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The role of GRP94 in regulating cell growth and apoptosis in pancreatic cancer cells

Zheng Pan

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LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECL	Enhanced chemiluminescence
FBS	Fetal Bovine Serum
Hsp	Heat shock proteins
H	Hour
KD	Kilodalton
MW	Molecular Weight
mM	Millimolar
MTT	3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
˚C	Grade Celcius
OD	Optical Density
PBS	Phosphate Buffered Saline
PDAC	Pancreatic ductal adenocarcinomas
PSC	Pancreatic stellate cell
SD	Standard Deviation
Sec	Second
SiRNA	Small interfering RNA
TBS	Tris Buffered Saline

1. INTRODUCTION

Carcinoma of the pancreas is an aggressive human cancer noted for its early metastatic potential and poor prognosis [1]. The disease accounts for around 37,170 new cases and 33,370 deaths a year in the United States [2], and around 59.900 new cases and 65,7 00 deaths a year in European Economic Area [3]. These nearly equal numbers of new cases and deaths illustrate the dismal prognosis generally associated with pancreatic carcinoma. Roughly 80% of cases occur between 60 and 80 years of age, whereas less than 2% occur in people younger than 40. Risk factors include a history of hereditary or chronic pancreatitis, cigarette smoking, and occupational exposure to carcinogens. The median survival time for patients with locally advanced disease is 6 to 10 months and for those with metastases, the median survival time is only 3 to 6 months. Most patients present with advanced disease and the symptoms of the disease cause substantial morbidity.

Nearly 90% of pancreatic neoplasms are ductal adenocarcinomas [4]. Roughly 70% of ductal cancers arise in the pancreatic head or uncinate process. These are characterized by tumor desmoplasia, early local extension to contiguous structures, metastases to regional lymph nodes and to the liver [5-7]. Moreover, pancreatic cancer cells are usually

resistant to the programmed cell death (apoptosis) mediated by conventional chemotherapeutic agents [8, 9]. It is generally believed that cancer cells have an altered cellular physiology characterized by abundance of growth signals, insensitivity to cycle arrest signals, and evasion of apoptosis [10]. Unresponsiveness to apoptotic stimuli can result in tumor progression and resistance to most oncological therapies [8].

1.1 Apoptosis and Necrosis

Cell proliferation must be balanced by an appropriate process of cell elimination to maintain tissue homeostasis. Cell death can occur in two main forms, apoptosis and necrosis, each with its own morphologic and biochemical manifestations[11–13]. Apoptosis or programmed cell death is a process with typical morphological characteristics including plasma membrane blebbing, cell shrinkage, chromatin condensation and fragmentation. Apoptosis has been implicated in various physiologic functions, including the remodeling of tissues during development, removal of senescent cells and cells with genetic damage beyond repair, and the maintenance of tissue homeostasis. Necrosis is a passive, adenosine triphosphate–independent form of cell death requiring an acute non-physiologic injury (i.e., ischemia, mechanical injury, and toxins) that

results in destruction of the cytoplasmic and organellar membranes with subsequent cellular swelling and lysis.[14，15] The lysis of necrotic cells releases cytoplasmic and organelle contents into the extracellular milieu, resulting in inflammation with surrounding tissue necrosis and destruction.

Mediated by a family of cysteine proteases known as caspases, two distinct pathways characterize apoptotic processes. The extrinsic pathway that is triggered by death receptors, such as FAS or the tumor necrosis factor (TNF) receptor with their respective ligands, results in caspase-8 activation. In response to growth factor withdrawal, hypoxia, or DNA damage, the intrinsic pathway is initiated, resulting in cytochrome c release, loss of mitochondrial membrane potential [12], and the apoptosome formation; a complex consisting of cytochrome c, apoptotic protease-activating factor-1 (Apaf-1), and procaspase-9 [16, 17]. Both pathways lead to the typical morphologic and biochemical changes of the apoptotic cell. In addition, two other apoptotic pathways are emerging: endoplasmic reticulum stress-induced apoptosis and caspase-independent apoptosis. Disturbances in apoptosis contribute to numerous diseases, including cancer [16, 18-21]. Tumor cells express several proteins that suppress apoptosis. Functional interactions of those proteins in apoptosis pathways are therefore of particular interest. Among them, the

antiapoptotic members of the Bcl-2 protein family, members of the inhibitor of apoptosis protein family, and heat shock proteins (Hsp) play a major role [16, 22-24]. Many key components of survival and apoptotic pathways are regulated by interactions with molecular chaperones. These proteins are primarily the Hsp70, Hsp90 and Hsp27. Although heat-shock proteins are only induced transiently after periods of cell stress, they are often constitutively overexpressed in tumor cells. Overexpression of Hsp70 or Hsp27 in transformed cell lines enhanced tumorigenic potential when the cells were transferred into syngenic mice [25, 26], and transgenic mice overexpressing Hsp70 were found to develop malignant T-cell lymphomas [27]. Although, overexpression of Hsp70 or Hsp27 can increase resistance to stress induced apoptosis in cultured cell lines, it is unclear whether the elevated expression of heat-shock proteins in tumor cells contributes to tumorigenesis by preventing stress-induced apoptosis.

1.2 GRPs in Cancer Treatment

It has been established that the glucose-regulated proteins (GRPs) play an important role in maintaining cellular homeostasis. As endoplasmic reticulum (ER) chaperones, they participate in ER protein translocation, chaperoning, protein quality control, ER-associated protein degradation, ER stress sensing and regulation, and ER calcium binding [28]. Recently,

the ER stress pathways and the GRPs have been linked to cancer growth and drug resistance [29, 30]. As such, they represent novel markers for cancer progression and chemo-responsiveness, as well as targets for cancer therapy. Some GRPs are also capable of modulating innate and adaptive immunity, and have been studied extensively in cancer immunotherapy [31-33].

