HSPA2 Expression Predicts Early Postsurgical Relapse and Promotes Tumor Cell Migration and Invasion in Pancreatic Cancer

Lu-Lu Zhai

Renmin Hospital of Wuhan University: Wuhan University Renmin Hospital <https://orcid.org/0000-0003-0839-7316>

Pei-Pei Qiao

Ningxia Medical University

Yue-Shen Sun

Peking Union Medical College Hospital

Zhi-Gang Tang (✉ tzg567@163.com)

Deptment of General Surgery, Renmin Hospital of Wuhan University, Wuhan, China.

<https://orcid.org/0000-0001-5390-9670>

Tong-Fa Ju

Affiliated Hangzhou First People's Hospital Zhejiang University School of Medicine: Hangzhou First People's Hospital

Research article

Keywords: HSPA2, Pancreatic cancer, Recurrence, Migration, Invasion

Posted Date: March 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-218267/v2>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Heat shock protein A2 (HSPA2) is known to relate to the pathogenesis and progress of cancer. This study aimed to investigate the connection between HSPA2 and early postsurgical relapse, and the influences of HSPA2 on cell biological behaviors in pancreatic cancer (PC).

Methods: Expression of HSPA2 in cancerous and noncancerous samples was determined by bioinformatics and immunostaining methods. The significance of HSPA2 expression in predicting early postsurgical recurrence was assessed. The biological role and potential mechanisms of HSPA2 in PC progression were uncovered by enrichment analysis. HSPA2 expression in human PC cells was detected by immunoblotting. Silencing of HSPA2 in BxPC-3 cells and upregulation of HSPA2 in PANC-1 cells were achieved with siRNA and overexpression plasmid, respectively, and then the impacts of HSPA2 downregulation and upregulation on cancer cell biological behaviors were estimated.

Results: HSPA2 expression in tumor specimens was markedly elevated compared with nontumor specimens. HSPA2 overexpression could independently predict early postsurgical recurrence, and combined with malignant clinicopathological characteristics had greater predictive power and potential application value. Enrichment analysis revealed that HSPA2 expression was closely associated with PC progression. HSPA2 was expressed in BxPC-3, PANC-1 and SW1990 cells, with the highest and lowest levels in BxPC-3 and PANC-1 cells, respectively. Cell migration and invasion abilities were remarkably restrained in HSPA2-silenced BxPC-3 cells and significantly enhanced in HSPA2-overexpressed PANC-1 cells.

Conclusion: HSPA2 expression predicts early postoperative recurrence and promotes cell migration and invasion, and is expected to become a useful molecular target of antitumor treatment in PC.

Introduction

Pancreatic cancer (PC) is an extremely invasive and quickly progressive human digestive system malignant tumor, which is the seventh main reason of cancer-associated deaths globally [1]. Statistic data from the American Cancer Society indicate that the disease has jumped to the fourth main reason of malignancy-associated deaths domestically, with a five-year relative survival rate of approximately 9% for all stages and races from 2009 to 2015 [2]. Although radical surgery is currently the most effective treatment for PC, the incidence of early tumor recurrence after surgery is high, which leads to a reduction in long-term survival. Therefore, the identification of specific biomarkers to predict early postoperative recurrence is of great value in improving the prognosis of PC. Furthermore, the discovery of molecules that promote tumor invasiveness is also of great importance for the development of treatment strategies in PC.

Heat shock-related 70-kDa proteins (HSP70s) are a class of hot shock proteins (HSPs) with the molecular weight of about 70-kD, they usually play the role of molecular chaperones, and also have antioxidant, antiapoptotic and immunomodulatory effects [3]. HSPA2, also named HSP70-2, is one of the significant

members of the HSP70s family, originally found in male germ cells and described as a testicle-specific protein that is essential in spermatogenesis [4-6]. The HSPA2 gene is located on chromosome 14 (14q24.1), and its abnormal expression in the testis causes meiosis to be unsuccessful, leading to male sterility [4,7]. Previous studies have reported that HSPA2 was overexpressed in several human solid neoplasms, including cervical malignancies [8], bladder urothelial cancer [9], esophageal squamous cell cancer (ESCC) [10], non-small cell lung cancer (NSCLC) [11], hepatocellular cancer (HCC) [12,13], colorectal cancer [14], breast cancer [15-17], and PC [18,19]. These reports show that HSPA2 overexpression acts as a vital function in the genesis and progress of carcinoma.

It has been demonstrated that the upregulation of HSPA2 in cancerous tissues tightly interrelated with the shortened postoperative survival of PC [18,19]. However, the relationship between HSPA2 and early postoperative recurrence of PC and the impact of HSPA2 on the biological behaviors of human PC cells remain unclear. Therefore, this study assessed HSPA2 expression in PC tissues by immunohistochemistry and analyzed its [correlation](#) with early postoperative recurrence of PC. This study also evaluated the expression of HSPA2 mRNA in tumor samples and the biological role and underlying mechanism of HSPA2 in PC progression using bioinformatics methods. Moreover, the present study investigated the influences of HSPA2 on the propagation, apoptosis, migration, invasion and vascular endothelial growth factor (VEGF) expression of PC cells.

Materials And Methods

Tissue samples and clinicopathological data

The cancerous and adjacent paracancerous specimens were gathered from 85 PC cases that achieved radical excision between January 2008 and December 2011. All patients were confirmed as PC by pathology and hadn't received presurgical anticancer treatment, including chemotherapy, radiotherapy, chemoradiotherapy, biologically targeted treatment, and/or immunotherapy. The samples were immersed in formaldehyde solution within 30 minutes after excision for fixation and stored in the Biobank after embedding with paraffine. Paracancerous tissues refer to normal pancreas tissues which situate 2 cm or more away from the edge of the confirmed neoplasms. The implementation of this study complies with the Helsinki Declaration and has been ratified by the Ethics Committee. All patients signed written consent.

The clinicopathologic parameters of PC were collected retrospectively from the medical records (Electronic Medical Record), including age, sex, tumor site, tumor size, presurgical sera carbohydrate antigen 19-9 (CA19-9) measurement values, tumor differentiation grade, lymph node metastasis (LNM) pattern, peripheral nerve infiltration (PNI) pattern, tumor staging status and early postsurgical relapse state. The tumor differentiation was histologically categorized into moderate-poor and well differentiation. The tumor staging was determined with the 8th edition of the AJCC TNM staging system [20]. All cases were followed up continuously after radical resection. Early postsurgical recurrence of PC was designated as the neoplasm recurred within one year following radical surgery [21].

Immunohistochemical analysis

The expression of HSPA2 in clinical samples was detected by immunohistochemical staining, as formerly reported [19]. The paraffin-embedded specimens were made into 4-micron contiguous tissues and attached to glass slides. Next, the sections with tissues were placed in a heater at 60°C for 60 min to melt the paraffin. The tissue sections were then treated with xylene to dewax and with different concentrations of alcohol to hydrate. After that, the tissue slices were immersed into sodium citrate-hydrochloric acid buffer solution (0.01 mol/L, PH 6.0) and heated by microwave at 80 °C for 20 min to achieve antigenic retrieval. After quenching the endogenous peroxidase function with 3% deionized H₂O₂ solution, the tissue sections were incubated with monoclonal rabbit anti-human HSPA2 primary antibody (Abcam Company, Cambridge, England, UK) at 4 °C overnight. After rewarming, the sections were cleaned in phosphate-buffered saline (PBS) and then incubated with goat anti-rabbit HRP-labeled IgG secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at room temperature for 30 minutes. Finally, the sections were sequentially subjected to chromogenic reaction, counterstaining, dehydration, cleaning, and mounting. The section was not incubated with the HSPA2 antibody served as a negative control.

