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Expression of sortilin in pancreatic carcinoma and influence of pancreatic cancer cells by sortilin siRNA knockdown

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SUMMARY

Background: Currently, there are few effective therapeutic options for pancreatic cancer patients.Sortilin is a member of vps10p receptor family , reported in many types of cancers.However, the underlying mechanism and prognostic value of sortilin in pancreatic cancer are still unclear. Objective: Understand the expression of sortilin in pancreatic cancer, and analyze its mechanism that affects the occurrence and development of pancreatic cancer.

Key words: Pancreatic cancer, sortilin, proliferation, invasion, migration

Abbreviations: PC: Pancreatic cancer; ROC: the receiver operating characteristic; SEIN: Sortilin expression in nucleus; NTs: Neurotrophic factor family;min:minute;sec:second; BLR: Binary Logistic Regression; siRNA:small interference RNA; IHC: Immunohistochemistry; RTPCR: Reverse Transcription Polymerase Chain Reaction

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INTRODUCTION

Pancreatic Cancer (PC) remains one of the most lethal types of cancer. It is the 11th most common cancer worldwide. There are no current screening recommendations for pancreatic cancer; thus primary prevention is of utmost importance[1]. More than 90% of pancreatic cancers are ductal adenocarcinoma and its variants[2]. It has been reported that inactivated mutations of tumor suppressor genes (such as *CDKN2A/p16*, *TP53* and *Smad4*) and *KRAS* mutations can cause the growth of invasive PDAC tumors[3], which is far from meeting the clinical needs. Neurotrophic factor family (NTs) not only plays an important role in regulating the strength, number of synaptic connections and neurotransmission, but also has a good performance in cancer[4]. Sortilin is a member of NTS factor family receptors and has been reported in a number of cancers such as: human adenocarcinoma epithelial cell line (*HT29*) [5], breast cancer[6], lung cancer *A549* cells[7] and prostate cancer cells. But it is rare in pancreatic cancer. In this study, we focused on the role of *sortilin* in pancreatic cancer and explored its relationship with the pathogenesis of pancreatic cancer.

MATERIALS AND METHODS

A tissue microarray of 115 pancreatic cancer metastases was analysed by immunohistochemistry. All data were analyzed by univariate analysis and multivariate analysis. Multivariate logistic regression analysis and the area under the Receiver Operating Characteristic (ROC) curve were used to analyze the ability of sortilin in predicting pancreatic cancer. Next, survival analysis was performed to compare the survival time between high-risk and low-risk patients to validate the prognosis prediction efficacy of sortilin. The effects of sortilin on the invasion, metastasis and proliferation of pancreatic cancer cells were investigated both *in vitro* [8,9]. investigate the anti-cancer effect of soritlin on human pancreatic cancer *Capna1* and *Bxpc3* cells, and its possible molecular mechanism.

Immunohistochemistry (IHC)

The PC tissue microarray (hpan-ade120sur-01) consisted of 115 points in 60 cases were bought from Shanghai core super.

The interpretation is according to TPS[10]. TPS=(The number of viable tumor cells positive for sortilin/total number of viable neoplastic cells) × 100%. 0 points (negative), 1 point (1%-25%), 2 points (26%-50%), 3 points (51%-75%), 4 points (76%-100%), take the median(90%) as the standard, and score less or equal than 90% was defined as low expression, and more than 90% as high expression.

Cell culture

The pancreatic cancer cells, *Capan1* and *Bxpc3*, are from the Imaging Laboratory of North Sichuan Medical College. *Capan1* cells were grown in IMDM (SH30228.01, Hyclone) with 20% FBS. *Bxpc3* cells were cultured in RPMI-1640 Medium (SH30809.01, Hyclone), supplemented with 10% fetal bovine serum (11011-8611, Sijiqing, Zhejiang Tianhang Biotechnology Co., Ltd.) at 37℃ in a 5% CO_2 incubator. Cells were dissociated with 0.25% Trypsin-EDTA (PYG0015, Boster Biological Technology Co. ltd) at 37° for 5min and collected by centrifugation at 1000 rpm for 5 min. Cells were resuspended in a new complete medium and placed in an incubator for further culture.

