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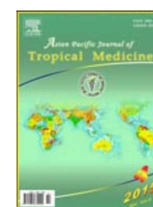

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Effects of gene silencing of *CypB* on gastric cancer cells

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ABSTRACT

Objective: To determine the effect of gene silencing of *cyclophilin B* (CypB) on growth and proliferation of gastric cancer cells. **Methods:** CypB siRNA lentivirus (LV-CypB-si) and control lentivirus (LV-si-con) were produced. CypB expression in gastric cancer cell lines was detected by Western blot. BGC823 and SGC7901 cells were chosen to be infected with LV-si-con and LV-CypB-si, and stable transfectants were isolated. The cell groups transfected with LV-CypB–siRNA, LV-siRNA-con and transfected no carrier were served as the experimental group, the implicit control group and the blank control group respectively. MTT and colony formation assays were used to examine the effect of CypB on the cell growth and proliferation *in vitro*. Cell cycle was analyzed with flow cytometry. The expression of VEGFR of BGC823-si and SGC7901-si was detected by Western blot. **Results:** Gene silencing of *CypB* can inhibit gastric cancer cell growth, proliferation, cell cycle progress and tumorigenesis. *CypB* expression level was obviously higher in SGC7901 and BGC823 than MKN28 and GES. These two cell lines were infected with LV-si-con and LV-CypB-si respectively. MTT and cloney formation assays showed a significantly decreased rate of cell proliferation from the forth day or the fifth day in cells transfected with LV-CypB-si (P<0.05). Down-regulation of *CypB* resulted in slightly decreased percentage of S phase and increased percentage of G_1 (P<0.05). These findings indicated that CypB could promote the G_1-S transition of gastric cancer cell. In addition, the expression of VEGF of BGC823 and SGC7901 transfected with CypB siRNA was reduced in comparison with the implicit control group and the blank control group. **Conclusions:** Gene silencing of *CypB* decreases gastric cancer cells proliferation and *in vivo* tumorigenesis. These findings indiccate CypB could be a potential biomarker and therapeutic target for gastric cancer.

1. Introduction

Multi-genes involvement and abnormality of multi-signal transduction pathway are found to play an important role in the genesis, development, invasion and metastasis of gastric carcinoma, which provides a theoretical basis for treating gastric cancer with gene therapy[1–5]. The expression of vascular endothelial growth factor receptor (VEGFR), to a certain extent, is controlled by the expression of cyclophilinB (CypB). VEGFR expression in gastric cancer has a close relationship with lymph node metastasis in patients suffered from gastric cancer, and its high expression in gastric cancer cells often indicates poor prognosis of patients[6]. Hence, down regulating the expression level of CypB and thereby inhibiting the activity of VEGFR could be an important means to treat gastric cancer. CypB also plays an important role in endonuclear transcription activation[7–10]. Dieriks *et al* found that within the fibrillar centers CypB interacts with RNA polymerase, dyskerin (DCK1) and fibrillarin and is involved in ribosome biogenesis and RNA transcription[11]. In addition, CypB is involved in various cancer-promoting signaling pathways in liver cancer, pancreatic cancer, breast cancer and other tumors[12–14], significantly influencing the malignant development of tumors.

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In tumors like breast tumor, proliferation and migration of tumor cells can be remarkably inhibited through *CypB* gene silencing, which undoubtedly point out a new direction for the treatment of gastric cancer[15]. However, at present the specific role of CypB in gastric cancer remains unclear, and the reports on using *CypB* down regulation as gene therapy for cancer are scarce. Hence, to determine the effect of gene silencing of *CypB* on the growth and proliferation of gastric cancer cells CypB siRNA lentivirus (LV-CypB-siRNA) and corresponding control lentivirus (LV-siRNA-con) were produced and transfected into BGC823 and SGC7901 cell lines. And then detection and comparison of the growth, proliferation and monoclonal forming capacity of these cells were carried out to confirm our hypothesis that gene silencing of *CypB* may have impact on the growth and proliferation of gastric cancer cells. At the same time, the expression of VEGFR in CypB-silenced BGC823 and SGC7901 cell lines was also explored so as to provide a basis for further research on mechanism of the effect of CypB on gastric cancer.

