Expression of lncRNAs in children with pancreaticobiliary maljunction: functional analysis and potential biomarkers

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Abstract

Introduction: Pancreaticobiliary maljunction (PBM) leads to higher rates of complications, including cholangitis, pancreatitis, and malignancies. The aim of the present study was to investigate the expression profile of long non-coding RNAs (lncRNAs) and their potential role as biomarkers in children with pancreaticobiliary maljunction.

Material and methods: The differential expression of lncRNAs and messenger RNA (mRNAs) from pediatric patients with pancreaticobiliary maljunction and control subjects was analyzed using a commercial microarray and later validated with qRT-PCR. The potential biological functions of differentially expressed genes were explored based on Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment. The ability of potential lncRNA biomarkers to predict pancreaticobiliary maljunction was assessed based on the area under the receiver operating characteristic curve (AUC).

Results: There were 2915 mRNAs and 173 lncRNAs upregulated, and 2121 mRNAs and 316 lncRNAs downregulated in PBM cases compared to controls. The enriched Gene Ontology categories associated with differentially expressed mRNAs were extracellular matrix, extracellular region, and kinetochore. The most enriched Kyoto Encyclopedia pathway was protein digestion and absorption, which was associated with cancer and PI3K-Akt signaling. Analysis of *cis*- and *trans*-target genes predicted that a single lncRNA was able to regulate several mRNAs. The qRT-PCR results for *NR_110876, NR_132344*, *XR_946886*, and *XR_002956345* were consistent with the microarray results, and the difference was statistically significant for *NR_132344*, *XR_946886*, and *XR_002956345* ($p < 0.05$). AUC was significant only for *XR_946886* (0.837, $p < 0.001$).

Conclusions: Our results implicate lncRNAs in common bile duct pathogenesis in PBM, and they identify *XR_946886* as a potential biomarker for the disease.

Key words: pancreaticobiliary maljunction, long non-coding RNA, gene ontology, biomarker, differentially expressed genes.

Introduction

Pancreaticobiliary maljunction (PBM) is a rare congenital anomaly in which the junction of the pancreatic and biliary ducts is located outside the duodenal wall, with a long common channel [1–3]. The resulting two-way reflux of bile and pancreatic juice leads to several complications,

including bile retention with cholangitis, pancreatitis, and malignancies [4–6].

Cyst excision and Roux-en-Y hepaticojejunostomy are the first choice for PBM treatment [7–9]. However, these procedures are associated with postoperative complications affecting the residual bile ducts, such as cholangitis, hepatolithiasis, and carcinogenesis [8, 10–13]. Identification of biomarkers associated with PBM complications of the bile duct could enable the early detection of these complications and support the development of effective diagnostic and therapeutic methods.

Long noncoding RNAs (lncRNAs), which are non-coding RNAs longer than 200 nucleotides, play an important role in regulating the occurrence and progression of many diseases, such as infectious and malignant tumors [14–16]. They exert these effects by modulating biological processes such as cellular proliferation, motility, and immune response to diseases [17–19]. In addition, some lncRNAs have been identified as biomarkers for diagnosis and prognosis of diseases [20–22]. However, the genome-wide expression and functional roles of lncRNAs in PBM are unclear.

To the best of our knowledge, this is the first study focused on lncRNAs in the common bile duct of PBM. Here, we investigated the expression profile of lncRNAs in the common bile duct of patients with PBM, and we identified lncRNAs differentially expressed relative to controls. We explored the potential functions of the differentially expressed lncRNAs and assessed their ability to serve as biomarkers.

Table I. Clinicodemographic characteristics of study subjects

Characteristic	Todani types I (n = 7)	Todani types IV (n = 8)
Abdominal pain	6	4
Jaundice	1	4
Mass	0	0
Fever	1	1
Vomiting	5	3
Sex (male)	0	3
Age [months]	9–71	4.5–50
Pathological findings:		
Cyst wall hyperplasia	7	8
Gallbladder wall	7	8
Congestion	–	–