Immunostaining results were scored independently and semi-quantitatively by two researchers participating in the experiment based on the percent of staining cells and the degree of color, as mentioned formerly [19]. The scoring range for the percent of staining cells was as following: 0 marks means nonstained cells, 1 mark means 1-10% staining cells, 2 marks means 10-30% staining cells, and 3 marks means >30% staining cells [19]. The scoring range of immunostaining degree was recorded as: 0 for colorless, 1 for weak coloring, 2 for middle coloring, 3 for strong coloring [19]. The final total score for each section was the staining percentage score multiplied by the staining degree score. A total point of less than 3 was considered a low expression of HSPA2, while no less than 3 was denoted as a high expression. The difference in scores was resolved through a joint evaluation.

Bioinformatics analysis

Before December 6, 2020, the mRNA expression data of 178 PC samples and 77 nontumor samples were obtained from the Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>). The HSPA2 mRNA data was then extracted using perl software, and the difference in expression between tumor and normal tissues was analyzed by R software. The Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (<http://gepia2.cancer-pku.cn/>), a database that matches TCGA normal and Genotype-Tissue Expression (GTEx) data with online analysis function, was used for analyzing the expression differences of HSPA2 mRNA in cancer and noncancerous tissues [22].

LinkedOmics (<http://www.linkedomics.org/login.php>) is an online analysis platform for analyzing cancer data from TCGA, including two modules, LinkFinder and LinkInterpreter [23]. The LinkFinder module was used to analyze the coexpressed genes associated with HSPA2 in the TCGA dataset by Spearman correlation test. The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8

(<https://david.ncifcrf.gov/home.jsp>) was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses on those coexpressed genes to understand the biological role of HSPA2 in PC. GO functional annotation includes biological process (BP), cell component (CC), and molecular function (MF). The results of GO and KEGG pathway enrichment analyses were visualized by R software.

To identify the biological processes of HSPA2 and HSPA2-associated signal pathways in PC, gene set enrichment analysis (GSEA) with single-gene method was implemented on the TCGA dataset. The median HSPA2 mRNA level was used as a cutoff value to group the TCGA dataset into two parts (high HSPA2 expression versus low HSPA2 expression). GSEA v3.0 software was used for enrichment analysis. Two gene sets from the molecular signatures database (MSigDB), Hallmark ([h.all.v6.2.symbols.gmt](#)) and KEGG ([c2.cp.kegg.v6.2.symbols.gmt](#)), were designated as references. The graphs of top 50 sets in the enrichment analysis were plotted. The cutoff criteria were defined as nominal *P* value less than 0.05 and false discovery rate (FDR) *q*-value less than 0.25. All significantly enriched gene sets were plotted to one coordinate by R software.

Cell culture

The human PC cell lines (BxPC-3, PANC-1 and SW1990) were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultivated in DMEM (Gibco Inc., Grand Island, NY, USA) complemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries Ltd., Kibbutz Beit Haemek, Israel) under a humid gas environment with 5% CO₂ at 37 °C.

Transfection of siRNA and recombinant plasmid

To silence and overexpress HSPA2, siRNAs targeting HSPA2 and recombinant plasmids carrying human HSPA2 gene were transferred into PC BxPC-3 cells and PANC-1 cells, respectively. For the silencing of HSPA2 expression in PC BxPC-3 cells, the RNA interference technology was performed using siRNA. The candidate siRNA sequences targeting HSPA2 mRNA were bought from the Shanghai GenePharma Co., Ltd. (Shanghai, China), which were introduced into BxPC-3 cells for 24h and 48h, according to the siRNA transfection manual. The siRNA without a specific target sequence was designed as a negative control. For the overexpression of HSPA2 in PC PANC-1 cells, the overexpression vector containing the human HSPA2 gene was transfected into the cells. The constructed recombinant HSPA2 vector plasmid and non-recombinant plasmid (empty vector) were bought from Thermo Fisher Scientific Inc. (Waltham, USA). PANC-1 cells (1×10^5 cells/well) were plated in 12-well plates. Cell transfection was performed in compliance with the supplier's direction.

Western blot analysis

Cells were gathered, total protein was extracted, and the product levels were measured by BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, USA) in the light of the instructions provided by the manufacturer. The quantified protein (25 µg) was split in SDS-PAGE (AMRESCO, Solon, OH, USA), treated

in 5% nonfat milk at normal temperature for 1 hour, and incubated in rabbit anti-HSPA2 primary antibody (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C all night. After incubating in the HRP-conjugated goat anti-rabbit IgG secondary antibody (Biosharp Life Sciences, Hefei, Anhui, China) at normal temperature for 2 hours, the signal from the membrane was determined using an ECL (Bio-Rad, Hercules, CA, USA) method. Image J software was used for the quantitative analysis of protein gray values. Protein loading was normalized using the antibody against β -actin, and the expression levels of the protein were shown as relative values from that of β -actin.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay was implemented to estimate the proliferation ability of tumor cells. The cells (1×10^4 /100 μ L) were inoculated in a 96-well plate and then incubated in an incubator with 5% CO₂ at 37°C for 0, 1, 2, 3, and 4 days. After that, 10 μ L of CCK-8 reagent (Dojindo, Kyushu Island, Japan) was sequentially dropped into the cell culture medium at different times, and the culture was continued for 4 hours at 37°C. Finally, the optical density (OD) at 450 nm was measured, and the cells' growth curves were plotted according to the average number of cell counts in each group.

Cell apoptosis assay

Apoptosis of tumor cells was analyzed with flow cytometry (FCM). The cells were harvested 24 hours after transfection, rinsed twice with cold PBS, and diluted to 1×10^6 /mL with binding buffer. One hundred microliters of the cell suspension were filled into a 5 mL flow tube, and then 5 μ L Annexin V/FITC (Beijing 4A Biotech Co., Ltd, Shanghai, China) was mixed in and incubated for 5 min at normal temperature without light. Next, 10 μ L of 20 μ g/mL PI (Beijing 4A Biotech Co., Ltd, Shanghai, China) solution and 400 μ L of PBS were dropped in sequence, and flow detection was performed immediately by a flow cytometer (BD Bioscience, San Jose, USA).

Cell migration and invasion assay

The migratory and invasive capabilities of the PC cells were assessed using the Transwell method. For the migration assay, 300 μ L of serum-free medium and 800 μ L of serum-containing medium was put into the upper and lower layers of the chamber, respectively, and placed in an incubator at 37 °C for 2 hours. Next, the cells were incubated in serum-free medium for 24 hours and then diluted in serum-free medium to the cell density of 1×10^5 /mL. After the chamber was thoroughly washed with PBS, 800 μ L of 10% FBS culture medium was loaded onto the bottom layer, 300 μ L of cell suspension (1×10^5 /mL) was loaded onto the upper layer, and the cells were then incubated for 24 hours at 37°C with 5% CO₂. The cells moved to the bottom of the chamber were stained with crystal violet reagent. The count of staining cells was performed in three random microscope fields. For the invasion experiment, the upper layer of the chamber was precoated with 20 μ L of Matrigel (BD Bioscience, San Jose, USA).