2. Materials and methods

2.1. Reagents and instruments

GES, SGCSGC7901, MKN-28 and BGC823 cell lines were all procured from Institute of Biochemistry and Cell Biology, SIBS, CAS. DMEM and 1604 cell culture medium were purchased from HYCLONE Ltd., USA. Fetal calf serum was from GIBCO Ltd., USA. ECL Western Blot kits were purchased from Santa Cruz Ltd., USA. PCR primer (R&F) was produced by Shanghai Genechem Ltd., China and TEMED was obtained from Beijing Beyotime Ltd., China.

2.2. Construction of LV–CypB–siRNA

In the present study, interference or random sequences of siRNA were inserted in the corresponding restriction enzyme cutting site of lentivirus vector GV115 and the sequence of the components were as follow: hU6-MCS-CMV-EGFP. The vector contains green fluorescent protein sequences and EGFP can be used as an indicator of transfection efficiency. The sequence of CypB siRNA was GGTGGAGAGCACCAAGACA and the vector was GV115. Single-stranded DNA oligo containing interference sequence was chemically synthesized, then annealing to produce double strands. The synthesized DNA was connected to the digested lentiviral vector RNAi at the restriction enzyme cutting sites, *Age* Ⅰ and *Eco*R Ⅰ. and the synthesized product was transfected into the prepared cells. When positive clone of LV-CypB-siRNA was confirmed by PCR, the sequences was detected and the results were compared.

2.3. Cell culture

Gastric cancer cell lines SGC7901 and BGC823 were cultured in a medium containing 10% fetal bovine serum, vaccinated in 10 cm cell culture dishes, and kept in incubator under 37 ℃ and 5% CO_2. SGC7901 and BGC823 cells were inoculated onto 24-well cell culture plates with 4×10^4 cells per well. The virus were taken out of the refrigerator, centrifuged and diluted 10 times on the ice. A volume of 10 μL lentiviral vector dilution was added to the cell culture at a MOI of 10. The culture solution was renewed after the overnight incubation. After 72 h fluorescence was observed under inverted fluorescence microscope to estimate the infection efficiency of the lentiviral vector into cells. When the cells full filled the well, they were gradually subcultured into 6-well plates and 25 cm culture flasks. The cell groups transfected with LV-CypB-siRNA, LV-siRNA-con and transfected no carrier were served as the experimental groups (BGC823-si group and SGC7901-si group), the implicit control groups (BGC823-con group and SGC7901-con group) and the blank control groups (BGC823 group and SGC7901-con group) respectively, frozen for further use.

2.4. Western blot detection of the CypB expression in gastric cancer cell lines

After transfection about 5×10^6 of gastric cancer cells in logarithmic phase were collected, washed twice with cold PBS. Cell lysis buffer (100 μL) was added into the collected cells, kept in ice-bath for 30 min and centrifuged at 12 000 *g*/min at 4 ℃ for 10 min. The total proteins were extracted by BCA protein assay kit and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were transferred onto nitrocellulose membranes under 80 V constant voltage. Membranes were blocked using 10 mL 10% defatted milk and corresponding primary antibody CypB (Abcam 1:1 000) were added. After incubated overnight at 4 ℃ membranes were washed with TBST. Defatted milk (2%) was used to dilute the corresponding HRP-conjugated secondary antibody (1:2 000), and the membranes were incubated at room temperature for 1-2 h, and developed using ECL after washing with TBST. Bandscan 5.0 software was employed to detect the grey level of the bands. The ratio of grey levels of the targeted band and the corresponding β-actin was regarded as the indicator of the expression level of the targeted protein samples.

