

Original Research Article

Heparin-binding epidermal growth factor inhibits apoptosis in cisplatin-resistant pancreatic cancer cells via upregulation of EGFR and ERCC 1 expressions

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Abstract

Purpose: To investigate the influence of heparin-binding epidermal growth factor (HB-EGF) on apoptosis in cisplatin-resistant pancreatic cancer cells, as well as its mechanism of action.

Methods: Pancreatic cancer cisplatin-resistant cells (BXPC-3/CDDP) were transfected with HB-EGF small interfering RNA (siRNA). The cells were randomly assigned to four groups, namely, BXPC-3 group (group A), BXPC-3/CDDP group (group B), transfected group A (group Asi) and transfected group B (group Bsi). Cell proliferation was determined using MTT assay, and the levels of expression of HB-EGF, epidermal growth factor receptor (EGFR) and excision repair cross-complementation group 1 (ERCC 1) were determined using Western blotting. The extent of apoptosis was determined by flow cytometry.

Results: Cell proliferation was increased in group B, relative to group A, but was significantly decreased after transfection with HB-EGF siRNA ($p < 0.05$). The half-maximal inhibitory concentration (IC₅₀) of group Bsi was reduced, relative to group Asi ($p < 0.05$). The expression of HB-EGF was significantly upregulated in group B, relative to group A ($p < 0.05$). In contrast, HB-EGF siRNA transfection of the cells significantly down-regulated HB-EGF expression ($p < 0.05$). Early apoptosis was significantly higher in group A than in groups B and Bsi. Higher levels of apoptosis were seen in group Bsi, relative to group B after inhibition of HB-EGF expression ($p < 0.05$).

Conclusion: These results indicate that HB-EGF is resistant to cisplatin, and it inhibits apoptosis in pancreatic cancer cells via the upregulation of EGFR and ERCC 1 expressions.

Keywords: Heparin-binding epidermal growth factor, Cisplatin resistance, Pancreatic cancer, Apoptosis, Proliferation, EGFR

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INTRODUCTION

Pancreatic cancer (PC) is a highly malignant gastrointestinal tumor characterized by high mortality and poor prognosis. In the United States, less than 6 % of PC patients survive

beyond 5 years. In China, PC is ranked ninth in the hierarchy of malignant tumors [1]. Each year, more than 200,000 people worldwide die from PC, and the overall mortality keeps rising. Approximately 20 % of patients diagnosed with PC go through surgical resection with a high

probability of recurrence, and only 5 % survive beyond 5 years [2].

Treatment strategies for PC include surgery, chemotherapy, radiation therapy and other comprehensive treatments. The disease is not easily detected at its early stage due to its high degree of malignancy. It is mostly diagnosed in the advanced stage, at which time radical surgery cannot be employed. Chemotherapy is an extremely important treatment for advanced PC. Cisplatin is usually used in PC chemotherapy, but its use is limited due to resistance. Reducing cisplatin resistance is an effective way to improve its chemotherapeutic effect in patients with PC.

Heparin-binding epidermal growth factor (HB-EGF) is a peptide growth factor expressed in cultures of human macrophages, and is so named because of its strong affinity for heparin [3]. As an ubiquitous molecule, HB-EGF is present in various organs and tissues. Its expression is upregulated in various tumors. Studies have demonstrated a link between HB-EGF and tumor progression since it accelerates cell cycle progression and proliferation [4]. Indeed, HB-EGF inhibits apoptosis via the activation of Ras/Raf/MAPK and AKT signaling pathways, and this phenomenon is evident in both tumor growth and drug resistance. Studies have also shown that HB-EGF can be used as a drug target in tumor chemotherapy and that it can reverse resistance associated with most chemotherapy drugs [5]. This study was carried out to investigate the effect of HB-EGF on apoptosis in cisplatin-resistant pancreatic cancer cells, and the underlying mechanism.

EXPERIMENTAL

Materials

Human PC cell line (BXPC-3) was provided by the Third Military Medical University Laboratory, while RPMI 1640 cell culture medium was purchased from Thermo Fisher Co., Ltd. (USA). Human HB-EGF gene-specific siRNA and non-specific sequence siRNA were purchased from Sigma Co., Ltd. (USA). Cisplatin injection and MTT assay kit were products of Sigma-Aldrich (Germany), while lipofectamine reagent was obtained from Invitrogen (USA).