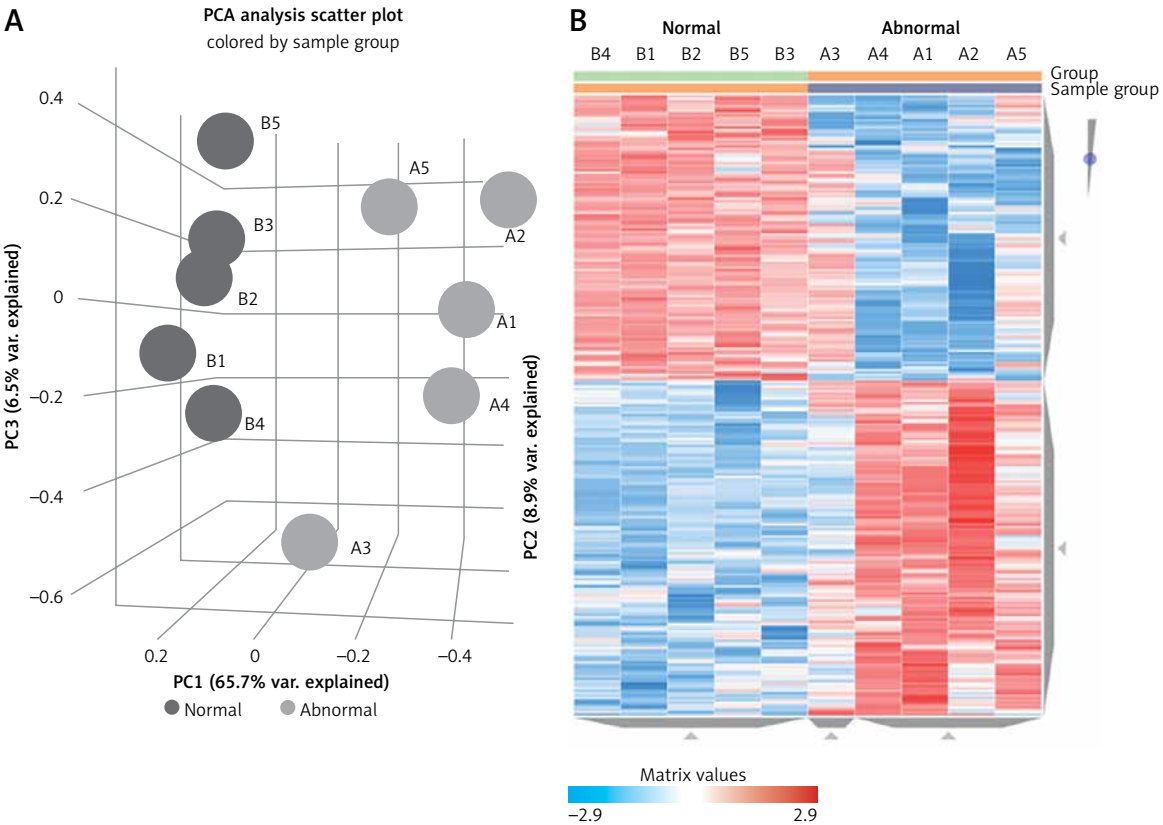

Figure 1. A – Principal component analysis of differentially expressed lncRNAs. Principal component 1 explained 65.7% of the observed variance; component 2, 8.9%; and component 3, 6.5%. **B** – Heatmap of differentially expressed lncRNAs

Table II. The top 20 differentially expressed lncRNAs in pancreaticobiliary maljunction (PBM) (up and down regulated)