2.5. Determination of cell proliferation by MTT test

BGC823-si, BGC823-con, BGC823, SGC7901-si, SGC7901-con and SGC7901 cells were inoculated. Single-cell suspension was prepared using 1640 cell culture medium containing 10% fetal calf serum. Then the suspension was inoculated onto 96-well plates with 1 000 cells per well and the volume was 200 μL per well. After 3 days culture in the incubator at 37 ℃ and 5% CO_2, 20 μL of MTT solution (5 mg/mL) was added to each well. After 4 h incubation at 37 ℃, the culture of cells was terminated. The supernatant in the wells was removed. The 150 μL DMSO was added into each well, shaking for 10 min to sufficiently dissolve the crystal substance.

Then enzyme-linked immunosorbent assay was used to detect the absorbance at 490 nm for each well. Cell growth curves in each groups were plotted with time as x-axis and the mean absorbance in three wells as y-axis.

2.6. Colony formation assays

Cells in logarithmic phase in each group (SGC7901-si group, SGC7901-con group, BGC-si group and BGC-con group) were selected, digested by 25% trypsin, separated into single cell and suspended in 1640 culture medium containing 10% fetal calf serum for further use. Gradient dilution of the cell suspensions was performed. Cells in each group were gradiently inoculated into 37 ℃ preheated culture medium (10 mL) with 500 cells per dish, gently shaking to make the cells homogeneous spread. Then cells were cultured in incubator under 37 ℃, 5% CO_2 and saturated humidity for 2-3 weeks. Culture medium was changed every two days until the formation of typical clone of the cells. The supernatants were removed and the cells were washed with PBS buffer twice. Cells were fixed with 4% paraformaldehyde (4%) for 15 min. After removing of the fixing liquid and 30-min GIMSA staining, cells were washed slowly with running water, dried in air and counted with naked eye.

2.7. Cell cycle assay

Cells in each group (experimental, implicit control and blank control groups) were inoculated onto 60 mm culture plates (1×10^6 for each group of cells). After 48 h cells were collected by tyrisin digestion, washed by PBS buffer and the supernatants were removed. Precooled ethyl alcohol (1 mL, 70%) was added; cells were homogenized and kept in refrigerator for fixing for more than 12 h. Then cells were washed with PBS to remove ethyl alcohol, centrifuged twice at 100 rpm for 5 min. Cells were resuspended with 0.5 mL PBS. PI and RNaseA were added and adjusted to the final concentration of 50 g/mL, kept in warm bath at 37 ℃ for 30 min. Cell cycle was determined using flow cytometry.

2.8. VEGFR expression in gastric cancer cells by Western blot assay

After adding RIPA lysis buffer the collected cells were placed on the ice for 30 min, centrifuged at 12 000 rpm under 4 ℃ for 5 min, and the supernatants were collected. The total proteins were extracted by BCA protein assay kit and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were transferred onto nitrocellulose membranes under 80 V constant voltage. Membranes were blocked using 10 mL 10% defatted milk and corresponding primary antibody were added. After incubated overnight at 4 ℃ membranes were washed with TBST. The corresponding HRP-conjugated secondary antibody (1:5 000) were added into the membranes, incubated for 1 h, and developed using ECL after washing with TBST. Bandscan 5.0 software was employed to detect the grey level of the bands. The ratio of grey levels of the targeted band and the corresponding β-actin was regarded as the indicator of the expression level of the targeted protein samples.

2.9. Statistical analysis

All the experiments were repeated for at least three times. Graph-Pad Prism 4 (GraphPad Software, La Jolla, CA) was employed for statistical analysis. Results in each group were analyzed by Student's *t* test and $P<0.05$ was regarded as significantly difference.

3. Results

3.1. Construction of LV–CypB–siRNA

The sequencing assay showed that the target sequence has been successfully inserted, suggesting that the pGV115-CypB-siRNA plasmid was successfully constructed (Figure 1).