Cell siRNA transfection

The Capan1 and *Bxpc3* cells were seeded in 6-well plates at 50% confluence 1 day prior to transfection. We used a ratio of 50 pmol siRNA (SR304211, OriGeneTechnologies) Rfect 10μl(11013, Changzhou Baidai Biotechnology Co., Ltd) to mix them in 500 μl of serum-free medium for 20 min and then the siRNA/Rfect mixture was added to the cells with 2 ml of complete medium overnight. The targets equences for siRNA are *siRNA-A: GCAGAGCUAGAUAUAGCAC, siRNA-B: CGCAAGGACA GGGUUAGC,siRNA C: AGACGUAGGAAACUCAAUAUUCUTC*.

Transfection of cells were performed in triplicate. The transfection effect of *sortilin* knockdown was evaluated by RT-PCR and verified by western blot.

Reverse Transcription Polymerase Chain Reaction(RT-PCR)

Cells were collected at 24 hours (*Bxpc3*) and 48 hours (*Canpan-1*) after transfection. The cells in a well of 6-well plate were mixed with 1 mL of TRIzol(DP405-02, Tiangen Biotech (Beijing) Co.,Ltd.). Total RNA was extracted according to the manufacturer's instructions using a Total RNA Extraction Kit(TR201-100, Beijing Tianmo Technology Development Co., Ltd.). Total RNA concentration and OD value were measured, and reverse transcribed into cDNA by Reverse Transcription Kit (*K1622*, Thermo Fisher Scientific). The qPCR reactions (20 μl final volume) were conducted using the Bestar® SybrGreen qPCR Mastermix (DBI2043, DBI®Bioscience). for primer sequence. Reaction conditions for RT-PCR were denaturation for 5 min at 95℃, 95℃ for 30 sec, 55℃ for 30 sec, and 72℃ for 40 sec for 35 cycles.2-ΔΔct were used to analyze the results. Each experiment was repeated three times.

Cell scratch test

A marker pen was used to draw an uniform line every 0.5 cm-1 cm at the back of the 6-well plate, with three transverse lines crossed over each well. After 24 h transfection, *Capan1* and *Bxpc3* cells were overpaved to 12-well plates. Three horizontal lines were drawn perpendicular to the reference line. The cells were washed three times with PBS and added serum-free medium, at 37°C with 5% CO_2 for 24 hours. Samples were taken at t = 0, 12 hours. Each result was repeated three times.

CCK8 cell proliferation experiment

Cells were plated on 96 well plates at concentration of 5000 cells/well. The cells were cultured in an incubator at 37°C 5% CO_2 for 24 h. CCK8 reagents (boster, China) were prepared in fresh medium (100 μl medium containing 10 μl *CCK8* solutions) and applied to the cells. After cell treatments, the incubation was continued for 2 hours at 37°C 5% CO_2. Absorbance was measured at 450/540 nm (Sunrise™ Absorbance Reader).Each result was repeated three times.

Transwell cell invasion experiment

Matrigel (BD Biosciences, Beijing, China) was thawed overnight at 4℃ and then kept on ice. Matrigel was diluted by serum-free medium (1:8 dilution) on ice and added 45 μl to transwell chambes for 2 h at 37°C. The transfected cells (20×10^4) in 100 μl were added to the upper chamber of the transwell chamber, and 600 μl of medium containing 20% fetal bovine serum was added to the lower chamber at 3℃ 5% CO_2 for 72 hours. The lower chamber was washed with PBS for three times, following which a cotton bud was used to remove cells and medium from the upper chambers, fixed with methanol for 15 min, washed with PBS and stained with Giemsa solution (Gibco BRL) for 30 min. The stained cells were photographed with a digital camera. The number of colonies in each well was counted with Image J software. Each result was repeated three times.