Cell grouping

Human PC cell line (BXPC-3) was used in this study. The pancreatic cancer cisplatin-resistant cells (BXPC-3/CDDP) were obtained by culturing some of the BXPC-3 cells in RPMI-1640 medium

containing increasing concentrations of cisplatin. Some of the BXPC-3 and BXPC-3/CDDP cells were transfected with HB-EGF siRNA. The cells were randomly assigned to four groups: BXPC-3 group (group A), BXPC-3/CDDP group (group B), transfected group A (group Asi), and transfected group B (group Bsi).

Establishment of cisplatin-resistant PC cells (BXPC-3/CDDP)

The parental BXPC-3 cells were maintained in RPMI-1640 medium to which were added FBS (10 %), as well as penicillin and streptomycin (each at a concentration of 100 µg/mL), and incubated at 37 °C in a humidified incubator with 5 % CO₂. The culture medium was changed for fresh medium at intervals of 48 – 72 h. At 80 % confluency, trypsinization was applied to the cells. Cells in logarithmic growth phase were inoculated into RPMI-1640 medium containing increasing concentrations of cisplatin. The cells were incubated for 2 days, after which the cisplatin-containing supernatant was discarded and replaced with fresh medium. This procedure was repeated several times until cells that tolerated 1.0 µg/mL of cisplatin were obtained.

Transfection of BXPC-3 and BXPC-3/CDDP cells with HB-EGF siRNA

This was performed following Lipofectamine 2000 instructions. The BXPC-3 and BXPC-3/CDDP cells were seeded into 96-well plates 24 h prior to being transfected. At 60 - 70 % confluency, HB-EGF siRNA was transfected into the cells using liposomes, and the cells were incubated for 2 days.

Western blotting

This was carried out to determine the levels of EGFR, HB-EGF, and ERCC in the cells. The cells were lysed using chilled RIPA buffer for 180 min. The cell lysate was rinsed two times in PBS, and then treated with 10 µg/mL leupeptin, Nonidet P-40 (1 %), Triton X-100 (0.1 %), Na₂PO₄ (30 mM) mixed with 1 nM sodium orthovanadate, 2.5 mM Tris-HCl, 100 mM NaCl, and aprotinin for 45 min at 4 °C. Thereafter, the cell suspension was subjected to centrifugation for 160 s at 12,000 g at 4 °C.

Protein concentration of the supernatant was estimated with bicinchoninic acid (BCA) assay kit. A portion of total cell protein (35 µg) from each sample was electrophoresed using 10 % SDS-PAGE, followed by transfer onto a fixed PVDF membrane at 110 V and 90 °C for 120 min. Subsequently, skimmed milk powder (0.05

%) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Thereafter, the blot was subjected to incubation with primary antibodies of HB-EGF, EGFR, ERCC 1 and β-actin at a dilution of 1 to 200 at 4 °C overnight.

Then, the PVDF membrane was washed thrice with TBS-T and further subjected to incubation with HRP-linked goat anti-rabbit IgG secondary antibody (1:3000) for 60 s at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ImageJ analysis software (4.6.2). Respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

Assay of cell proliferation

The cells were cultured in a 96-well plate at a density of 5 × 10⁴ cells/well by adding FBS supplemented RPMI-1640 medium, and were incubated for 24 h. Varied concentrations of cisplatin (up to 200 µg/ml) were introduced, and the cells were subjected to incubation for 48 h. Thereafter, MTT solution (5 mg/mL, 20 µL) was put into each well, and the wells were incubated for 4 h. Following medium removal, the resultant formazan crystals were solubilized in 150 µl of DMSO, followed by agitation for 10 min. Optical density of each sample was measured at 570 nm in a microplate reader. All experiments were done in triplicates and cell proliferation (P) was calculated as shown in Equation 1.

$$P (\%) = \{(1 - Abs)/Abc\}100 \dots\dots\dots (1)$$

where Abs and Abc are absorbance values of sample and control wells, respectively.