CTAGAGAGATAaTTGGaATTAATTTGACTGTAAACACAAAGATATTA
GTACAAAA
TACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAA
AATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGA
TTTCTTGGC
TTTATATATCTTGTGGACAGGACGAAACACCGGAAGGTGGAGAGCA
CCAAGAC
ACTCGAGTGTCTTGGTGCTCTCCACCTTTTTTTTGAATTTCGGGATC
CATTTAGG
GCGGGCCGGCGTGGAATAAACCGTATTACCGGCCATGCCATAAGTT
TATTAAAT
AAGTAATTCAATTTACGGGGCTCTTTAGTTTCATAAGCCCATATATT
GGAGTTTC
GGCGTTTACATACCTTACGGAAATGGCCCGCCTGGTTGACCGCCCA
ACGACCCC
GGTCTATTGACGTCATAATGACGTATGTTCCCATAGTAACGCGATAG
GGACTTTC
CACTGACGTACTGGGTGGAGGAC

(Sequence marked with red is designed siRNA sequence).

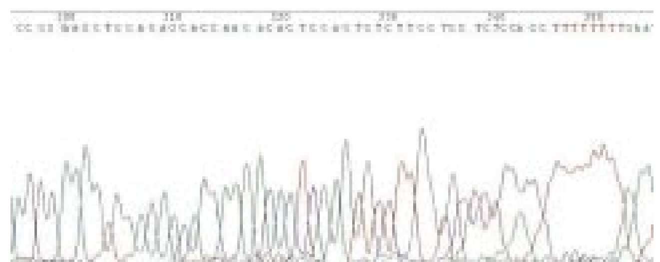

Figure 1. Sequencing results of lentivirus vector after inserted with isRNA.

3.2. CypB expression in gastric cancer cell lines

Western blot was used to detect the expression of *CypB* in immortalized gastric epithelial cells line GES, gastric cancer cell line MKN28, SGC7901 and BGC823. It was found that the expression of *CypB* was lower in GES and MKN28 but higher in SGC7901 and BGC823 cell lines (Figure 2). Therefore we chose SGC7901 and BGC823 cells to infect with lentivirus CypB-siRNA CypB for further functional study on CypB.

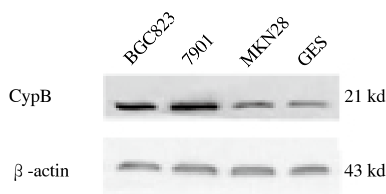

Figure 2. Expression of *CypB* in gastric cancer cell line BGC823, SGC7901, MKN28 and immortalized gastric epithelial cells line GES.
Compared with expression of β *–action* (43 kd), the expression of *CypB* (21 kd) was lower in GES and MKN28 and higher in SGC7901 and BGC-823 cells.

3.3. Determination of cell proliferation by MTT assay

According to the results of MTT assay, it was found that the growth rate of LV-CypB-siRNA infected BGC823 and SGC7901 cells, compared with the blank control group and implicit control group, started to significantly slow down from the fourth and fifth days, respectively (P < 0.05) (Figure 3A and 3B), while there was no statistically significant differences between the blank control group and implicit control group.

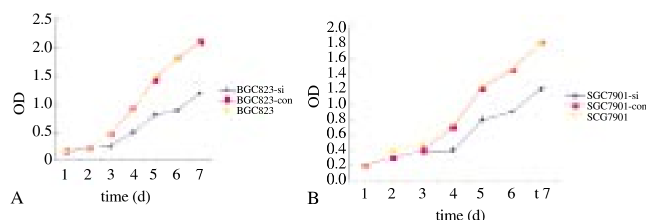

Figure 3. Determination of cell proliferation by MTT assay.
A: Cell growth (OD value) in BGC823-si group was significantly slow down from the fourth day compared with BGC823 group and BGC823-con group (P<0.05); B: Cell growth (OD value) in SGC7901-si group was significantly slow down from the fifth day compared with SGC7901 group and SGC7901-con group (P<0.05).

3.4. Determination of clonality using colony formation assay

The clonality of the single cell in experimental group, negative control and blank control groups were ca were detected using colony formation assay. After infected with LV-CypB-siRNA the clonality of the BGC823 and SGC7901 cells were reduced compared with that in control groups (P<0.05) (Figure 4).

3.5. Determination of cell growth and proliferation capacity by cell cycle assay

After transfection of CypB siRNA in BGC823 and SGC7901 the proportion of cells in G_1 phase increased while cells in S phase significantly decreased compared with that in negative control group and blank control group (P<0.05) (Figure 5A and 5 B).