GRP94, which is a member of the Hsp90 family, is the most abundant glycoprotein in the endoplasmic reticulum. GRP94 knockdown mouse embryos die in early gestation [34] and GRP94 is up-regulated in transformed cells, suggesting an expanded role for this chaperone beyond the maturation of immune response proteins. Overexpression of GRP94 is associated with tumorigenicity, and decreased sensitivity to radiation, whereas suppression of GRP94 sensitizes cells to etoposide treatment [35-38]. GRP94, as in the case of the antiapoptotic protein Bcl-2, can be a target of proteolytic cleavage itself during the apoptotic process [36]. Overexpression of GRP94 suppresses ER stress-induced apoptosis of neuronal cells [39], whereas downregulation of GRP94 expression accelerates ER stress-induced apoptosis [36, 39]. A recent report has shown that GRP94 blocks apoptosis induced by HCV infection [40]. These findings suggest that GRP94 protects against ER stress-induced apoptosis. Despite these observations, effects of GRP94 expression in

pancreatic cancer have not been extensively investigated.

A previous study has shown that actinomycin D is a potent inducer of apoptosis in a variety of pancreatic cancer cells in vitro [41]. It binds to DNA and inhibits RNA and protein synthesis. Actinomycin D may act via JNK/SAPK and Bax to promote apoptosis in Panc-1 cells. Moreover, several lines of evidence indicate that actinomycin D causes apoptotic and not necrotic cell death [41].

2. AIMS

In vitro systems in combination with xenograft models strongly suggest that GRP78 plays an important role in tumor growth and survival, drug resistance and dormancy. GRP94, although less well characterized in these aspects, may also be an active participant.

In the present study, the expression of GRP94 was analyzed in the normal pancreas, pancreatic cancer and chronic pancreatitis tissues. We also investigated the role of GRP94 in regulating cell growth and apoptosis in pancreatic cancer cells.

3. MATERIAL AND METHODS

3.1 Materials

3.1.1 Laboratory Equipments

Analytic balance	MERRLER
Balance	SCALTEC
Centrifuge	Eppendorf
CO_2 incubator	SANYO
Computer Hardware	Fujitsu SIEMENS
Freezer -20℃	LIEBHERR
Freezer -80℃	Heraeus
Gel chamber, glass plate	Bio-Rad
Microplate Reader	Thermo Labsystems
Microscope	LEICA
Microwave oven	Bosch
pH-meter	BECKMAN
Refrigerator 4℃	Bosch
Roller mixer	STUART
Scanner	Canon
Table Centrifuge	Eppendorf
Vortex	Heidolph

Water bath	LAVDA
Tissue embedding machine	Leica
Tissue processor	Leica

3.1.2 Consumables

BCA Protein Assay Kit	PIERCE
Cell scraper	BD Falcon
Coverslips	Assistant
Filter(0.2 μ M)	Neo Lab
Hyperfilm	Kodac Inc
Pure nitrocellulose menbrane	Bio-Rad
Sterile needles	BD Falcon
Tissue culture dishes(60×15㎜;100×20㎜)	Cell Star
Tissue culture flasks(25cm^2; 75cm^2; 125cm^2)	Cell Star
Tissue culture plates(6-well;24-well;96-well)	Cell Star
Tubes(15ml; 50ml)	Falcon
Blotting paper	Whatman

3.1.3 Chemicals

Actinomycin D	Sigma
Albumin bovine	Sigma
Dimethyl sulfoxide	Sigma
ECL detection reagent	Invitrogen
EDTA	Sigma
Ethanol	Roth
Protein molecular weight marker	Amersham
Gemcitabine	Sigma
Glycine	Roth
GRP94 siRNA	Qiagen
IgG control siRNA	Qiagen
SDS	Sigma
Liquid nitrogen	Tec-Lab
Methanol	Roth
MTT	Sigma
Permount	Vector Laboraories
Protease inhibitor cocktail tablets	Roth
RNAi HiperFect Transfection Reagent	Qiagen
Sodium Chloride	Merck
Sodium Citrate	Merck

Tris Base	Merck
Tween 20	Merck
Western blotting Detection Reagent	Amersham

3.1.4 Cell Culture Medium

DMEM	Invitrogen
RPMI 1640	Invitrogen
Ham’s F12 medium	Invitrogen
fetal bovine serum	PAN Biotech
Trypsin-EDTA	Invitrogen
penicillin-streptomycin	Invitrogen

3.1.5 Antibodies and Cell-lysates

GRP94 polyclonal rabbit antibody and GAPDH polyclonal rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

β-actin rabbit antibody was purchased from BD Biosciences (Erembodegem, Belgium)

Hela cell-lysate was purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

3.1.6 Buffer and Solutions

MTT Solution (in PBS): 5 mg/ml

Cell lysis buffer: 50 mM Tris-HCl
150 mM NaCl
2 mM EDTA
1% SDS
1 Tbl Protease inhibitor cocktail for 10ml buffer

1× TBS: 150 mM Tris-HCl
50 mM NaCl
pH 7.4

SDS PAGE running buffer: 25 mM Tris-HCl
192 mM Glycine
0.1% SDS

Wash buffer (TBS/T): 1× TBS
0.1% Tween 20

Blocking buffer: 1× TBS
0.1% Tween 20

	5% w/v	nonfat dry milk
Transfer buffer:	25 mM	Tris Base
	0.2 M	Glycine
	20%	Ethanol
SDS Sample buffer:	2%	SDS
	30%	Glycerol
	0.45 M	Tris-HCl (PH 6.8)
	10% v/v	2-Mercaptoethanol
	0.03%	Bromphenoblue

3.1.7 Pancreatic Cancer Cell Lines

Aspc-1, Bxpc-3, Capan-1, Colo-357, MiaPaCa-2, Panc-1, SU86.86 and T3M4 cell lineS were obtained from American Type Culture Collection

3.2 Methods

3.2.1 Tissue Specimens and Cell Cultures

Tissues of pancreatic ductal adenocarcinoma (PDAC) and chronic pancreatitis (CP) were collected at the University of Heidelberg and

Technischen Universität München (TUM), Germany. Normal human pancreatic tissue samples were obtained from previously healthy individuals through an organ donor procurement program whenever there was no suitable recipient for pancreas transplantation. All samples were confirmed histologically. Freshly removed tissues were fixed in 4% paraformaldehyde solution for 12-24 h and then paraffin embedded for histological analysis. In addition, a portion of the tissue samples was preserved in RNAlater (Ambion Europe Ltd., Huntingdon, Cambridgeshire, UK), or snap-frozen in liquid nitrogen immediately upon surgical removal and maintained at -80˚C until use. The Human Subjects Committee of the University of Heidelberg and TUM, Germany, approved the use of human tissues for molecular research. Written informed consent was obtained from all patients.