Apoptosis assay

Induction of apoptosis in the cells was determined using a flow cytometer. To each group of cells were added 500 µL of digestive juice (a solution of 0.1 % trypsin and 0.02 % EDTA) until the cells appeared rounded and enlarged on microscopic examination. The cells were incubated for 72 h, harvested and subsequently treated with HEPES binding buffer which contained propidium iodide (PI) and Annexin V-FITC.

The treatment was carried out at room temperature for 20 min, after which the cells were placed on a flow cytometer and read. The measurements were performed in triplicates and the mean was taken.

Statistical analysis

Data are expressed as mean ± SEM, and statistical analysis was performed using SPSS, version 20.0. Two-group comparison was done using t-test. Statistical significance was fixed at p < 0.05 significant.

RESULTS

Effect of treatments on cell proliferation

There were only few viable cells in cisplatin-treated wells. Cisplatin significantly and dose-dependently reduced cell proliferation (p < 0.05). Cell proliferation was markedly increased in group B, when compared to group A, but was markedly decreased after transfection with HB-EGF siRNA (p < 0.05; Figure 1).

Higher IC₅₀ was seen in group B cells than in group A, but IC₅₀ was significantly decreased after transfection (p < 0.05). However, IC₅₀ was markedly lower in group Bsi than in group Asi (p < 0.05; Table 1).

Table 1: Effect of cisplatin on cell proliferation

Parameter	Group			
	A	Asi	Bsi	B
IC ₅₀ (µg/mL)	66.00	52.00*	270.20	90.10 [#]

*P < 0.05, compared with group A; [#]p < 0.05, compared with group B

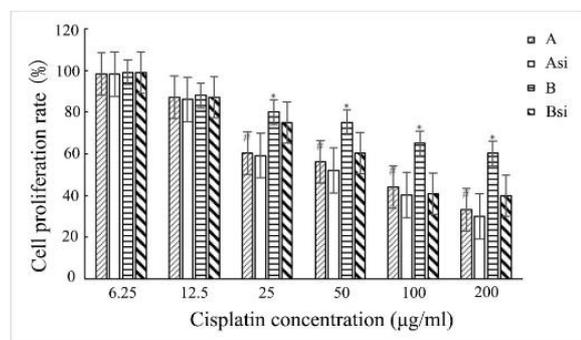


Figure 1: Effect of cisplatin on cell proliferation; [#]p < 0.05, relative to group B; *p < 0.05, relative to Bsi group

Expression of HB-EGF

Figure 2 shows that in group B, HB-EGF expression was markedly elevated, relative to that of group A (p < 0.05). However, when the cells were transfected with HB-EGF siRNA, HB-EGF expression was markedly decreased (p < 0.05). A significant reduction was seen in HB-EGF expression in group Bsi, relative to group Asi (p < 0.05).



Figure 2: HB-EGF levels in the cells

Expressions of EGFR and ERCC 1 proteins in cisplatin-resistant cells

Figure 3 shows that the levels of expression of EGFR and ERCC1 were markedly less in group Bsi, when compared with group B ($p < 0.05$).

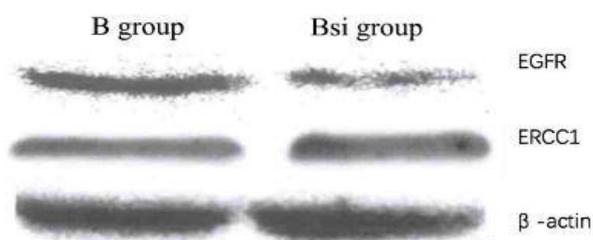


Figure 3: Degrees of protein expressions of EGFR and ERCC 1 in cisplatin-resistant cells

Outcomes of flow cytometric analysis

Early apoptosis was markedly increased in group A (21.16 ± 3.85 %), relative to groups B (0.47 ± 0.12 %) and Bsi (1.13 ± 0.08 %). Apoptosis was also markedly higher in group Bsi than in group B after inhibition of HB-EGF expression ($p < 0.05$; Figure 4).

