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Original Article

Comparison of the gene expression profiles between gallstones and gallbladder polyps

Quanfu Li[1], Xin Ge[2], Xu Xu[1], Yonggang Zhong[1], Zengwang Qie[1]

[1]Department of Hepatobiliary Surgery, The Second Hospital of Baoding, Baoding 071051, China; [2]National Hepatobiliary and Enteric Surgery, Central South University, Changsha 410008, China

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Abstract: Background: Gallstones and gallbladder polyps (GPs) are two major types of gallbladder diseases that share multiple common symptoms. However, their pathological mechanism remains largely unknown. The aim of our study is to identify gallstones and GPs related-genes and gain an insight into the underlying genetic basis of these diseases. Methods: We enrolled 7 patients with gallstones and 2 patients with GP for RNA-Seq and we conducted functional enrichment analysis and protein-protein interaction (PPI) networks analysis for identified differentially expressed genes (DEGs). Results: RNA-Seq produced 41.7 million in gallstones and 32.1 million pairs in GPs. A total of 147 DEGs was identified between gallstones and GPs. We found GO terms for molecular functions significantly enriched in antigen binding (GO:0003823, *P*=5.9E-11), while for biological processes, the enriched GO terms were immune response (GO:0006955, *P*=2.6E-15), and for cellular component, the enriched GO terms were extracellular region (GO:0005576, *P*=2.7E-15). To further evaluate the biological significance for the DEGs, we also performed the KEGG pathway enrichment analysis. The most significant pathway in our KEGG analysis was Cytokine-cytokine receptor interaction (*P*=7.5E-06). PPI network analysis indicated that the significant hub proteins containing S100A9 (S100 calcium binding protein A9, Degree=94) and CR2 (complement component receptor 2, Degree=8). Conclusion: This present study suggests some promising genes and may provide a clue to the role of these genes playing in the development of gallstones and GPs.

Keywords: Gallstones, gallbladder polyps, RNA-Seq, differentially expressed genes

Introduction

Diseases of the gallbladder commonly manifest as gallstones, gallbladder polyps and gallbladder cancer. Gallstones are common with incidence ranging from 10% to 20% of the world population, which increases with age and is higher among women than men [1-3]. It is well accepted that gallstones are associated with gallbladder carcinoma [4-6]. The pathogenic mechanism whereby gallstones are related to gallbladder carcinoma remains unclear.

Gallbladder polyps (GPs) are common gallbladder lesions and require no treatment unless they are symptomatic. In patients with age ≥ 50 the presence of polyps larger than 10 mm has been reported as a risk factor for malignancy [7, 8], so GP should be attached importance because of their association with malignancy. Based on the common association with gallbladder carcinoma, gallstones and GPs were supposed to be share some aberrantly expressed genes or pathways involved the pathophysiological processes in these diseases.

Determining global levels of global genomic expressions may be particularly important for understanding the pathological basis of diseases. Recently, next-generation sequencing technology, RNA-Seq, provides a powerful way over conditional microarrays to measure global genomic expressions with high resolution and low cost [9, 10]. In this paper, gene expression analysis was performed by RNA-seq to compare gene expression profiling of gallstones to that of GPs to identify DEGs and other biological functions that contribute to the development of gallstones and GPs.

Materials and methods

Patients and tissues

This study used tissue specimens obtained from the Second Hospital of Baoding with

informed consent from patients, which included 7 samples from gallstones and 2 samples from GPs who underwent surgery. The tissue specimens were grossly dissected and preserved in liquid nitrogen immediately after surgery. All protocols were approved by the Local Ethics Committee.