Pancreatic cancer cell lines were grown in DMEM medium (Panc-1 and MiaPaCa-2) or RPMI-1640 (Aspc-1, BxPc-3, Capan-1, Colo-357, SU86.86, and T3M4), supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin (complete medium), and incubated at 37 ℃ in a 5% CO_2 humidified atmosphere.

3.2.2 Human Pancreatic Stellate Cell Isolation and Culture

Human Pancreatic Stellate Cell (PSC) isolation and culture were

performed as described by Bachem et al[42]. Pancreatic tissue was obtained during surgery from patients with chronic pancreatitis and pancreatic cancer. For the isolation of PSCs, the outgrowth method was used. Cell populations between passage 3 and 6 were used. A 1:1 (vol/vol) mixture of low glucose (1000 mg/L) Dulbecco's modified Eagle medium with Ham's F12 medium supplemented with 20% FBS, L-glutamine (2 mmol/L), penicillin/streptomycin, and amphotericin was the standard medium (SM 20%).

3.2.3 Quantitative real-time PCR

RNA was reverse transcribed into cDNA using the cDNA synthesis kit for reverse transcription polymerase chain reaction (PCR) according to the manufacturer's instructions. Quantitative real-time PCR (QRT-PCR) was performed with the Light Cycler Fast Start DNA SYBR Green kit. The number of specific transcripts was normalized to the levels of the housekeeping gene HPRT. Specific primers were used: GRP94 forward 5´-TTGCCAGACCATCCGTACTG-3´; GRP94 reverse 5´-GAATTGGATGAAAGATAAAGCCCTTA-3´.

3.2.4 Immunohistochemistry

Immunohistochemistry was performed using the Dako Envision System, as published previously [43]. Briefly, consecutive paraffin-embedded

tissue sections (3 μm thick) were deparaffinized and rehydrated using routine methods. Antigen retrieval was performed by pretreatment of the slides in citrate buffer (pH 6.0) in a microwave oven for 15 minutes. Endogenous peroxidase activity was quenched by incubation in deionized water containing 3% hydrogen peroxide at room temperature for 10 min. Sections were incubated with rabbit anti-human GRP94 polyclonal antibodies (1:250) diluted with antibody diluent at 4˚C overnight and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody and counterstaining with Mayer's hematoxylin. In addition, to confirm the specificity of the primary antibodies, tissue sections were incubated with negative control rabbit IgG. Under these conditions, no specific immunostaining was detected.

3.2.5 Semi-quantitative Analysis of GRP94 Expression in Tissues

Semiquantitative analysis was performed as published previously [43]. Scores were given separately for the stained area and for the intensity of staining. Quantification was made as follows: <33% of the cancer cells, 1; 33%–66% of the cells, 2; >66% of the cancer cells, 3. Intensity of staining was quantified as follows: absent, 1; weak/moderate, 2; strong, 3. Each section had a final grade that derived from the multiplication of the area and intensity scores. GRP94 expression was considered to be absent/low for grade 1, moderate for grades 2, 3, and 4, and strong for

grades 6 and 9.

3.2.6 Immunoblot Analysis

Cultured pancreatic cancer cells were lysed in ice-cold buffer containing 20 mM Tris-HCl (pH = 7.4), 150 mM NaCl, 0.1% TritonX-100, 2.5 mM sodium pyrophosphate, and 1 tablet EDTA-free protease inhibitor cocktail for 30 minutes. Cell lysates were then collected after centrifugation at 13500 rpm for 10 minutes at 4˚C. 20 μg of the total protein were loaded on 10% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 20 ml TBS, 5% skim milk and 0.05% Tween-20 for 1 h and incubated with rabbit anti-GRP94 polyclonal antibody (1:400) overnight at 4˚C. Membranes were washed three times with 0.05% Tween-20-TBS and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (1:5000) for 2 min at room temperature. Signals were detected using the enhanced chemiluminescence system (ECL, Amersham Life Science Ltd., Bucks, UK). GAPDH (1:5000) was used to verify equal loading.

3.2.7 siRNA Transfection

Synthetic siRNA oligonucleotides for GRP94 were prepared and stored according to the manufacturer’s instructions. The target sequences are 5 ́-TCGCCTCAGTTTGAACATTGA-3 ́;

5´-AAGTTGATGTGGATGGTACAT-3´. Cells were grown until 70% confluence and transfections were carried out with HiPerFect trqansfection reagent according to the manufacturer's instructions. The final concentration of both the control and specific oligonucleotides was 5 nM. The efficacy of the siRNA transfection was ascertained by immunoblot analysis 48 h to 120 h after transfection.

3.2.8 Cell Growth Assay

Cell growth was determined using the 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric growth assay. Cells were seeded at a density of 5000 cells/well in 96-well plates for up to five days. All assays were performed in triplicate and repeated four times.

3.2.9 Growth Assays and Response to Gemcitabine

Normal and transfected Panc-1 cells were seeded triplicate in 96-well plate at densities of 5000/well. For assessment of growth, cells were kept under standard conditions for 48 hours. For the assessment of chemotherapeutic resistance, gemcitabine was added 12 hours after seeding at gradient concentrations; 0.01% dimethyl sulfoxide was used as controls. After 48 hours of incubation, the MTT test was performed to assess cell viability, as described previously [8]. Growth assays were

repeated three times and reported as percent change compared with control. The median effective doses of gemcitabine on cancer cells grown were calculated from 5 independent experiments normalized to matching day-0 observations.

3.2.10 Actinomycin D Treatment

Transfected cells (5,000/well) were seeded overnight in 96-well plates and incubated for 48 hours under increasing concentrations of actinomycin D. Cell growth was assessed by the MTT assay, as published previously [8]. All assays were performed in triplicate and repeated four times.

3.2.11 Statistical Analysis

Statistical analysis and graph presentation were carried out using the GraphPad Prism 4 Software (GraphPad, San Diego, CA). mRNA results of QRT-PCR analyses are presented as mean ± SEM. The Shapiro-Wilk test was used to evaluate data distribution. The Mann-Whitney test was used for comparison of two groups with independent samples. One-way analysis of variance (ANOVA) and Bonferroni's multiple comparison tests were used to compare three groups. The Kaplan-Meier method and log-rank test were used for survival analysis. $P<0.05$ was taken as the level of significance.

4. RESULTS

4.1 Expression of GRP94 in Pancreatic Tissues

To quantify the mRNA levels of GRP94, QRT-PCR was performed using tissues of the normal pancreas (n=10), chronic pancreatitis (n=15) and pancreatic ductal adenocarcinoma (n=20). Compared to normal pancreatic tissues, median mRNA levels of GRP94 were 1.5-fold and 3.7-fold ($p<0.05$) lower in chronic pancreatitis and pancreatic cancer tissues, respectively **(Fig. 1).** Importantly, chronic pancreatitis tissues also had 2.5-fold ($p<0.05$) higher GRP94 mRNA than pancreatic cancer tissues. To determine the localization of GRP94 protein in tissues, immunohistochemistry was performed in 20 normal pancreatic tissue samples, 20 chronic pancreatitis samples, and 44 pancreatic cancer samples. In normal pancreas **(Fig. 2A-C)** and chronic pancreatitis **(Fig. 2D-F)** tissues, GRP94 was absent or weakly present in ductal cells and strongly present in acinar cells. In pancreatic cancer tissues, strong staining was detected in 20% of sections, while in 32% there was moderate staining and in 48% no staining **(Fig. 2G-I)**. GRP94 expression was also detected in the stellate cells of tumor tissues and to some extent in stellate cells of chronic pancreatitis tissues.

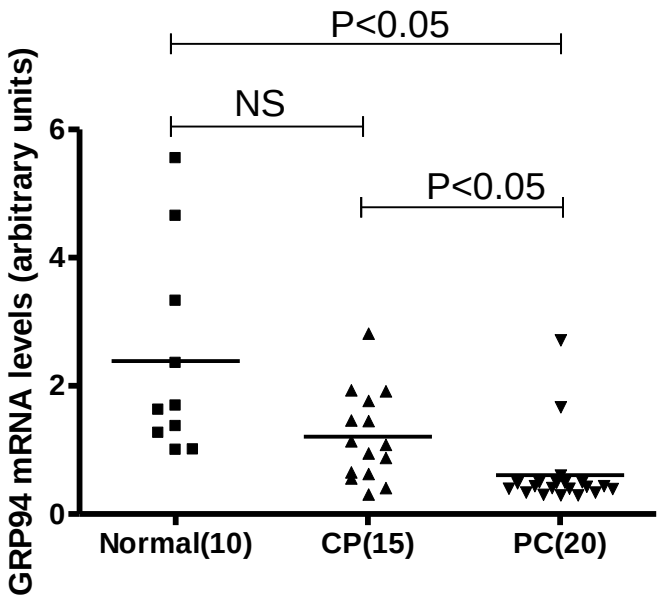


Figure 1. GRP94 mRNA expression in pancreatic tissues. Real-time quantitative RT-PCR analysis of GRP94 mRNA levels in normal pancreas, chronic pancreatitis, and pancreatic cancer tissues was carried out as described in the Materials and Methods section. RNA input was normalized to the average expression of the housekeeping gene HPRT. Horizontal lines represent the median values.

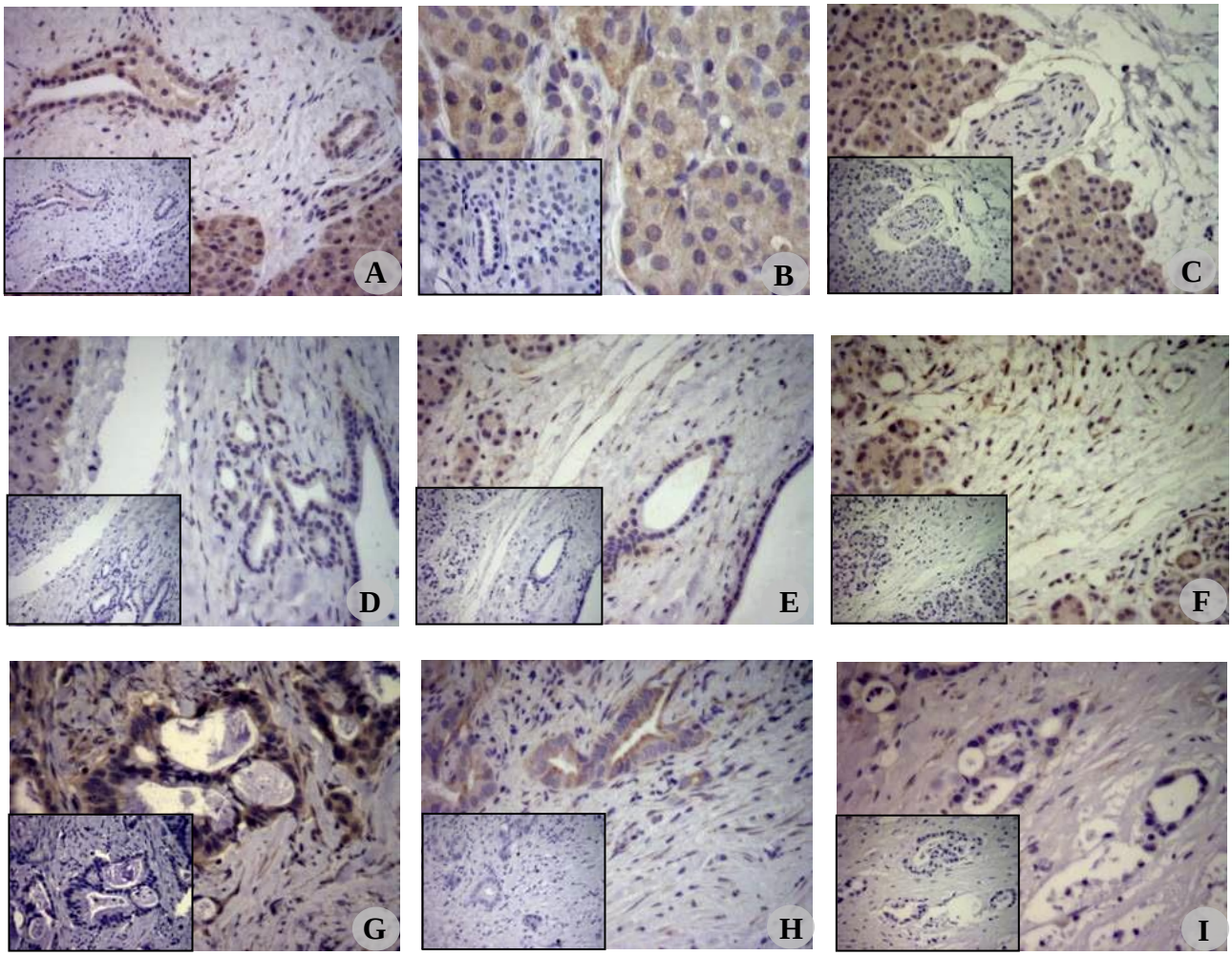
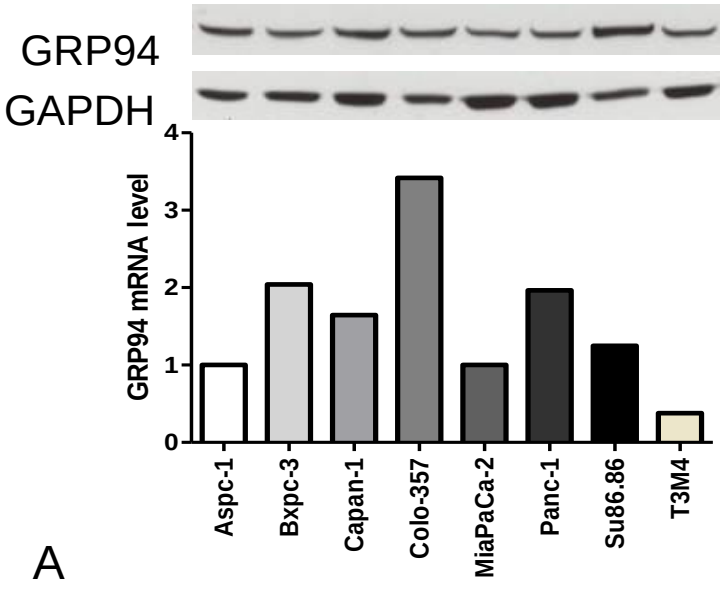


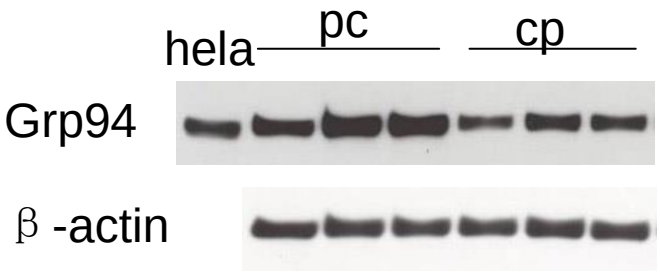
Figure 2. Localization of GRP94 in pancreatic tissues. Immunohistochemistry using a specific GRP94 antibody was carried out as described in the Materials and Methods section. GRP94 expression in the normal pancreas (A, B, C), chronic pancreatitis (D, E, F) and pancreatic ductal adenocarcinoma (G, H, I) is shown. A-I-inserts: negative control sections using isotype-matched IgG are shown.

4.2 Expression of GRP94 in Pancreatic Cancer Cell Lines

Next we quantified GRP94 mRNA and protein levels in eight human pancreatic cancer cell lines (Aspc-1, BxPc-3, Capan-1, Colo-357, MiaPaCa-2, Panc-1, SU86.86 and T3M4). All cancer cells analyzed expressed various degrees of GRP94 mRNA and protein **(Fig. 3A).** We also inspected GRP94 protein levels in three pancreatic stellate cells isolated from cancer cells and three from chronic pancreatitis. Hela cell-lysate was used as positive control. All six PSCs analyzed expressed GRP94 protein.(**Fig. 3B)**



A



B

Figure 3. Expression of GRP94 in pancreatic ductal adenocarcinoma cell lines. Real-time quantitative RT-PCR analysis of GRP94 mRNA levels in 8 pancreatic cancer cell lines, as described in the Materials and Methods section. RNA input was normalized to the average expression of the housekeeping gene HPRT. Immunoblot analysis was carried out to detect expression of GRP94 in eight pancreatic cancer cell lines and PSC. Equal loading of the protein samples was confirmed using a GAPDH or β-actin antibody.

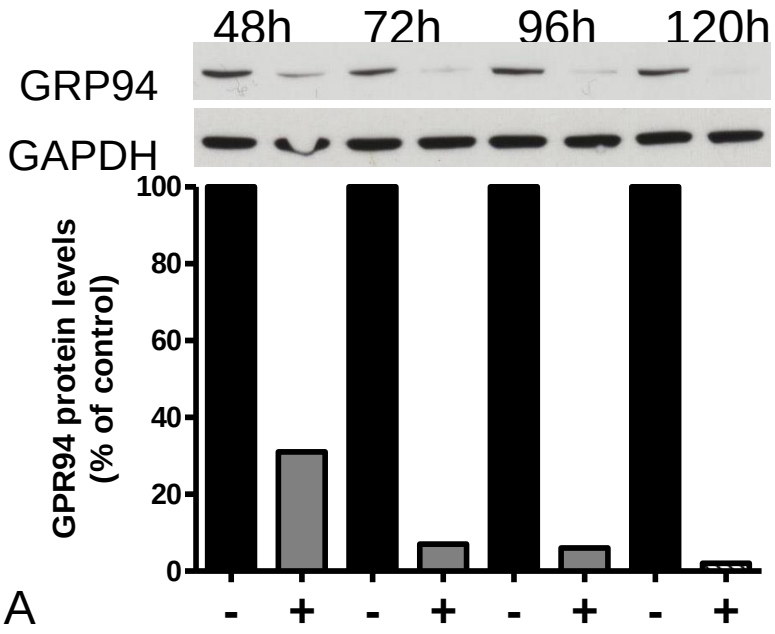
4.3 Effects of GRP94 Silencing on Chemoresponsiveness of Pancreatic Cancer Cells

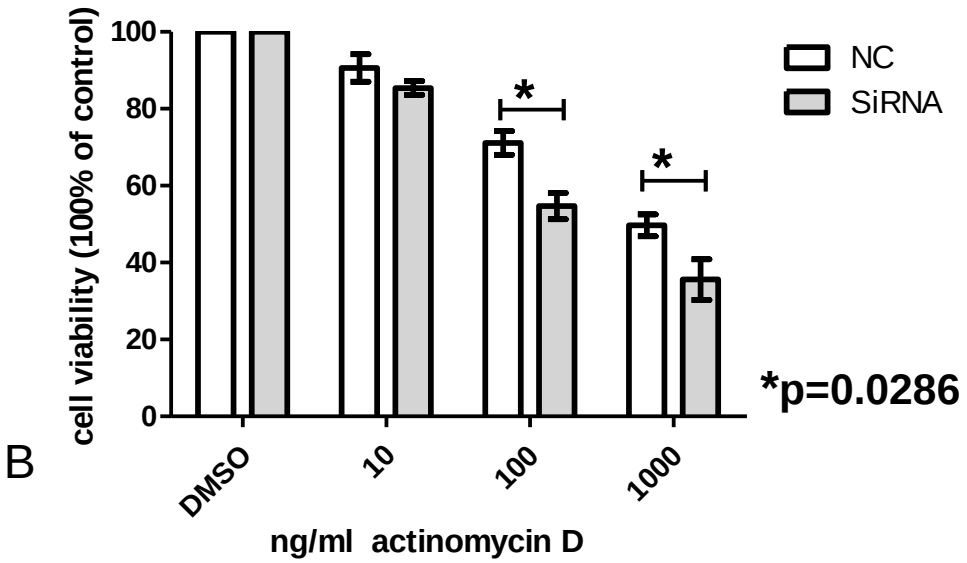
Transfection of Panc-1 cells with GRP94 siRNA for 24 h to 120 h reduced GRP94 expression significantly in a time-dependent way **(Fig. 4A).** Silencing of GRP94 had no significant effect on cell growth (DATA NOT SHOWN).

Next, actinomycin D, which is known to induce apoptosis in pancreatic cancer cells [41], was used to induce apoptosis. Panc-1 cells were treated with GRP94 siRNA or negative control siRNA for 48 h before treatment with actinomycin D. After 24 h of incubation (72 h after transfection) with actinomycin D, there was a dose-dependent decrease in cell number,

with a significant decrease of 45% in GRP94 siRNA-treated cells and only a 29% decrease in control cells (p=0.03) at a concentration of 100 ng/ml actinomycin D as well as at a concentration of 1000 ng/ml actinomycin D (64% vs. 50%, p=0.03) **(Fig. 4B)**.

We also inspected the effects of GRP94 silencing on gemcitabine treatment of Panc-1 cells. After 48 hours of incubation with gemcitabine, there was a dose-dependent decrease in both normal and GRP94 siRNA-treated cells. But the median effective dosed of gemcitabine on GRP94 siRNA-treated cells had no dignificant change compared with control **(Fig. 4C)**.





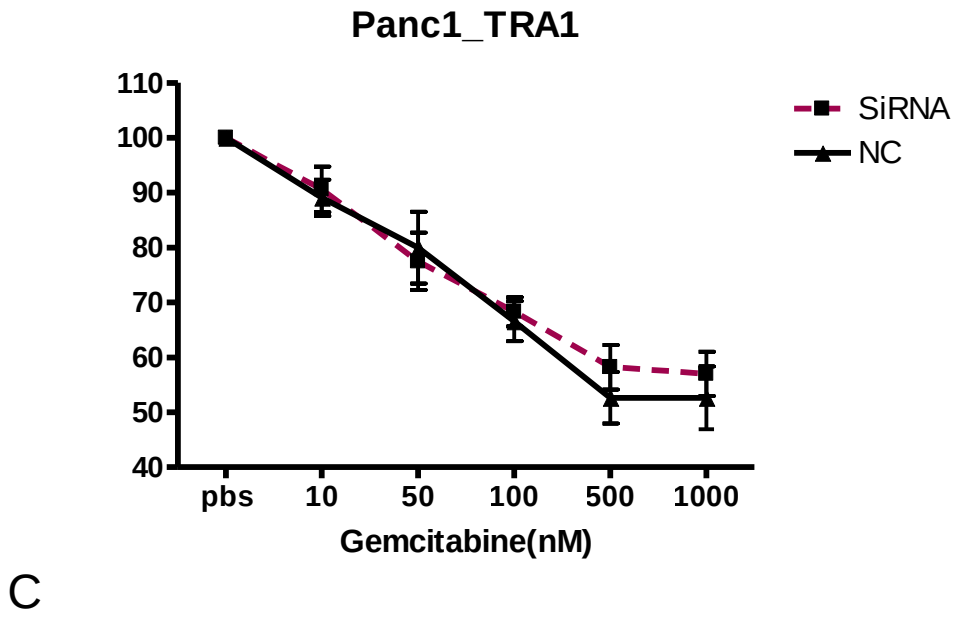
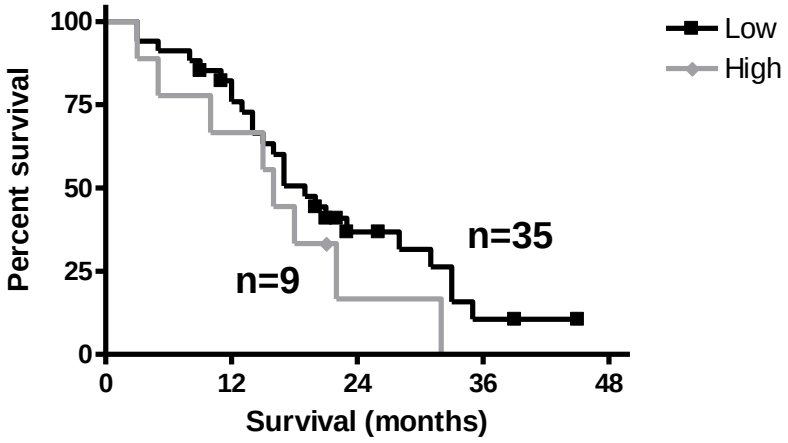


Figure 4.The effect of silencing of GRP94 in Panc-1 on apoptosis resistance. (A) Expression of GRP94 after transfection with GRP94 siRNA (+) and siRNA control (-) at different time points in Panc-1 cells. Equal loading of the protein samples was confirmed using a GAPDH antibody. (B) Cells were incubated for 24 h in the absence (DMSO was used as control) or presence of 10, 100 and 1000 ng/ml actinomycin D

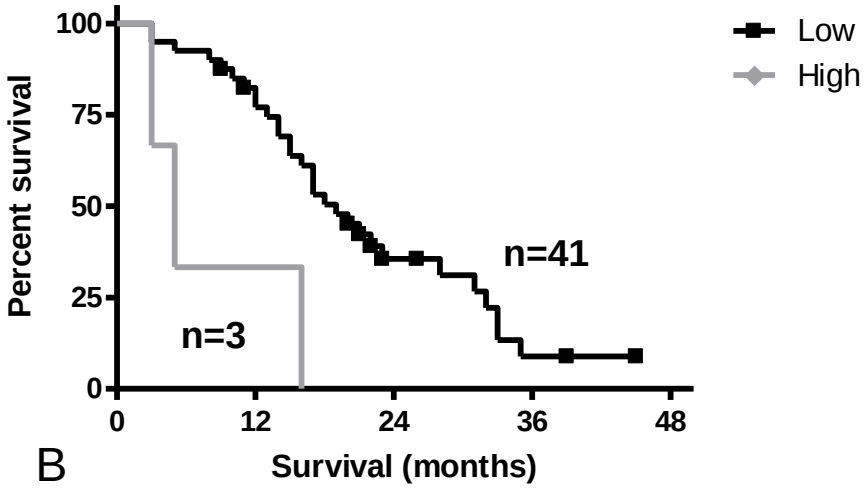
dissolved in DMSO. Cell growth was determined by the MTT assay, as described in Materials and Methods. Data are expressed as percent decrease of the respective untreated controls and are mean ± SEM obtained from four independent experiments. (C) Cells were incubated for 48 h in the absence (PBS was used as control) or presence of 10,50,100,500 and 1000 nM gemcitabine. Cell growth was determined by the MTT assay, as described in Materials and Methods. Data are expressed as percent change compared with control.

4.4 Correlation of GRP94 Protein Expression and Patient Survival

When the patients were categorized according to the staining scores, patients with strong staining (scores of 6 and 9) showed a tendency toward worse survival (19 months vs. 16 months, p=0.24) **(Fig. 5A)**. There were only 3 patients with a score of 9. Interestingly, these patients had a median survival of 5 months (p=0.006). However, due to the small number of patients, the data should be carefully interpreted **(Fig. 5B)**.



A



B

Figure 5. Correlation of GRP94 immunopositivity with survival in pancreatic cancer patients. Semi-quantitative immunohistochemistry was used to evaluate GRP94 immunopositivity in PDAC tissues. Scores were given separately for the stained area and for the intensity of staining. Quantification was made as follows: <33% of the cancer cells, 1; 33%–66% of the cells, 2; >66% of the cancer cells, 3. Intensity of

staining was quantified as follows: absent, 1; weak/moderate, 2; strong, 3. Each section had a final grade that was derived from the multiplication of the area and intensity scores. GRP94 expression was considered to be absent/low for grade 1, moderate for grades 2, 3, and 4, and strong for grades 6 and 9. (A) Comparison of survival in patients with low/absent and moderate (n=35) vs. high (n=9) IHC scores was made using the Kaplan-Meier method and Log rank analysis. (B) A subgroup analysis of survival for patients with a score of 9 (n=3) vs. all other patients.

5. DISCUSSION

Our results show that there is a significant reduction of GRP94 expression in pancreatic ductal adenocarcinoma patients. This finding is contradictory to several reports in the literature showing increased GRP94 expression in a variety of malignant tumors or cancer cell lines, such as breast [44], oral [45], lung [46], gastric [47], esophageal [48] and colorectal carcinomas [49]. Using immunohistochemistry we observed that GRP94 was absent or weakly present in ductal cells but strongly present in acinar cells in chronic pancreatitis and normal pancreatic tissues. This reduction in cancer can partially be explained by the loss of acinar component in tumorous areas. However, the mRNA levels of GRP94 in PDAC was also significantly lower than those of chronic pancreatitis tissues, therefore it is likely that there are other mechanisms involved in the downregulation of GRP94 in PDAC.

Although GRP94 expression was lost in 48% of cancer tissues, it was strongly present in 20% of the cases. Low or absent expression of GRP94 showed a tendency toward better survival, but the difference was not significant in our group of 44 patients.

We quantified GRP94 mRNA and protein levels in eight human

pancreatic cancer cell lines and six PSCs culture in vitro. Interestingly, all cells analyzed expressed various degrees of GRP94 mRNA and protein. It is likely that there are different mechanisms involved in the expression of GRP94 in tissues and cells cultured in vitro.

Recently, the endoplasmic reticulum stress pathways and the GRPs have been linked to cancer growth and drug resistance. They represent novel markers for cancer progression and chemo-responsiveness, as well as targets for cancer therapy. Previous studies have shown that overexpression of GRP94 is associated with cellular transformation, tumorigenicity and decreased sensitivity to radiation, whereas suppression of GRP94 sensitizes cells to etoposide treatment. Although several studies strongly suggest that GRP78 plays an important role in tumor growth and tumor metastasis [50, 51], GRP94 is still less well characterized in these aspects. Here, cell growth assays were performed to observe the effects of GRP94 silencing on cell growth. Our results show that down-regulation of GRP94 did not have a significant effect on Panc-1 pancreatic cancer cell growth.

Gemcitabine, which is a novel necleoside analogue, exerts its action by inhibiting DNA synthesis, has replaced 5-FU based chemotherapy as the preferred choice for first line palliative chemotherapy in patients with

advanced pancreatic cancer. We inspected the effects of GRP94 silencing on gemcitabine treatment of Panc-1 cells. But the median effective dose of gemcitabine on GRP94 siRNA-treated cells had no significant change compared with control.

We also examined the effects of GRP94 on apoptosis induced by treatment with actinomycin D. Actinomycin D has been reported to cause apoptosis in Panc-1 cells in conjunction with JNK/SAPK activation and enhanced expression of Bax [41]. Our data demonstrate that silencing GRP94 increases the cell death induced by actinomycin D in Panc-1 cells. The mechanisms of the antiapoptotic effect of GRP94 are not known. However, previous reports have indicated that GRP94 reduces cell death in SH-SY5Y cells, perturbs calcium homeostasis, and suppresses ischemic neuronal cell death induced by ischemia/reperfusion injury [39, 52]. Moreover, honokiol-induced calpain-II-mediated GRP94 cleavage causes human gastric cancer cell apoptosis [53]. It has also been shown that GRP94, with its calcium-binding and anti-apoptotic properties, is a proteolytic target of calpain during etoposide-induced apoptosis [36].

Since all patients in the study received postoperative chemotherapy, our results show that GRP94 plays only a partial role in resistance to apoptosis in pancreatic cancer, as the tendency toward better survival in

patients with absent/low expression in cancer cells did not reach statistical significance. Similarly, in esophageal cancer the level of expression of GRP94 had no correlation with clinico-pathologic parameters [54]. While one study demonstrated the association of high expression levels of GRP78, GRP94 and HSP60 in esophageal adenocarcinomas with a more aggressive clinical behavior [55]. One possible explanation could be the dual function of GRP94. On the one hand, GRP94 is involved in apoptosis resistance [36, 39, 40]. On the other hand, GRP94 has a significant role in promoting immunity against tumors. GRP94 may bind tumor antigens in the ER [34] which are captured during cell lysis by antigen-presenting cells and cross-primes cytotoxic $CD8^+$ T cells [56, 57]. Alternatively, release of GRP94 from the ER of stressed tumor cells activates the innate immune system through Toll-like receptors on antigen-presenting cells [32]. Therefore, paradoxical to its effect in creating apoptosis resistance, its absence may help tumor cells to escape from immune surveillance [28]. Furthermore, the role of GRP94 in human diseases such as cancer, diabetes and neurodegeneration can be also achieved through the use of the mutant mouse models [58].

Recently, novel therapeutic approaches targeting GRP94 have been studied and in part introduced for cancer treatment [59-61].

6. SUMMARY

In this study, we investigated the expression of GRP94 in the normal pancreas, pancreatic cancer and chronic pancreatitis tissues. We also investigated the role of GRP94 in regulating cell growth and apoptosis in pancreatic cancer cells. In conclusion, our current data show that GRP94 is lost in a significant fraction of pancreatic cancer tissues, and that it might be involved in the apoptosis resistance of pancreatic cancer cells. Further studies are warranted to explore the role of GRP94 in tumor immunity in PDAC.

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8. CURRICULUM VITAE

Family Name:	Pan
First Name:	Zheng
Gender:	Male
Date of Birth:	28 Dec 1977
Place of Birth:	Jiangsu Province, China
Nationality:	Chinese
Marital Status:	Married
Tel:	015237622282/0086-15251879136
E-mail:	panzheng_hd@yahoo.com

Education and Work Experience:

05.2009-	M.D. student, Technischen Universität München
08.2007-04.2009	Research fellow in pancreas research group, Technischen Universität München
08.2004-	The department of general surgery, Zhongda hospital, Southeast University, China
09.2001-07.2004	Master of Surgical Science Medical School, Nanjing University, China
09.1996-07.2001	Bachelor of Medicine Medical School, Jiangsu University, China

Publications:

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