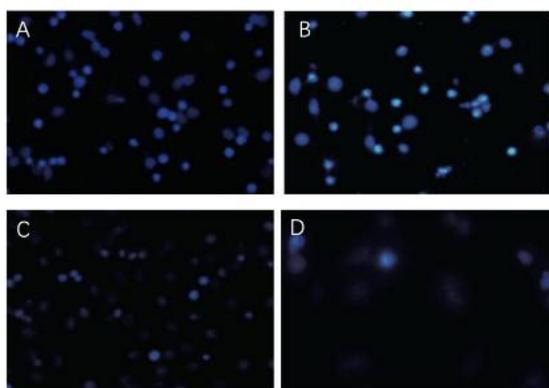


Figure 4: Morphology of cells under fluorescence microscopy ($\times 200$). Groups Asi (A), A (B), Bsi (C), and B (D)

DISCUSSION

The current treatment for advanced PC is not very effective, and the mean survival is only 9 - 10 months [5]. The use of chemotherapy in the treatment of advanced PC can significantly

prolong patients' survival. Due to structural similarities between cisplatin and its derivatives, its use as a chemotherapeutic agent is accompanied by serious resistance which limits its therapeutic effectiveness [6]. In order to overcome cisplatin resistance in PC treatment, it is important to find new targets.

Apoptosis, which is a programmed cell death, is regulated at the genetic level. Studies have shown that some anti-apoptotic proteins are involved in cisplatin resistance. It is known that HB-EGF, one of the seven ligands of EGFR, is highly expressed in a variety of tumor cells, and takes part in several physiological and pathological events.

In the present study, Western blotting showed that the expression of HB-EGF was markedly upregulated in group B, relative to group A. However, HB-EGF siRNA transfection down-regulated HB-EGF expression. Moreover, HB-EGF was expressed markedly less in Bsi group, when compared to group Asi. Thus, HB-EGF may be highly expressed in cisplatin-resistant cells. The IC_{50} was markedly higher in group B than in group A, an indication that group B cells were resistant to cisplatin. The IC_{50} of the cells was significantly decreased after their transfection with HB-EGF siRNA. Flow cytometric analysis showed that early apoptosis was markedly increased in group A, relative to groups Bsi and B. In addition, apoptosis was markedly accentuated in group Bsi, when compared with group B after inhibition of HB-EGF expression.

Inhibition of HB-EGF expression increases the ability of cells to undergo apoptosis. Reduction in cell viability means increased apoptosis. In this study, there were only few viable cells in cisplatin-treated wells, and cisplatin significantly and dose-dependently reduced cell proliferation. Compared to group A, there was a greater degree of cell proliferation in group B but proliferation was markedly decreased after transfection with HB-EGF siRNA.

After activation by ligands, epidermal growth factor receptor (EGFR) initiates intracellular signal transduction, participates in cell migration, adhesion, proliferation, differentiation and apoptosis, and it is closely related to the degree of growth of malignant tumors [8]. As a receptor for HB-EGF, EGFR is highly expressed in PC cells. Excision repair cross-complementation group 1 (ERCC1) plays a key role in the repair of damaged human DNA [9]. Studies have suggested that high ERCC1 expression is implicated in poor clinical prognosis of cancers

[10]. Overexpression of ERCC1 can rapidly repair damaged DNA arrested in G2/M phase of the cell cycle [11]. The expressions of EGFR and ERCC1 were decreased in group Bsi, relative to group B. It is likely that cisplatin resistance in PC cells is associated with EGFR and ERCC1 expressions.

Heparin-binding epidermal growth factor (HB-EGF) may inhibit apoptosis of PC cells via the up-regulation of ERCC1 expression.

CONCLUSION

The results obtained in this study indicate that HB-EGF is resistant to cisplatin and inhibits death of PC cells through the upregulation of the expressions of EGFR and ERCC 1.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors aver that this study was carried out by those whose names appear in the manuscript. All liabilities appertaining to the manuscript content or claims arising therefrom are the sole responsibilities of the authors. All those whose names appear in this manuscript read and gave approval for its publication. Lingyang Zhou conceived and designed the study, Lingyang Zhou, Yuming Wang, Shenghong Lan, Mingfu Hu, Lipeng Lu collected and analyzed the data. Lingyang Zhou wrote the manuscript.

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