

RESEARCH ARTICLE

Prohibitin Induces Apoptosis in BGC823 Gastric Cancer Cells Through the Mitochondrial Pathway

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Abstract

Prohibitin (PHB), an evolutionarily-conserved protein, has been found to be over-expressed in gastric cancer and be closely related with tumor malignancy. In this study, to investigate the relationship between PHB expression and cell apoptosis in the BGC823 gastric cancer cell line, low and high expression PHB in BGC823 cells was accomplished using RNA interference technology and gene transfer techniques. Cell proliferation, cell cycling, apoptosis, Bax, Bcl-2 and Cyt.c protein expression and the activation of Caspase-3,9 were assessed after 48h. Over-expression of PHB gene in BGC823 cells resulted in slow cell growth, cell arrest in G2 phase, and an increased apoptosis ratio while the opposite was found for PHB under-expressing cells. In PHB over-expressing cells, the expression of Bax gene was increased, the expression of Bcl-2 was decreased, the activation level of Caspase-3,9 was increased, but the activation level of Caspase-8 demonstrated no change. These results indicate that PHB induced apoptosis through the mitochondrial pathway.

Keywords: Prohibitin - apoptosis - BGC823 - mitochondrial pathway

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Introduction

Gastric cancer is a very serious disease in China and worldwide. About 160 000 deaths of gastric cancer patients occur every year in China (Ren et al., 2006). In the United States, gastric cancer is the fourth leading cause of cancer deaths with the lowest survival rate among all cancers (Sun et al., 2007). Previous studies have revealed several genes related to human gastric cancer, but the common molecular mechanisms of gastric cancer remain to be elucidated. Gastric cancer is a complex genetic disease, in which the expressions of many specific genes, known as oncogenes or tumor suppressors, are abnormally changed.

Prohibitin (PHB), an ubiquitous 30-kDa evolutionarily-conserved protein, located in a wide range of organisms like bacteria, plant, yeast, protozoon and mammalian (Leo et al., 2000). It was found that PHB might be closely related with the development of cancer. The expression level of PHB in cancer patients' serum was obviously higher than that in normal serum (Jorg et al., 2004). Spurdle et al (Spurdle et al., 2002) found that a C to T transition within the 3' untranslated region of the prohibitin gene increased risk of breast cancer in North American women. In lung and mammary cancer, PHB was also found to highly express (Jue et al, 1996).

In our previous study, PHB protein and mRNA were both found to be over-expressed in gastric cancer tissue. Moreover their elevated expression patterns paralleled the differentiation degree of gastric cancer (Kang et al, 2006; Kang et al., 2008; Zhang et al., 2009; Ji et al.,

2010). However, the relationship between PHB with the development of gastric cancer was not clear at present. The current study aimed to investigate the difference of cell proliferation, cell cycle, the expression of Bax, Bcl-2, cytoplasm Cyt.c and the activity of Caspase-3, 8, 9 in gastric cancer cell which had the different expression level of PHB. PHB can be founded to induce apoptosis in gastric cancer cell BGC823, Cyt.c were leaked into cytoplasm and the activity of Caspase-3, 9 were increased greatly. This results showed that Prohibitin-induced apoptosis in gastric cancer cell BGC823 through the mitochondrial pathway.

Materials and Methods

Cells and cell culture

Gastric cancer cells (BGC823) were cultured and maintained in 1640 (Invitrogen, USA), supplemented with 10% heated-inactivated fetal calf serum (FCS), 100 U/mL penicillin, 10 µg/mL streptomycin sulfate and then held at 37 °C in a humidified 5% CO₂ incubator.

Materials

T4 DNA Ligase, protein markers, restriction enzymes (BamHI and EcoRI) were all purchased from Takara (Dalian, China). Lipofectmin2000, Taq DNA-polymerase, M-MLV Reverse Transcriptase, gel-extraction kits were purchased from Qiagen (Shanghai, China). Trizol was purchased from Watson (Shanghai, China). Caspase-3, 8, 9 kits were purchased from Beyotime Institute of

Biotechnology (Shanghai,China). Mouse monoclonal antibody against PHB was purchased from Fermentas (Shanghai, China). Mouse monoclonal antibody against Bax, Bcl-2 and Cyt.c were purchased from Labvision(CA,USA). Gastric cancer cell BGC823 and pMD18-T-PHB plasmid were stored in our Laboratory.

Plasmid constructions

The pMD18-T-PHB plasmid was digested with BamHI and EcoRI to give a fragment of PHB, and ligated into the pEGFP-C1 vector (Novagen, Madison, USA) digested with the same endonucleases. E. coli DH5α cells were transformed with the ligation mixture and the colonies obtained were examined for the presence of PHB gene of by PCR amplification and sequencing analysis. The correct constructed plasmid was named pEGFP-C1-PHB.

Knockdown of PHB using siRNA

BGC-283 cells were plated in 6-well plates at a density of 1×10⁶ cells per well and incubated overnight at 37 °C, 5%CO₂. Cells were transfected with PHB siRNA at 10 nm concentration using Lipofectamine 2000 transfection reagent according to manufacturer`s Transfection and RNA interference. Cells were then grown for 72 hours at 37 °C, 5% CO₂.

Quantitative Real-Time PCR Analysis of PHB expression level

Real-Time PCR analysis was performed using the ABI7300. The reaction mixtures (25 ul) using the Taq-Man probe were: 10 × PCR buffer 2.5 ul, MgCl₂ (25 mM) 2 ul, primers (10 mM) 1 ul each, dNTPs (2.5mM) 2ul, TaqMan probe (10 mM) 1 ul, ddH₂O water 13 ul, cDNA 2 ul, Taq DNA polymerase 0.5ul. The following temperature/time program for Taqman-PCR was: 2 min at 94 °C, then 40 cycles with 30 s at 94 °C, and 30 s at 60 °C. Experiments were preformed in double for each sample. Real-time PCR data were analysed by computer and the threshold cycle (Ct) was calculated. The sequence of primers and probes were in Table 1.

Western blotting analysis of the expression levle of PHB,Bax,Bcl-2 and Cyt.c.

The cell which had been transfected or RNAi for 48h were collected, washed twice by PBS. The total protein and the cytoplasm protein without mitochondria protein were extracted respectively. The protein were separated on a 10% SDS-PAGE, followed by transferring to nitrocellulose membrane at 30 V for 2 h at room temperature. The nitrocellulose membrane was blocked with 10% dry milk and washed by PBS for two times. The membrane was then incubated with the primary antibody for 2h at room temperature, and washed by

PBS (contianing 20% Toween-20) for 3 times followed by incubation with secondary antibody (anti-mouse IgG horseradish peroxidase) for 2 h at room temperature. Finally, immunoreactive bands were detected by DAB. Relative expression level of target protein = Gray value of target protein/Gray value of Beta-actin.

Measurements of cellular viability and proliferation

BGC823 cells were seeded in 96-well plates with 5×10³ cells/well and incubated until the cells reached approximately 70–80% confluence. After transfection and RNA interference, the cell number were analysed by MTT assay at 24 h, 48 h, 72 h and 96 h.

Flow cytometric analysis

Kaiji Cell Cycle Detection Kit was used to analyse cell cycle and apoptosis. The cell which had been transfected or RNAi for 48h were collected, washed twice by PBS. 5×10⁵ cells were suspended with 500 μl binding buffer and incubated darkly with 5 μl APC and 5 μl 7-AAD. The cells were detected using FACSCalibur flow cytometry (BD Biosciences, USA) and CellQuest software package.

The protease activity of Caspase-3,8,9

The cell which had been transfected or RNAi for 48h were collected, washed twice by PBS. 2×10⁶ cells were suspended with 100 μl lysate liquid and lysated in ice for 15 min. The Supernatant were centrifugalized and detected.

Statistical analysis

All the detection items in this study were repeated at least 3 times. Statistical analysis was accomplished using SPSS software (Version 13.0, SPSS Inc, Chicago, IL, USA). The data were expressed as mean ± SD values. Statistical significance of the differences was determined by a two-tailed Student's t-test with a 95% confidence interval.

Results

PHB mRNA and protein expression levels after PHB transfection and RNA interference

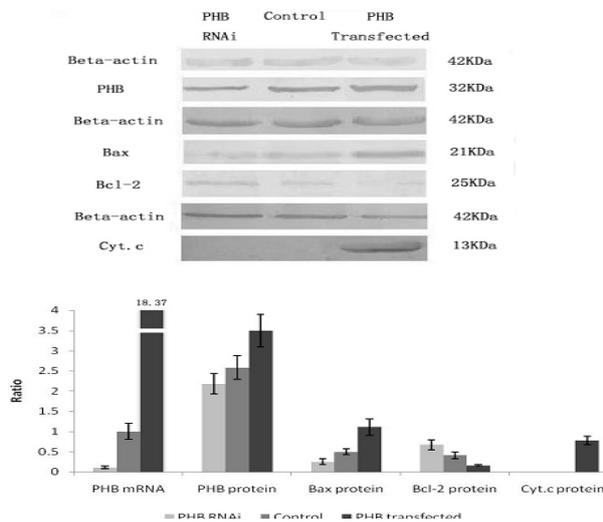
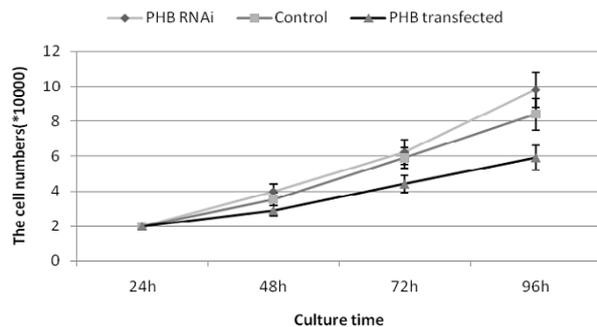
To confirm the effect of transfection and RNAi we measured the mRNA and protein expression level of PHB after PHB gene transfection and RNA interference after 48h by using quantitative RT-PCR (qRT-PCR) and western blotting. Compared with the normal gastric cancer cells BGC823, as expected, PHB mRNA expression level in transfected group increased deeply, but those in RNAi group decreased greatly (P<0.01);the PHB protein expression level has a similar trend with PHB mRNA expression level (Figure 1).

Table 1. The Sequence of the Primers and Probes for PHB and GAPDH

PHB	Forward Primer	5`-TCCCTACCAAAAATTGCCAAGT-3`
	Reverse Primer	5`-CCAGCAGGCCCCGAAT-3`
	Taqman Probe	5`-fam-CCTATGCAAACCCAGCTTTAGGTCCC-tamra-3`
GAPDH	Forward Primer	5`-GGTGGTCTCCTCTGACTTCAACA-3`
	Reverse Primer	5`-CCAAATTCGTTGTCATACCAGGAAATG-3`
	Taqman Probe	5`-fam-CGACACCCACTCCTCCACCTTTGACGC-tamra-3`

Table 2. The Cell Cycle and Apoptosis of BGC823 after Transfection and Interference by using Flow Cytometry(n=3);Compared with control, P<0.01; ## Compared with control, P<0.01**

Group	n	G0/G1(%)	S(%)	G2/M(%)	Apoptosis(%)
PHB RNAi	7	60.77±7.22	14.05±1.58	25.18±2.97*	1.23±0.21*
Control	7	64.19±8.46	15.26±2.01	20.56±2.89	3.65±0.27
PHB transfected	7	42.22±5.39##	18.06±1.72	39.71±3.02##	13.47±1.46##

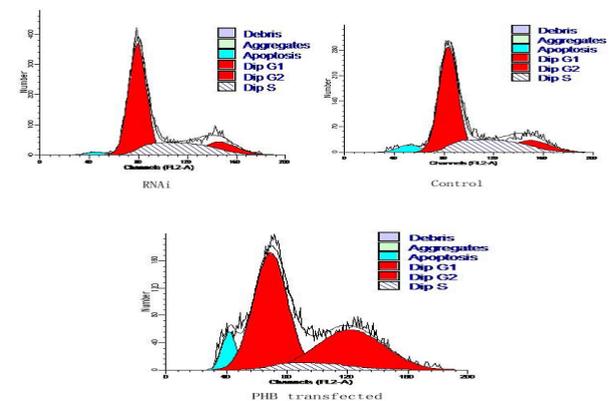
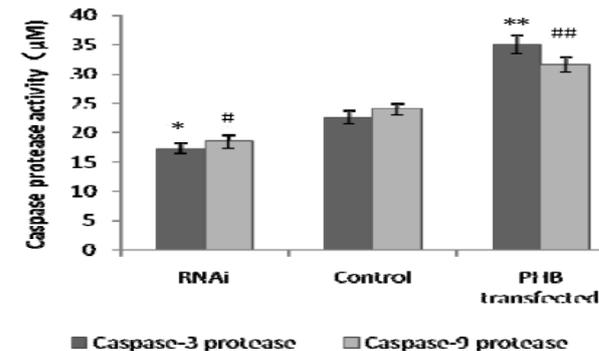
**Figure 1. The Change of Expression Level of PHB mRNA, PHB Protein, Bax Protein, Bcl-2 Protein and Cyt.c Protein after PHB Transfection and RNA Interference****Figure 2. The Cell Number of BGC823 after Transfection and RNA Interference (n=14).** The Results Show that the Cell Growth of BGC-823 Slowed in Transfected Group. However, the Cell Growth of BGC-823 Accelerated in RNAi Group

Expression levels of Bax, Bcl-2 and cytoplasm Cyt.c protein after PHB transfection and RNA interference

To confirm that PHB induced apoptosis was carried out through the mitochondrial pathway, we examined the changes of several protein expression level which were closely related to the mitochondrial apoptosis pathway after transfection and RNA interference for 48 h by Western Blotting. The Internal reference was Beta-actin. Compared with the normal gastric cancer cells BGC823, the content of cytoplasm Cyt.c and the expression level of PHB and Bax protein in transfected group increased deeply, but the expression level of PHB and Bcl-2 protein in RNAi group decreased greatly(P<0.01) (Figure 1).

Difference of proliferative viability of BGC823 cells after PHB transfection and RNA interference

After PHB gene transfection and RNA interference

**Figure 3. The Cell Cycle and Apoptosis of BGC823 after Transfection and RNA Interference by using Flow Cytometry****Figure 4. The Caspase-3, 9 Protease Activity of BGC823 after Transfection and RNA Interference (n=3); Compared with control, *0.01<P<0.05, **P<0.01; #0.01<P<0.05; ##P<0.01**

for 96h, the proliferative viability of BGC823 cells were detected by MTT assay. The results show that the cell growth of BGC823 slowed in transfected group. However, the cell growth of BGC823 accelerated in RNAi group. (Figure 2)

The cell cycle and apoptosis of BGC823 after transfection and RNA interference by using flow cytometry

The apoptotic bodies were observed in the BGC823 cells after PHB gene transfection and RNA interference for 48 h. According to the protocol of Kaiji Cell Cycle Detection Kit, the cell cycle and apoptosis were detected by using Flow cytometry. Compared with the normal gastric cells BGC823, G1 phase cells decreased significantly, G2 phase cells increased slightly, S phase cells and the apoptosis rate increased in transfected group; but G1 and G2 phase cells had little change, S phase cells increased slightly, the apoptosis rate decreased slightly in RNAi group (Figure 3, Table 2).

The difference of protease activity of Caspase-3,8,9

To determine the possible pathway in PHB- induce

apoptosis, the caspase cascade in BGC823 cells was analyzed. After PHB gene transfection and RNA interference for 48 hours, the protease activity of Caspase-3, 8, 9 were detected according to the protocol of Beyotime Caspase Activity Assay Kit. The activity of Caspase-3, 9 in transfected group were more than those of control group; but the activity of Caspase-3, 9 in RNAi group were less than those of control group ($P < 0.01$). The activity of Caspase-8 has no change (Figure 4).

Discussion

PHB is expressed as two transcripts, one is 1.2 kb and another is 1.9 kb in length. Both mRNAs code for the same 30-kDa protein, but the 1.9-kb transcript has a longer 3'UTR that contains two polyadenylation of cleavage signals at appropriate locations to produce the two mRNA species (Jupe et al., 1996). PHB comprise two evolutionarily conserved proteins, prohibitin-1 (PHB1) and prohibitin-2 (PHB2), which share more than 50% identical amino acid residues. Both proteins are present in organisms of all phylogenetic kingdoms. PHB1 was the first of the PHB to be discovered, and was identified on the basis that the expression of the gene was higher in normal cells compared with regenerating liver cells. PHB have been found to localize to mitochondria in all cell types examined to date. In vivo immunofluorescence studies, immunogold labeling and biochemical subcellular fractionation experiments in various cell types and in different organisms have been used to identify PHB as integral membrane proteins of the mitochondrial inner membrane (Ikonen et al., 1995; Coates et al., 1997; Snedden et al., 1997; Berger et al., 1998; Artal-Sanz et al., 2003).

In this study, the PHB expression level in gastric cancer cell BGC823 were regulated by using RNA interference and Transfection. The results from Real-time PCR and Western Blotting about PHB gene demonstrate that the up-regulated PHB expression can improve Bax expression, promote the cell apoptosis and inhibit Bcl-2 expression and the cell proliferation. Moreover, it can arrest cell cycle at S phase and enhance the activity of Caspase-3 and Caspase-9. On the contrary, the down-regulated PHB expression can decrease Bax expression, increase Bcl-2 expression, raise the cell proliferation, inhibit cell apoptosis and weaken the activity of Caspase-3 and Caspase-9.

Nineties of last century, Wang Xiaodong found the mitochondrial apoptosis pathway (Fang et al., 2002). In this pathways, basically, the release of the proapoptotic proteins from the intermembrane space triggers apoptosis, in a caspase-dependent (through cytochrome- c, Omi/HtrA2 and SMAC) as well as in a caspase independent form (through AIF and endonuclease G). Besides, activated caspases can cleave antiapoptotic Bcl-2 family members to render proapoptotic proteins, leading to more mitochondrial damage and, therefore, amplifying the apoptotic signal (Nora et al., 2005). Bcl-2 family proteins play an important role in regulating this pathway: Bcl-2 family proteins can form pore compounds in mitochondrial inter membrane and release the apoptotic

factors into the cytoplasm to cause apoptosis (Korsmeyer et al., 2000). Caspase-9, which was in the upstream of apoptosis cascade, was the important initiation factor of mitochondrial-dependent apoptosis pathway. The mitochondria released cytochrome C when the start signal reached them. Then Cytochrome C bound Apaf-1 and changed the conformation to octamer. The latter combined with the Caspase-9 and induced the self-catalysis. The activated Caspase-9 activated downstream Caspase-3 and made further amplification cascade reaction, leading to apoptosis (Wolf et al., 1999; Alenzi et al., 2010; Shi et al., 2011).

In summary, PHB could not only induce apoptosis in gastric cancer cells and its expression could change the expression level of Bcl-2 family protein and the activity of Caspase-3, 9 protein. Moreover, PHB itself located in the mitochondrial inner membrane and its high expression could cause mitochondrial damage to release the cytochrome C. Therefore, PHB probably induced apoptosis through mitochondrial pathway, but the details need further study.

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