RNA isolation and sequencing

Total RNA was isolated with the TRIzol according to the manufacturer's instructions, and its quality and quantity were verified spectrophotometrically (NanoDrop 1000 spectrometer; Thermo Scientific, Wilmington, DE, USA) and electrophoretically (Bioanalyzer 2100; Agilent Technologies, Palo Alto, CA, USA). To construct Illumina sequencing libraries, a TruSeq RNA library preparation kit (Illumina, San Diego, CA, USA) was utilized. Briefly, messenger RNA (mRNA) purified from total RNA using polyA selection was chemically fragmented and further converted into double-stranded (ds) cDNA after subjecting to complementary DNA (cDNA). Short ds-cDNA products were joined with sequencing adapters, and proper fragments were separated by agarose gel electrophoresis. TruSeq RNA libraries constructed by PCR amplification were quantified by using quantitative PCR (qPCR), then their quality was assessed electrophoretically (Bioanalyzer 2100; Agilent Technologies). A HiSeqTM 2000 platform (Illumina) was used to perform sequencing.

RNA-seq reads mapping

The cleaned sequencing reads were aligned to the UCSC human reference genome (build hg19) with TopHat v1.3.1 [11], which initially removes partial reads according to quality information accompanying each read. The prebuilt human UCSC hg19 index was downloaded from the TopHat homepage and used as the reference genome. Multiple alignments per read (up to 20 by default) and a maximum of two mismatches were allowable when mapping the reads to the reference genome. The default parameters were used for the TopHat method.

Transcript abundance estimation

The Cufflinks v1.0.3 [12] was used to process the original alignment file produced by TopHat, and Fragments per Kilobase of exon per Million fragments mapped (FPKM) was used to measure the normalized expression level of each gene. Confidence intervals for FPKM estimates were calculated by using a Bayesian inference method [13]. The reference gene transfer format (GTF) annotation file for Cufflinks was downloaded from the Ensembl database (Homo_sapiens.GRCh37.63.gtf). The downloaded GTF file was processed by Cuffdiff along with the original alignment files produced by TopHat. Cuffdiff calculated the abundance of the transcripts listed in the GTF file using alignments produced by TopHat and tested concurrently for differential expression. "q value" less than 0.01 and test status marked as "OK" in the Cuffidff output were selected as the criteria for significant differences.

Functional enrichment analysis of differentially expressed genes

We performed gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to investigate the biological significance of those differentially expressed genes by Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 which is a set of web-based functional annotation tools [4], and the cut-off of the false discovery rate (FDR) was set at 5%.

PPI network construction

The protein-protein interactions (PPIs) research could reveal the functions of proteins at the molecular level and uncover the rules of cell activities including growth, development, metabolism, differentiation and apoptosis [14]. The identification of protein interaction in a genome-wide scale is very important to interpret the cellular control mechanisms [15]. In this analysis, we constructed PPI network by using Biological General Repository for Interaction Datasets (BioGRID) (http://thebiogrid.org/) and visualized the distribution characteristics of the top 20 significantly up- and down-regulated DEGs in gallstones in the network with Cytoscape [16]. Nodes represent proteins; edges represent interactions between two proteins. The higher the node shape, the greater degree of connection.

Results

Characterization of sequencing and mapping

The gallstones and GPs simples were subjected to massively parallel paired-end cDNA sequencing. Totally we obtained 44.6 and 34.1 million read pairs respectively from gallstones and

Table 1. Statistics of transcriptome data mapping to human genome hg19

	Gallstones	GPs
Total reads	44653908 (100.00%)	34,048,012 (100.00%)
Uniquely Mapped Single Reads	2,733,711 (6.12%)	1,906,579 (5.60%)
Uniquely Mapped Paired Reads	38,949,598 (87.23%)	30,151,946 (88.56%)
Total Uniquely Mapped Reads	41,683,309 (93.35%)	32,058,525 (94.16%)