Measurement of VEGF level

The levels of VEGF were examined by enzyme-linked immunosorbent assay (ELISA) after silencing or upregulating HSPA2 expression to reflect the effect of HSPA2 on angiogenesis of PC. Briefly, after the transfection was completed, the cells were cultivated for 48 and 72 hours, and then the VEGF levels in the culture supernatants were detected by the Human VEGF ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) on the basis of the manufacturer's instructions.

Statistical analysis

The statistical analyses were conducted with SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA), Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA), R software version 4.0.3 and GraphPad Prism version 6.01 (GraphPad, Inc., San Diego, CA, USA). Categorical variables were expressed as the number of cases and percentages, and the statistic analysis between the groups was estimated by Pearson's chi-square test or Fisher's exact test. Logistic regression analysis was applied to determine risk variables related to early postoperative recurrence. Variables that were shown to be statistically significant in univariate logistic regression analyses were incorporated into a multivariate logistic regression model to identify independent predictors of early postoperative relapse. The Hosmer-Lemeshow test was used to estimate the goodness of fit of the multivariate logistic regression model. Receiver operating characteristic (ROC) curve analysis was applied to assess the predictive power of risk factors for early relapse of PC after surgery. The clinical utility evaluation of risk factors was performed by decision curve analysis (DCA). Continuous variables were presented as mean $\pm$ standard deviation (SD), and the statistic analysis between the groups was evaluated using the unpaired t-test. A two-tailed *P* value of less than 0.05 was suggested to be statistically significant.

Results

Clinicopathologic features of PC patients

A total of 85 PC patients were included in this study. The average age of these PC patients was 56.9 ± 6.5 years (ranging from 44 to 68 years), including 45 men and 40 women. There were 41 patients with tumor size less than 20mm, 44 patients with tumor size greater than or equal to 20mm, 71 patients with pancreatic head cancer, 14 patients with pancreatic body/tail carcinoma, 38 patients with serum CA19-9 ≤ 37 U/mL, 47 patients with >37 U/mL. Postoperative pathological examination confirmed that 29 patients with moderate/poor tumor differentiation, 56 patients with well tumor differentiation, 26 patients with non-metastatic lymph nodes, 59 patients with metastatic lymph nodes, 49 patients without PNI, 36 patients with PNI, 23 patients with stage I, and 62 patients with stage II. Thirty-five cases without early postsurgical recurrence and 50 cases with early postsurgical recurrence. A summary of clinicopathologic parameters is shown in **Supplementary Table 1**.

Expression of HSPA2 in PC tissues

Data from TCGA and GEPIA indicated that HSPA2 mRNA expression was evidently increased in PC specimens compared to nontumor samples ($P < 0.05$; **Fig. 1a-b**). Immunostaining indicated that HSPA2

was basically distributed in the cytoplasmic space, as shown in **Fig. 1c**. Of the 85 PC specimens, overexpressed HSPA2 was observed in 70.6% (60/85). However, only 25.9% (22/85) of HSPA2 overexpression was found in the matched nontumor specimens. Statistical analysis revealed that HSPA2 expression was signally increased in PC specimens relative to surrounding paracarcinoma specimens ($P=0.003$; **Fig. 1d** and **Supplementary Table 2**). HSPA2 expression was observably higher in tumors with early postsurgical relapse compared with those without early postsurgical relapse ($P<0.001$; **Fig. 1e** and **Supplementary Table 3**). The correlation between HSPA2 expression and clinicopathological features was shown in **Supplementary Table 4**.

Logistic regression analysis of risk variables potentially associated with early postsurgical relapse of PC

To ascertain the variates that preestimate the early postsurgical recurrence of PC, we carried out univariate and multivariate analyses with logistic regression models. Univariate logistic regression analysis revealed that early neoplasm relapse was observably correlated with high serum CA19-9 levels before surgery ($P=0.019$), moderate/poor tumor differentiation ($P=0.001$), the presence of LNM ($P<0.001$), the presence of PNI ($P=0.011$), high tumor staging ($P<0.001$), and overexpressed HSPA2 ($P<0.001$) in PC patients after resection (**Fig.2a** and **Supplementary Table 5**).

In the multivariate logistic regression model, upregulated HSPA2 proved to be an independent hazard variate for early recidivation of PC following surgery [odds ratio (OR) =6.601; 95% confidence interval (CI) 1.635-26.652; $P=0.008$]. High tumor stage was also shown to be an independent variable for predicting early relapse of PC after operation (OR =8.049; 95% CI 1.783-36.334; $P=0.007$) (**Fig.2b** and **Supplementary Table 5**). The Hosmer-Lemeshow test showed a P value of 0.642 (greater than 0.05), indicating that this multivariate logistic regression model had a good goodness of fit.

Predictive nomogram for early postsurgical relapse of PC

A nomogram was established based on these risk variables associated with early postsurgical recurrence of PC. The results indicated that HSPA2 overexpression was the greatest risk factor, followed by high tumor stage, metastatic lymph nodes, presence of PNI, poor tumor differentiation and high serum CA19-9 levels (**Fig. 2c**). The C-index for internal validation of the nomogram model was 0.843 (95% CI 0.753-0.932). Internal calibration curve demonstrated that nomogram prediction results were consistent with actual observation results (**Fig. 2d**).

Predictive performance of the nomogram

ROC curves were constructed to assess the reliability of these risk factors for predicting early relapse of PC following surgery. The area under the curve (AUC) was 0.760 (95% CI 0.649-0.871) for HSPA2, 0.630 (95% CI 0.509-0.751) for serum CA19-9, 0.671 (95% CI 0.552-0.791) for tumor differentiation, 0.750 (95% CI 0.638-0.862) for LNM, 0.641 (95% CI 0.522-0.760) for PNI, 0.756 (95% CI 0.643-0.868) for tumor stage. The combination of HSPA2 and the above-mentioned malignant clinicopathologic parameters improved the specificity and sensitivity over that of HSPA2 or these clinicopathologic parameters alone for

predicting early postsurgical relapse of PC, with an AUC of 0.843 (95% CI 0.753-0.932) (**Fig. 2e** and **Supplementary Table 6**).

DCA was employed to quantify the probability of net benefits of risk factors to further assess their predictive performance and potential clinical application value for early recurrence of PC after surgery. The results demonstrated that HSPA2 expression had a larger net benefit than other risk variables. The combination of all risk variables presented the highest net benefit relative to individual risk variables, indicating that HSPA2 expression combined with other malignant clinicopathological characteristics had a great advantage in predicting the possibility of early postoperative relapse (**Fig. 2f**).

Coexpression genes associated with HSPA2 in PC

To understand the biological function of HSPA2 in PC, we analyzed the coexpressed genes of HSPA2 using the LinkFinder module of LinkedOmics and performed GO and KEGG pathway analyses of coexpressed genes using the DAVID tool. The results of coexpression gene analysis suggested that there were 1,240 and 342 genes with distinct positive (red dots) and negative (blue dots) association with HSPA2, respectively (FDR <0.05; **Fig. 3a** and **Additional File 1**). The top 50 genes with positive and negative correlations with HSPA2 were displayed by heat maps (**Fig. 3b**).

GO and KEGG pathway analyses of HSPA2-related coexpressed genes

The GO functional annotation results showed that HSPA2-related coexpressed genes were principally involved in extracellular matrix organization, proteinaceous extracellular matrix, extracellular matrix, cell adhesion, focal adhesion, endoplasmic reticulum lumen, extracellular matrix binding, collagen trimer, collagen catabolic process, extracellular matrix structural constituent, integrin binding, calcium ion binding, extracellular space, Z disc, extracellular region, etc. (FDR <0.001; **Fig. 3c**). The KEGG pathway analysis indicated that HSPA2-related coexpressed genes were enriched in multiple tumor-related signaling pathways, including ECM-receptor interaction, focal adhesion, PI3K-Akt signaling pathway, proteoglycans in cancer, pathways in cancer, cGMP-PKG signaling pathway, regulation of actin cytoskeleton, MAPK signaling pathway, TGF- β signaling pathway, etc. (FDR <0.05; **Fig. 3d**).

Identification of HSPA2-associated gene sets by single-gene GSEA

To explore the potential mechanism of HSPA2 promoting the progression of PC (including biological processes and signaling pathway), we identified the gene sets enriched in the HSPA2 high expression dataset through single-gene GSEA based on the TCGA data. In the reference Hallmark gene set, NOM p value less than 0.05 indicated a significantly enriched gene set. The results revealed that there were 14 tumor-associated gene sets significantly enriched in the dataset with high HSPA2 expression, including epithelial mesenchymal transition, TGF- β signaling pathway, angiogenesis, apoptosis, IL6/JAK/STAT3 signaling, hypoxia, mitotic spindle, hedgehog signaling, peroxisome, Wnt β -catenin signaling, TNFA- α signaling via NF- κ B, Kras signaling up, heme metabolism and P53 pathway (**Fig 3e** and **Table 1**).

In the reference KEGG gene set, FDR q value less than 0.05 suggested significant enrichment. The results showed that there were 26 tumor-related gene sets involved in focal adhesion, extracellular matrix (ECM) receptor interaction, TGF- β signaling pathway, regulation of actin cytoskeleton, pathways in cancer, basal cell carcinoma, small cell lung cancer, adherens junction, renal cell carcinoma, prostate cancer, axon guidance, NOD-like receptor signaling pathway, neurotrophin signaling pathway, Wnt signaling pathway, melanoma, colorectal cancer, gap junction, Fc γ mediated phagocytosis, MAPK signaling pathway, pancreatic cancer, glioma, endometrial cancer, chemokine signaling pathway, tight junction, cell adhesion molecules (CAMs) and hedgehog signaling pathway were significantly enriched in the phenotype with high expression of HSPA2 (**Fig 3f** and **Table 2**).

Expression of HSPA2 protein in human PC cell lines

The above functional and pathway enrichment analyses have revealed that HSPA2 expression was strongly associated with PC progression. Therefore, we next performed several in vitro cellular experiments to further validate the effect of HSPA2 expression on the biological behavior of human PC cells.

We obtained several human PC cell lines (BxPC-3, PANC-1 and SW1990) and further identified the protein expression pattern of HSPA2. The results of Western blotting determined that HSPA2 protein could be detected in BxPC-3, PANC-1 and SW1990 cells, among which the protein expression intensity in BxPC-3 cells was highest, and that in PANC-1 cells was lowest (**Fig. 4a**). Therefore, BxPC-3 and PANC-1 cells were selected for the downregulation and upregulation of HSPA2 in subsequent experiments, respectively.

Downregulation and upregulation of HSPA2 expression in PC cells

To realize the function of HSPA2 in the biologic behaviors of PC cells, such as propagation, apoptosis, migration and invasion, the expression of HSPA2 in PC BxPC-3 cells and PANC-1 cells was downregulated and upregulated, respectively. The siRNAs (siRNA-HSPA2-1, siRNA-HSPA2-2 and siRNA-HSPA2-3) targeting three different regions of HSPA2 were introduced into PC BxPC-3 cells to downregulate the expression of HSPA2. The siRNA without a specific target sequence served as a control (siRNA-NC). The recombinant plasmid with the human HSPA2 gene was introduced into PC PANC-1 cells to upregulate the expression of HSPA2. Untransfected cells and cells transferred with the mock vehicle were utilized as controls. The protein expression quantities of HSPA2 were measured using immunoblotting to assess the knockdown and overexpressing efficiency. Of the three siRNAs used, two (siRNA-HSPA2-2 and siRNA-HSPA2-3) worked well, and the other (siRNA-HSPA2-1) did not (**Fig. 4b**). The two siRNAs with higher silencing efficiency were selected for the downregulation of HSPA2 in subsequent experiments. The expression of HSPA2 in PANC-1 cells transferred with the recombinant plasmid (HSPA2 vector) was enhanced relative to the non-transfection group (blank control) and the empty vector group (**Fig. 4c**).

Effects of HSPA2 on proliferation of PC cells

To estimate the influences of HSPA2 silencing and overexpressing on the multiplication of BxPC-3 and PANC-1 cells, respectively, we implemented a CCK-8 assay. The data manifested that the proliferative ability of BxPC-3 cells in the two HSPA2 downregulation groups decreased relative to siRNA-NC, but there were no conspicuous differences ($P>0.05$; **Fig. 5a**). Consistently, the proliferation ability of PANC-1 cells in the HSPA2 upregulation group was increased compared with the non-transfection group (blank control) and the empty vector group, but there were no obvious differences ($P>0.05$; **Fig. 5b**).

Effects of HSPA2 on apoptosis of PC cells

To appraise the impacts of HSPA2 silencing and overexpressing on the apoptosis of BxPC-3 and PANC-1 cells, respectively, we implemented FCM analysis. The data revealed that HSPA2 silencing had no obvious impact on apoptosis of BxPC-3 cells versus the controlled siRNA (siRNA-NC) ($P>0.05$; **Fig. 5c**). Likewise, HSPA2 overexpression also had no significant effect on the apoptosis of PANC-1 cells in comparison to the non-transfected group (blank control) and the empty plasmid group ($P>0.05$; **Fig. 5d**).

Effects of HSPA2 on migration and invasion of PC cells

To identify the impacts of HSPA2 silencing and overexpressing on the migratory and invasive capabilities of BxPC-3 and PANC-1 cells, respectively, we carried out cell migration and invasion experiments with transwell cell culture chambers. The results showed that HSPA2 knockdown remarkably reduced the migratory number of BxPC-3 cells ($P<0.01$ for siRNA-HSPA2-2 vs. siRNA-NC, $P<0.05$ for siRNA-HSPA2-3 vs. siRNA-NC; **Fig. 5e**), while HSPA2 overexpression significantly increased the migratory number of PANC-1 cells ($P<0.01$ for blank control vs. HSPA2 vector, $P<0.05$ for empty vector vs. HSPA2 vector; **Fig. 5f**). Similarly, HSPA2 downregulation significantly decreased the invasive number of BxPC-3 cells ($P<0.05$ for siRNA-HSPA2-2 or siRNA-HSPA2-3 vs. siRNA-NC; **Fig. 5e**), and HSPA2 overexpression significantly elevated the invasive number of BxPC-3 cells ($P<0.01$ for blank control vs. HSPA2 vector, $P<0.01$ for empty vector vs. HSPA2 vector; **Fig. 5f**).

Effect of HSPA2 on VEGF expression in PC cells

To preliminarily understand the influence of HSPA2 on the angiogenesis of PC, VEGF concentrations in the supernatant of cell culture medium were detected by the ELISA method after HSPA2 was downregulated in BxPC-3 cells and upregulated in PANC-1 cells, respectively. The results indicated that the VEGF value in the supernatant of BxPC-3 cells did not change significantly after HSPA2 silencing compared to siRNA-NC ($P>0.05$; **Fig. 5g**). Similarly, the VEGF value in the supernatant of PANC-1 cells did not change significantly after HSPA2 overexpressing relative to the untransfected group and the mock carrier group ($P>0.05$; **Fig. 5h**). These results revealed that HSPA2 had no significant influence on the expression of VEGF in PC cells.

Discussion

HSPA2 expression has been confirmed to be elevated in a variety of human solid malignancies, including PC [8-19]. Although overexpression of HSPA2 in PC tissues has been confirmed to be strongly correlative with shortened survival time, the relationship between HSPA2 and early postsurgical recurrence of PC and its effect on the biological behaviors of human PC cells remained unclear. Therefore, this study explored the associativity between HSPA2 expression and early reappearance of PC after surgery, and its effect on the propagation, apoptosis, invasion, migration and VEGF-mediated angiogenesis of PC cells. The findings showed that HSPA2 expression in PC specimens was markedly enhanced relative to the nontumor specimens; which was in accord with the former results [18,19]. Elevated HSPA2 expression served as an independent risk indicator for early postsurgical relapse of PC. ROC curve analysis showed that HSPA2 expression combined with other malignant clinicopathological parameters had a larger AUC value. DCA indicated that HSPA2 expression combined with other malignant clinicopathologic features had a higher net benefit than HSPA2 or clinicopathologic parameter alone. The enrichment results showed that HSPA2 expression was intimately associated with the progression of PC. Loss of HSPA2 expression dramatically impaired the migratory and invasive abilities of PC BxPC-3 cells, and the increasement of HSPA2 expression strongly raised the migratory and invasive capabilities of PC PANC-1 cells. However, the downregulation or upregulation of HSPA2 expression did not significantly affect the multiplication, apoptosis and VEGF expression of BxPC-3 or PANC-1 cells.

The correlation between HSPA2 overexpression and the outcome of patients with malignant tumors has been clarified. For instance, Zhang et al. [10] reported that increased HSPA2 expression apparently associated with a declining survival rate and independently forecasted the adverse outcome in ESCC individuals. Scieglińska et al. [11] demonstrated that HSPA2 upregulation was markedly relevant to shortened overall survival time in NSCLC population with stages I and II. Fu et al. [12] suggested that HCC with upregulated HSPA2 presented a less overall survival time than those with downregulated HSPA2, and HSPA2 overexpression could be used as an independent adverse variable for prognosis prediction. Yang et al. [17] showed that overexpression of HSPA2 was clearly connected with lessened survival of breast cancer patients. Moreover, previous studies have also shown that elevatory HSPA2 expression was significantly linked to the shortened recurrence-free survival and overall survival of PC cases [18,19]. The present work found that the upregulated HSPA2 was evidently correlated with early postoperative recrudescence of PC and was an independent hazard indicator of early postoperative recurrence. HSPA2 expression combined with other malignant clinicopathological characteristics significantly improved the reliability for prediction of early postsurgical recurrence, with a great potential application value. These findings together suggest that HSPA2 overexpression is tightly connected with the unfavorable outcome of human malignant tumors, which further reveals the important role of its gene in the progress and recurrence of malignancies.

There is mounting evidence that HSPA2 is closely associative with the malignant phenotype of cancer cells. In cervical cancer, bladder urothelial cancer, colorectal cancer, and breast cancer, the depletion of HSPA2 expression observably reduced the abilities of carcinoma cells to proliferate, invade and migrate, initiated apoptosis, and restrained the increase in the volume of transplanted tumors [8, 9, 14-16, 24]. In kidney cancer, ablated HSPA2 in tumor cells caused the attenuation of vitality, colony formation and

movement [25]. In ovarian cancer cells, HSPA2 silencing induced by shRNA resulted in the decrease of cell proliferation, viability, colony formation and cell motility capacities, and the cell cycle was arrested in the G0-G1 phase [26]. In lung adenocarcinoma cell lines A549 and H1975 cells, siRNA mediated downregulation of HSPA2 caused the reduction of cell activity and cell cycle arrest in the G1-S stage [27]. Additionally, some studies have shown that HSPA2 overexpression was prominently associated with malignant clinicopathological characteristics of several human malignant neoplasms, including ESCC [10], NSCLC [11], HCC [12], breast cancer [17] and PC [18, 19]. In the current work, we conducted GO and KEGG pathway enrichment analysis of HSPA2 coexpressed genes, and single gene GSEA of HSPA2 to understand the possible biological role and potential mechanisms of HSPA2 in PC. All these results revealed that HSPA2 expression was closely related to the malignant phenotype of PC cells. Furthermore, the experimental results proved that the silencing of HSPA2 expression caused an obvious reduction in the capabilities of PC BxPC-3 cells to migrate and invade, and the overexpression of HSPA2 brought about a prominent enhancement in the migratory and invasive abilities of PC PANC-1 cells. The current findings, together with previous data uncover the vital effect of HSPA2 in cancer aggressiveness. Nevertheless, Sojka et al [28] reported that the downregulation of HSPA2 had no clear influence on the proliferation and cloning abilities of NSCLC cell lines NCI-H1299 and NCI-H23 cells. Our current results also demonstrated that the interference of HSPA2 expression in BxPC-3 cells by specific siRNAs and the transfection of overexpression plasmids carrying human HSPA2 gene into PANC-1 cells had no remarkable impact on the multiplication and apoptosis. These inconsistent experimental data imply that HSPA2 has different tumor-promoting functions in different human cancer types and tumor cells with different biological characteristics of the same cancer type.

It is well known that tumor angiogenesis has a pivotal function in cancer progress and is considered closely related to the aggressive biological behaviors of neoplasm, which is an important cause of unsatisfactory prognosis [29-32]. Our previous research results also showed that high expression of HSPA2 was positively relevant to VEGF expression and microvessel count in PC tissues, revealing that it is closely correlated with tumor angiogenesis [19]. To investigate whether HSPA2 has the function of promoting PC angiogenesis, we detected the concentration of VEGF in the culture fluid of PC cells with different HSPA2 expression levels in the present study. VEGF is a specific angiogenic regulator that promotes angiogenesis by binding to corresponding receptors and has been recognized as an essential intermedium in the angiogenesis pathway, including tumor angiogenesis [33]. Unexpectedly, the data of this study demonstrated that the silencing and overexpressing of HSPA2 had no significant effect on the VEGF concentration in the culture supernatants of PC BxPC-3 and PANC-1 cells, respectively, suggesting that HSPA2 did not notably affect the expression of VEGF in PC cells. The current data indirectly indicate that HSPA2 has no significant [influence](#) on the VEGF-mediated angiogenesis of PC. Up to now, there are few reports on the impact of HSPA2 on tumor neovascularization. Therefore, more similar researches are required to further evaluate the role of HSPA2 in tumor neovascularization.

We have to acknowledge the deficiencies of this research. First, the establishment of logistic regression models that predict the risk factors for early postoperative relapse of PC was based on a retrospective analysis of a single institution with a relatively small sample capacity, so there is ineluctably a selection

bias. Second, the current study did not clarify the specific mechanism of HSPA2 overexpression leading to increased invasion and migration ability of PC cells. Finally, this study did not explore the influence of HSPA2 on the biological behaviors of transplanted tumours in animals. Consequently, it is necessary to collect more clinical data, apply more experimental techniques, and design more rigorous research protocols to further validate the current findings and reveal the mechanism of HSPA2 affecting the malignant biological behaviors of PC.