Western blot experiment

MIP-3α(10485-H07E, Sino Biological Solution Specialist) [11,12] was added to the culture medium at a final concentration of 100ng/ml, incubating for 24 h. After 48h-72h of transfection, the medium was discarded and 100μl of RIPA lysate (P0013B, Beyotime Biotechnology) was added into each well. After 48 hrs transfection, 2 μg/ml puromyc in was added into the medium. The primary antibodies MMP9(13667, Cell Signaling Technology) p53(2527, Cell Signaling Technology), NFκB p65(8242, Cell Signaling Technology) are rabbit monoclonal antibodies, and Sortilin (AF3154, R&D Bio-techne brand) is Antigen Affinity-purified Polyclonal Goat IgG. The secondary antibody uses HRP Conjugated AffiniPure Goat Anti rabbit IgG(BA1054, Boster Biological technology Co., Ltd.) and HRP Conjugated AffiniPure Rabbit Anti-goat IgG(BA1060 , Boster Biological technology Co., Ltd.).The test refers to the steps summarized by Sean C Taylor et al. The relative gray scale of the bands was analyse using Image J software.Each result was repeated three times.

Statistical analysis

Statistical analysis was performed using SPSS23.0 and Graphpad prism7 software.Measurement data was presented as mean±standard deviation (SD). Enumeration data and categorical variables were analyzed using c2 or Fisher's exact tests. The median survival time and the mean survival time were calculated by Kaplan-Meier method. No significant (P>0.05), Statistically significant difference was considered at *:P<0.05,**:P<0.01 ,***:P<0.001 and ****:P<0.0001 between groups.

The Receiver Operating Characteristic (ROC) curve and binary logistic regression were applied to IHC data.

RESULTS

Differential expression analysis of 115 tissue microarrays showed sortilin expression was up-regulated in pancreatic cancer tissues, and it mainly comes from the nucleus. Sortilin expression in nucleus (SEIN) was only negatively correlated with N stage, Binary logistic regression model showed that SEIN is a good diagnostic marker for predicting pancreatic cancer and the accuracy of prediction is as high as 79.1%. ROC curve analysis demonstrated a statistically significant diagnostic value of SEIN, and the diagnostic accuracy was 86.3%, the Youden Index was calculated to evaluate the diagnostic power, the cut-off value for SEIN in pancreatic cancer diagnosis was 0.85, with a sensitivity of 90.9% and a specificity of 68.3%. Univariate analysis showed that M stage (P=0.022), histological grade(P=0.021), clinic stage(P=0.030) and SEIN(P=0.039) were correlated with prognosis of pancreatic cancer patients, Multivariate regression analysis showed that M stage (P=0.036) and ESIN (P=0.004) were independent factors. The proliferation, invasion and migration of pancreatic cancer cells were inhibited *in vitro* by sortilin siRNA knockdown. It may have something to do with soritlin/P53/NFκB regulated the the proliferation function and sortilin/MMP9 regulated the invasion promotion of pancreatic cancer cells.

Differentexpressions of sortilin in pancreatic cancer and adjacent tissues

Firstly, the online database GEPIA was used to investigate *sortilin* different expressions between different cancer and normal tissues(Figure1). Including pancreatic cancer, the content of *sortilin* in pancreatic cancer was significantly higher than that in adjacent tissues (Figure2A). After immunohistochemical staining of 115 pancreatic cancer tissue chips (Figure 2B), sortilin is highly expressed in the nucleus of pancreatic cancer tissues (20%), and almoste not in adjacent normal tissue(0%). The difference was highly significant (P<0.0001). Sortilin are both highly expressed in the cytoplasm of pancreatic cancer tissues(98%) and adjacent normal tissue(95%).The difference was not significant (P =0.897).

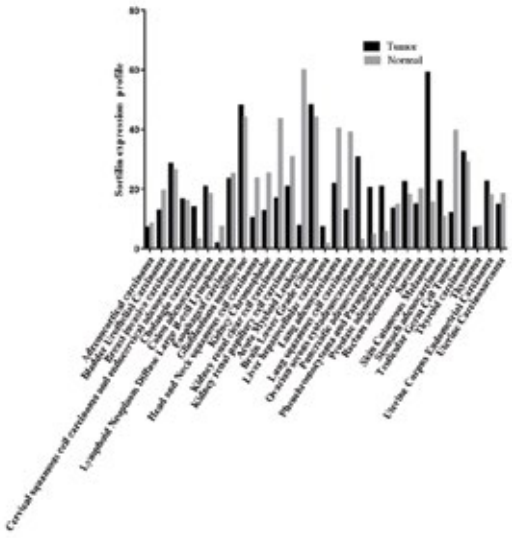

Fig. 1. GEPIA website analysis The gene expression profile across all tumor samples and paired normal tissues.The height of bar represents the median expression of certain tumor type or normal tissue.(http://gepia.cancer-pku.cn/detail.php?gene=SORT1)

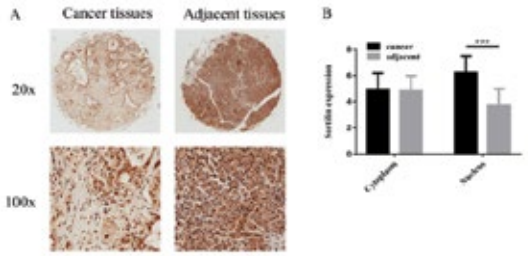

Fig. 2. The difference of sortilin between pancreatic cancer and adjacent tissues
A. Pancreatic cancer tissue chips were analyzed by IHC staining,magnification for the panel is 20X and 100X respectively
B. The expression level of sortilin in the cytoplasm and nucleus by a total score standard. There is no any difference in cytoplasm expression (P=0.897),There is a significant difference in nucleus expression(P<0.001).

Binary Logistic Regression (BLR)model for the estimation of Prediction of pancreatic cancer from SEIN

BLR modeling was used to predict pancreatic cancer.The diagnostic accuracy is 73.9 %.Moreover, the ROC curve analysis showed that SEIN had good diagnostic value for PC(Figure3). The cutoff point of 0.85, calculated by the Youden Index, had 90.9% sensitivity and 68.3% specificity (AUC=0.863,95% CI 0.852–0.922).

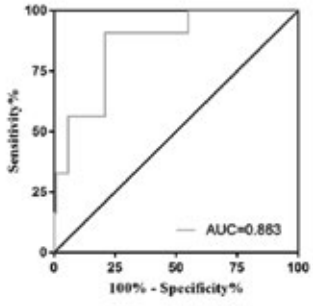

Fig.3. ROC curve for the predicted probabilities of SEINThe ROC statistical analysis of SEIN in the diagnosis of pancreatic cancer showed that the area under the curve was 0.863, sensitivity 90.9%, specificity 68.3%.

Correlation between SEIN and clinicopathological features of 60 PC patients

In view of the up regulation of SEIN, we further analyzed the relationship between SEIN in 60 pancreatic cancer tissues and various clinical indicators.Our results showed SEIN was only negative correlated with N stage(R=-0.254,P=0.040), which suggested, SEIN is related to lymphatic metastasis of pancreatic cancer.

Association of clinicopathological indicators and prognostic factors. Univariate survival analyses indicated that pathological grade,SEIN, M stage, and clinical stage were associated with prognosis, while gender, age, tumor size, T stage, and N stage were not associated with prognosis(Figure 4).

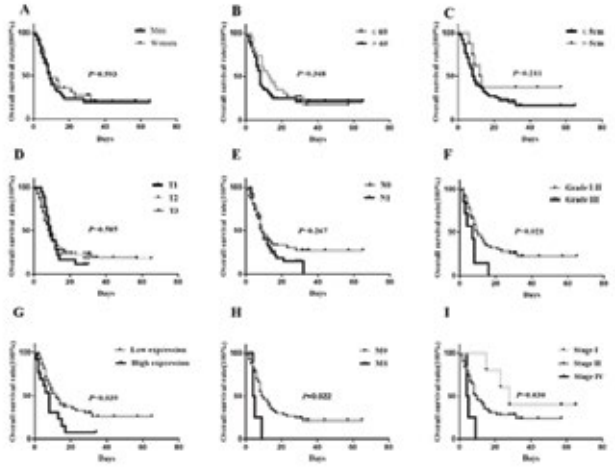

Fig. 4. Univariate analysis of prognostic factor Clinicopathological indicators and prognosis factors such as gender (A),age(B), tumor size (C), T stage(D), N stage(E), pathological grade(F), SEIN (G), M stage (H), clinical stage (I).

To determine the independent prognostic factors, we performed a multivariate analysis on the statistically significant factors in the univariate analysis. It is suggested that ESIN(P=0.004) and M stage (P=0.0.36)could be used as an independent prognostication factor for PC patients, but the pathological grade(P=0.248) and clinical stage(P=0.560) could not be used as an independent factor for the prognosis of patients (Figure 5). It is suggested that SEIN is related to the prognosis of patients. The higher the SEIN level was, the higher the risk of death was.

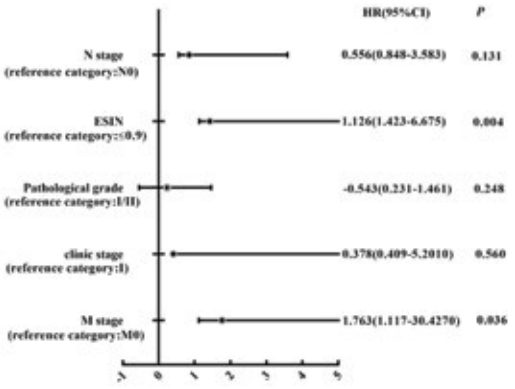

Fig.5. Forest plots of multivariate analysis

Multivariate analysis was performed on the indicators with significant correlation in univariate analysis, among which SEIN(P<0.004) and M staging(P<0.036).

Validation of siRNA knockdown of an effective fragment of sortilin from mRNA level and protein level

Cells were divided into five groups (blank control group,negative control group,siRNA-A group,siRNA-B group, and siRNA-C group).Capan1 cells and Bxpc3 cells were collected afte48 and 24 hoursr transfection respectively,then knockdown fragment were screened. It was identified that siRNA-B and siRNA-C had knockdown effect on the sortilin of both cells, while siRNA-A had no knock down effect from the mRNA level(Figure 6).

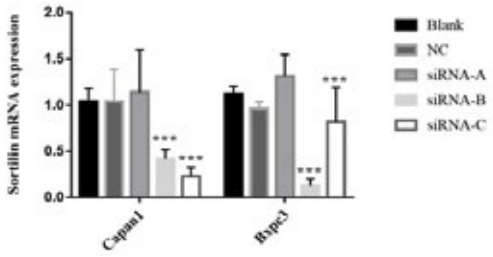

Fig.6. RT-PCR verification of sortilin knock down level Sortilin mRNA expression was detected significantly decreased in siRNA-B(P<0.001) and siRNA-C (P<0.0001) compared with that in Blank,siRNA-A(P=0.769) knockdown had no obvious effect on sortilin mRNA expression in Capan1 cells. Sortilin mRNA expression was detected significantly decreased in siRNA-B(P<0.0001) and siRNA-C (P<0.0001) compared with that in Blank,siRNA-A(P=0.344) knockdown had no obvious effect on sortilin mRNA expression in Bxpc3 cells.

Migration of Pancreatic cancer cells after soritlin knockdown

Aafter 12 hours transfection, the cells in blank group, negative group and mock group showed different degree of migration, but there was no significant migration in transfection group. It is suggested that the inhibition of sortilin can effectively control the migration of pancreatic cancer cells (Figure 7).

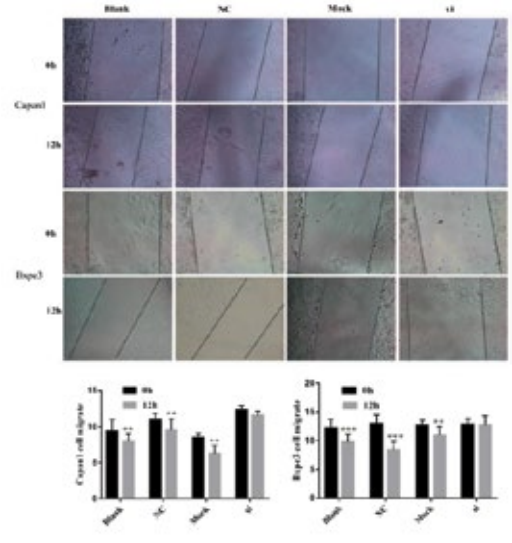

Fig.7. Migration of two cell lines after knocking down sortilin

A. After sortilin inhibition,the migration of Capan1 cells(100x).

B. After sortilin inhibition,the migration of Bxpc3 cells(100x).

C. The migration of Capan1 cells showed significant changes in the Blank group (P=0.0004), negative group (P<0.0001) and Mock group (P=0.0366),but no changes in the knockdown group (P=0.1251).

D. The migration of Bxpce cells showed significant changes in the Blank group (P=0.0074), negative group (P=0.0022) and Mock group (P=0.0021),but no changes in the knockdown group (P=0.7791).

Proliferation of pancreatic cancer cells after sortilin knockdown

After transfection, cells were collected at different time points. After 48 hours transfection, the proliferation of capan1 cells decreased in knockdown group, with a inhibition rate of 71.21%. After 24 hours transfection, the proliferation of Bxpc3 cells decreased in knockdown group, with a inhibition rate of 52.28%.The OD values of the two cells in each time period were shown in (Figure 8).After sotilin was inhibited, the proliferation ability of two cell lines decreased at different time, suggesting that sortilin is related to the proliferation of pancreatic cancer cells, which can be inhibited by intervention of sotilin.

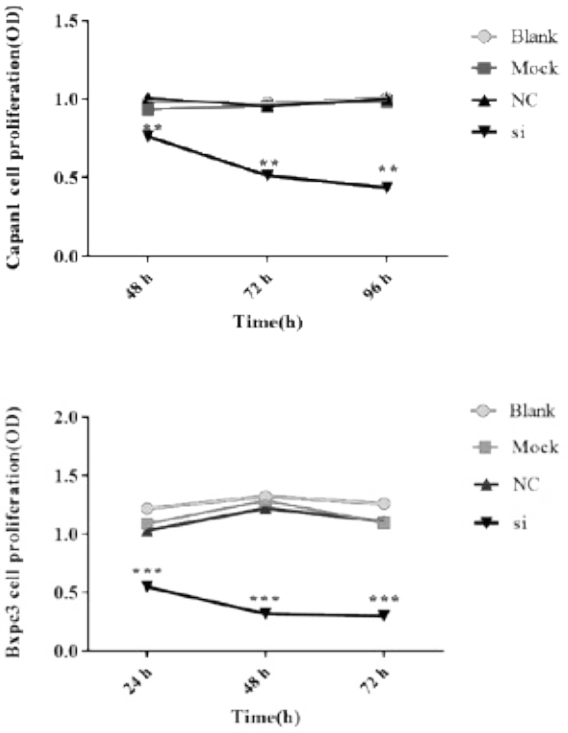

Fig. 8. Proliferation of two cell lines after knocking down sortilin

A.The proliferation of Capan1 cells.The transfection group(P<0.0001) compared with Blank groups has significant difference at 48 hours after transfection,however there was no difference among the negative groups(P=0.640),Mock groups(P=0.136) and Blank control groups.

B.The proliferation of Bxpc3 cells.The transfection group(P<0.0001) compared with Blank groups has significant difference at 24 hours after transfection,however there was no difference among the negative groups(P=0.505),Mock groups(P=0.096) and Blank control groups

Invasion ability of pancreatic cancer cells after sortilin knockdown

Our data showed that after sotilin inhibition, the invasiveness of the two cells in the inhibition group (si) was significantly lower than that in the Blank group. It is suggested that the inhibition of sortilin can effectively reduce the invasiveness of pancreatic cancer cells(Figure 9).

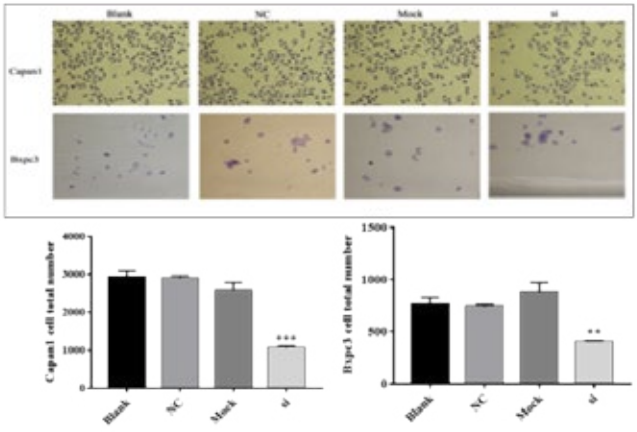

Fig. 9. Invasion of two cell lines after knockdown sortilin

A. The invasiveness of capan1 and bxpc3 cells in each group after inhibition of sortilin.

B. B.In capan1 cells, the invasion ability of sortilin inhibition group (P = 0.0057) was significantly lower than that of Blank group, while there was no significant difference between negative group (P = 0.333) and Mock Group (P = 0.333).C. In Bxpc3 cells, the invasion ability of sortilin inhibition group (P = 0.0006) was significantly lower than that of Blank group, while there was no significant difference between negative group (P = 0.333) and Mock Group (P = 0.333).

After sortilin inhibition mRNA expression of P53, NFκB and MMP9

After siRNA transfection, the expression of P53, NFκB and MMP9 mRNA was detected. NFκB and MMP9 decreased, while P53 increased. There was no significant difference between blank group, negative control group and mock group,The blank group and knockdown group were statistically significant difference(Figure 10).It is suggested that sortilin may affect the migration, invasion and proliferation of pancreatic cancer cells in relation to P53, NFκB and MMP9. The inhibition of sortilin may affect the migration, invasion and proliferation of pancreatic cancer cells through the increase ofP53 and the decrease of NFκB and MMP9.

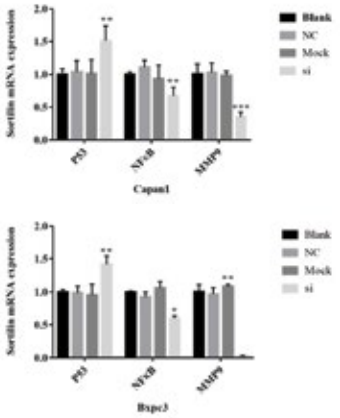

Fig.10. mRNA expression of P53, NFκB and MMP9 in pancreatic cancer cells.

A. mRNA expression levels of three molecules in capan1 cells.The expression of three molecules in soritlin knockdown group(si) was statistically significant compared with Blank group, reagent control group(Mock) and negative control group(NC).

B. mRNA expression levels of three molecules in Bxpc3 cells.The same situation as capan1 cells.

DISCUSSION

Pancreatic cancer is one of the pancreatic cancers with very poor prognosis. The main risk factors of pancreatic cancer include smoking, obesity, long-standing diabetes and family history of disease.At the same time, people still have insufficient knowledge about the development of pancreatic cancer[13]. So, it is still important to explore the mechanism of PC and find new biomarkers at an early stage. In this study, The expression of sortilin in various cancers was retrieved using the bioinformatics website GEPIA, including pancreatic cancer tissues among them.Sortilin was indeed up-regulation in 115 cases of pancreatic cancer tissues which was verified by IHC. This is consistent with the report by Fangfang Gao et al[14]. In this study, we analyzedfor the first time that the up-regulation of soritlin comes from SEIN. We continue to analyze the results of IHC in depth. The predictive ability of sortilin was evaluated by BLR and ROC curve, The predictive value of SEIN was 73.9%, and the diagnostic accuracy was 86.3%. The Youden Index was calculated to evaluate the diagnostic power. The cut-off value of SEIN for PC diagnosis was 0.85, with the corresponding sensitivity of 90.9% and the specificity of 68.3%. Univariate Survival analysis showed that the expression level of SEIN, M stage, Pathological grade and Clinical stage had a significant impact on the survival time, and patients with higher expression level of SEIN had poor prognosis. Multivariate analysis suggested that SEIN might be an independent prognostic indicator for the survival of PC patients.

Based on IHC research results we further verified the role of sortilin in the development of PC through in vitro. After we specifically knocked down sortilin with small interfering siRNA fragments, both pancreatic cancer cells showed reduced proliferation, invasion and migration.This observation is consistent with the results from IHC characterization.Target reduction of sortilin is somewhat helpful for controlling the progression of pancreatic cancer and improving the survival rate of PC patients.

This study also further analyzed the mechanism of sortilin in controlling the proliferation, migration and invasion of pancreatic cancer cells. We introduced P53, NFκB and MMP9. It was found that p53 was up-regulated, and NFκB and MMP9 in a differen degress of down-regulated after reduction of sortilin. We preliminarily estimated that the decrease of sortilin may be caused by sortilin/NFκB/p53 pathway affecting cell proliferation and sortilin-MMP9 pathway affecting cell invasion. A large number of reports have pointed out that sortilin binds to different ligands and has different effects on cells.When it is combined with ProNGF, it induces tumor cell apoptosis and arterial remodeling[15]; high affinity binding with ProNGF and p75NTR complex, mediates cell death of central neurons, natural killer cells, and retinal photoreceptors[16] and activates substantia nigra Caspase-mediated dopamine neuronal death signaling pathway in the striatum[17]. Binding to the p75NTR/TrκB complex plays a key role in cancer cell survival [18]. Most reports are about the close association between sortilin and neurotrophic factor family. We have opened a new way for sortilin to act in pancreatic cancer, but more data is still needed to support, also it is necessary to further expand the analysis that whether it has similar performance in other cell lines of PC.

CONCLUSION

These fndings demonstrated that the low expression of SEIN indicates better prognosis in pancreatic cancer and supplemented the effect mechanism of sortilin on pancreatic cancer cells. SEIN expression may serve as a potential diagnostic indicator of pancreatic cancer.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethics Committee of North sichuan Medical college (Nanchong, China) in accordance with the principles of the Declaration of Helsinki.

The patient's informed consent was obtained and their medical data wereused for research.

CONSENT FOR PUBLICATION

All authors have seen and agreed to publish.

COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AUTHORS' CONTRIBUTIONS

Conception

DX designed the work and wrote the manuscript.

Interpretation or analysis of data

DX is mainly responsible for interpretation and analysis of Data; FY, RC, and SQL participated in data analysis and the discussion and language editing.

Preparation of the manuscript

Revision for important intellectual content

RC and SQL reviewed the manuscript.

Supervision

RC and SQL are responsible for the supervision.

AVAILABILITY OF DATA AND MATERIALS

The datasets supporting the conclusions of Figure 1 are available in [GEPIA dataset] at (http://gepia.cancerpku.cn/detail.php?gene=SORT1). Please contact the author for the rest of the data.

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