Gene symbol	Gene ID	Accession	Description	Length	Regulation (A vs. B)	FC (abs) (A vs. B)	*P*-value (A vs. B)	Type
LOC105371291	105371291	XR_933627.3	Uncharacterized LOC105371291, transcript variant X3	1138	down	12.056273	0.01451713	ncRNA
LOC105374803	105374803	XR_940242.2	Uncharacterized LOC105374803	1305	down	9.0651	0.00494713	ncRNA
LINC00261	140828	NR_001558.3	Long intergenic non-protein coding RNA 261	4912	down	7.9187694	0.01998967	ncRNA
PSORS1C3	100130889	NR_152831.1	Psoriasis susceptibility 1 candidate 3 (non-protein coding), transcript variant 5	624	down	6.993259	0.01583394	ncRNA
LOC112267956	112267956	XR_002956345.1	Uncharacterized LOC112267956	2005	down	6.9718432	0.01816426	ncRNA
LOC158434	158434	NR_132344.1	Uncharacterized LOC158434	2662	down	6.0362997	0.00271874	ncRNA
LOC105372653	105372653	NR_134564.1	Uncharacterized LOC105372653	573	down	5.8000107	2.83E-04	ncRNA
LINC01320	104355288	NR_126404.1	Long intergenic non-protein coding RNA 1320	1131	down	5.6668267	0.01277497	ncRNA
LOC105371825	105371825	XR_001752940.1	Uncharacterized LOC105371825	3133	down	5.4514194	0.02630284	ncRNA
PP7080	25845	NR_024158.1	Uncharacterized LOC25845	1743	down	5.4081116	0.01141873	ncRNA
LOC105369381	105369381	XR_001748496.1	Uncharacterized LOC105369381, transcript variant X2	5254	down	5.392252	0.00472638	ncRNA
EMX2OS	196047	NR_144378.1	EMX2 opposite strand/antisense RNA, transcript variant 2	7201	down	5.325151	0.00589902	ncRNA
SNORA25B	109623459	NR_145801.1	Small nucleolar RNA, H/ACA box 25B	127	down	5.319602	0.00461494	ncRNA
LOC101928875	101928875	XR_001748392.2	Uncharacterized LOC101928875	728	down	5.1403937	0.006804	ncRNA
GBA3	57733	NR_102356.1	Glucosylceramidase beta 3 (gene/pseudogene), transcript variant 2, non-coding	1243	down	5.103703	0.00271457	ncRNA
LOC101927269	101927269	NR_110075.1	Uncharacterized LOC101927269	507	down	4.978601	0.03097457	ncRNA
LOC105377924	105377924	NR_134603.1	Uncharacterized LOC105377924	415	down	4.93889	0.0150742	ncRNA
LOC105374510	105374510	XR_001741602.1	Uncharacterized LOC105374510, transcript variant X3	4802	down	4.9087567	0.03875371	ncRNA
LOC102724834	102724834	XR_429171.4	Uncharacterized LOC102724834	3131	down	4.588613	0.0016174	ncRNA
LOC107985242	107985242	XR_001738352.1	Uncharacterized LOC107985242, transcript variant X2	1899	down	4.5234885	0.00128315	ncRNA
LOC105378608	105378608	XR_946886.2	Uncharacterized LOC105378608	3022	up	14.387957	0.0011432	ncRNA
LOC105376384	105376384	XR_930616.3	Uncharacterized LOC105376384, transcript variant X3	1188	up	6.8661094	9.25E-04	ncRNA
HECW1-IT1	100127950	NR_135295.1	HECW1 intronic transcript 1	1839	up	6.84244	0.03381596	ncRNA
LOC105371401	105371401	XR_002957935.1	Uncharacterized LOC105371401	3094	up	6.693866	0.0253292	ncRNA
CASC23	103581031	NR_125366.1	Cancer susceptibility 23 (non-protein coding)	1583	up	6.3594065	0.00331883	ncRNA
LOC105374641	105374641	XR_925740.2	Uncharacterized LOC105374641	294	up	5.8032284	0.00589144	ncRNA

Table II. Cont.

Gene symbol	Gene ID	Accession	Description	Length	Regulation (A vs. B)	FC (abs) (A vs. B)	*P*-value (A vs. B)	Type
LOC105379057	105379057	XR_001742782.1	Uncharacterized LOC105379057, transcript variant X3	8261	up	5.3560147	4.11E-04	ncRNA
LOC107986570	107986570	XR_001743976.1	Uncharacterized LOC107986570, transcript variant X2	770	up	5.3314576	0.01600899	ncRNA
LINC00578	100505566	NR_047568.1	Long intergenic non-protein coding RNA 578	1222	up	5.320564	0.03225355	ncRNA
MIR4435-2HG	541471	NR_136164.1	MIR4435-2 host gene, transcript variant 6	2046	up	5.3023853	0.00741963	ncRNA
LOC107984270	107984270	XR_001747591.1	Uncharacterized LOC107984270, transcript variant X1	3786	up	5.1539755	0.01410949	ncRNA
MIR503HG	84848	NR_024607.1	MIR503 host gene	786	up	5.049058	0.00200934	ncRNA
ADAM5	255926	NR_001448.1	ADAM metallopeptidase domain 5 (pseudogene)	1718	up	5.048116	9.81E-04	ncRNA
MIR8061	102466251	NR_107028.1	MicroRNA 8061	75	up	4.8443885	5.84E-04	ncRNA
LOC105375855	105375855	XR_928920.2	Uncharacterized LOC105375855	7758	up	4.6685147	0.01672733	ncRNA
SH3RF3-AS1	100287216	NR_029193.1	SH3RF3 antisense RNA 1	2793	up	4.144925	0.01781914	ncRNA
LOC100506688	100506688	NR_104615.1	Uncharacterized LOC100506688, transcript variant 2	3451	up	4.080634	0.00154016	ncRNA
LOC107986185	107986185	XR_001741392.1	Uncharacterized LOC107986185	513	up	3.9532552	4.36E-05	ncRNA
LOC107986821	107986821	XR_001745275.1	Uncharacterized LOC107986821, transcript variant X1	3257	up	3.8777435	0.0174942	ncRNA
LOC105373592	105373592	XR_001739685.1	Uncharacterized LOC105373592, transcript variant X2	877	up	3.8124175	0.00616176	ncRNA

A – PBM, B – controls, FC (abs) – absolute value of the fold change.

