A transcriptome profile in gallbladder cancer based on annotation analysis of microarray studies

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Abstract. The purpose of the present study was to identify aberrantly expressed genes for gallbladder cancer based on the annotation analysis of microarray studies and to explore their potential functions. Differential gene expression was investigated in cholesterol polyps, gallbladder adenoma and gallbladder cancer using microarrays. Subsequently, microarray results were comprehensively analyzed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to determine the affected biological processes or pathways. Differentially expressed genes (DEGs) of cholesterol polyps, gallbladder adenoma and gallbladder cancer were identified. Following comprehensive analysis, 14 genes were found to be differentially expressed in the gallbladder wall of both gallbladder cancer and gallbladder adenoma. The 20 most significantly upregulated genes were only upregulated in the gallbladder wall of gallbladder cancer, but not in the gallbladder wall of cholesterol polyps and gallbladder adenoma. In addition, 182 DEGs were upregulated in the gallbladder wall of gallbladder adenoma compared with the gallbladder wall of cholesterol polyps. A total of 20 most significant DEGs were found in both the tumor and gallbladder wall of gallbladder cancer. In addition, the most significant DEGs that were identified were only upregulated in the tumor of gallbladder cancer. GO and KEGG analysis indicated that the aforementioned DEGs could participate in numerous biological processes or pathways associated with the development of gallbladder cancer. The present findings will help improve the current understanding of tumorigenesis and the development of gallbladder cancer.

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Abbreviations: DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; aRNA, amplified RNA; FC, fold-change

Key words: gallbladder diseases, gallbladder cancer, cholesterol polyps, gallbladder adenoma, biomarkers

Introduction

Gallbladder cancer is the most common malignant tumor of the biliary tract and is the third most common gastrointestinal malignancy worldwide (1). Due to the vague clinical symptoms and signs of gallbladder cancer, most patients are diagnosed at an advanced stage (2). Since the etiology and pathogenesis of gallbladder cancer are still unclear, it is essential to research the molecular mechanism of the disease and explore novel potential biomarkers that may assist early diagnosis and treatment.

Gallbladder adenoma is a rare disease and rarely malignant, but transformation may occur (3). Previous evidence has proposed that adenomas are the premalignant lesions of gallbladder cancer (3,4). However, the genetic evidence is still poorly defined (5). Due to the poor prognosis of gallbladder cancer, it is crucial to distinguish benign and malignant gallbladder adenoma (6). There is a need for accurate diagnostic methods to distinguish between benign and malignant diseases. Currently, the size and number of gallbladder polyps, along with patient age, are typically used to assist with distinguishing benign and malignant diseases (7). For example, previous research has found that conjugated bile acids (glycochenodeoxycholic and taurochenodeoxycholic) could be identified as possible biomarkers for cholesterol polyps and adenomatous polyps, and the gallbladder bile acids glycochenodeoxycholic acid and taurochenodeoxycholic acid are highly expressed in cholesterol polyps (8). For patients with gallbladder carcinoma, compared with healthy individuals and patients with cholesterol polyps, serum vascular endothelial growth factors (SVEGF)-C are closely related with lymph node metastasis, distant metastasis and stage, in addition, SVEGF-D has a positive relationship with the tumor depth, lymph, distant metastasis and stage that could represent available biomarkers for the diagnosis of gallbladder carcinoma (6).

The present study aimed to comprehensively analyze a transcriptome profile and identify DEGs in gallbladder cancer based on annotation analysis of microarray studies.

Materials and methods

Patients and tissue samples. Gallbladder stones (two men and one woman; age range, 60-62 years), gallbladder adenoma (two men and one woman; age range, 60-62 years) and gallbladder carcinoma (two men and one woman; age range, 60-63 years) tissues (n=3 each) were obtained from the Department of Pancreaticobiliary Surgery, the First Affiliated Hospital of China Medical University between September 2018 and December 2019. All cases were reviewed by two or more independent pathologists. No patients received radiation or chemotherapy before surgery. During the surgery, fresh tumor tissues or gallbladder wall tissues were collected in the operating room and immediately frozen in liquid nitrogen within 15 min and then stored in RNA Fixer reagent (Thermo Fisher Scientific, Inc.) at -80˚C for total RNA extraction. The present study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University (2018075). All patients who participated in the study signed written informed consent.

RNA extraction and transcript analysis. RNA extraction was performed using an RNeasy kit (Qiagen, Inc.) according to the manufacturer's protocol. Total RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and the RNA integrity was assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies, GmbH). Total RNA samples were analyzed on the Agilent Bioanalyzer 2100 and amplified RNA (aRNA) was prepared using the GeneChip 3'IVT Express kit (Affymetrix; Thermo Fisher Scientific, Inc.). Briefly, cDNA was synthesized by reverse transcription, and a double-stranded DNA template was then obtained by second-strand synthesis. Subsequently, an aRNA labeled with biotin was inverted *in vitro* utilizing GeneChip 3'IVT Express kit (Affymetrix; Thermo Fisher Scientific, Inc.) at 40˚C for 16 h and stored at 4˚C. The aRNA was purified, fragmented and hybridized with the chip probe (Beckman Coulter, Inc.). Following hybridization, the chip was automatically washed using a GeneChip Hybridization Wash and Stain kit (Affymetrix; Thermo Fisher Scientific, Inc.) and dyed using GeneChip Fluidics Station 450 instrument (Affymetrix; Thermo Fisher Scientific, Inc.). Finally, it was scanned to obtain the image and the Affymetrix microarray data using a GeneChip Scanner 3000 (Affymetrix; Thermo Fisher Scientific, Inc.).

To obtain the raw data, the Feature Extraction function in GeneSpring (version 10.5.1.1; Agilent Technologies, GmbH) was utilized to analyze the array image. Briefly, the raw data were normalized with the quantile algorithm. In the experiment, probe groups in the lowest 20% of the signal strength in the two sample groups were filtered as background noise. The coefficient of variation of the probe group was calculated in the sample group, and the probe group with a coefficient of variation >25% in both groups were also filtered out. Finally, DEG transcripts were identified.

To explore DEGs in different gallbladder diseases, further analysis was conducted, as shown in Table I. In the present study, gallstones served as normal samples compared with cholesterol polyps, gallbladder adenoma and gallbladder cancer.

Differential expression analysis. In the present study, linear models for microarray data (version 3.44.3; Bioconductor) were performed based on empirical Bayesian distribution to calculate the P-value (9). The screening criteria for DEGs was as follows: |Fold change (FC)|>1.5 and P-value <0.05. To probe out DEGs in cholesterol polyps, gallbladder adenoma and gallbladder cancer, differential expression analyses including scatter plot analysis, volcano plot analysis and hierarchical clustering analysis were performed using GraphPad Prism (version 7.0; GraphPad Software, Inc.).

Comparative analysis. To explore differentially expressed genes between different diseases a comparative analysis was undertaken, as shown in Table II and Fig. 1. In group II, the gallbladder wall of gallbladder adenoma and gallbladder wall of gallbladder stones were compared. In group III, the gallbladder wall of gallbladder cancer and gallbladder wall of gallbladder stone were compared. In group IV, comparative analysis was performed between gallbladder wall of gallbladder adenoma and gallbladder wall of cholesterol polyps. For group V, comparative analysis between gallbladder wall of gallbladder cancer and gallbladder wall of gallbladder adenoma was presented.

Functional enrichment analysis. To explore the biological processes or pathways involved in DEGs, Gene Ontology (GO; http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.kegg.jp/kegg/) pathway enrichment analyses were performed (10,11). GO terms include 'biological process' (BP), 'molecular function' (MF) and 'cellular component' (CC).

Validation of the differential expression and prognostic value of key genes using gene expression profiling interactive analysis (GEPIA). Key genes were verified by GEPIA (http://gepia.cancer-pku.cn/) in The Cancer Genome Atlas (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) and Genotype-Tissue Expression dataset (GTEx; http://commonfund.nih.gov/GTEx/) (12). Differential expression and overall survival (OS) analyses were performed.

Reverse transcription-quantitative PCR (RT-qPCR) assay. A total of 10 pairs of gallbladder cancer tissues and normal tissues (five men and five woman; age range, 55-65 years) were collected from the Department of Pancreaticobiliary Surgery, the First Affiliated Hospital of China Medical University between September 2018 and December 2019. All patients signed written informed consent. Total RNA was extracted from tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). According to the manufacturer's instructions, reverse transcription was performed using a TaqMan Real-Time PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-qPCR was run on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). The following primer pairs were used for the qPCR: HLA class II histocompatibility

Table I. Six groups for differential expression analysis via microarray analysis.

Groups	Tissue type	Number of samples	Comparison
Group I	Gallbladder wall	3	Cholesterol polyps vs. gallbladder stones
Group II	Gallbladder wall	3	Gallbladder adenoma vs. gallbladder stones
Group III	Gallbladder wall	3	Gallbladder cancer vs. gallbladder stones
Group IV	Gallbladder wall	3	Gallbladder adenoma vs. cholesterol polyps
Group V	Gallbladder wall	3	Gallbladder cancer vs. gallbladder adenoma
Group VI	Tumor tissue	3	Gallbladder cancer vs. gallbladder adenoma

Table II. Comprehensive analysis of DEGs in the six groups.

Comparative groups	Gallbladder wall of gallstone	Cholesterol polyps (gallbladder wall)	Gallbladder adenoma (gallbladder wall)	Gallbladder cancer (gallbladder wall)	Gallbladder adenoma (tumor wall)	Gallbladder cancer (tumor wall)
I	○	√				
II	○		√			
III	○			√		
IV		○	√			
V			○	√		
VI					○	√
A						
a=I∩II∩III		√	√	√		
b=II∩III-a			√	√		
c=II-b-I∩II			√			
d=III-b-I∩III				√		
B=IV			√			
C=V∩VI				√		√
D=VI-V∩VI						√

∩ represents the common DEGs between two groups, √ and ○ represent different samples (gallbladder wall of gallstone, gallbladder wall of cholesterol polyps, gallbladder wall of gallbladder adenoma and tumor wall of gallbladder adenoma) used for intersection. DEGs, differentially expressed genes.

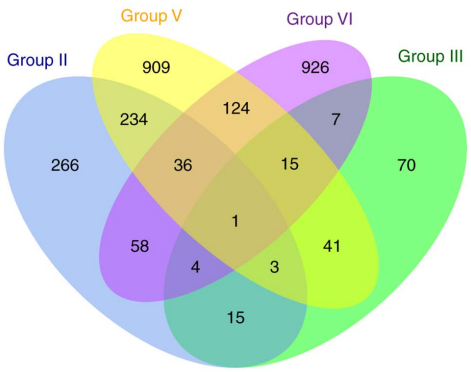

Figure 1. Venn diagram showing comprehensive analysis methods, which involves the comparison of different groups, of DEGs in the six groups. Group II: Gallbladder wall of gallbladder adenoma vs. gallbladder wall of gallbladder stones. Group III: Gallbladder wall of gallbladder cancer vs. gallbladder wall of gallbladder stones; Group IV: Gallbladder wall of gallbladder adenoma vs. gallbladder wall of cholesterol polyps; Group V: Gallbladder wall of gallbladder cancer vs. gallbladder wall of gallbladder adenoma. The DEGs in groups I and VI were not compared in this analysis.

antigen, DP a1 chain (HLA-DPB1) forward, 5'-ATGACACTC TTCTGAATTGACTG-3' and reverse, 5'-GGTAATGATAAA ACATGCTCTC-3'; nuclear receptor subfamily 4 group A member 2 (NR4A2) forward, 5'-TCATCTCCTCAGACTGGG GG-3' and reverse, 5'-TGTACCAAATGCCCCTGTCC-3'; ephrin-B2 (EFNB2) forward, 5'-TATGCAGAACTGCGA TTTCCAA-3' and reverse, 5'-TGGGTATAGTACCAGTCC TTGTC-3'; four and a half LIM domains protein 1 forward (FHL1), 5'-AAATGCACAAAGTGTGCCCG-3' and reverse, 5'-TCGTTTGGGACACTCAGCAC-3'; insulin-like growth factor-binding protein 7 (IGFBP7) forward, 5'-ACAGTGGTT GATGCCTTAC-3' and reverse, 5'-CCCTTATGGGTTGCT AACTAC-3'; Rho Family GTPase (RND) forward, 5'-CTA TGACCAGGGGGCAAATA-3' and reverse, 5'-TCTTCGCTT TGTCCTTTCGT-3'; E3 ubiquitin-protein ligase NEURL1B (NEURL1B) forward, 5'-ACAGCAGCTTCCAAGACACA-3' and reverse, 5'-GTTGGGCAGGCTGTAGTAGG-3'; and GAPDH forward, 5'-ACTCCCATTCTTCCACCTTTG-3'

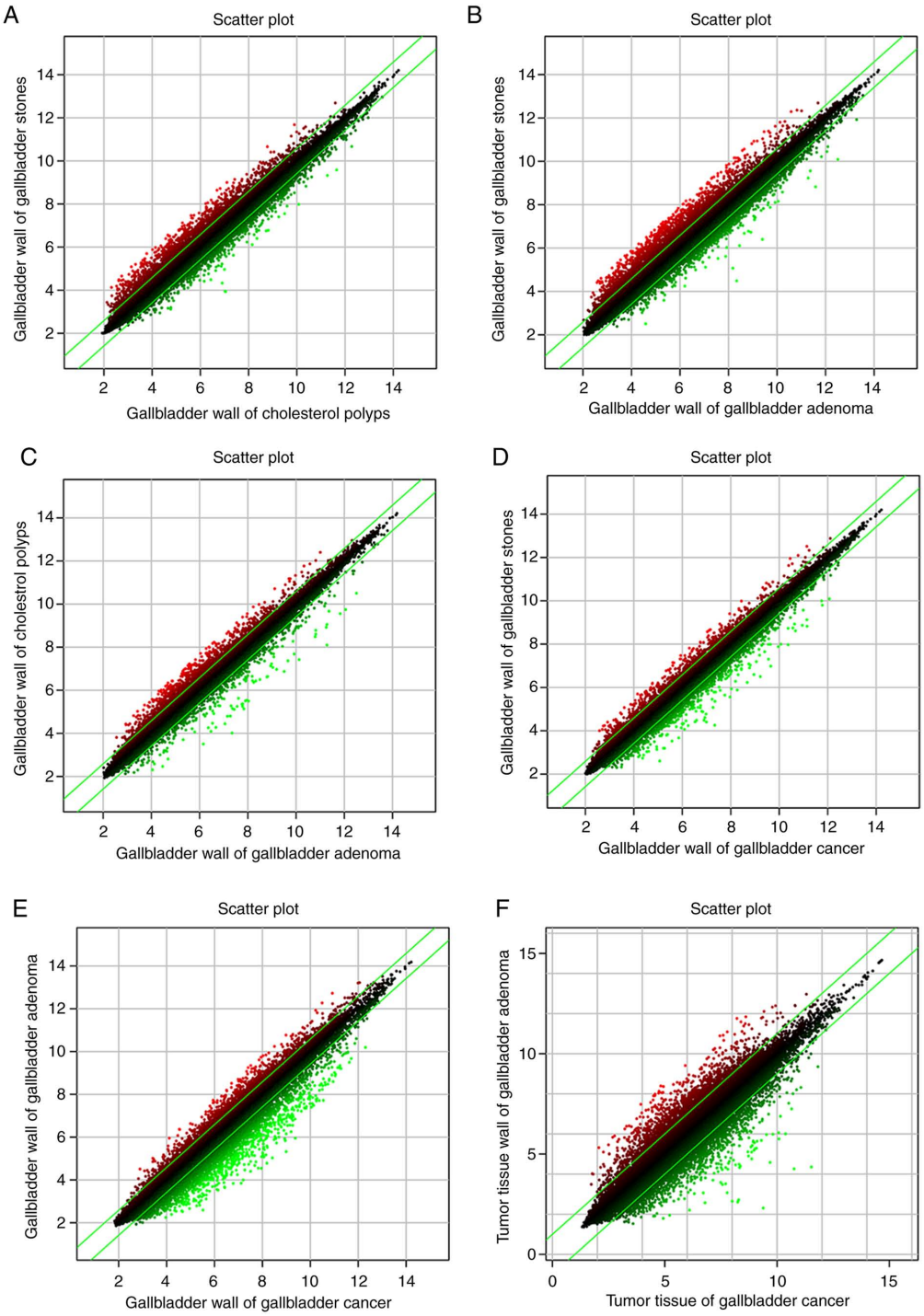

Figure 2. Scatter plots of genes in different compared groups. (A-F) Represent comparison I, II, III, IV, V and VI, respectively. Red represents upregulated genes and green represents downregulated genes. Comparison I: Gallbladder wall of gallstone vs. gallbladder wall of cholesterol polyps; Comparison II: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder adenoma; Comparison III: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder cancer; Comparison IV: Gallbladder wall of cholesterol polyps vs. gallbladder wall of gallbladder adenoma; Comparison V: Gallbladder wall of gallbladder adenoma vs. gallbladder wall of gallbladder cancer; Comparison VI: Tumor wall of gallbladder adenoma vs. tumor wall of gallbladder cancer.

and reverse, 5'-CCCTGTTGCTGTAGCCATATT-3'. GADPH served as an internal control. The relative expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (13).

Statistical analysis. All statistical analysis was conducted on R (14) or GraphPad Prism 7.0 (GraphPad Software, Inc.). Data were expressed as the mean ± SD. Each experiment was repeated at least three times. Comparisons between groups were analyzed by a Student's t-test. GO and KEGG annotation enrichment analyses were evaluated using a Fisher's exact test. For OS analysis, the samples were divided into high and low expression groups according to the median expression value of key genes. Differences between two groups were compared with Kaplan-Meier curves, followed by log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

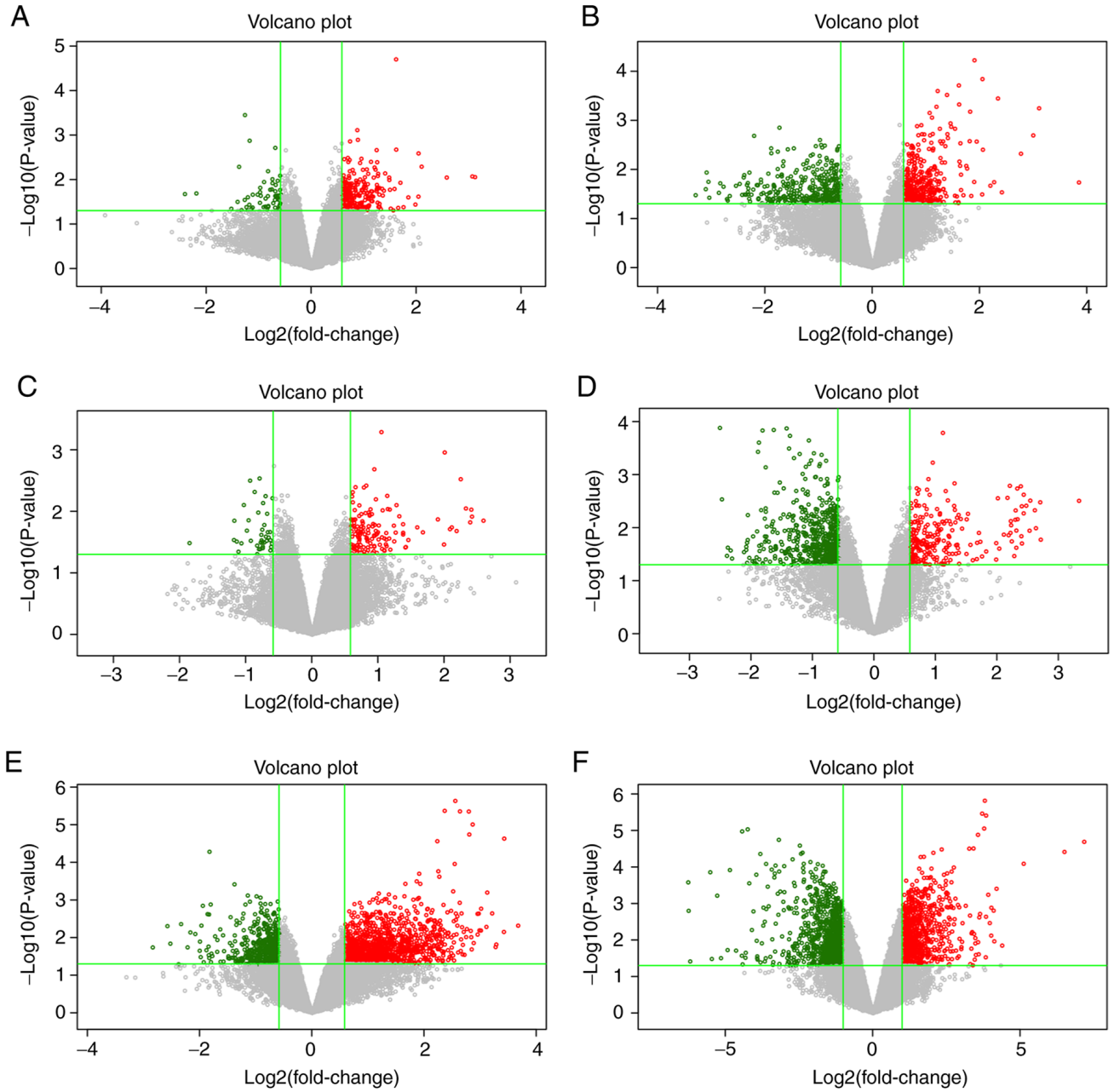

Figure 3. Volcano plots of compared groups. (A-F) Represent comparison I, II, III, IV, V and VI, respectively. Red represents upregulated genes and green represents downregulated genes. Comparison I: Gallbladder wall of gallstone vs. gallbladder wall of cholesterol polyps; Comparison II: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder adenoma; Comparison III: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder cancer; Comparison IV: Gallbladder wall of cholesterol polyps vs. gallbladder wall of gallbladder adenoma; Comparison V: Gallbladder wall of gallbladder adenoma vs. gallbladder wall of gallbladder cancer; Comparison VI: Tumor wall of gallbladder adenoma vs. tumor wall of gallbladder cancer.

Results

Transcript analysis results. In the present study, the gene expression profiles in the compared groups were analyzed via microarray analysis. The differential expression analysis results were shown in Table III according to |FC|>1.5 and P-value <0.05. The DEGs were marked for further analysis (Tables SI-SVI listed all DEGs in the six compared groups). The scatter plots show the distribution of upregulated genes in the two groups (Fig. 2). The volcano plots were used to show the DEGs between different compared groups. In Fig. 3, the red and green dots represented DEGs with the criteria of | FC |>1.5 and P-value <0.05, and the gray dot indicates genes with no significant difference.

Table III. Differentially expressed genes in six different comparative groups.

Comparative groups	Total number of upregulated genes	Total number of downregulated genes
I	198	43
II	346	271
III	116	40
IV	182	502
V	830	533
VI	540	632

Table IV. Common DEGs in the gallbladder wall of gallbladder cancer and gallbladder adenoma.

A, Upregulated genes

Entrez accession no.	Gene symbol	Gene name	Fold-change	P-value	False discovery rate
1906	EDN1	Endothelin 1	1.549618769	0.029390674	0.955609729
83661	MS4A8	Membrane-spanning 4-domains, subfamily A, member 8	2.003454854	0.016940921	0.955609729
213	ALB	Albumin	2.246885592	0.044086126	0.955609729
10232	MSLN	Mesothelin	3.577553479	0.034944882	0.955609729
1515	CTSV	Cathepsin V	1.810360067	0.018438263	0.955609729
573	BAG1	BCL2 associated athanogene 1	1.619984489	0.045294551	0.955609729
100507412	LOC100507412	Uncharacterized LOC100507412	2.405560916	0.00588484	0.955609729
55283	MCOLN3	Mucolipin 3	2.597985418	0.035436453	0.955609729
7586	ZKSCAN1	Zinc finger with KRAB and SCAN domains 1	2.303988209	0.010795501	0.955609729

B, Downregulated genes

Entrez accession no.	Gene symbol	Gene name	Fold-change	P-value	False discovery rate
6926	TBX3	T-box 3	-1.97381941	0.024886922	0.955609729
25987	TSKU	Tsukushi, small leucine rich proteoglycan	-1.506934454	0.049505143	0.955609729
6319	SCD	Stearoyl-coa desaturase (delta-9-desaturase)	-1.692411038	0.031149526	0.955609729
4857	NOVA1	Neuro-oncological ventral antigen 1	-1.754139316	0.041834676	0.955609729
5209	PFKFB3	6-phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3	-1.859592699	0.015450602	0.955609729

To effectively distinguish DEGs in the different comparison groups, unsupervised hierarchical clustering analysis was performed. This analysis could distinguish between the different samples in the six comparison groups (Figs. S1-S6).

Comparative analysis. In comparison A (Groups I-III; Table II): i) A total of eight commonly upregulated genes [including Uncharacterized LOC101928168 (LOC101928168), 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (HMGCS2), Secretagogin, EF-Hand Calcium Binding Protein (SCGN), Chimerin 2 (CHN2), X-Linked Kx Blood Group (XK), Mucin 6, Oligomeric Mucus/Gel-Forming (MUC6), Phospholipid Phosphatase 5 (PLPP5) and Heat Shock Protein Family H Member 1 (HSPH1)] and one downregulated gene [ST8 α-N-Acetyl-Neuraminide α-2,8-Sialyltransferase 4 (ST8SIA4)] were identified in cholesterol polyps, gallbladder adenoma and gallbladder cancer for gallbladder walls (data not shown); ii) A total of 14 common DEGs were found to overlap in the gallbladder wall of gallbladder cancer and gallbladder adenoma (Table IV); iii) A total of 273 differentially upregulated genes were only expressed in the gallbladder wall of gallbladder adenoma, of which the 20 most significantly DEGs, according to the FC, were selected to continue further analysis (Tables IV and V). A total of 85 upregulated genes were identified in the gallbladder wall of gallbladder cancer (Table VI). The 20 most significantly DEGs were selected, as shown in Table VI.

In comparison B (Group IV; Table I), 684 DEGs in the gallbladder wall of gallbladder adenoma, of which 182 were upregulated and 502 were downregulated, shown in Table SIV. The top 20 DEGs are shown in Table VII. In comparison C, it was revealed that 177 DEGs were expressed both in the tumor tissue and gallbladder wall in gallbladder cancer. The 20 most significantly DEGs were selected according to the FC (Table VIII). In comparison D, 459 upregulated genes were found in the tumor of gallbladder cancer. The top 20 upregulated genes that were identified according to FC in Table IX.

Function enrichment analysis. To better understand the biological pathways that were affected in the gallbladder walls of cholesterol polyps, gallbladder adenoma and gallbladder cancer, GO analysis was conducted on the DEGs. Fig. 4A shows that the GO terms that experienced the most significant

Table V. Top 20 upregulated genes in the gallbladder wall of gallbladder adenoma.

Entrez accession no.	Gene symbol	Gene name	Fold-change	P-value	False discovery rate
3115	HLA-DPB1	Major histocompatibility complex, class II, DP β1	14.39294587	0.018542717	0.95561
10321	CRISP3	Cysteine-rich secretory protein 3	8.596046776	0.000569554	0.95561
9153	SLC28A2	Solute carrier family 28 (concentrative nucleoside transporter), member 2	7.972874721	0.002038587	0.95561
1733	DIO1	Deiodinase, iodothyronine, type I	4.13402372	0.000144984	0.95561
2568	GABRP	γ-aminobutyric acid (GABA) A receptor, pi	4.11863611	0.032541353	0.95561
6555	SLC10A2	Solute carrier family 10 (sodium/bile acid cotransporter), member 2	3.729761379	5.99474E-05	0.95561
6819	SULT1C2	Sulfotransferase family 1C member 2	3.716642697	0.018104673	0.95561
4496	MT1H	Metallothionein 1H	3.527957872	0.000668336	0.95561
4494	MT1F	Metallothionein 1F	3.505661422	0.002675451	0.95561
54346	UNC93A	Unc-93 homolog A (C. Elegans)	3.355156356	0.008516354	0.95561
148523	CIART	Circadian associated repressor of transcription	3.136234278	0.026466979	0.95561
388561	ZNF761	Zinc finger protein 761	3.111132847	0.002949854	0.95561
100127888	SLCO4A1-AS1	SLCO4A1 antisense RNA 1	3.072422599	0.006932207	0.95561
4495	MT1G	Metallothionein 1G	3.050121402	0.00019533	0.95561
2069	EREG	Epiregulin	3.031503175	0.048716806	0.95561
7364	UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	2.971195084	0.035730027	0.95561
3821	KLRC1	Killer cell lectin-like receptor subfamily C, member 1	2.902803001	0.00149172	0.95561
3822	KLRC2	Killer cell lectin-like receptor subfamily C, member 2	2.902803001	0.00149172	0.95561
10562	OLFM4	Olfactomedin 4	2.899525179	0.03455814	0.95561
990	CDC6	Cell division cycle 6	2.763474564	0.001361724	0.95561

enrichment of comparison A was the BP 'Immune system process', MF 'Integrin binding' and the CC 'Anchoring junction'. Fig. 4B shows that the DEGs of gallbladder walls in cholesterol polyps vs. gallbladder adenoma participate in a number of GO pathways, the number of DEGs with the highest count had roles in the BP 'Tissue development', the CC 'Cell junction' and the MF 'Receptor binding'. Fig. 4C demonstrates that the number of DEGs of the tumor tissues in cholesterol polyps compared with gallbladder adenoma had the highest count in the BP 'Positive regulation of gene expression', the CC 'Cytoskeleton' and the MF 'Enzyme binding'.

KEGG pathway enrichment analysis. The biological pathways that were enriched by DEGs were also analyzed with KEGG. Fig. 5A shows that the most significantly enriched pathways in the gallbladder walls of cholesterol polyps, gallbladder adenoma and gallbladder cancer compared with normal groups was 'cell adhesion molecules (CAMs)'. Fig. 5B shows that the most significantly enriched pathway with DEGs in the gallbladder walls of gallbladder adenoma vs. gallbladder cancer was 'Cell adhesion molecules (CAMs)'. Fig. 5C indicates that the most significantly enriched pathway in the tumor tissues of gallbladder adenoma vs. gallbladder cancer was the 'Systemic lupus erythematosus'.

Validation of key genes in gallbladder cancer. Among the top 20 DEGs in the gallbladder wall and tumor of gallbladder cancer, seven novel DEGs, including HLA-DPB1 (Fig. 6A), NR4A2 (Fig. 6B), EFNB2 (Fig. 6C), FHL1 (Fig. 6D), IGFBP7 (Fig. 6E), RND3 (Fig. 6F) and NEURL1B (Fig. 6G) for gallbladder cancer were validated using GEPIA. HLA-DPB1, EFNB2, IGFBP7 and NEURL1B had a significantly higher expression in gallbladder cancer tissues compared with normal tissues. The prognostic value of these DEGs were further analyzed. There was no significant difference between low HLA-DPB1 expression and prognosis of gallbladder cancer (Fig. 7A). The low expression of NR4A2 (Fig. 7B) indicated poorer OS for patients with gallbladder cancer. No significant difference was found between patients with low EFNB2 expression and those with high EFNB2 expression (Fig. 7C). Low FHL1 expression predicted significantly less favorable OS (Fig. 7D). There was no significant difference observed for different expression levels of IGFBP7 (Fig. 7E), RND3 (Fig. 7F) and NEURL1B

Table VI. Top 20 upregulated genes in the gallbladder wall of gallbladder cancer.

Entrez accession no.	Gene symbol	Gene name	Fold-change	P-value	False discovery rate
54474	KRT20	Keratin 20, type I	6.027529	0.014263	0.99994
11075	STMN2	Stathmin 2	4.545556	0.021002	0.99994
22943	DKK1	Dickkopf WNT signaling pathway inhibitor 1	3.983841	0.034789	0.99994
3790	KCNS3	Potassium voltage-gated channel, modifier subfamily S, member 3	3.193245	0.022064	0.99994
3606	IL18	Interleukin 18	2.740635	0.029388	0.99994
8174	MADCAM1	Mucosal vascular addressin cell adhesion molecule 1	2.657304	0.038834	0.99994
1305	COL13A1	Collagen, type XIII, α1	2.593326	0.016279	0.99994
7171	TPM4	Tropomyosin 4	2.492788	0.012088	0.99994
84189	SLITRK6	SLIT and NTRK like family member 6	2.419904	0.007957	0.99994
79966	SCD5	Stearoyl-CoA desaturase 5	2.399926	0.02229	0.99994
56892	C8orf4	Chromosome 8 open reading frame 4	2.363066	0.030948	0.99994
81671	VMP1	Vacuole membrane protein 1	2.354891	0.017192	0.99994
406991	MIR21	MicroRNA 21	2.354891	0.017192	0.99994
55612	FERMT1	Fermitin family member 1	2.268497	0.042115	0.99994
55816	DOK5	Docking protein 5	2.254499	0.009369	0.99994
24147	FJX1	Four jointed box 1	2.241426	0.027788	0.99994
2043	EPHA4	EPH receptor A4	2.181555	0.036922	0.99994
1908	EDN3	Endothelin 3	2.174776	0.02892	0.99994
1316	KLF6	Kruppel-like factor 6	2.17353	0.022611	0.99994
440712	C1orf186	Chromosome 1 open reading frame 186	2.165575	0.034576	0.99994
5318	PKP2	Plakophilin 2	2.134774	0.043853	0.99994
63923	TNN	Tenascin N	2.081014	0.040874	0.99994
78989	COLEC11	Collectin subfamily member 11	2.067131	0.000518	0.99994
100131541	LOC100131541	Uncharacterized LOC100131541	2.032802	0.035034	0.99994
5727	PTCH1	Patched 1	2.01107	0.017085	0.99994
1359	CPA3	Carboxypeptidase A3 (mast cell)	1.992477	0.022002	0.99994
3775	KCNK1	Potassium channel, two pore domain subfamily K, member 1	1.975891	0.02328	0.99994
5732	PTGER2	Prostaglandin E receptor 2	1.95077	0.006402	0.99994
51751	HIGD1B	HIG1 hypoxia inducible domain family member 1B	1.9257	0.025133	0.99994
23705	CADM1	Cell adhesion molecule 1	1.907871	0.044745	0.99994

(Fig. 7G). Therefore, the data presented here indicated that only NR4A2 and FHL1 could represent potential prognostic markers for patients with gallbladder cancer. Following validation using RT-qPCR, HLA-DPB1 (Fig. 8A), NR4A2 (Fig. 8B) and EFNB2 (Fig. 8C) had significantly higher expression levels in gallbladder cancer tissues compared with normal tissues. However, there was no statistical difference in FHL1 expression between gallbladder cancer tissues and normal tissues (Fig. 8D). Moreover, high IGFBP7 expression was determined in gallbladder cancer tissues compared with normal tissues (Fig. 8E). As shown in Fig. 8F, RND3 mRNA expression was significantly decreased in gallbladder cancer tissues compared with normal tissues. A significantly higher expression level of NEURL1B was also detected in gallbladder cancer tissues compared with normal tissues (Fig. 8G).

Discussion

Gallbladder disease is one of the most common causes of upper abdominal pain (15). It is critical to focus on gallbladder diseases due to the potential for malignant degeneration of any gallbladder lesion (15). Gallbladder adenomas and primary adenocarcinomas have been identified as the most common benign and malignant tumors, respectively (16). Nevertheless, efforts have been put into elucidating the pathophysiological mechanisms leading to the development of gallbladder cancer, however, most of these mechanisms remain unknown.

Table VII. Top 20 upregulated genes in the gallbladder wall of gallbladder adenoma compared with the gallbladder wall of cholesterol polyps.

Entrez accession no.	Gene symbol	Gene name	Fold-change	P-value	False discovery rate
8076	MFAP5	Microfibrillar associated protein 5	10.055828	0.00313194	0.930859211
8483	CILP	Cartilage intermediate layer protein	5.7556811	0.01148426	0.930859211
8839	WISP2	WNT1 inducible signaling pathway protein 2	5.0126842	0.001836636	0.930859211
4495	MT1G	Metallothionein 1G	4.958911	0.024060491	0.930859211
4496	MT1H	Metallothionein 1H	4.7119253	0.018260689	0.930859211
2202	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	3.9536176	0.019679555	0.930859211
7364	UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	3.9512615	0.040165838	0.930859211
3489	IGFBP6	Insulin like growth factor binding protein 6	3.3672636	0.035074166	0.930859211
10562	OLFM4	Olfactomedin 4	3.3045794	0.018570013	0.930859211
4494	MT1F	Metallothionein 1F	3.2510145	0.01264233	0.930859211
64167	ERAP2	Endoplasmic reticulum aminopeptidase 2	3.1751689	0.039260457	0.930859211
1543	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	3.0349092	0.041312515	0.930859211
388561	ZNF761	Zinc finger protein 761	2.8743053	0.00550548	0.930859211
683	BST1	Bone marrow stromal cell antigen 1	2.8327043	0.00702193	0.930859211
55057	AIM1L	Absent in melanoma 1-like	2.8267351	0.031481094	0.930859211
100127888	SLCO4A1-AS1	SLCO4A1 antisense RNA 1	2.689454	0.01048406	0.930859211
30835	CD209	CD209 molecule	2.6028341	0.048163532	0.930859211
148523	CIART	Circadian associated repressor of transcription	2.5922233	0.029837355	0.930859211
10720	UGT2B11	UDP glucuronosyltransferase 2 family, polypeptide B11	2.5871195	0.005778714	0.930859211
2199	FBLN2	Fibulin 2	2.5660098	0.024397318	0.930859211

Therefore, it is crucial to disclose the molecular mechanisms of gallbladder cancer to promote the development of new cancer biomarkers and appropriate treatment strategies.

Different from other microarray studies, in the present study, the microarrays were comprehensively analyzed (17-19). Firstly, eight differentially expressed upregulated genes were found, which included LOC101928168, HMGCS2, SCGN, CHN2, XK, MUC6, PLPP5 and HSPH1 both in cholesterol polyps and gallbladder adenoma from gallbladder walls. Secondly, 14 common DEGs were identified in the gallbladder walls of gallbladder cancer and gallbladder adenoma. It is important to distinguish benign and malignant gallbladder adenoma due to the poor diagnosis of gallbladder cancer. T-Box Transcription Factor 3, Tsukushi, Small Leucine Rich Proteoglycan, Stearoyl-CoA Desaturase (SCD), NOVA Alternative Splicing Regulator 1 and 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 were downregulated in the gallbladder wall of gallbladder cancer and gallbladder adenoma; EDN1, MS4A8, ALB, MSLN, CTSV, BAG1, LOC100507412, MCOLN3 and ZKSCAN1 were upregulated in the gallbladder wall of gallbladder cancer and gallbladder adenoma. Thirdly, 273 upregulated genes were expressed in the gallbladder wall of gallbladder adenoma. Fourthly, the 20 most significantly DEGs that were upregulated in the gallbladder wall of gallbladder cancer were identified including KRT20, STMN2, DKK1, KCNS3, IL18, MADCAM1, COL13A1, TPM4, SLITRK6, SCD5, C8orf4, VMP1, MIR21, FERMT1, DOK5, FJX1, EPHA4, EDN3, KLF6 and C1orf186. Among them, DKK1 is known to regulate tumor angiogenesis, which is essential for tumor invasive growth and metastasis (20). IL18 has been reported to be a candidate cytokine that may provide a new insight into the development of next generation cancer immunotherapy (21). Desaturated fatty acids are essential for tumor cell survival, and SCD5 may represent a viable target for the development of novel agents for cancer treatment (22), which could become a candidate for the treatment of gallbladder cancer. KLF6 is a member of the Kruppel-like family of zinc finger transcription factors, which has been identified as a mutated tumor inhibitor in selective human cancer types, but not gallbladder cancer (23).

A total of 182 upregulated DEGs in the gallbladder walls of gallbladder adenoma were obtained and compared with that of cholesterol polyps. The top 20 most significantly expressed genes included MFAP5, CILP, WISP2, MT1G,

Table VIII. Top 20 upregulated genes both in the tumor and gallbladder wall in gallbladder cancer.

Entrez accession no.	Gene symbol	Gene name	Fold-change	P-value	False discovery rate
1490	CTGF	Connective tissue growth factor	12.721	0.0049	0.663
3115	HLA-DPB1	Major histocompatibility complex, class II, DP β1	8.0389	0.0066	0.663
3400	ID4	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	6.919	4E-06	0.044
3399	ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	5.8099	0.0001	0.435
1282	COL4A1	Collagen, type IV, α1	5.3601	0.0398	0.663
4929	NR4A2	Nuclear receptor subfamily 4 group A member 2	5.0011	0.0113	0.663
2669	GEM	GTP binding protein overexpressed in skeletal muscle	4.6392	0.0094	0.663
1948	EFNB2	Ephrin-B2	4.5216	0.0087	0.663
3397	ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	4.3673	0.0121	0.663
2919	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, α)	4.2191	0.0453	0.663
390	RND3	Rho family GTPase 3	4.1688	0.0017	0.663
2273	FHL1	Four and a half LIM domains 1	4.1366	0.011	0.663
3490	IGFBP7	Insulin like growth factor binding protein 7	4.1039	0.0369	0.663
54492	NEURL1B	Neuralized E3 ubiquitin protein ligase 1B	3.8487	0.0083	0.663
301	ANXA1	Annexin A1	3.811	0.0242	0.663
10631	POSTN	Periostin, osteoblast specific factor	3.7339	0.0224	0.663
79772	MCTP1	Multiple C2 domains, transmembrane 1	3.6779	0.0247	0.663
3486	IGFBP3	Insulin like growth factor binding protein 3	3.4581	0.0304	0.663
5327	PLAT	Plasminogen activator, tissue	3.288	0.0023	0.663
26353	HSPB8	Heat shock protein family B (small) member 8	3.2012	0.0416	0.663

MT1H, EFEMP1, UGT2B7, IGFBP6, OLFM4, MT1F, ERAP2, CYP1A1, ZNF761, BST1, AIM1L, SLCO4A1-AS1, CD209, CIART, UGT2B11 and FBLN2. The overexpression of CCN5/WISP2 in adipose tissue has previously been secreted and circulated in the blood in a transgenic mouse model, which suggests that WISP2 could become a biomarker in blood for gallbladder adenoma and cholesterol polyps (24). The gene expression of MT1G, MT1H and MT1F in human peripheral blood lymphocytes can be used as potential biomarkers for cadmium exposure (25). Cadmium exposure could contribute to the development of gallbladder cancer (26). EFEMP1 expression accumulates angiogenesis and accelerates the growth of cervical cancer *in vivo* (27). Patients with UGT2B7*1/*2 genotypes, UGT2B7 genetic variation are at risk for suboptimal immune recovery due to significant long-term autologous induction (28). The expression of IGFBP-6 in vascular endothelial cells is upregulated by hypoxia and IGFBP-6 suppresses angiogenesis *in vitro* and *in vivo* (29), but this has not been reported in the context of gallbladder cancer. OLFM4 expression is associated with cancer differentiation, stage, metastasis and prognosis in a variety of cancer types, such as breast cancer, esophageal adenocarcinoma and gastrointestinal cancer, suggesting that it has underlying clinical value as an early cancer biomarker or therapeutic target (30). CYP1A1/1A2 isoenzymes are involved in EROD activity in blood lymphocytes (31); however, there is currently no previous report on the functions of this gene in gallbladder cancer. The production of extracellular cADPR, catalyzed by BST-1, followed by concentrating the uptake of cyclic nucleotides by hemopoietic progenitors, may be physiologically relevant in normal hematopoiesis (32), but its function in gallbladder cancer remains unknown. CD209 has been identified to present on monocyte-derived DCs, a cell adjuvant for cancer immunotherapy (33). FBLN2 is a novel gene associated with hypertension (34).

The top 20 upregulated genes were expressed both in tumors and gallbladder walls of gallbladder cancer, which included CTGF, HLA-DPB1, ID3, ID4, COL4A1, NR4A2, GEM, EFNB2, ID1, CXCL1, RND3, FHL1, IGFBP7, NEURL1B, ANXA1, POSTN, MCTP1, IGFBP3, PLAT and HSPB8. The study focused on whether these genes expression levels could be assessed using blood or bile. CTGF could play an important role in the inflammation of gallbladder cancer (35). Therefore, CTGF has the potential to become a future biomarker for gallbladder cancer, circulating in the blood and bile. It has been revealed that the dynamic changes of growth centers and plasma cell differentiation are determined by ID3 and E protein activity (35). Following validation

Table IX. Top 20 upregulated genes in the tumor of gallbladder cancer.

Entrez accession no.	Gene symbol	Gene name	Fold-change	P-value	False discovery rate
1048	CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5	143.4154524	2.06062×10^{-5}	0.0894515
10562	OLFM4	Olfactomedin 4	90.22299879	3.89271×10^{-5}	0.0960529
7020	TFAP2A	Transcription factor AP-2 α (activating enhancer binding protein 2 α)	18.24994233	3.9829×10^{-4}	0.1609015
1015	CDH17	Cadherin 17, LI cadherin (liver-intestine)	16.14818371	1.31492×10^{-3}	0.1871078
10451	VAV3	VAV guanine nucleotide exchange factor 3	16.0872594	7.651281×10^{-3}	0.2974407
1604	CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	14.25260113	3.93845×10^{-6}	0.0512905
1373	CPS1	Carbamoyl-phosphate synthase 1	14.14519343	2.998467×10^{-2}	0.3979446
3872	KRT17	Keratin 17, type I	11.21846461	2.5881794×10^{-2}	0.3854011
5268	SERPINB5	Serpin peptidase inhibitor, clade B (ovalbumin), member 5	11.07304422	1.2500212×10^{-2}	0.3344667
9843	HEPH	Hephaestin	11.07030706	8.947495×10^{-3}	0.3090802
213	ALB	Albumin	10.91257754	0.034730042	0.408424
3158	HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	10.74010853	6.900844×10^{-3}	0.2884597
63928	CHP2	Calcineurin-like EF-hand protein 2	7.987754539	1.5774911×10^{-2}	0.3509474
7031	TFF1	Trefoil factor 1	7.9601374	0.017823277	0.3616271
11074	TRIM31	Tripartite motif containing 31	7.589614806	1.280721×10^{-3}	0.1871078
23213	SULF1	Sulfatase 1	7.505284011	2.8219398×10^{-2}	0.3929094
3216	HOXB6	Homeobox B6	7.355635549	1.101194×10^{-3}	0.1861479
84419	C15orf48	Chromosome 15 open reading frame 48	7.266966891	3.1186816×10^{-2}	0.4004668
2982	GUCY1A3	Guanylate cyclase 1, soluble, α3	7.041356237	1.9708261×10^{-2}	0.3677601
8329	HIST1H2AI	Histone cluster 1, h2ai	6.935644729	7.41761×10^{-4}	0.1737322

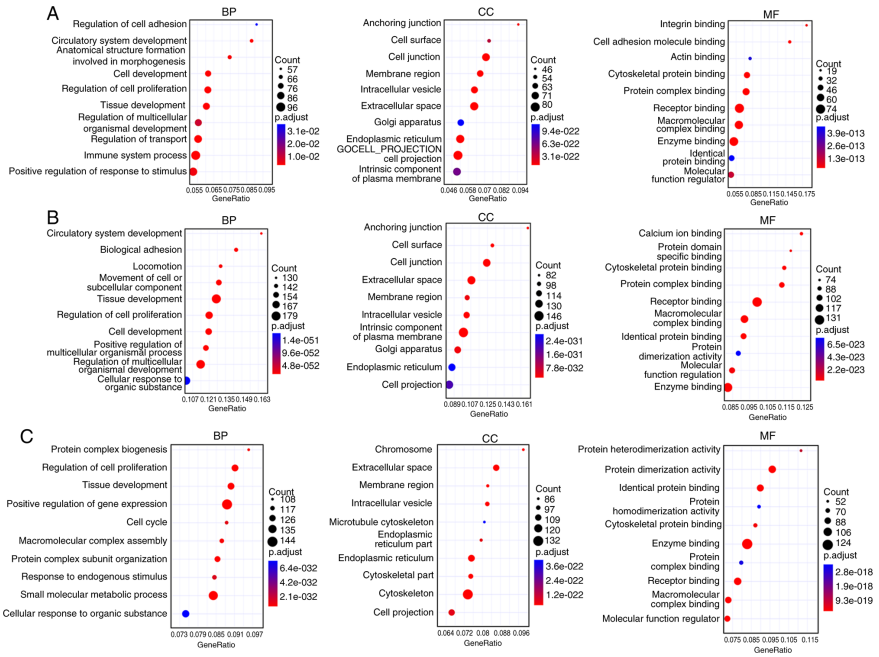

Figure 4. GO function enrichment analysis of all the compared groups. (A) GO function enrichment analysis of (A) comparison I, II, III and IV, (B) comparison V and (C) comparison VI. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function. Red represents upregulated genes and green represents downregulated genes. Comparison I: Gallbladder wall of gallstone vs. gallbladder wall of cholesterol polyps; Comparison II: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder adenoma; Comparison III: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder cancer; Comparison IV: Gallbladder wall of cholesterol polyps vs. gallbladder wall of gallbladder adenoma; Comparison V: Gallbladder wall of gallbladder adenoma vs. gallbladder wall of gallbladder cancer; Comparison VI: Tumor wall of gallbladder adenoma vs. tumor wall of gallbladder cancer.

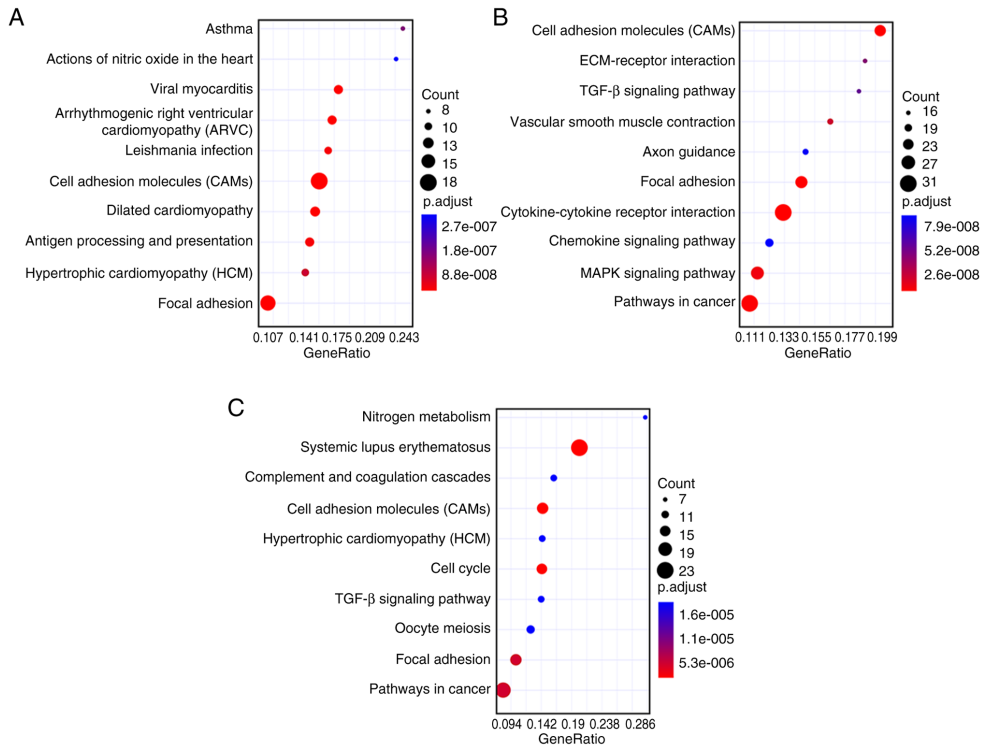

Figure 5. Top 10 pathways in KEGG pathway analysis of all compared groups. op 10 pathways in KEGG pathway analysis of (A) comparison I, II, III and IV, (B) comparison V and (C) comparison VI. KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix; TGF, transforming growth factor. Comparison I: Gallbladder wall of gallstone vs. gallbladder wall of cholesterol polyps; Comparison II: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder adenoma; Comparison III: Gallbladder wall of gallstone vs. gallbladder wall of gallbladder cancer; Comparison IV: Gallbladder wall of cholesterol polyps vs. gallbladder wall of gallbladder adenoma; Comparison V: Gallbladder wall of gallbladder adenoma vs. gallbladder wall of gallbladder cancer; Comparison VI: Tumor wall of gallbladder adenoma vs. tumor wall of gallbladder cancer.

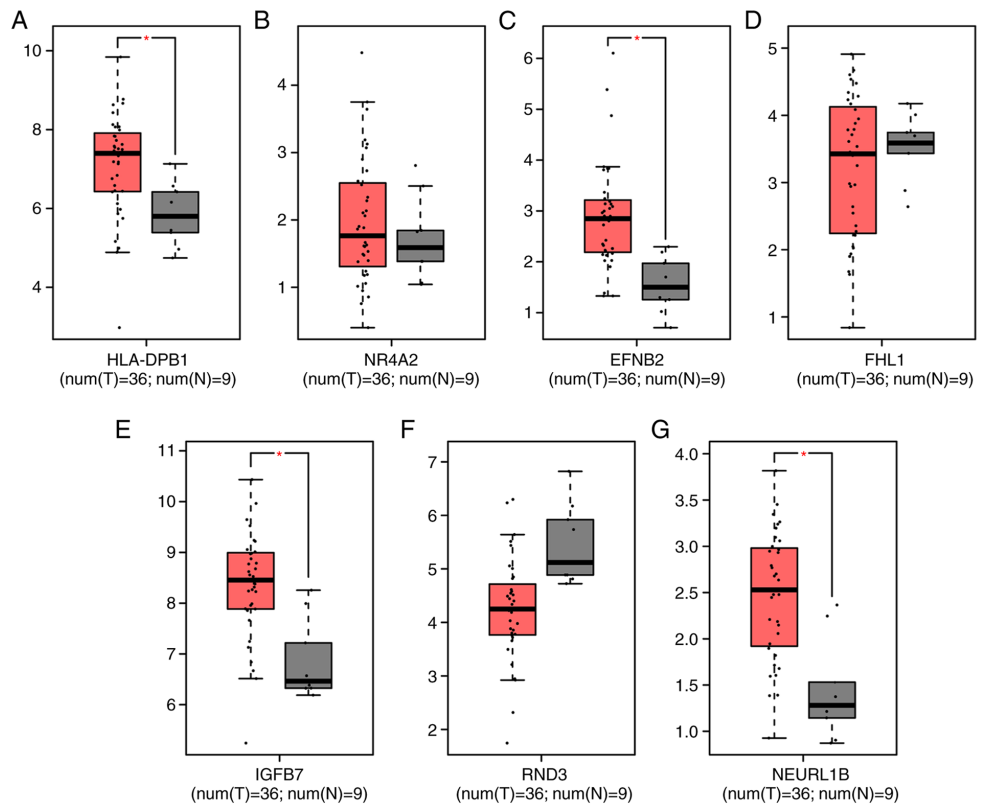

Figure 6. Validation of key genes in gallbladder cancer tissues using Gene Expression Profiling Interactive Analysis. (A) HLA-DPB1, (B) NR4A2, (C) EFNB2, (D) FHL1, (E) IGFBP7, (F) RND3 and (G) NEURL1B. HLA-DPB1, Major Histocompatibility Complex, Class II, DP β1; NR4A2, nuclear receptor subfamily 4 group a member 2; EFNB2, ephrin B2; FHL1, four and a half LIM domains 1; IGFBP7, insulin like growth factor binding protein 7; RND3, Rho family GTPase 3; NEURL1B, neutralized E3 ubiquitin protein ligase 1B; T, tumor; N, normal. *P<0.05.

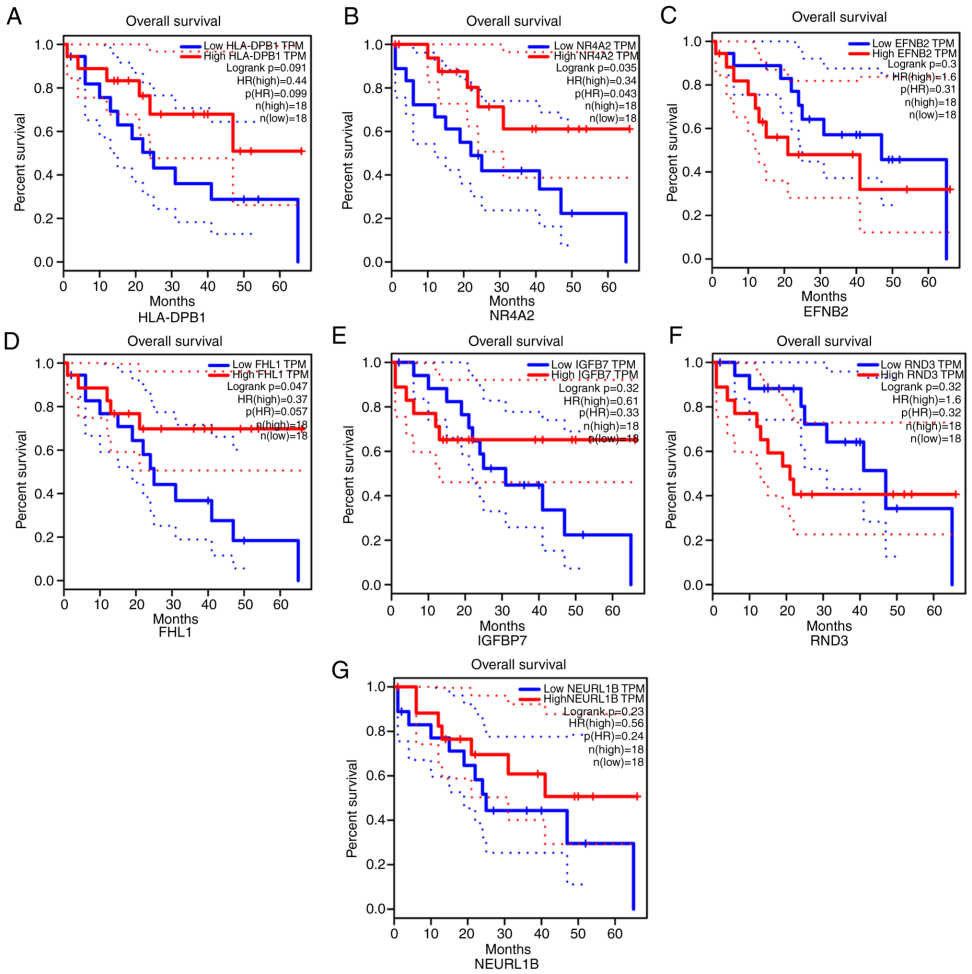

Figure 7. Overall survival analysis of key genes in gallbladder cancer. (A) HLA-DPB1, (B) NR4A2, (C) EFNB2, (D) FHL1, (E) IGFBP7, (F) RND3 and (G) NEURL1B. Red represents high expression and blue represents low expression. HLA-DPB1, Major Histocompatibility Complex, Class II, DP β1; NR4A2, nuclear receptor subfamily 4 group a member 2; EFNB2, ephrin B2; FHL1, four and a half LIM domains 1; IGFBP7, insulin like growth factor binding protein 7; RND3, Rho family GTPase 3; NEURL1B, neutralized E3 ubiquitin protein ligase 1B; HR, hazard ratio.

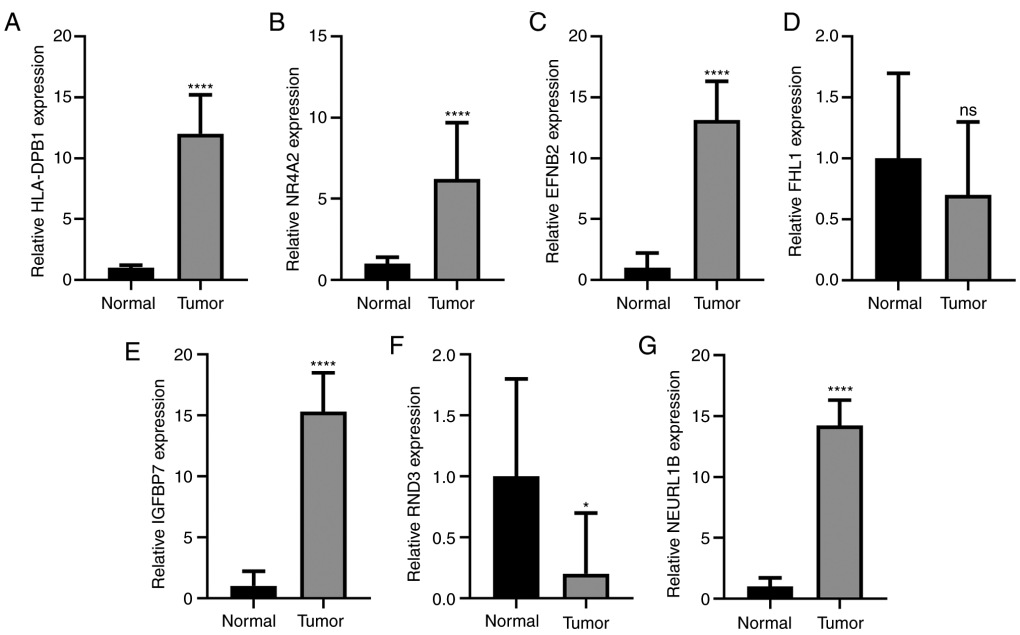

Figure 8. Validation of key genes in gallbladder cancer tissues using a reverse transcription-quantitative PCR assay. (A) HLA-DPB1, (B) NR4A2, (C) EFNB2, (D) FHL1, (E) IGFBP7, (F) RND3 and (G) NEURL1B. *P<0.05; and ****P<0.0001; ns, not significant; HLA-DPB1, Major Histocompatibility Complex, Class II, DP β1; NR4A2, nuclear receptor subfamily 4 group a member 2; EFNB2, ephrin B2; FHL1, four and a half LIM domains 1; IGFBP7, insulin like growth factor binding protein 7; RND3, Rho family GTPase 3; NEURL1B, neutralized E3 ubiquitin protein ligase 1B.

using RT-qPCR, the genes HLA-DPB1, NR4A2, EFNB2, IGFBP7 and NEURL1B were found to be highly expressed in gallbladder cancer. RND3 was significantly decreased in gallbladder cancer. HLA-DPB1, NR4A2 and FHL1 could be underlying prognostic markers for gallbladder cancer.

In the present study, a transcriptome profile was comprehensively analyzed enabling the identification of DEGs in gallbladder cancer, based on an annotation analysis of microarray studies. The present findings could provide a novel understanding on the tumorigenesis and development of gallbladder cancer.

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Availability of data and materials

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

Authors' contributions

CG conceived and designed the study. XZ and XN conducted most of the experiments and data analysis and wrote the manuscript. BZ and LC participated in acquiring data and helped draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of China Medical University (2018075). All patients who participated in the study signed written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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