Conclusions

In conclusion, the current results indicate that high expression of intratumoral HSPA2 is strongly linked to early relapse of PC after resection and acts as an independent predictor of early postsurgical recurrence. The combination of HSPA2 expression and other malignant clinicopathological parameters shows better performance and greater potential clinical value in predicting early postsurgical recurrence of PC. The inhibition and upregulation of HSPA2 expression notably weakens and enhances the migratory and invasive abilities of PC BxPC-3 and PANC-1 cells, respectively, but has no significant influences on cell multiplication, apoptosis, and VEGF levels in the cell supernatants. Therefore, HSPA2 expression predicts early tumor recurrence after surgery and promotes cancer cell migration and invasion in PC. These data reveal that HSPA2 has a significant part in carcinogenesis and cancer progress, and may be viewed as a hopeful tumor marker for predicting early postoperative recurrence and a potential molecular target for antitumor therapy in PC.

Abbreviations

PC: pancreatic cancer; HSPA2: Heat shock protein A2; TCGA: the Cancer Genome Atlas; GEPIA2: Gene Expression Profiling Interactive Analysis 2; CA19-9: carbohydrate antigen19-9; LNM: lymph node metastasis; PNI: peripheral nerves infiltration; OR: odds ratio; CI: confidence interval; ROC: receiver operating characteristic; AUC: area under the curve; DCA: decision curve analysis; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: false discovery rate; GSEA: gene set enrichment analysis; NES: normalized enrichment scores; NOM: nominal; CCK-8: Cell Counting Kit-8; FCM: flow cytometry; ELISA: enzyme-linked immunosorbent assay; VEGF: vascular endothelial growth factor

Declarations

Acknowledgments

The authors thank the Anhui Provincial Hospital and the Anhui Province Key Laboratory of Hepatopancreatobiliary Surgery for providing experimental materials and equipment, and technical support from Core Facilities, Zhejiang University School of Medicine.

Authors' contributions

Lu-Lu Zhai, Zhi-Gang Tang and Tong-Fa Ju contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lu-Lu Zhai, Pei-Pei Qiao and Yue-Shen Sun. The first draft of the manuscript was written by Lu-Lu Zhai and all authors reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Funding

This study was supported by the Zhejiang Provincial Science and Technology project for Medicine and Health of China (No. 2018KY579), the Zhejiang Provincial Natural Science Foundation of China (No. LGF19H290004), the Science and Technology Development Foundation of Nanjing Medical University (No. 2017NJMU084), and the National Natural Science Foundation of China (No. 81272740).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The implementation of this study complies with the Helsinki Declaration and has been ratified by the Ethics Committee.

Consent for publication

Not applicable.

Conflicts of interest/Competing interests

The authors declare that they have no competing interests.

References

1. 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 Cancer J Clin* (2018) 68:394–424. doi:10.3322/caac.21492
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* (2020) 70:7–30. doi:10.3322/caac.21590
3. Glover JR, Lindquist S. Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* (1988) 94:73–82. doi:10.1016/s0092-8674(00)81223-4
4. Bonnycastle LL, Yu CE, Hunt CR, Trask BJ, Clancy KP, Weber JL, et al. Cloning, sequencing, and mapping of the human chromosome 14 heat shock protein gene (HSPA2). *Genomics* (1994) 23:85–doi:10.1006/geno.1994.1462

5. Dix DJ, Allen JW, Collins BW, Poorman-Allen P, Mori C, Blizard DR, et al. HSP70-2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. *Development* (1997) 124:4595–
6. Son WY, Han CT, Hwang SH, Lee JH, Kim S, Kim YC. Repression of hspA2 messenger RNA in human testes with abnormal spermatogenesis. *Fertil Steril* (2000) 73:1138– doi:10.1016/s0015-0282(00)00496-9
7. Dix DJ, Allen JW, Collins BW, Mori C, Nakamura N, Poorman-Allen P, et al. Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proc Natl Acad Sci U S A* (1996) 93:3264– doi:10.1073/pnas.93.8.3264
8. Garg M, Kanojia D, Saini S, Suri S, Gupta A, Surolia A, et al. Germ cell-specific heat shock protein 70-2 is expressed in cervical carcinoma and is involved in the growth, migration, and invasion of cervical cells. *Cancer* (2010) 116:3785– doi:10.1002/cncr.25218
9. Garg M, Kanojia D, Seth A, Kumar R, Gupta A, Surolia A, et al. Heat-shock protein 70-2 (HSP70-2) expression in bladder urothelial carcinoma is associated with tumour progression and promotes migration and invasion. *Eur J Cancer* (2010) 46:207– doi: 10.1016/j.ejca.2009.10.020
10. Zhang H, Chen W, Duan CJ, Zhang CF. Overexpression of HSPA2 is correlated with poor prognosis in esophageal squamous cell carcinoma. *World J Surg Oncol* (2013) 11:141. doi:10.1186/1477-7819-11-141
11. Scieglińska D, Gogler-Pigłowska A, Butkiewicz D, Chekan M, Malusecka E, Harasim J, et al. HSPA2 is expressed in human tumors and correlates with clinical features in non-small cell lung carcinoma patients. *Anticancer Res* (2014) 34:2833–
12. Fu Y, Zhao H, Li XS, Kang HR, Ma JX, Yao FF, et al. Expression of HSPA2 in human hepatocellular carcinoma and its clinical significance. *Tumour Biol* (2014) 35:11283–7. doi:10.1007/s13277-014-2430-y
13. Yang Z, Zhuang L, Szatmary P, Wen L, Sun H, Lu Y, et al. Upregulation of heat shock proteins (HSPA12A, HSP90B1, HSPA4, HSPA5 and HSPA6) in tumour tissues is associated with poor outcomes from HBV-related early-stage hepatocellular carcinoma. *Int J Med Sci* (2015) 12:256–63. doi:10.7150/ijms.10735
14. Jagadish N, Parashar D, Gupta N, Agarwal S, Suri V, Kumar R, et al. Heat shock protein 70-2 (HSP70-2) is a novel therapeutic target for colorectal cancer and is associated with tumor growth. *BMC Cancer* (2016) 16:561. doi:10.1186/s12885-016-2592-7
15. Rohde M, Daugaard M, Jensen MH, Helin K, Nylandsted J, Jäättelä M. Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. *Genes Dev* (2005) 19:570– doi:10.1101/gad.305405
16. Jagadish N, Agarwal S, Gupta N, Fatima R, Devi S, Kumar V, et al. Heat shock protein 70-2 (HSP70-2) overexpression in breast cancer. *J Exp Clin Cancer Res* (2016) 35:150. doi:10.1186/s13046-016-0425-9

17. Yang YL, Zhang Y, Li DD, Zhang FL, Liu HY, Liao XH, et al. RNF144A functions as a tumor suppressor in breast cancer through ubiquitin ligase activity-dependent regulation of stability and oncogenic functions of HSPA2. *Cell Death Differ* (2020) 27:1105– doi:10.1038/s41418-019-0400-z
18. Zhang H, Gao H, Liu C, Kong Y, Wang C, Zhang H. Expression and clinical significance of HSPA2 in pancreatic ductal adenocarcinoma. *Diagn Pathol* (2015) 10:13. doi: 10.1186/s13000-015-0253-9
19. Zhai LL, Xie Q, Zhou CH, Huang DW, Tang ZG, Ju TF. Overexpressed HSPA2 correlates with tumor angiogenesis and unfavorable prognosis in pancreatic carcinoma. *Pancreatol* (2017) 17:457–63. doi:10.1016/j.pan.2017.04.007
20. Amin MB, Edge S, Greene F, Byrd DR, Brookland RK, Washington MK, et al. American Joint Committee on Cancer. AJCC cancer staging 8th ed. New York, NY: Springer (2017).
21. Yamamoto Y, Ikoma H, Morimura R, Konishi H, Murayama Y, Komatsu S, et al. Optimal duration of the early and late recurrence of pancreatic cancer after pancreatectomy based on the difference in the prognosis. *Pancreatol* (2014) 14: 524– doi: 10.1016/j.pan.2014.09.006
22. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* (2017) 45:W98– doi:10.1093/nar/gkx247
23. Vasaikar SV, Straub P, Wang J, Zhang B. LinkedOmics: analyzing multi-omics data within and across 32 cancer types. *Nucleic Acids Res* (2018) 46:D956– doi:10.1093/nar/gkx1090
24. Daugaard M, Kirkegaard-Sørensen T, Ostenfeld MS, Aaboe M, Høyer-Hansen M, Orntoft TF, et al. Lens epithelium-derived growth factor is an Hsp70-2 regulated guardian of lysosomal stability in human cancer. *Cancer Res* (2007) 67:2559– doi:10.1158/0008-5472.CAN-06-4121
25. Singh S, Suri A. Targeting the testis-specific heat-shock protein 70-2 (HSP70-2) reduces cellular growth, migration, and invasion in renal cell carcinoma cells. *Tumour Biol* (2014) 35:12695– doi: 10.1007/s13277-014-2594-5
26. Gupta N, Jagadish N, Surolia A, Suri A. Heat shock protein 70-2 (HSP70-2) a novel cancer testis antigen that promotes growth of ovarian cancer. *Am J Cancer Res* (2017) 7:1252–69.
27. Cao L, Yuan X, Bao F, Lv W, He Z, Tang J, et al. Downregulation of HSPA2 inhibits proliferation via ERK1/2 pathway and endoplasmic reticular stress in lung adenocarcinoma. *Ann Transl Med* (2019) 7:540. doi: 10.21037/atm.2019.10.16
28. Sojka DR, Gogler-Piğłowska A, Vydra N, Cortez AJ, Filipczak PT, Krawczyk Z, et al. Functional redundancy of HSPA1, HSPA2 and other HSPA proteins in non-small cell lung carcinoma (NSCLC); an implication for NSCLC treatment. *Sci Rep* (2019) 9:14394. doi: 10.1038/s41598-019-50840-7
29. Folkman J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* (2002) 29:15– doi: 10.1053/sonc.2002.37263
30. Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* (2011) 473:298– doi:10.1038/nature10144
31. Benckert C, Thelen A, Cramer T, Weichert W, Gaebele G, Gessner R, et al. Impact of microvessel density on lymph node metastasis and survival after curative resection of pancreatic cancer. *Surg*

32. Hoem D, Straume O, Immervoll H, Akslen LA, Molven A. Vascular proliferation is associated with survival in pancreatic ductal adenocarcinoma. *APMIS* (2013) 121:1037– doi:10.1111/apm.12057
33. Hoebe A, Landuyt B, Highley MS. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev* (2004) 56:549– doi:10.1124/pr.56.4.3

Tables

Table 1 Hallmark gene sets enriched in the high HSPA2 expression phenotype

Gene set name	NES	NOM <i>p</i> -value	FDR <i>q</i> -value
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	2.091	0.000	0.004
HALLMARK_TGF_BETA_SIGNALING	1.675	0.001	0.052
HALLMARK_ANGIOGENESIS	1.484	0.006	0.076
HALLMARK_APOPTOSIS	1.451	0.000	0.084
HALLMARK_IL6_JAK_STAT3_SIGNALING	1.405	0.017	0.093
HALLMARK_HYPOXIA	1.394	0.001	0.091
HALLMARK_MITOTIC_SPINDLE	1.359	0.035	0.100
HALLMARK_HEDGEHOG_SIGNALING	1.347	0.019	0.103
HALLMARK_PEROXISOME	1.303	0.018	0.139
HALLMARK_WNT_BETA_CATENIN_SIGNALING	1.282	0.021	0.145
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.276	0.043	0.145
HALLMARK_KRAS_SIGNALING_UP	1.272	0.028	0.141
HALLMARK_HEME_METABOLISM	1.237	0.022	0.157
HALLMARK_P53_PATHWAY	1.206	0.029	0.166

NES, normalized enrichment scores; NOM, nominal; FDR, false discovery rate. NOM *P* value <0.05 and FDR *q*-value <0.25 were set as cutoff criteria. Pathways with NOM *P* value <0.05 were considered significant

Table 2 KEGG gene sets enriched in the high HSPA2 expression phenotype

Gene set name	NES	NOM <i>p</i> -value	FDR <i>q</i> -value
KEGG_FOCAL_ADHESION	2.178	0.000	0.000
KEGG_ECM_RECEPTOR_INTERACTION	2.172	0.000	0.000
KEGG_TGF_BETA_SIGNALING_PATHWAY	2.041	0.000	0.002
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON	2.025	0.000	0.003
KEGG_PATHWAYS_IN_CANCER	2.010	0.000	0.002
KEGG_BASAL_CELL_CARCINOMA	1.917	0.000	0.013
KEGG_SMALL_CELL_LUNG_CANCER	1.883	0.000	0.017
KEGG_ADHERENS_JUNCTION	1.878	0.004	0.016
KEGG_RENAL_CELL_CARCINOMA	1.878	0.002	0.014
KEGG_PROSTATE_CANCER	1.875	0.002	0.014
KEGG_AXON_GUIDANCE	1.865	0.002	0.015
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.859	0.002	0.014
KEGG_NEUROTROPHIN_SIGNALING_PATHWAY	1.858	0.002	0.014
KEGG_WNT_SIGNALING_PATHWAY	1.851	0.000	0.014
KEGG_MELANOMA	1.843	0.006	0.015
KEGG_COLORECTAL_CANCER	1.821	0.000	0.019
KEGG_GAP_JUNCTION	1.815	0.002	0.019
KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	1.808	0.004	0.019
KEGG_MAPK_SIGNALING_PATHWAY	1.793	0.002	0.020
KEGG_PANCREATIC_CANCER	1.753	0.014	0.029
KEGG_GLIOMA	1.742	0.012	0.029
KEGG_ENDOMETRIAL_CANCER	1.707	0.015	0.037
KEGG_CHEMOKINE_SIGNALING_PATHWAY	1.703	0.018	0.037
KEGG_TIGHT_JUNCTION	1.686	0.004	0.041
KEGG_CELL_ADHESION_MOLECULES_CAMS	1.679	0.030	0.043
KEGG_HEDGEHOG_SIGNALING_PATHWAY	1.663	0.010	0.048

NES, normalized enrichment scores; NOM, nominal; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes. NOM *P* value <0.05 and FDR *q*-value <0.25 were set as cutoff criteria. Pathways

with FDR q -value <0.05 were considered significant

Figures

Figure 1

Expression of HSPA2 in PC tissues. (a-b) TCGA and GEPIA data showed that HSPA2 mRNA expression was significantly higher in tumor specimens than in nontumor specimens. (c) Representative photographs of HSPA2 immunostaining in cancer and paracancerous tissues were taken at different magnifications (Top panel $\times 200$, bottom panel $\times 400$). High expression of HSPA2 was observed in cancer tissues, mainly in the cytoplasm, while the expression of HSPA2 was low in paracancerous tissues. The HSPA2 aggregate score <3 was defined as low expression, and ≥ 3 as high expression. (d) High expression rate of HSPA2 in cancer tissues was significantly higher than that in adjacent paracancerous tissues. (e) High expression rate of HSPA2 in tumors with early postoperative relapse was significantly higher than that of tumors without early postoperative relapse. HSPA2, heat shock protein A2; PC, pancreatic cancer; TCGA, the Cancer Genome Atlas; GEPIA2, Gene Expression Profiling Interactive Analysis 2. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Figure 2

Prediction of risk variables for early postsurgical recurrence of PC. (a, b) Forest plots for univariate and multivariate logistic regression analysis. High HSPA2 expression, high preoperative serum CA19-9 levels, moderate/poor tumor differentiation, the presence of LN, the presence of PNI, and late tumor stage were risk factors for early postoperative recurrence. High HSPA2 expression and late tumor stage were independent risk factors. (c) The nomogram suggested that the scores predicting early postoperative

relapse were, in descending order, high HSPA2 expression, high tumor stage, presence of LNM, presence of PNI, poor tumor differentiation and high serum CA19-9 concentration. (d) The internal validation curve established by bootstrap method demonstrated that the nomogram model had good discrimination ability. (e) ROC curves analysis of risk variables for predicting the early postsurgical relapse. The AUC for HSPA2 expression, serum CA19-9, tumor differentiation, LNM, PNI, tumor stage and nomogram (combination of six variables) were 0.760, 0.630, 0.671, 0.750, 0.641, 0.756, and 0.843, respectively. (f) DCA of risk variables for predicting the early postsurgical relapse. The Y-axis and X-axis represent the net benefit and the threshold probability, respectively. The two curves of "All" and "None" mean respectively assuming that all patients have early postoperative relapse and that no patients have early postsurgical recurrence. HSPA2 expression, serum CA19-9, tumor differentiation, LNM, PNI, tumor stage and nomogram (combination of six variables) had more net benefits than the two extreme cases (All and None) across the area of appropriate threshold probabilities. HSPA2 expression was superior to the other five clinicopathological features in predicting early postoperative relapse. The nomogram was better than HSPA2 expression or clinicopathological parameters alone. Net benefit = (number of true positives/overall number of cases) - (number of false positives/overall number of cases) × [Threshold probability / (1 - threshold probability)]. Abbreviation: PC, pancreatic cancer; CA19-9, carbohydrate antigen 19-9; LNM, lymph node metastasis; PNI, peripheral nerves infiltration; HSPA2, Heat shock protein A2; OR, odds ratio; CI, confidence interval; ROC, receiver operating characteristic; AUC, area under the curve; DCA, decision curve analysis. The HSPA2 aggregate score <3 was defined as low expression, and ≥3 as high expression. P <0.05 indicates statistically significant

Figure 3

GO and KEGG pathway enrichment analyses of HSPA2 coexpressed genes and single-gene GESA of HSPA2. (a) Coexpressed genes significantly associated with HSPA2 identified by the LinkedOmics platform using the Spearman test. Red dots show genes positively associated with HSPA2 and green dots represent genes negatively associated with HSPA2. (b) Heat maps of the top 50 genes significant positive and negative correlations with HSPA2. (c) Bar graph of strongly enriched GO annotation for HSPA2 coexpression genes (FDR <0.001). The y-axis indicates the GO annotation item and the x-axis indicates the number of genes enriched in the GO item. (d) Bubble plot of prominently enriched KEGG

pathway for HSPA2 coexpressed genes (FDR <0.05). The y-axis indicates the KEGG pathway name and the x-axis indicates the fold enrichment (GeneRatio divided by BgRatio). Higher values of fold enrichment indicate higher levels of enrichment. The size and color of the bubbles in the graph represent the number of enriched genes and the P value, respectively. (e, f) Enrichment plots of Hallmark and KEGG gene sets with NES obtained by single-gene GSEA. NOM P value <0.05 and FDR q-value <0.25 were set as cutoff criteria. Hallmark gene sets with NOM P value <0.05 were considered significant. KEGG pathways with FDR q-value <0.05 were considered significant. Abbreviation: PC, pancreatic cancer; HSPA2, heat shock protein A2; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate; GSEA, gene set enrichment analysis; NES, normalized enrichment scores; NOM, nominal

Figure 4

Expression, knockdown and upregulation of HSPA2 in human PC cell lines. (a) HSPA2 protein was expressed to varying degrees in BxPC-3, PANC-1 and SW1990 cells. Among them, HSPA2 protein had the highest expression in BxPC-3 cells and the lowest expression in PANC-1 cells. (b) Three specific siRNAs (siRNA-HSPA2-1, siRNA-HSPA2-2 and siRNA-HSPA2-3) and one non-specific siRNA (siRNA-NC) were transfected into BxPC-3 cells for 24h and 48h, Western blotting results showed that siRNA-HSPA2-2 and siRNA-HSPA2-3 had conspicuous knockdown effects compared with siRNA-HSPA2-1 and siRNA-NC. (c)

Recombinant plasmid containing human HSPA2 gene (HSPA2 vector) and non-recombinant plasmid (empty vector) were transfected into PANC-1 cells for 48h, Western blotting results showed that the expression level of HSPA2 in the overexpression vector group was upregulated relative to the non-transfected group (blank control) and the empty vector group. Abbreviation: HSPA2, heat shock protein A2; PC, pancreatic cancer

Figure 5

CCK-8, FCM, Transwell and VEGF ELISA assays after silencing or overexpressing HSPA2. CCK-8 assay, FCM analysis, Transwell assay and ELISA detection of VEGF levels after silencing or overexpressing HSPA2. CCK-8, FCM, Transwell and ELISA assays after HSPA2 silencing or overexpression(a) The

proliferation ability of BxPC-3 cells in the siRNA-HSPA2-2 and siRNA-HSPA2-3 groups was depressed relative to the siRNA-NC group, but there were no distinct differences ($P > 0.05$). (b) The proliferation ability of PANC-1 cells in the HSPA2 overexpression group was raised compared with the non-transfection group (blank control) and the empty vector group, but there were no marked differences ($P > 0.05$). (c) HSPA2 silencing had no distinct influence on the apoptosis of BxPC-3 cells compared with the siRNA-NC ($P > 0.05$). (d) HSPA2 overexpressing had no conspicuous impact on the apoptosis of PANC-1 cells versus the two control groups ($P > 0.05$). (e) The migration and invasion capabilities of BxPC-3 cells in the two HSPA2 silencing groups were remarkably weaker than those in the control group (siRNA-NC). (f) The migratory and invasive abilities of PANC-1 cells in the HSPA2 overexpression group were markedly stronger than those in the non-transfection group (blank control) and the empty vector group. (g) The VEGF levels in the cell culture supernatants did not change significantly after culturing the BxPC-3 cells with HSPA2 downregulation for 48 and 72 hours ($P > 0.05$). (h) There were no notable changes in VEGF concentrations in the cell supernatants of PANC-1 cells with HSPA2 upregulation after culturing for 48 hours and 72 hours ($P > 0.05$). Abbreviation: PC, pancreatic cancer; HSPA2, heat shock protein A2; CCK-8, Cell Counting Kit-8; FCM, flow cytometry; ELISA, enzyme-linked immunosorbent assay; VEGF, vascular endothelial growth factor. * $P < 0.05$, ** $P < 0.01$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFile1.xls](#)
- [SupplementaryTables.doc](#)