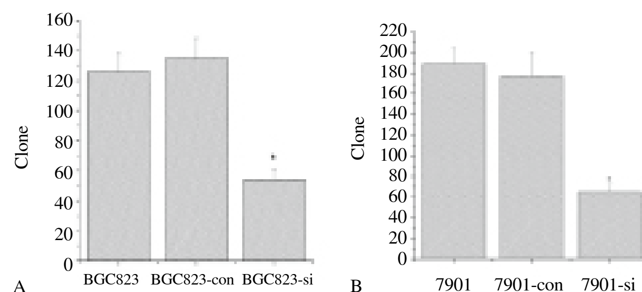

Figure 4. Effect of CypB on the clone formation capacity of BGC823 and SGC7901 gastric carcinoma cells.
A: The clone formation capacity of LV-CypB-siRNA infected BGC823 cells were significantly reduced compared with BGC823 (t=15, P=0.04) and BGC823-con group (t=21, P=0.03); B: The clone formation capacity of LV-CypB-siRNA infected SGC7901 cells were significantly reduced compared with SGC7901 (t=25, P=0.01) and SGC7901-con group (t=22, P=0.03).

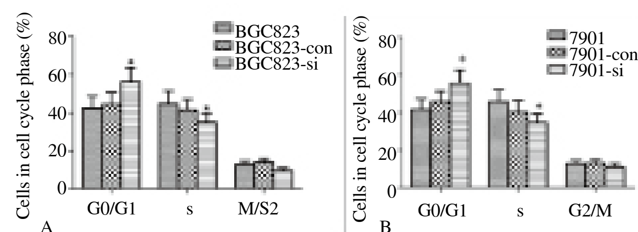

Figure 5. Effect of *CypB* gene silencing on the cell cycles of BGC823 and SGC7901 cells.
A: Compared with BGC823-con and BGC823 groups, cells in G_1 phase increased and cells in S phase significantly decreased in BGC823-si group (P<0.05); B: Compared with SGC7901-con and SGC7901 groups, cells in G_1 phase increased and cells in S phase significantly decreased in SGC7901-si group (P<0.05).

3.6. VEGFR expression in gastric cancer cells by Western blot assay

CypB and CD147 are mutually ligand and receptor. And it is known that CD147 is closely related to tumor. It can regulate matrix metalloproteinases (MMP2, MMP9, and VEGFR) and involves in regulating the growth, proliferation and metastasis of tumors. We detected the expression of VEGFR in CypB silenced BGC823 and SGC7901 gastric cancer cells (Figure 6). And the results showed that compared with negative control group and blank control group, the expression of VEGFR in CypB siRNA transfected BGC823 and

SGC7901cells were reduced, especially in CypB siRNA transfected BGC823 cells (BGC823-si group).

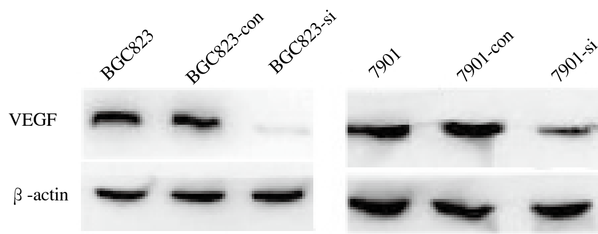

Figure 6. Western blot detection of VEGFR expression in *CypB* gene silenced BGC823 and SGC7901 cells .

The expression of VEGFR in BGC823-si group was significantly decreased compared with BGC823-con and BGC823 groups. The expression of VEGFR inSGC7901-si group was slightly decreased compared with SGC7901-con and SGC7901 groups. And the expression of β -action in each group did not show much difference.

4. Discussion

Many studies have shown that Cyp is a kind of multifunctional protein which is likely to play an important role in diseases such as tumor[16–18]. For example, CypA, highly homologous with CypB, shows significantly increasing amount of expression in hepatocellular carcinoma, lung cancer, pancreatic cancer and endometrial cancer expression[19–22]. The high expression of the members of Cyp proteins in malignancy tissue is likely to be closely correlated with the genesis and development of the malignant tumor. However, relevant research on the effects of CypB expression level on the cancer cells is rare, and its specific role and possible mechanism of action remains unclear. By silencing the expression of CypB, the present study detected its effect on the growth, proliferation and monoclonal formation ability, and also verified that *CypB* gene silencing can be used as a new method of gastric cancer treatment. At the same time its possible mechanism of action was explored.

4.1. CypB may be closely associated with the growth and migration of gastric cancer cells

In the present study LV-CypB-siRNA and the corresponding control carrier (LV-siRNA-con) were used to transfect gastric cancer cell lines BGC823 and SGC7901. Results showed after LV-CypB-siRNA was stablely transfected into BGC823 and SGC7901, the growth and proliferation ability, monoclonal formation ability and *in vivo* tumor formation ability were significantly diminished compared with control group. The cell cycle in G_1/S phase was blocked, suggesting *CypB* gene silencing can effectively inhibit the growth, invasion and migration of gastric cancer cells. At the same time, CypB siRNA inhibited the development of the cell cycle, blocking the cell phase in G_1 and S phases. Meza-Zepedu *et al* found that CypD and FK506 binding protein4 (FKBP4), CypAL-4A and NIMA - interacting protein l (Pinl) were highly expressed in breast cancer, osteosarcoma and squamous cell carcinoma of the head and neck, respectively[23–25]. In addition, Leong *et al* reported that by down regulating the expression of CypB in breast cancer cells, cell growth, proliferation and migration were inhibited[26]. By studying the expression profile of *CypB* gene silenced breast cancer cells and control cells, it was found that expression of 663 genes were changed, among which expression of 297 genes were up regulated and 366 genes were down regulated including stathminlike 3 (STMN3) which is closely related to the invasion of tumors. In addition, expression of S100A4 whose relation with breast cancer has been studied in many researches was also significantly decreased, and it plays an important role in tumor invasion and metastasis[14,27]. The results of the effect of CypB in breast cancer indirectly support our experimental conclusion, namely CypB plays an important role in gastric cancer, and may be closely related with tumor growth and migration.

4.2. CypB gene silencing can effectively decrease the expression of VEGFR in gastric cancer cells

To study the progress mechanism of CypB involved in gastric cancer, the proteins of CypB siRNA infected BGC823 cells and control cells were collected, and the VEGFR expression was detected by using western blot method. It was found that after decrease the expression of CypB, the expression of VEGFR was also significantly decreased compared with that of control group. Kim *et al* found that when oxygen is deficient, hypoxia-inducible factor HIF-1 can directly act on CypB promoter, leading to its increasing expression[28]. They also found that CypB works together with STAT3 in the promoter region of HIF-1, producing a feedback regulation of HIF-1 expression. CypB can protect tumor cells (including p53-absent cells) from apoptosis induced by anti-hypoxic and cis-platinum. In addition, under the hypoxic condition, overexpressed CypB can induce more release of VEGFR and generation of new vessels[29,30]. The present study verified that decreasing the expression of CypB in gastric cancer will simultaneously decrease the expression of VEGFR, suggesting that CypB may be involved in regulating the signaling pathway of VEGFR. The results show that CypB may be involved in promoting development of gastric cancer, and CypB could be a potential therapeutic target in gastric cancer treatment. However, its specific mechanism remains to be further studied and discussed.

4.3. Deficiency of the present study

Our work is imperfect. We have just performed an *in vitro* study of *CypB* gene silencing in gastric cancer, and further *in vivo* study is needed to verify its effects. In addition, the mechanisms of CypB involved in development of gastric cancer was not deeply studied, which is a deficiency of the present study. Hence further research is needed.

In conclusion, CypB may be related with the growth, invasion and migration of gastric cancer cells. *CypB* gene silence may influence the growth and proliferation of the gastric cancer cells, providing a new prospect for the gene therapy of gastric cancer. Furthermore, *CypB* gene silience also decreases the expression of VEGFR, which provides a basis for further study on the mechanism of CypB in gastric cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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