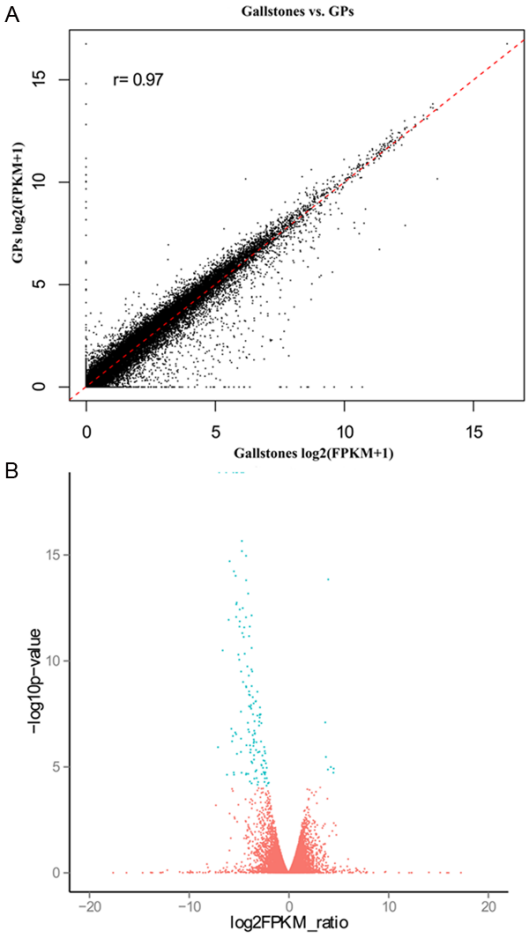

Figure 1. Differential expression analysis of gallstones and GPs. A. The scatter plot for global expression between samples; the Pearson correlation coefficient is shown; B. Volcano plots for all the genes in each comparison. The red and blue dots indicate that up- and down-regulated DEGs were significant at q values less than 0.01.

GPs. We mapped the reads to the UCSC (the University of California Santa) reference human genome Hg19 with TopHat. The uniquely aligned reads for gallstones and GPs are 41.7 million and 32.1 million pairs, and the proportion of reads that aligned to the Ensembl reference genes is 93% and 94% respectively. The average coverage of our sequencing depth in our analysis was approximately 70 times of human transcriptome. Additionally only 1% reads were mapped to rRNA, suggesting that our libraries are properly established and represent the expression of RNA with ployA tails reliably. The detail result is shown in **Table 1**.

Analysis of differentially expressed genes

To identify the differentially expressed genes (DEGs) between gallstones and GPs, we estimated the gene expression and identified significantly dysregulated genes with the method of Cuffdiff. By requiring that the FPKM was greater than 1, we detected 11721 and 10511 expressed genes in gallstones and GPs samples respectively. Pearson correlation coefficient for correlation of the gene expression among the samples was conducted, and we found that the global profiles of gene expression were generally highly associated with the Pearson correlation coefficient (Gallstones vs. GPs 0.97) (**Figure 1A**).

We detected 147 DEGs between gallstones and GPs, the complete lists of the DEGs are summarized in Table S1. It is noteworthy that 143 genes are up-regulated in gallstones and only 5 genes are up-regulated in GPs, as shown in the "volcano plot" of the gene expression profiles (**Figure 1B**). The top significantly up- and down-regulated 15 genes in gallstones are displayed in **Table 2**.

Functional enrichment analysis of differentially expressed genes

To better understand the function of DEGs, we conducted an enrichment analysis of Gene Ontology for the dysregulated genes. GO categories are organized into three groups: biological process, cellular component, and molecular function. To identify the enriched functional categories, we first performed parallel enrichment tests for significantly differentially regulated genes using online tools from DAVID. The GO categories that were significantly enriched in the dysregulated genes from the gallstones and GPs was selected. In total, the DEGs between gallstones and GPs were categorized into 20 functional categories (**Table 3**). We

Table 2. The top significantly up- and down-regulated 15 genes

Gene Symbol	FPKM (Gallstones)	FPKM (GPs)	*F.D.R*	Fold Change
Up-regulated genes in gallstones				
CST1	111.70	2.97	0.00E+00	37.64
IGHV3-7	118.73	1.57	0.00E+00	75.46
IGKV2-28	140.00	3.97	0.00E+00	35.23
IGLV2-8	141.09	3.75	0.00E+00	37.63
IGHV3-23	185.52	4.05	0.00E+00	45.86
IGKV2-30	203.37	5.59	0.00E+00	36.39
IGLV2-14	228.43	8.73	0.00E+00	26.16
IGKV1-17	230.65	1.88	0.00E+00	122.91
IGKV3-20	421.00	18.36	0.00E+00	22.93
IGKV1-5	114.63	4.51	4.19E-13	25.39
IGHA2	2640.97	104.30	1.14E-12	25.32
S100A9	267.02	14.04	1.75E-12	19.02
IGLV2-11	89.97	1.51	2.90E-12	59.62
IGHV3-30	74.15	1.68	8.08E-12	44.14
IGKV1-9	79.10	2.02	1.23E-11	39.17
Down-regulated genes in gallstones				
CR2	0.170712	2.21314	2.17E-05	0.08
FDCSP	0.427935	8.19021	1.66E-03	0.05
HERC2P5	0.228785	5.31174	1.89E-03	0.04
ASB5	0.0937923	1.45932	2.12E-03	0.06

Table 3. Significantly enriched GO terms of DEGs

GO category	GO ID	GO Term	No. of genes	FDR
BP	GO:0006955	immune response	28	2.6E-15
	GO:0042330	taxis	15	3.3E-11
	GO:0006935	chemotaxis	15	3.3E-11
	GO:0006954	inflammatory response	18	2.0E-10
	GO:0009611	response to wounding	21	4.4E-10
	GO:0007626	locomotory behavior	16	3.5E-09
	GO:0007610	behavior	19	6.5E-09
	GO:0006952	defense response	20	6.4E-08
	GO:0030595	leukocyte chemotaxis	7	3.1E-05
	GO:0060326	cell chemotaxis	7	4.4E-05
	GO:0050900	leukocyte migration	7	4.5E-04
	GO:0030593	neutrophil chemotaxis	5	2.2E-03
CC	GO:0005576	extracellular region	42	2.7E-15
	GO:0005615	extracellular space	18	2.9E-05
	GO:0044421	extracellular region part	18	3.4E-03
MF	GO:0003823	antigen binding	11	5.9E-11
	GO:0005125	cytokine activity	15	1.6E-10
	GO:0008009	chemokine activity	9	2.9E-08
	GO:0042379	chemokine receptor binding	9	5.0E-08
	GO:0008083	growth factor activity	8	9.1E-03

Note: 'BP', 'CC' and 'MF' represent biological process, cellular component and molecular function, respectively.

found GO terms for molecular functions significantly enriched in antigen binding (GO:0003823, *P*=5.9E-11), while for biological processes, the enriched GO terms were immune response (GO:0006955, *P*=2.6E-15), and for cellular component, the enriched GO terms were extracellular region (GO:0005576, *P*=2.7E-15). To further evaluate the biological significance for the DEGs, we also performed the KEGG pathway enrichment analysis. The most significant pathway in our KEGG analysis was Cytokine-cytokine receptor interaction (*P*=7.5E-06). Furthermore, Chemokine signaling pathway (*P*=7.6E-03) and NOD-like receptor signaling pathway (*P*=0.1) are found to be highly enriched (**Table 4**).

Protein-protein interaction (ppi) network construction

We just obtained 7 up-regulated and 1 down-regulated DEGs among the top 20 significantly up- and down-regulated DEGs in gallstones in BioGRID dataset, and then the PPI networks of these DEGs was established by Cytoscape software including 121 nodes, 140 edges. The significant hub proteins containing S100A9 (S100 calcium binding protein A9, Degree=94), CR2 [complement component (3d/Epstein Barr virus) receptor 2, Degree =8] (**Figure 2**).

Table 4. KEGG pathway of enriched DEGs

Pathway ID	Pathway Name	Fold Enrichment	FDR (%)	Genes
hsa04060	Cytokine-cytokine receptor interaction	9.10	7.5E-06	CXCL1, IL6, CCL3, CCL2, IL8, CXCL3, CXCL2, CXCR1, CCL4L1, CCL8, CXCR2, TNFSF9, OSM, CCL20, IL1B
hsa04062	Chemokine signaling pathway	9.35	7.6E-03	CXCL1, CCL3, CCL2, IL8, CCL20, CXCL3, CXCL2, CCL8, CCL4L1, CXCR1, CXCR2
hsa04621	NOD-like receptor signaling pathway	17.94	0.1	CXCL1, IL6, CCL2, IL8, CXCL2, CCL8, IL1B

Discussion

Gallstones and GPs are the most commonly observed types of gallbladder diseases, both of which are risk factors for gallbladder carcinoma. However, the underlying pathogenesis of gallstones and GPs remain largely unknown, which have important implications for drug development and treatment for these diseases. RNA-Seq is a powerful tool to identify differences in transcription of thousands of genes on a genome-wide scale. To our knowledge, no other comparative study of gene profiling has been performed to investigate the differences and similarities of gene expression between gallstones and GPs. This present study suggests some promising genes and may provide a clue to the role of these genes played in the development of these diseases.

In this study we identified 148 DEGs between gallstones and GPs (143 up-regulated in gallstones and only 5 up-regulated in GPs) by using RNA-Seq. The large amount of up-regulated DEGs in gallstones may arise from gallbladder epithelium inflammation associated with the presence of gallstones. CST1, a member of type 2 cystatin subfamily, showed the greatest up-regulation in gallstones, and is expressed in gallbladder and other organs including submandibular gland and uterus [17]. CST1, a potent allegen, has been displayed to bind to the cysteine protease and papain to inhibit the cysteine protease activity of papain [18]. There is no report on its role in gallstones or GPs forming. CR2, a integral membrane glycoprotein, showed the greatest up-regulation in GPs. CR2 was shown to be associated with some immunologically mediated diseases including systemic lupus erythematosus [19, 20] and multiple sclerosis [21]. CR2 may be involved in the development of GPs due to its function of regulating the immune response.

In order to research the biological roles of the DEGs, we performed a GO categories enrichment analysis. We found GO terms for molecular functions significantly enriched in antigen binding, while for biological processes, the enriched GO terms were immune response, and for cellular component, the enriched GO terms were extracellular region. To further evaluate the biological significance for the DEGs, we also performed the KEGG pathway enrichment analysis. The most significant pathway in our KEGG analysis was Cytokine-cytokine receptor interaction. These results may be explained by the chronic inflammation of the gallbladder, which is frequently associated with the presence of gallstones, and it was shown to lead to some mutation of genes such as p53 [22, 23], resulting in an increase in the incidence of gallbladder carcinoma.

Furthermore the results from PPI network analysis of the top 20 up-regulated and down-regulated DEGs indicated the significant hub proteins containing S100A9 and CR2, although only 8 DEGs was available in BioGRID dataset for PPI analysis. S100A9 and other S100 proteins including S100A8 and S100A12 identified in our study strictly correlated to the regulation of immune system, the overexpression of which was observed in acute and chronic inflammatory diseases and tumors [24-26]. More importantly S100A8 and S100A9 proteins were shown to be present in the gallbladder mucosa with higher amounts in acute cholecystitis than in chronic disease, suggesting these important role in the development of calculous cholecystitis by involving in inflammation [27]. This result also suggests that our analysis is acceptable.

In summary, we compared gene expression profiling between gallstones and GPs by RNA-Seq and identified the expression of 147 DEGs, and functional analysis of these genes on immune response and cytokine-cytokine receptor interaction may provide insights into the complex process of these diseases. In addition PPI network analysis identified an important