Material and methods

Material

The study protocol was approved by the Ethics Committee of our hospital and complied with the Helsinki Declaration. Written informed consent was obtained from the guardian of each subject before surgery. A total of 15 pediatric subjects with PBM and 15 control subjects were included in the study between January 2017 and September 2020. In all patients, diagnosis was confirmed by imaging and surgical pathological examination. PBM was diagnosed based on the following criteria: (1) the union of the pancreatic and biliary duct was located outside the sphincter of Oddi, based on magnetic resonance cholangiopancreatography (MRCP) or intraoperative cholangiography (IOC); (2) the common duct was longer than 5 mm; (3) the biliary amylase level was greater than 1000 U/l [23].

RNA microarray analysis

Microarray experiments to investigate lncRNAs and messenger RNA (mRNAs) differentially expressed between the PBM and control groups were performed by Western SCI Biotech Company (Chongqing, China, www.westernsci.cn). Agilent Human lncRNA Microarray 2018 Version (4*180k, Design ID: 085630), Agilent Technologies, Inc., was used.

The common bile duct tissue from PBM subjects was collected and stored at –80°C before RNA extraction. Total RNA was isolated using TRIZOL (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and quantified using the NanoDrop ND-2000 (Thermo Scientific). RNA integrity was assessed using the Agilent Bioanalyzer 2100. Sample labeling, microarray hybridization, and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA was transcribed to double-stranded cDNA, then synthesized into cRNA and labeled with cyanine-3-CTP. The labeled cRNAs were then hybridized onto the microarray. After washing, the arrays were scanned using the Agilent Scanner G2505C (Agilent Technologies).

Array images were analyzed using Feature Extraction software (version 10.7.1.1, Agilent Technologies), and the raw data were further processed using Genespring (version 13.1, Agilent Technologies). First, the raw data were normalized using the quantile algorithm. The probes that had flags in "P" in at least 1 out of 2 conditions were chosen for further analysis. Differentially expressed mRNAs and lncRNAs were defined as those whose expression differed by ≥ 2.0-fold between PBM and control groups ($p < 0.05$). The potential roles of these differentially expressed

mRNAs and lncRNAs were explored based on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (see section 2.3). Finally, the differentially expressed genes were organized using hierarchical clustering.

Prediction of lncRNA function

Following the approach in a previous study [24], we examined differentially expressed mRNAs for enrichment of KEGG functional pathways, then we attributed the same functional pathways to lncRNAs that were co-expressed with those mRNAs. Co-expression of mRNAs and lncRNAs was defined as a Pearson correlation p-value < 0.05.

The enrichment of functional terms was analyzed using a hypergeometric cumulative distribution function, and the 200 and 500 most reliable predicted relationships were displayed in order to understand the distribution of functions of differentially expressed lncRNAs.

Fine mapping of co-expression of lncRNAs and adjacent coding genes

For each differentially expressed lncRNA, associated "*cis*-regulated mRNAs" were identified based on the following criteria: (1) the loci encoding the mRNAs were within 300 kb up- or downstream of the given lncRNA; and (2) the Pearson correlation coefficient for lncRNA-mRNA co-expression was significant ($p \leq 0.05$). The co-expressed genes and enrichment significance for differentially expressed genes in each transcription factor (TF) entry were calculated for each differentially expressed lncRNA, using the hypergeometric distribution test. The calculated results return a p-value of enrichment significance, where a small p-value indicates that the gene is enriched.

The intersection between the coding gene set co-expressed by lncRNAs and the target gene set of the TF/chromatin regulatory complex was calculated, and the enrichment degree of the intersection was calculated using a hypergeometric distribution test. This calculation identified the TFs significantly related to differentially expressed lncRNAs, and thereby the TFs and chromatin regulatory factors that may regulate those lncRNAs. The network diagram was visualized based on the results of the hypergeometric distribution analysis.

According to hypergeometric distribution calculations, multiple lncRNA-TF pairs were obtained for each differentially expressed lncRNA. