Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;34:1152-1165
DOI: 10.1159/000366328
Published online: September 22, 2014
Accepted: August 13, 2014

1421-9778/14/0344-1152$39.50/0

Original Paper

Expression of the RIP-1 Gene and its Role in Growth and Invasion of Human Gallbladder Carcinoma

Guangwei Zhu[a,b] Xiao Chen[a,b] Xiaoqian Wang[a] Xiujin Li[a] Qiang Du[a,b] Haijie Hong[a,b] Nanhong Tang[a] Feifei She[b] Yanling Chen[a,b]

[a]Department of Hepatobiliary Surgery and Fujian Institute of Hepatobiliary Surgery, Union Hospital, Fujian Medical University, [b]Key Laboratory of Ministry of Education for Gastrointestinal Cancer and Key Laboratory of Tumor Microbiology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, China

Key Words
Gallbladder cancer • Receptor interacting protein-1 • siRNA, growth • Invasion • Xenograft tumor

Abstract
Background and Aim: Receptor interacting protein(RIP)-1 is thought to have a significant role in inflammation signaling pathways; however, the role of RIP-1 in malignant tumors is largely unknown. ***Methods:*** The present study examined the functions and underlying mechanisms of RIP-1 in gallbladder cancer *in vitro* and *in vivo*. In this study we determined the expression and role of RIP-1 in 60 clinical specimens from patients with gallbladder cancer and 3 gallbladder cancer cell lines. Using siRNA targeting RIP-1, plasmid vectors (phU6-EGFP-puro/siRIP-1) were constructed and transfected into the gallbladder cells to characterize the biological effect of RIP-1. ***Results:*** *In vitro* experiments indicated that silencing of RIP-1 in NOZ cells significantly suppressed growth and invasion. Furthermore, silencing of RIP-1 affected the RIP1-NF-κB/c-jun(AP-1)-VEGF-C pathways in NOZ cells. Silencing of RIP-1 *in vivo* inhibited tumor growth in a NOZ cell subcutaneous xenograft model. Immunohistochemstry analysis of the tumor in thesubcutaneous xenograft model also suggested that RIP-1 mediates the expression of VEGF-C. ***Conclusion:*** We have elucidated therelationship between RIP-1 overexpression and the growth and invasion of gallbladder cancer from clinical specimens using a xenograft model. We provide evidence that a reduction in the expression of RIP-1 in gallbladder cancer cells can exert inhibitory effects on the ability of cells to grow and invade *in vitro*. Thus, targeting RIP-1may be useful in the treatment of gallbladder cancer.

Yanling Chen
and Feifei She

Dept Hepatobiliary Surgery and Fujian Institute of Hepatobiliary Surgery, Union Hospital, Fujian Medical University, 29 Xin-Quan Road, Fuzhou, 350001, (China) and Key Lab of Ministry of Education for Gastrointestinal Cancer and Key Laboratory of Tumor Microbiology, Fujian Medical University, 1 Xue-Yuan Road, Fuzhou, 350001 (China)
E-Mail ylchen@medmail.com.cn E-Mail shefeifei@yeah.net

Introduction

Gallbladder cancer is a very rare, highly-lethal disease. Gallbladder cancer is the most common malignant tumor of the extrahepatic biliary tract and the seventh common gastrointestinal carcinoma [1]. Gallbladder cancer is highly invasive and aggressive and carries a dismal prognosis. The 5-year survival rate for all stages of gallbladder cancer is approximately 5% [2, 3]. A total of 10310 cases of gallbladder cancer were diagnosed in 2013 [4]. Although, gallbladder cancer is an uncommon disease, there is a very high incidence of gallbladder cancer in some regions, such as Chile, where gallbladder cancer has the highest mortality rate [5]. Thus, there it is important to study gallbladder cancer in relation to human health. The etiology of gallbladder cancer is unknown, although genetic and environmental factors, infections, and calculi are considered to play important roles. Indeed, most of the risk factors associated with gallbladder carcinoma are related to inflammation [6, 7].

The relationship between inflammation and cancer dates back to ancient time. Rudolf Virchow first advanced the hypothesis that inflammation and cancer co-existed in 1863 [8]. Subsequent research has shown that inflammation promotes the growth and metastasis of cancer [9, 10].

The specific mechanism by which inflammation promotes the progression of malignant tumors has not been elucidated. The death domain-containing kinase receptor interacting protein(RIP-1, RIPK-1) was first described in a yeast two-hybrid screen, hence the designation 'receptor-interacting protein' [11]. RIP-1 plays an important role during cellular stress caused by different factors, such as inflammation and DNA damage. Different cell signals are transmitted by various cellsurface receptors, then the cascade of events are initiated by activated RIP-1. Among these events, the most important response is RIP-1 activation of transcription factors (NF-κB [12-14]and AP-1 [15, 16]), which trigger a gene expression and promotes cell survival and differentiation. Therefore, we concluded that RIP-1 is a crucial regulator of cell survival and is a pivotal component of the inflammation-signaling pathway [12, 17].

The activation of NF-κB in human cancer is very common, and as a result, promotes growth and invasion of cancer cells [18-20]. NF-κB activation is involved in cancer cross-talk inflammation, and the inflammation in turn promotes tumor progress, as evidenced by results in an animal model and cell experiments [21, 22]. In our previous study [23], we demonstrated that activation of NF-κB plays an important role in gallbladder cancer progression.

Thus, RIP-1 may play an important role in the occurrence and development of tumors. Park et al. [24] reported that patients with glioblastomas who had increased expression of RIP-1 had a markedly worse prognosis. Park et al. [24] reported that RIP-1 is overexpressed with an increase in the malignant grade of glioblstoma. A recent study reported that the K562 and HL60 leukemia cell lines with knockdown of RIP-1 were sensitized by induced apoptosis [25]. When RIP-1 was silenced using a selective siRNA in A549 and H460 cells lines, the migration and invasion abilities of these cells lines were reduced [26].

However, the role of RIP-1 mediating the migration and invasion of neoplasm cells is largely unknown. The precise mechanism underlying RIP-1 promotion of cancer migration and invasion is not well understood. Therefore, the purpose of this study was to characterize the role of RIP-1 using gallbladder cancer as an example. We analyzed the biological functions and underlying mechanisms of RIP-1 in human gallbladder cancer cell lines and addressed the role of RIP-1using a xenograft mouse model in the present study.

Materials and Methods

Patient Samples and data collection

The study of human gallbladder cancer tissue was approved by the Ethics Committee of the Affiliated Union Hospital of Fujian Medical University. 60 formalin-fixed and paraffin-embedded gallbladder cancer

samples and 17 samples of normal gallbladder tissues that were located away from the cancer were used as contorls. These samples were provied by Pathology Department and Fujian Institute of Hepatobiliary Surgery of the Affiliated Union Hospital of Fujian Medical University. The patient group samples were conserved during the period from 2000 to 2012, and the patient group was composed of 24 men and 36 women with amedian age of 59 years. All gallbladder cancer patients included in present study had not been given any preoperative chemotherapy or other therapy such as radiotherapy.

Immunohistochemistry and evalution

Serial 4μm slices were obtained from formalin-fixed and paraffin-embedded tissues specimens. After deparaffinization in turpentine and rehydration in alcohol gradient, tissue sections were incubated in 3% hydrogen peroxide(H_2O_2) for 25min at room temperature to block the activity of endogenous peroxidase. Then the tissue sections antigen-retrieved by boiling the sections in a microwave for 12 min in citrate buffer (PH 6.0) and cooled at room temperature for 45min and then followed by a wash step with phosphate buffered saline(PBS). The non-specific antigen of sections were blocked by 20-min in appropriate normal serum that were diluted in PBS. The tissue sections were then incubated in a 1:500 dilution of mouse monoclonal anti-human RIP-1 antibody or a 1:350 dilution of rabbit polyclonal anti-human VEGF-C antibody(in the mice model parts) and placed on the sections overnight in humidified boxes at 4°C. Then the sections were washed with PBS for 3min and 3 times followed by an incubation with an UltraSensitive S-P kit (Maixin-bio, Fuzhou, China) according to the manufacturer's instructions. The sections were visualized with 3,3-diaminobenzidine(DAB) for 4-6min. Counterstaining was carried out with hematoxylin and the sections were dehydrated, then mounted. For a negative control, PBS was substituted the primary antibody. Cells with a deposition of buffy-colored granules in the cytoplasm were scored as RIP-1 or VEGF-C positve. The expression of RIP-1 and VEGF-C were evaluated using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc. USA). Three slices were randomly selected for each group and five fields were selected for each slice. Semiquantitatively analysed the expression of RIP-1 and VEGF-C in each group using Mean Optical Density (MOD)= the Integral Optical Density(IOD)/the positive area, in fifteen fields respectively.

Cell culture

The gallbladder cancer cell line, SGC-996, was provided by the Tumor Cytology Research Unit, Medical College, Tongji University, Shanghai, China. GBC-SD cells were purchased from Shanghai Institutes for Biological Sciences in China. NOZ cells were obtained from the Health Science Research Resources Bank in Japan, which were isolated from ascites derived from a 48-year-old female gallbladder cancer patient [27]. All three cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) included with 10% fetal bovine serum(FBS). All of the cells were incubated in at 37°C in a humid atmosphere of 5% CO_2/95% air.

RNA preparation, reverse transcription, and real-time PCR ampification

Semiquantitative RT-PCR was used to detect the expression of RIP-1 gene in gallbladder cell lines SGC-996, GBC-SD and NOZ respectively. Total RNA was isolated from the cultued cells grown in 6-well plates using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and quantized by U.V.260/280nm to an absorption ratio of >1.8.Total RNA (2μg), according different the concentrations of samples RNA, were reverse transcribed to cDNA in a final volume of 20μl using the AVM First Strand cDNA synthesis kit (Invitrogen) following the manufacture's instructions. And, used these cDNA as the template for PCR. The PCR primers to use for amplification of RIP-1 and VEGF-C were as follows: forward, 5'-GTCCTGGTTTGCTCCTTCCC-3', reverse, 5'-GTCTCCTTTCCTCCTCTCTGTT G-3', and forward, 5'-TGTGTGTCCGTCTACAGATGTG-3', reverse, 5'-TCGGCAGGAAGTGTGATT GG-3' and they yielded 170bp and 165bp products respectively. The PCR primers to use for amplification of β-actin were as follows: forward, 5'-CTGTCTGGCGGCACCACCAT-3' and reverse, 5'-GCAACTAAGTCATAGTCCGC-3', and it yielded a 254bp product. All PCRs were performed with Thermo Scientific SYBR Green qPCR kit on an Applied Biosystems StepOne Real-time PCRSystem. PCR cycling were under the conditions: 95°C for 2 min, 95°C for 15s, and 60 °C for 30s for 40 cycles. All genes transcripts were quantified by the $^{\Delta\Delta}C_t$ method.

Western blot analysis

Western blot analysis was performed as described before [23]. The following primary antibodies were used from the different companies under listing respectively: mouse monoclonal anti-human RIP-

1 antibody(1:1000), rabbit polyclonal anti-human VEGF-C antibody(1:1000), monoclonal rabbit anti-human c-Jun(AP-1)(1:1000), and monoclonal rabbit anti-human p-c-Jun(p-AP-1)(1:1000) (all from Abcam biotechnolgoy), monoclonal mouse anti-human AKT (1:500), monoclonal mouse anti-human p-AKT (1:500), mono-clonal mouse anti-human nuclear factor-κB (NF-κB) (p65)(1:500), monoclonal mouse anti-human p-NF-κB (p-p65) (1:500) (all from Cell Signaling Technology, Danvers, MA, USA), monoclonal mouse anti-human β-actin(1:1500) (from Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Primary antibodies behind figures in brackets stand for the antibody dilution multiple.

RIP-1 siRNA plasmid construction and teansfection

Appropriate siRNA target sequences were seeked in the human RIP-1 sequence (Genebank accession number:NM-003804). DNA template oligonucleotides corresponding to four different siRNA sequences (P-1,P-2,P-3and P-4) were designed, according to the siRNA design guidelines. And the siRNA sequences were listed as follows respectively: P-1: GCACAAATACGAACTTCAA, P-2: GGCCAATTCCAAGTCATAT, P-3: TACAACAGAGAGGAGGAAA, and P-4: TTGTGATAATG- ACTTCCA. A small hairpin RNA(shRNA) of human RIP-1 in a phU6 gene transfer vector and the negative control(NC) sequence encoding a enhanced green fluorescent protein(EGFP) sequence with the Puromycin resistance were constructed by Genechem Co.Ltd. (Shanghai, China). And all the plasmids were verified by DNA sequencing. The NOZ cells were cultured in DMEM medium supplemented with 10%FBS. We transfected the plasmids into the NOZ cells, when the NOZ cells were at approximately 90% confluency. We used Lipofectamine 2000(Invitrogen) to transfect the NOZ cellls according to the manufacturer's instructions. And the transfection efficiency was quantified by counting the percentage of cells that EGFP-positive using a microscope. The culture medium was replaced with a selection medium containing puromycin at concentrations of 2μg/ml(Sigma,St.Louis,MO,USA)72h later. When we obtained the stably transfected cells, the cells were continuously maintained in 1μg/ml of puromycin for later experiments.

Cell proliferation assay

To analyze cell proliferation, the untransfected NOZ, RIP-1(P-1) siRNA-transfected NOZ and NOZ-NC/siRNA-transfected cells were seeded into 96-well plates at dendity of 10^3 cells/well with 100μl of DMEM medium supplemented with 10%FBS. The proliferative activity was determined after 1, 2, 3, 4, and 5 days by the addition of 10μl of sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/ml; Sigma) to each well. The reaction was terminated after 4h of incubation at 37°C through the addition of 100μl of dimethyl sulfoxide (DMSO; Sigma). The optical density(OD)value was obtained by measuring absorbance at a wavelength of 450 nm. Each well test was repeated 6 times.

Flow cytometric analysis.

To determine the apoptosis induced by RIP-1siRNA in NOZ cells. The flow cytometric analysis and the analysis results were carried out as described previously [23].

Cell invasion assay in vitro

Ability of cell invasion was assayed using Transwells (24-well format) with 8μm polycarbonate membranes (BD Biosciences, San Jose, CA, USA). In briefly, the upper side of the membranes was coated with Matrigel matrix (20μg/well) and the membranes were then air-dried for 1h of incubation 37°C. Then the lower side of the membranes was covered with 5μg fibronectin(FN)(BD Biosciences). To test cell invasion induced by RIP-1siRNA, the treated and untreated NOZ cells (2×10^5) in the 200μl of DMEM medium with 2.5% FBS were placed in the upper chamber. The lower chamber was filled with 700μl DMEM medium with 10%FBS as the chemoattractant. After 48h incubation at 37°C and 5%CO_2 , cells on the upper surface of the membrane were wiped off by gentle scrubbing with a cotton swab. Cell had invaded to the lower surface of the membrane were fixed with a stationary liquid of 95% ethanol and 5% acetic acid for 30min and then stained with hematoxylin and eosin(H&E). The number of cells on the lower surface of the membrane was counted in 5 random different fields (×400) using a bright field light microscope. And each assay was repeated in triplicate.

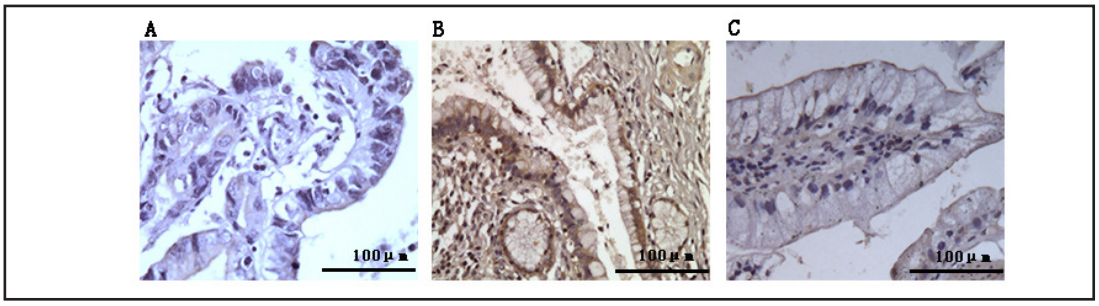


Fig. 1. Different expression of RIP-1 in human gallbladder tissues.(A) PBS replaced the primary antibody of RIP-1 as a negative control in gallbladder cancer(400×).(B) Strong expression of RIP-1in tumor cells from-gallbladder cancer(400×).(C) Mild expression of RIP-1 in normal gallbladder samples located away from the cancer(400×).

Table 1. The immunohistochemistry result of gallbladder cancer samples and normal tissues of the expression of RIP-1. [a]MOD±SD:Mean Optical Density ±Standard Deviation

Patients	N	RIP-1 (MOD±SD)[a] expression	P	
Gallbladder cancer	60	0.3134±0.0802	t=4.746	<0.0001
Normal tissue	17	0.1345±0.1220		

Animal experiments

Male four-to 6-week-old athymic BALB/c nu/nu mice were obtained from the Shanghai SLAC laboratory Animals Co, (Shanghai, China) and kept in the Experimental Center of the Fujian Medical University. All the mice were handled according to the recommendations of the institutional guidelines and were approved by the Ethics Committee of the Medical Faculty of the Fujian Medical University. To build the subcutaneous gallbladder cancer nude mice model, NOZ-blank control group, NOZ-NC/siRNA group and NOZ-P-1/siRNA group were randomly divided into three groups using 15 mice (five mice/group). Three groups of different NOZ cells (10^6) in 100μl serum-free DMEM medium were seeded subcutaneously into the upper flank region of nude mice. Tumor growth was monitored and measured in two dimensions (width and length respectively) every 3days. The size of tumor growth was estimated suing the formula=width2 (mm2) × length(mm)/2(the width and length are the shortest and longest diameters of the subcutaneous tumor). After 5weeks, nude mice were killed and the tumors were collected and weight immediately. Then all samples were fixed in 10%formalin solution for the immunohistochemical analysis.

Statistical analysis

All statistical analysis used GraphPad Prism 5 software. Data were analyzed by the one-way ANOVA or Student's t-test. The data were expressed as the means ±standard deviation(SD). A P-value<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of RIP-1 expression in human gallbladder cancer by immunohistochemistry staining and clinicopathologic factors

We analyzed the expression of RIP-1 in 60 clinical samples from gallbladder cancer patients and 17 normal gallbladder tissues located away from the cancer site using immunohistochemistry technology. In the normal gallbladder tissues,low levels of RIP-1 expression were found in the cytoplasm of gallbladder mucosa cells(Fig. 1C); however, the gallbladder cancer tissues had high levels of expression of RIP-1 in the cytoplasm(Fig.1B). The MOD of RIP-1 in normal gallbladder tissues (0.1345±0.1220) was significantly lower than gallbladder cancer tissues (0.3134±0.0802; P<0.0001; Table1). Thus, RIP-1 overexpression may promote progression of gallbladder cancer.

Table 2 shows the relationship between RIP-1 expression and select clinicopathologic parameters.RIP-1 expression did not vary significantly with age,gender, tumor size, and

Table 2. Relationship between RIP-1 expression and gallbladder cancer clinicopatholigic parameters. [a]MOD±SD: Mean Optical Density ±Standard Deviation. [b]the clinical stage using the Nevin stage.*P<0.05

Fctors	N	RIP-1 Expression (MOD±SD)[a]		P
Age(year)				
< 60	32	0.2329±0.0585	t=0.1028	0.9189
≥60	28	0.2351±0.0569		
Gender				
Male	24	0.3038±0.0719	t=0.8067	0.4266
Female	36	0.3347±0.1230		
Tumor size(cm)				
≤5	48	0.3175±0.0682	t=1.038	0.3083
> 5	12	0.3457±0.0804		
Clinical stage[b]				
I~III	20	0.2267±0.1264	t=2.442	0.0021*
IV~V	40	0.3463±0.1312		
Lymph node metastasis				
Negative	27	0.2143±0.1332	t=2.071	0.047*
Positive	33	0.3185±0.1423		
Histological grade				
Poorly	20	0.2616±0.1167	F=0.2866	0.7522
Moderately	18	0.2899±0.1282		
Well	22	0.2914±0.1194		
Gallstone				
Negative	21	0.2485±0.1366	t=2.176	0.0382*
Positive	39	0.3427±0.0971		

histologic grade; however, there was a marked relationship between RIP-1 overexpression and clinical stage(I~III vs. IV~V; P=0.0021<0.05), lymph node metastasis(negative vs.positive; P=0.047<0.05), and gallstones (negative vs.positive; P=0.0382<0.05).

Expression of RIP-1 mRNA and protein in gallbladder cancer cell lines

To assess the expression of RIP-1 in human gallbladder cancer cell lines (SGC-996, GBS-SD, and NOZ), we used qRT-PCR and Western blot technology. RIP-1 mRNA was detected in all three cell lines by qRT-PCR.In addition, expression of RIP-1protein in all three cell lines was confirmed. The intensity of expression of RIP-1mRNA and protein in NOZ cells was higher when compared to the SGC-996 and GBC-SD cell lines (Fig. 2a). Thus, we used the NOZ cells to further examine the role of RIP-1 in gallbladder cancer.

Expression of RIP-1 mRNA and protein after siRNA transfection

An RNAi-mediated method was used to silence RIP-1 to detect the biological effects of RIP-1 in NOZ cells. The DNA sequencing results verified that RIP-1 siRNA plasmid construction was successful. We used qRT-PCR and Western blot to determinethe level of RIP-1levels 72 h after transfection in NOZ cells. Expression of the siRNA plasmids (NC/P-1/P-2/P-3/P-4/siRNA) enhanced green fluorescent protein(EGFP) 72h after transfection, as shown by fluorescence microscopy, in the NOZ cells(Fig. 2d). qRT-PCR and Western blot revealed a decrease in RIP-1 mRNA and protein levels in siRNA-transfected cells. The P-1/siRNA vector resulted in a higher suppression of the levels of RIP-1 mRNA and protein expression than the other vectors (P-2/siRNA, P-3/siRNA, and P-4/siRNA), while non-transfected and NC/siRNA vectors had no effect on the levels of RIP-1 mRNA and protein expression. Semi-quantitative analysis showed that P-1/siRNA, P-2/siRNA, P-3/siRNA, P-4/siRNA, and NC/siRNA inhibited the levels of RIP-1mRNA and protein expression (Fig. 2b). There were marked differences between P-1/siRNA and non-transfected siRNA groups(P<0.05). Because P-1/siRNA was the most effective siRNA, we selected P-1/siRNA to knockdown the RIP-1 in our further studies.

To generate stably transfected P-1/siRNA and NC/siRNA NOZ cells, the culture medium was replaced with a selective medium containing puromycin at concentrations of 2μg/ml(Sigma, St. Louis, MO, USA)72h later. We obtained stably-transfected cells using puromycin after 2 weeks; these cells were then used for the following cell and animal experiments.

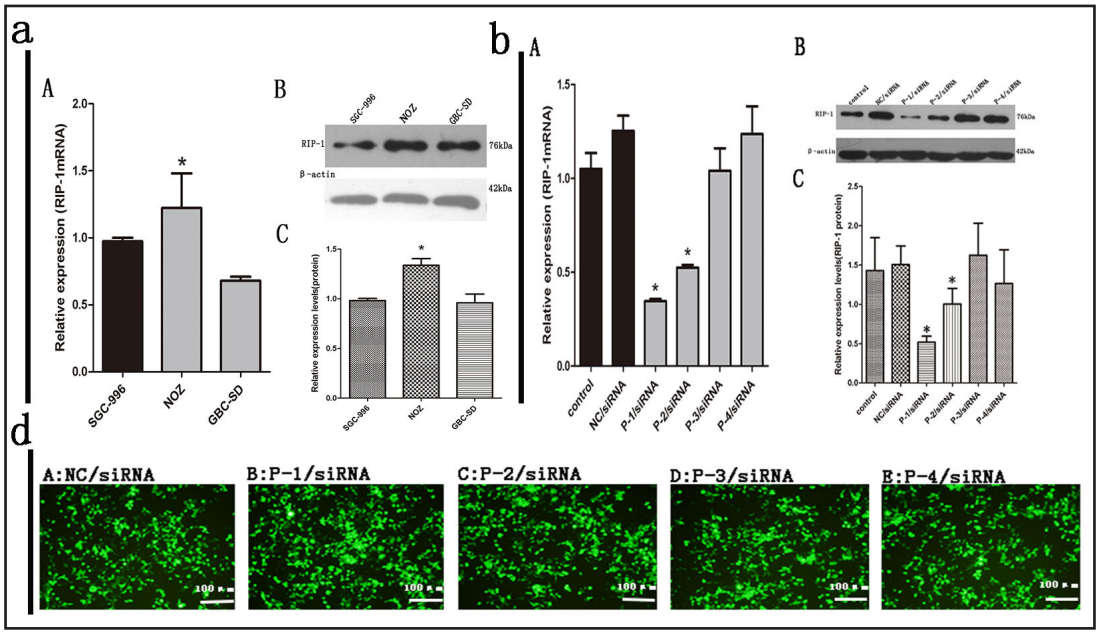
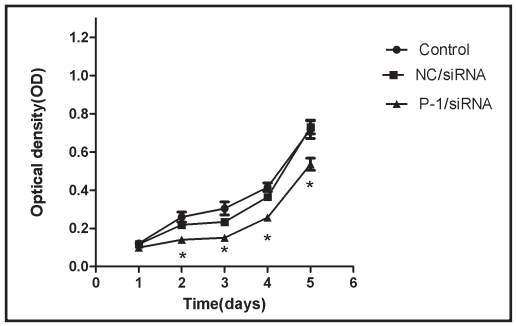


Fig. 2. (a). Expression of RIP-1 in human gallbladder cancer cell lines.(A) Total RNA from human gallbladder cancer cell lines was assessed by qRT-PCR using RIP-1 primers.β-actin was amplified for an internal control. (B) RIP-1 protein in human gallbladder cell lines was detected by Western blotting. β-actin protein was used as an internal control in qRT-PCR. Semi-quantitative analysis showed that RIP-1 protein(C) levels in NOZ cells were markedly higher compared with SGC-996 and GBC-SD cells.The densitometric value for all groups was normalized to the internal control and relative expression with the following equation: normalization of all groups / the first normalized value. Data represent the means±standard(SD). n=3;*P<0.05. (d) RIP-1 siRNA plasmid transfection. (A-E) Expression of the siRNA plasmids (NC/P-1/P-2/P-3/P-4/siRNA) enhanced green fluorescent protein(EGFP) 72h after transfection, as demonstrated by fluorescence microscopy, in NOZ cells. Fluorescent light (200X). NC/siRNA represents the negative control. (b) Effects of siRIP-1 transfection on the expression of RIP-1. The level of RIP-1 mRNA (A) and protein(C) expression was analyzed by qRT-PCR and Westernblot after transfection with siRNA in NOZ cells. β-actin was used as an internal control. The densitometric value for all groups wasthe same as (a). Semi-quantitative analysis showed that the level of RIP-1 protein (B) was significantly inhibited by P-1/siRNA in NOZ cells.

Fig. 3. Effects of siRIP-1 on the viability of non-transfected NOZ(control), NOZ NC/siRNA-transfected, and NOZ P-1/siRNA-transfected cells. The proliferation ability of NOZ cells was inhibited by P-1/siRNA(*P<0.05). Control, NOZ cells non-transfected with siRNA; NC/siRNA, NOZ cells transfected with negative control; P-1/siRNA, NOZ cells transfected with P-1/siRNA plasmids.



Knockdown of RIP-1 decreases the proliferation of NOZ cells in vitro

To determine whether or not RIP-1 promotes NOZ cell proliferation, we first treated human NOZ cells with siRNA directed against RIP-1. We transfected the NOZ cells with P-1/siRNA to induce the down-regulation of RIP-1 gene expression with NC/siRNA, and mock-transfected groups were used as controls. We tested cell proliferation daily (days 1-5) using the MTT assay. Cell proliferation in the P-1/siRNA groups was slower(P<0.05) when compared to the NC/siRNA and non-transfected groups (Fig. 3). The data from the *in*

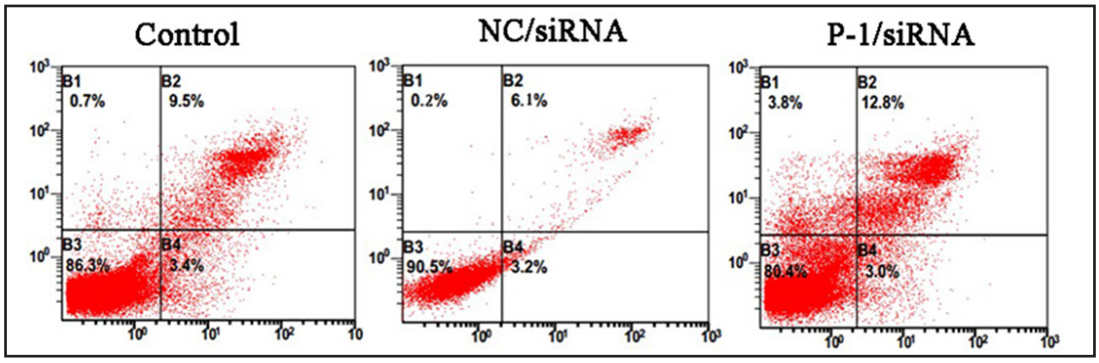


Fig. 4. Effects of siRIP-1 on apoptosis of NOZ cells. Non-transfected NOZ cells (control), NC/siRNA NOZ cells (NC/siRNA), and P-1/siRNA NOZ cells(P-1/siRNA) as assessed by flow cytometry (FCM). Control cells, NC/siRNA cells, and P-1/siRNA cells exhibited a similar rate of apoptosis.

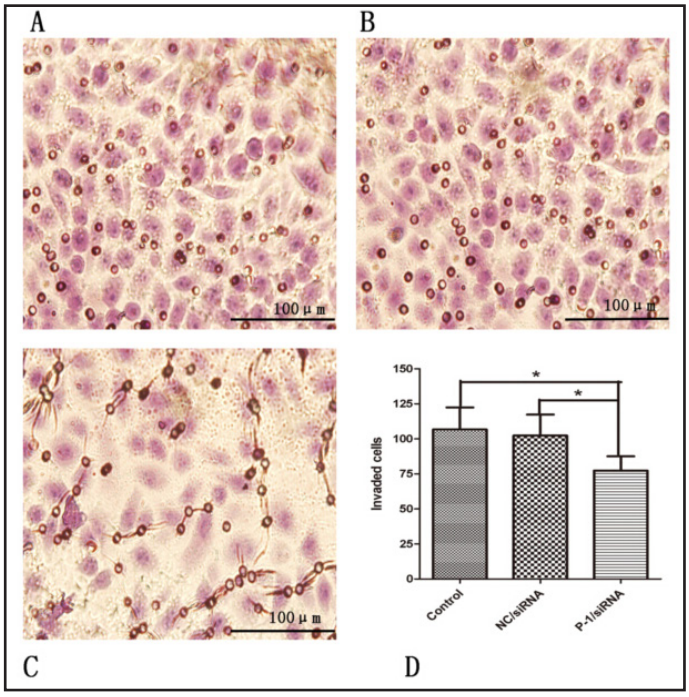


Fig. 5. Effects of siRIP-1 on NOZ cell invasion. (A) Invasion ability of non-transfected NOZ cells(control). (B) Invasion ability of NC/siRNA NOZ cells(NC/siRNA). (C)Invasion ability of P-1/siRNA NOZ cells(P-1/siRNA). (D) Number of invaded cells in all three cell groups (*P<0.05).

vitro cell proliferation assay indicated that the growth of cells in the P-1/siRNA group was significantly reduced compared with the NC/siRNA group, which was the same as the growth in the non-transfected group. The data support the role of RIP-1 in affecting the proliferation of NOZ cells.

RIP-1 knockdown does not affect apoptosis of NOZ cells

To detect whether or not the RIP-1 gene silencing-induced effects on cell viability were due to apoptosis, we used flow cytometry(FCM) after cells were stained with Annexin V-PE/7-aminoactinomycin D. We transfected the NOZ cells with P-1/siRNA to induce the down-regulation of RIP-1gene expression with NC/siRNA, and mock-transfected groups were used as controls. The control cells, NC/siRNA cells, and P-1/siRNA cells exhibited a similar rate of apoptosis (Fig. 4).

Knockdown of RIP-1 influences gallbladder cancer cell invasiveness

To determine whether or not the invasiveness of NOZ cells depends on RIP-1overexpression, we used the Transwell assay to test the effects of RIP-1 gene knockdown in NOZ cells. After staining with H&E, 5 different fields(×400magnification) were counted

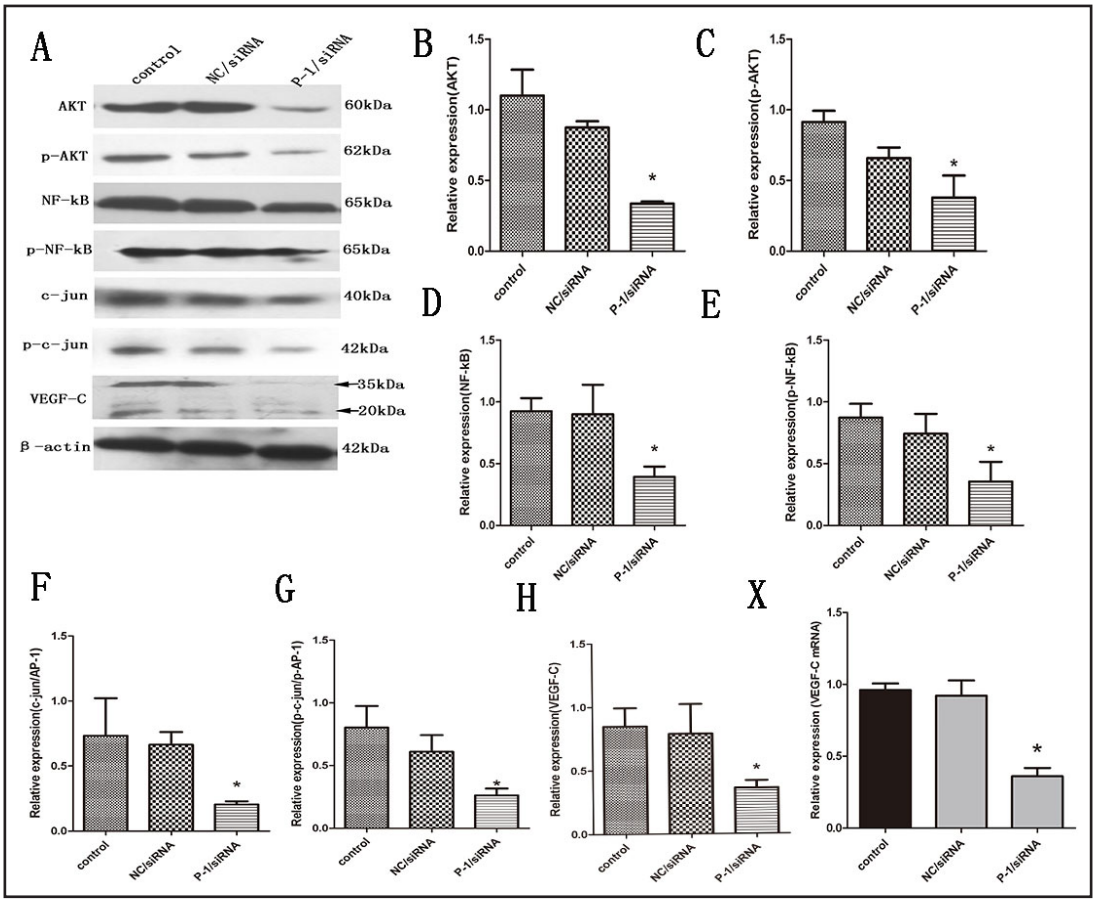


Fig. 6. Western blot analysis suggested that the silencing of RIP-1markedly inhibited the expression of AKT, p-AKT, NF-κB(p65), p-NF-κB(p-p65), c-jun(AP-1), p-c-jun(p-AP-1), and VEGF-C protein, and qRT-PCR analyzed the level of VEGF-C mRNA in NOZ P-1/siRNA cells. β-actin protein was used as an internal control. The densitometric value for all groups was normalized to the internal control and the relative level of expression with the following equation: normalization of all groups/ the first normalized value. Data represent the mean±standard(SD). n=3;*P<0.05.

to examine the numbers of invaded cells. The total number of cells in the P-1/siRNA group invaded through the Transwell polycarbonate filter was marked lower than the cells in the non-transfected NOZ group, which was similar to the number of cells in the NC/siRNA group (Fig. 5). These data suggest that RIP-1 overexpression plays an key role in the invasion of NOZ cells.

RIP-1 knockdown decreases the activity of NF-κB(p65) and C-jun(AP-1)and reduces VEGF-C gene expression

To investigate the mechanisms of RIP-1silencing responsible for the decrease in proliferation and invasion, we assessed the changes in levels of AKT, p-AKT, NF-κB(p65), p-NF-κB(p-p65), c-jun(AP-1), p-c-jun(p-AP-1), and VEGF-C protein and VEGF-C mRNA; these factors are vital to the survival of cancer cells. Western blot analysis showed that the AKT, AKT, p-AKT, NF-κB(p65), p-NF-κB(p-p65), c-jun(AP-1), p-c-jun(p-AP-1), and VEGF-C protein levels in the P-1/siRNA cells decreased when compared with the non-transfected and NC/siRNA cells (Fig. 6). The changes in VEGF-CmRNA expression in the three groups were the same as the levels of VEGF-C protein (Fig. 6X).

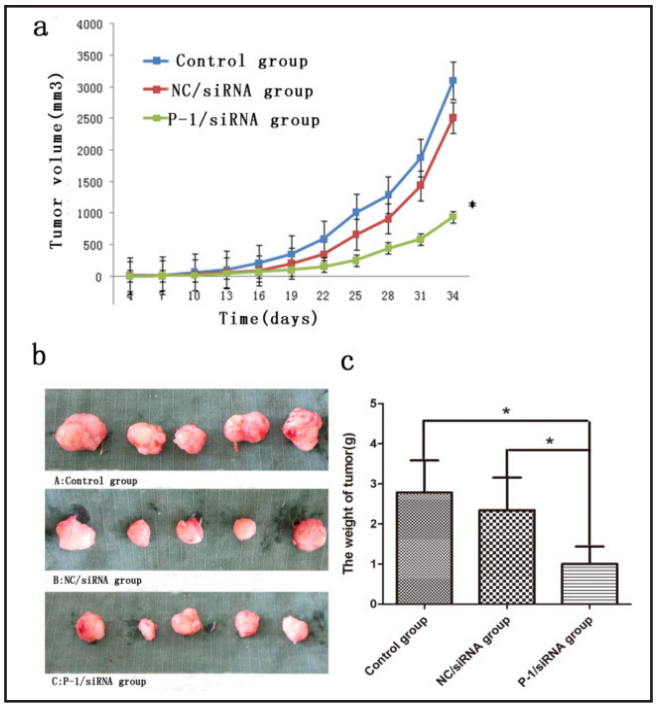


Fig. 7. Suppression of tumor growth by silencing the RIP-1 gene in NOZ cell subcutaneous xenografts. Three groups (control group, NC/siRNA group, and P-1/siRNA group) of NOZ cells were subcutaneously xenografted onto nude mice. (a) The size of primary tumors was measured every 3days. (b) Mice were killed after 5 weeks. (c) The weights of tumors were weighed. Significantly different from P-1/siRNA group(*P<0.05).

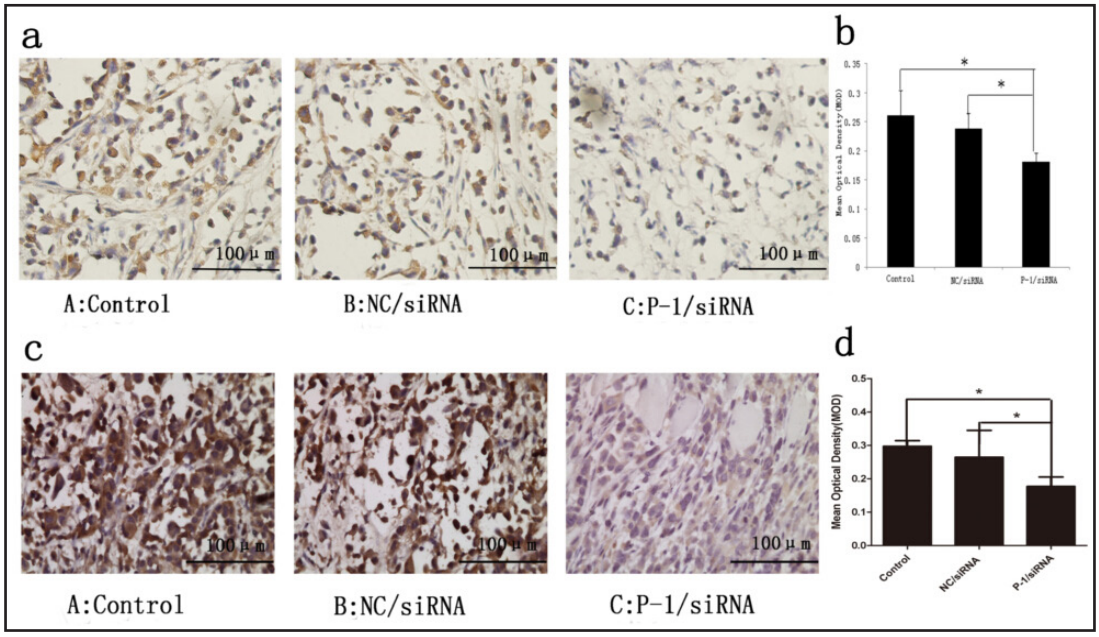


Fig. 8. Immunohistochemstry analysis of the expression of RIP-1 and VEGF-C in NOZ cell subcutaneous xenograft tumors. (a,c) showed the expression of RIP-1(400×) and VEGF-C (400×) in all three groups (control group, NC/siRNA group, and P-1/siRNA group). (b,d) shows quantitative evaluation of the expression of RIP-1 and VEGF-C, respectively. Bars represent the mean optical density(MOD)±standard deviation (SD). Markedly different from P-1/siRNA group (*P<0.05).

RIP-1 promotes tumor growth in nude mice with subcutaneous xenograft tumors

To assess the influence of RIP-1 expression on gallbladder cancer biology *in vivo*, we used NOZ cells which we obtained from the stably-transfected P-1/siRNA and NC/siRNA vectors and non-transfected NOZ cells to build three groups in the subcutaneous xenograft tumor nude mice model (5 mice /group). Fifteen nude mice were all successfully established in the subcutaneous model. None of the mice had ascites or metastases to the liver, lungs, and

lymph nodes at the time of autopsy. At the end of the 5-week experimental period, the growth of subcutaneous xenograft tumor sizes was as follows (mean±SD): 944.6±510.2mm^3 in the P-1/siRNA group; 3100.9±1035.3mm^3 in the non-transfected group; and 2513.3±959.1mm^3 in the NC/siRNA group. There were significant differences between the P-1/siRNA and NC/siRNA groups, which was similar to the non-transfected group. We found that xenograft growth was markedly slower from the second week until the day on which the mice were sacrificed (Fig. 7a). Scales were used immediately to weigh the samples after the animals were sacrificed.Compared with the non-transfected and NC/siRNA groups, the weights of the tumor samples were significantly less in the P-1/siRNA group(Fig. 7b,c).

Because VEGF-C expression was decreased after P-1/siRNA transfection in NOZ cells, we used immunohistochemstry technology to analyze the expression of RIP-1(Fig. 8a,b) and VEGF-C (Fig.8c,d)in the three nude mice groups. Compared with the non-transfected and NC/siRNA groups, the levels of RIP-1 and VEGF-C expression were markedly reduced in the P-1/siRNA group.

Discussion

RIP-1 is a crucial molecule for NF-κB and MAPKs-c-jun (AP-1) signaling [12-16, 28]. RIP-1 consists of a N-terminal kinase domain, an intermediate domain, a RIP homotypic interaction motif, and a C-terminal death domain motif [28]. RIP-1 is a kinase with important roles in inflammation, cell survival, and cell apoptosis [28, 29]. Inflammation can induce the expression of RIP-1 in T cells [11, 30]. Thus, the complicated functions of RIP-1render cells at the crossroads of life or death.

Many details pertaining to the role of RIP-1 in cancer cells are unknown. Some recent reports address the functions of RIP-1 in cancers, such as glioblastomas [24], leukemia [25], and lung cancer [26]. Because the specific interactions between RIP-1 and cancers are largely unclear, we next explored the biological functions and underlying mechanisms *in vitro* and *in vivo* using gallbladder cancer as a representative.

The current study is the first study to demonstrate the role of RIP-1 in promoting growth and invasion of human gallbladder carcinoma. In the present studywe used immunohistochemical techniques to investigate the expression of RIP-1 in 60 gallbladder cancer specimens and 17 samples of normal gallbladder tissues which were located away from the cancer site. One major finding in the current study was that RIP-1 is overexpressed in gallbladder cancer tissues, and RIP-1 has low expression in normal tissues. Increased expression of RIP-1 is common in gallbladder cancer tissues. The overexpression of RIP-1 in gallbladder tissues suggests that RIP-1 may be an important molecule and be of clinical significance in forecasting prognosis. A prior study reported that RIP-1 is an independent prognostic factor in glioblastomas [24]. The correlation with clinicopathologic factors was also analyzed. The expression of RIP-1 was significantly correlated with the clinical stage of gallbladder carcinoma patients, which suggests that RIP-1 may promote tumor growth and invasion. We also found that RIP-1 expression is markedly increased in gallbladder cancer patients with gallstones. Of note, inflammation and a number of studies have reported that gallstones and gallbladder cancer have a direct relationship [31-33]. Gallstones induces inflammation in the gallbladder, which can increase the expression of RIP-1 [30]; our research team also confirmed this finding in gallbladder cancer cells using TNF-α, which is an important inflammation factor that stimulates NOZ cells (data not shown). Interestingly, we found a significant difference in the expression of RIP-1 between gallbladder cancer patients with and without lymph node metastasis. For this reason, we speculated that high expression of RIP-1 may promote lymph node metastasis. In addition, our research team also showed that VEGF-C /D are very important factors which can promote lymph node metastasis and lymphangiogenesis in gallbladder cancer [34, 35]. Thus, we proposed the following question: can RIP-1 regulate the expression of VEGF-C?

In the present study we also analyzed the expression of RIP-1 in three gallbladder cancer cell lines(SGC-996, NOZ, and GBC-SD). Similar to the detection of RIP-1 in most gallbladder

cancer specimens, the levels of RIP-1 mRNA and protein were detectedin all three cell lines. This found finding further confirmed the importance of RIP-1 in the growth and invasion of gallbladder cancer. The levels of RIP-1 mRNA and protein expression in NOZ cells were higher compared to SGC-996 cells, which were similar to GBC-SD cells. Our previous research showed that the levels of VEGF-C/D expression were highest in all three cell lines [34, 35], thus indicating our hypothesis that RIP-1 could regulate the expression of VEGF-C was feasible.

In recent years, RNAi technology has served as the most important and widely used technique for gene silencing, and provides a new method to investigate gene expression and function [36]. In addition, siRNA technology has the properties of specificity, stability, and efficiency in knockdown of target gene [37]. Therefore, we used siRNA to assess the effect of inhibiting the RIP-1 gene in gallbladder cancer in cell and animal experiments. In the present study, we successfully selected P-1/siRNA as the most effective siRNA to silence RIP-1 expression in the NOZ cell line. The levels of RIP-1 mRNA and protein expression were suppressed by P-1/siRNA. We obtained stably-transfected cells using puromycin after 2 weeks, and we used these cells for our animal experiments, which showed that silencing the RIP-1 by P-1/siRNA inhibited the growth and invasion of NOZ cells *in vitro*. Our results were in agreement with a prior study, in which Azijli et al. [26] reportedt hat RIP-1 was silenced using a selective siRNA in A549 and H460 cells lines and clearly showed decreased migration and invasion of these cell lines. We found that RIP-1 gene silencing did not affect NOZ cell apoptosis. Indeed, the results indicate that RIP-1 has an important influence in the biological behaviors of gallbladder cancer cells.

To investigate the mechanisms underlying RIP-1 promotion of growth and invasion of NOZ cells, we used the Western blot method to assess the changes in the expression of AKT, p-AKT, NF-κB(p65), p-NF-κB(p-p65), c-jun(AP-1), p-c-jun(p-AP-1), VEGF-C protein and mRNA. These proteins are vital to the survival of gallbladder cancer cells. These proteins are also very important in the inflammation signaling pathways, in which RIP-1 can activate the activity of AKT, p-AKT, NF-κB(p65), p-NF-κB(p-p65), c-jun(AP-1), p-c-jun(p-AP-1), and VEGF-C [12-16, 28, 38]. In addition, our research team previously reported that inflammation factors could up-regulate the expression of VEGF-C in NOZ cells (manuscript in preparation), which is important in gallbladder cancer cell proliferation and invasion [34]. In present study, the Western blot analysis results indicated that the levels of AKT, p-AKT, NF-κB(p65), p-NF-κB(p-p65), c-jun(AP-1), p-c-jun(p-AP-1), and VEGF-C protein and mRNA levels were decreased in P-1/siRNA NOZ cells compared with non-transfected and NC/siRNA NOZ cells. These results suggest that RIP-1 may up-regulate the expression of VEGF-C through the RIP-1-NF-κB/AP-1signaling pathway.

In our *in vivo* model of gallbladder cancer, we observed that silencing the expression of RIP-1 by siRNA suppressed the growth of subcutaneous xenograft tumors, which was consistent with our *in vitro* study. In our murine model of gallbladder cancer, however, we did not find liver, lung, and lymph node metastases, and ascites had not developed. This result may explain why the subcutaneous xenograft gallbladder cancer model can not provide a suitable microenvironment for tumors [39]. Our laboratory also confirmed the view that orthotopic xenograft models of gallbladder cancer are better than subcutaneous xenograft gallbladder cancer models in studying the biological characteristics of gallbladder cancer [40].Therefore, we will establish an orthotopic xenograft modelof gallbladder cancer to further study RIP-1 and its influence on the biological behavior of gallbladder cancer.

For the first time, we have verified the relationship between RIP-1 overexpression and the growth and invasion of gallbladder cancer from clinical specimens using a murine model. We provided evidence that a reduction in RIP-1 expression in gallbladder cancer cells can exert inhibitory effects on the ability of the cells to grow and invade *in vitro*. Together, our findings provide evidence that targeting RIP-1may prove to be useful in the treatment of gallbladder cancer.

Disclosure statement

The authors declare that they have no potential conflict of interest.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (no.81272373), the Key Project of Science and Technology Research Program in Fujian Province (no.2009Y0024) and the Key Project of Science Research in Fujian Medical University (no.09ZD017), and the National Clinical Key Specialty Construction Project (General Surgery) of China.

Reference

1 Donohue JH, Stewart AK, Menck HR: The National Cancer Data Base report on carcinoma of the gallbladder, 1989-1995. Cancer 1998;83:2618-2628.
2 Bartlett DL, Fong Y, Fortner JG, Brennan MF, Blumgart LH: Long-term results after resection for gallbladder cancer. Implications for staging and management. Ann Surg 1996;224:639-646.
3 Cubertafond P, Gainant A, Cucchiaro G: Surgical treatment of 724 carcinomas of the gallbladder. Results of the French Surgical Association Survey. Ann Surg 1994;219:275-280.
4 Siegel R, Naishadham D, Jemal A: Cancer statistics, 2013. CA Cancer J Clin 2013;63:11-30.
5 Randi G, Malvezzi M, Levi F, Ferlay J, Negri E, Franceschi S, La Vecchia C: Epidemiology of biliary tract cancers: an update. Ann Oncol 2009;20:146-159.
6 Wernberg JA, Lucarelli DD: Gallbladder cancer. Surg Clin North Am 2014;94:343-360.
7 Albores-Saavedra J, Alcantra-Vazquez A, Cruz-Ortiz H, Herrera-Goepfert R: The precursor lesions of invasive gallbladder carcinoma. Hyperplasia, atypical hyperplasia and carcinoma in situ. Cancer 1980;45:919-927.
8 Mantovani A, Allavena P, Sica A, Balkwill F: Cancer-related inflammation. Nature 2008;454:436-444.
9 Bryant T: Remarks on Some Cases of Inflammation of the Breast Simulating Cancer. Br Med J 1868;2:608-609.
10 Coussens LM, Werb Z: Inflammation and cancer. Nature 2002;420:860-867.
11 Stanger BZ, Leder P, Lee TH, Kim E, Seed B: RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 1995;81:513-523.
12 Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV: TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity 1996;4:387-396.
13 Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P: The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 1998;8:297-303.
14 Hur GM, Lewis J, Yang Q, Lin Y, Nakano H, Nedospasov S, Liu ZG: The death domain kinase RIP has an essential role in DNA damage-induced NF-kappa B activation. Genes Dev 2003;17:873-882.
15 Devin A, Lin Y, Liu ZG: The role of the death-domain kinase RIP in tumour-necrosis-factor-induced activation of mitogen-activated protein kinases. EMBO Rep 2003;4:623-627.
16 Liu ZG, Hsu H, Goeddel DV, Karin M: Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kappaB activation prevents cell death. Cell 1996;87:565-576.
17 Bradley JR, Pober JS: Tumor necrosis factor receptor-associated factors (TRAFs). Oncogene 2001;20:6482-6491.
18 Karin M, Cao Y, Greten FR, Li ZW: NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2002;2:301-310.
19 Pacifico F, Leonardi A: NF-kappaB in solid tumors. Biochem Pharmacol 2006;72:1142-1152.
20 Wang CY, Cusack JC, Jr., Liu R, Baldwin AS, Jr.: Control of inducible chemoresistance: enhanced anti-tumor therapy through increased apoptosis by inhibition of NF-kappaB. Nat Med 1999;5:412-417.
21 Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M: IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004;118:285-296.

22 Karin M, Greten FR: NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 2005;5:749-759.
23 Zhu G, Du Q, Wang X, Tang N, She F, Chen Y: TNF-alpha promotes gallbladder cancer cell growth and invasion through autocrine mechanisms. Int J Mol Med 2014;33:1431-1440.
24 Park S, Hatanpaa KJ, Xie Y, Mickey BE, Madden CJ, Raisanen JM, Ramnarain DB, Xiao G, Saha D, Boothman DA, Zhao D, Bachoo RM, Pieper RO, Habib AA: The receptor interacting protein 1 inhibits p53 induction through NF-kappaB activation and confers a worse prognosis in glioblastoma. Cancer Res 2009;69:2809-2816.
25 Han W, Xie J, Fang Y, Wang Z, Pan H: Nec-1 Enhances Shikonin-Induced Apoptosis in Leukemia Cells by Inhibition of RIP-1 and ERK1/2. Int J Mol Sci 2012;13:7212-7225.
26 Azijli K, Yuvaraj S, Peppelenbosch MP, Wurdinger T, Dekker H, Joore J, van Dijk E, Quax WJ, Peters GJ, de Jong S, Kruyt FA: Kinome profiling of non-canonical TRAIL signaling reveals RIP1-Src-STAT3-dependent invasion in resistant non-small cell lung cancer cells. J Cell Sci 2012;125:4651-4661.
27 Homma S, Hasumura S, Nagamori S, Kameda H: [Establishment and characterization of a human gall bladder carcinoma cell line NOZ]. Hum Cell 1988;1:95-97.
28 Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P: RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell Death Differ 2007;14:400-410.
29 Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Bazan NG, Blagosklonny MV, Blomgren K, Borner C, Bredesen DE, Brenner C, Castedo M, Cidlowski JA, Ciechanover A, Cohen GM, De Laurenzi V, De Maria R, Deshmukh M, Dynlacht BD, El-Deiry WS, Flavell RA, Fulda S, Garrido C, Golstein P, Gougeon ML, Green DR, Gronemeyer H, Hajnoczky G, Hardwick JM, Hengartner MO, Ichijo H, Jaattela M, Kepp O, Kimchi A, Klionsky DJ, Knight RA, Kornbluth S, Kumar S, Levine B, Lipton SA, Lugli E, Madeo F, Malomi W, Marine JC, Martin SJ, Medema JP, Mehlen P, Melino G, Moll UM, Morselli E, Nagata S, Nicholson DW, Nicotera P, Nunez G, Oren M, Penninger J, Pervaiz S, Peter ME, Piacentini M, Prehn JH, Puthalakath H, Rabinovich GA, Rizzuto R, Rodrigues CM, Rubinsztein DC, Rudel T, Scorrano L, Simon HU, Steller H, Tschopp J, Tsujimoto Y, Vandenabeele P, Vitale I, Vousden KH, Youle RJ, Yuan J, Zhivotovsky B, Kroemer G: Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ 2009;16:1093-1107.
30 Lee TH, Shank J, Cusson N, Kelliher MA: The kinase activity of Rip1 is not required for tumor necrosis factor-alpha-induced IkappaB kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. J Biol Chem 2004;279:33185-33191.
31 Miquel JF, Covarrubias C, Villaroel L, Mingrone G, Greco AV, Puglielli L, Carvallo P, Marshall G, Del Pino G, Nervi F: Genetic epidemiology of cholesterol cholelithiasis among Chilean Hispanics, Amerindians, and Maoris. Gastroenterology 1998;115:937-946.
32 Andia ME, Hsing AW, Andreotti G, Ferreccio C: Geographic variation of gallbladder cancer mortality and risk factors in Chile: a population-based ecologic study. Int J Cancer 2008;123:1411-1416.
33 Diehl AK: Gallstone size and the risk of gallbladder cancer. JAMA 1983;250:2323-2326.
34 Chen Y, Jiang L, She F, Tang N, Wang X, Li X, Han S, Zhu J: Vascular endothelial growth factor-C promotes the growth and invasion of gallbladder cancer via an autocrine mechanism. Mol Cell Biochem 2010;345:77-89.
35 Lin W, Jiang L, Chen Y, She F, Han S, Zhu J, Zhou L, Tang N, Wang X, Li X: Vascular endothelial growth factor-D promotes growth, lymphangiogenesis and lymphatic metastasis in gallbladder cancer. Cancer Lett 2012;314:127-136.
36 Bantounas I, Phylactou LA, Uney JB: RNA interference and the use of small interfering RNA to study gene function in mammalian systems. J Mol Endocrinol 2004;33:545-557.
37 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494-498.
38 Cha HS, Bae EK, Koh JH, Chai JY, Jeon CH, Ahn KS, Kim J, Koh EM: Tumor necrosis factor-alpha induces vascular endothelial growth factor-C expression in rheumatoid synoviocytes. J Rheumatol 2007;34:16-19.
39 Horiuchi H, Kawamata H, Fujimori T, Kuroda Y: A MEK inhibitor (U0126) prolongs survival in nude mice bearing human gallbladder cancer cells with K-ras mutation: analysis in a novel orthotopic inoculation model. Int J Oncol 2003;23:957-963.
40 Du Q, Jiang L, Wang XQ, Pan W, She FF, Chen YL: Establishment of and Comparison between Orthotopic Xenograft and Subcutaneous Xenograft Models of Gallbladder Carcinoma. Asian Pac J Cancer Prev 2014;15:3747-3752.