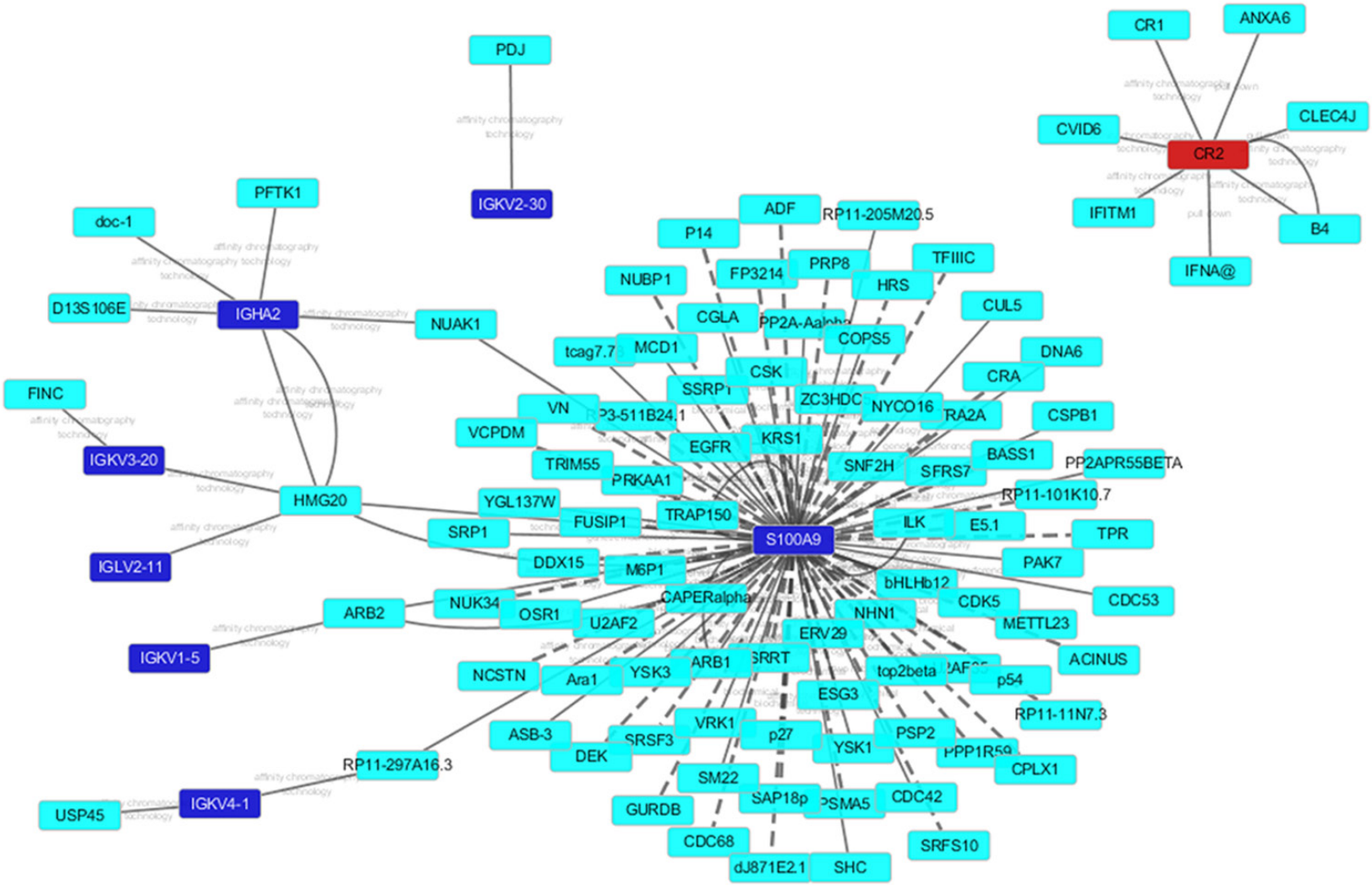

Figure 2. The constructed protein-protein interaction networks of the top 20 up- and down-regulated DEGs. Red- and green-color nodes represent products of up- and down-regulated DEGs, respectively. Blue nodes denote products of genes predicted to interact with the DEGs.

role of S100A9 on gallstone by involving in inflammation. This study may help to improve the diagnosis and treatment of these diseases.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zengwang Qie, Department of Hepatobiliary Surgery, The Second Hospital of Baoding, Baoding 071051, China. Tel: +86-15532275777; Fax: +86- 312-3099686; E-mail: zengwang_qie115@163.com

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