2G-I).

3.3 Overexpression of BAP-1 inhibited cell migration, invasion, and EMT

We further examined the effects of BAP-1 on migration, invasion and EMT of GC cells. As shown in Fig. 3A-D, overexpression of BAP-1 significantly inhibited cell migration and invasion in HGC-27 and AGS cells, compared with NC group ($P < 0.05$). Besides, overexpression of BAP-1 remarkably reduced the protein expression of N-cadherin and vimentin, and increased E-cadherin protein expression, compared with NC group ($P < 0.05$, Fig. 3E-G).

3.4 BAP-1 regulated the PI3K/AKT pathway by suppressing FOXK1

As shown in Fig. 4A and B, the mRNA and protein expression of FOXK1 were remarkably higher in MKN-1, AGS, and HGC-27 cells than GES-1 cells ($P < 0.05$). Our results showed that mRNA and protein expression of FOXK1 were significantly decreased in BAP-1-transfected cells, compared with negative control group ($P < 0.05$, Fig. 4C and D). Then we investigated the effect of FOXK1 overexpression on BAP-1-induced GC cells by transfecting FOXK1. The results of qPCR and Western blot assay confirmed the efficiency of FOXK1 overexpression vectors transfection (Fig. 4E and F). Moreover, phosphorylation of PI3K and AKT were suppressed by BAP-1 overexpression, and promoted by FOXK1 overexpression (Fig. 4G-I). Compare

with BAP-1 alone, co-transformation of FOXK1 and BAP-1 increased the ratio of p-PI3K/PI3K and p-AKT/AKT (Fig. 4G-I).

3.5 FOXK1 mediated the anticancer effect of BAP-1 via PI3K/AKT signaling pathway

As shown in Fig. 5, FOXK1 counteracted the inhibitory effects of BAP-1 on cell viability (Fig. 5A), migration and invasion (Fig. 5C-F), and the promotion effect of BAP-1 on cell apoptosis (Fig. 5B). Moreover, activation of the PI3K/AKT pathway with insulin-like growth factors 1 (IGF-1) also markedly inversed the anticancer effect of BAP-1 on HGC-27 cells ($P < 0.05$, Fig. 5A-F).

3.6 BAP-1 affects gastric cancer cell growth in vivo

Next, we performed Xenograft assay *in vivo* to confirm the effect of BAP-1 on gastric tumorigenesis. As shown in Fig. 6, BAP-1 significantly inhibited tumor volume in a time dependent manner and tumor weight. Additionally, the tumor volume and weight in BAP-1 + FOXK1 and BAP-1 + IGF-1 groups were significantly much larger/heavier than BAP- group.

4. Discussion

BAP-1 is a member of the UCH family and decreased BAP-1 expression is associated with higher histologic grade, tumor infiltration, metastasis status, and Tumor Node Metastasis (TNM stage)(Yan et al., 2016, Yang et al., 2016). Here, we confirmed that the expression of BAP-1 was decreased in GC cell lines. Overexpression of BAP-1 could inhibit GC cell viability, migration, invasion and EMT while promoting cell apoptosis.

The crucial role of EMT in tumor metastasis is attracting increasing people's attention(Chen et al., 2019a, Hebert et al., 2017). EMT endows tumor cells with higher ability to invasion and metastasis(Thiery et al., 2009). During EMT process, epithelial cells became mesenchymal cells by losing the expression of epithelial markers (E-cadherin, catenin) and acquiring mesenchymal markers (N-cadherin, Vimentin, ZEB1); thus, these transformed epithelial cells acquired fibroblast-like characteristics and showed decreased intercellular adhesion and increased activity(Li et al., 2019b). Reducing or blocking the EMT process may be an effective treatment strategy for GC. In this study, we reported that BAP-1 significantly inhibited EMT in GC cells, this process was characterized by an increase in E-cadherin and a decrease in N-cadherin and Vimentin. These changes reduced the motility of GC cells, which is evidenced by the reduction of migration and invasion. Similarly, it was reported that BAP-1 negatively regulated the EMT in Cervical Cancer (Wang et al., 2017). Our results are consistent with previous studies showing that BAP-1 is involved in the tumor metastatic process *in vivo* by regulating the occurrence of EMT.

FOXK1 is a member of the FOX transcription factor superfamily, and its high expression is associated with several cancers. Recent studies have found that FOXK1 was overexpressed in 16 human tumor tissue types, including gastric cancer(Peng, Zhang, 2016). Okino et al. found that BAP-1 could interact with FOXK1 *in vitro* through coimmunoprecipitation and pull-down experiments(Okino et al., 2015). In the

present study, our results show that FOXK1 expression is regulated by BAP-1 and silence of FOXK1 inhibited the viability, migration, and invasion of gastric cancer cells. These data indicated that BAP-1 regulated the progression of gastric cancer through FOXK1.

The involvement of the PI3K/AKT pathway in processes of gastrointestinal cancers has been reported in former researches(Akrami et al., 2016, Riquelme et al., 2016a). The PI3K/AKT pathway plays an important role in carcinogenesis(Lien et al., 2016, Riquelme et al., 2016b). It has been found that the phosphorylation of PI3K and AKT were upregulated in GC tissues of patients and mice(Chen et al., 2019b, Li et al., 2019a). Activation of the PI3K/AKT pathway elevated the clonogenicity and proliferation of GC cells(Wang et al., 2019). Interestingly, recent studies have indicated that FOXK1 has a positive effect on the AKT signaling pathway, and FOXK1 might promoted the proliferation and inhibited cell apoptosis of breast cancer by regulating the PI3K/AKT/mTOR signaling pathway(Li, Qu, 2019c). Moreover, FOXK1 promoted the malignant behavior of GC by activation of the PI3K/AKT pathway(Wang et al., 2020). We uncovered novel findings that the BAP-1, a UCH family member, inhibited phosphorylation of PI3K and AKT by suppressing FOXK1 expression in GC cells. It is coincident with previous studies that FOXK1 interacted with BAP-1 and activated the PI3K/AKT pathway.

In conclusion, we primarily identified that BAP-1 expression was decreased in AGS and HGC-27 cells. Overexpression of BAP-1 decreased GC cells viability, migration, invasion and EMT process and promoted cell apoptosis. Further, we found FOXK1 mediated the anticancer effect of BAP-1 via PI3K/AKT signaling pathway. Therefore, BAP-1 may be a potential new target for the treatment of GC.

Abbreviations

BRCA1-Associated Protein-1 (BAP-1); gastric cancer (GC); pithelial-mesenchymal transition (EMT); Forkhead-box K1 (FOXK1); ubiquitin c-terminal hydrolase (UCH); Fetal bovine serum (FBS); Tumor Node Metastasis (TNM stage)

Declarations

Ethics approval and consent to participate

All procedures were approved by the Committee of the first affiliated hospital of xi'an jiaotong university.

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Competing interests

The authors declare no conflict of interest.

Availability of data and material

All datasets for this study are included in the manuscript/supplementary files.

Author Contributions

CM designed the experiments and wrote the paper; YZ, LR and DZ carried out the experiments; YZ the statistical analysis.

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None

Consent for publication

Not applicated

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Figures

Figure 1

BAP-1 was downregulated in GC cell lines. (a) BAP-1 mRNA expression in GES-1, MKN-1, AGS, and HGC-27 cell lines were investigated by RT-qPCR. (b and c) BAP-1 protein expression in GES-1, MKN-1, AGS, and HGC-27 cell lines were detected by Western blot assay, * $P < 0.05$, compared with GES-1 cells.

Figure 2

Overexpression of BAP-1 inhibited cell proliferation and promoted apoptosis. (a-c) BAP-1 mRNA and protein expression were analyzed by qPCR and Western blot assay in AGS and HGC-27 cells. (d and e) Cell viability was evaluated by MTT assay in AGS and HGC-27 cells. (f) Cell apoptosis was detected by Annexin V-PI double staining test in AGS and HGC-27 cells. (g-i) The expression of Cleaved-caspase-3 (C-caspase-3), Bax, and Bcl-2 were assayed by Western blot assay in AGS and HGC-27 cells, * $P < 0.05$, compared with NC group.

Figure 3

Overexpression of BAP-1 inhibited cell migration, invasion and EMT. (a-d) Cell migration and invasion were detected by Transwell assay (e-g) The expression of N-cadherin, vimentin, and E-cadherin were assayed by Western blot assay, * $P < 0.05$, compared with NC group.

Figure 4

BAP-1 regulates the PI3K/AKT pathway via suppressing FOXK1 expression. (a) The FOXK1 mRNA expression was investigated by RT-qPCR in GES-1, MKN-1, AGS, and HGC-27 cells, * $P < 0.05$, compared with GES-1 cells. (b) The FOXK1 protein expression was investigated by Western blot assay in GES-1, MKN-1, AGS, and HGC-27 cells (c and d) The FOXK1 mRNA and protein expression were analyzed in BAP-1-transfected HGC-27 cells, * $P < 0.05$, compared with NC group. (e and f) qPCR and Western blot assay were used to analyze the expression of FOXK1 mRNA and protein in FOXK1-transfected HGC-27 cells, * $P < 0.05$, compared with NC group. (g-i) The protein expression of p-PI3K, PI3K, p-AKT, and AKT were detected by Western blot assay, * $P < 0.05$, compared with NC group; # $P < 0.05$, compared with BAP-1 group.

Figure 5

FOXK1 mediated the anticancer effect of BAP-1 via PI3K/AKT signaling pathway. (a and b) Cell viability and apoptosis were evaluated by CCK-8 assay and Annexin V-PI double staining test. (c-f) Cell migration and invasion were detected by Transwell assay. * $P < 0.05$, compared with NC group; # $P < 0.05$, compared with BAP-1 group.

Figure 6

BAP-1 suppresses gastric cancer cells tumor growth in vivo. (A) The tumor volume was calculated every week after injection. (B) Tumor weights was detected after injection for 35 d . (The data were presented as the mean $\pm$ SEM, n = 6, P < 0.05, compared with NC group; # P < 0.05, compared with BAP-1 group.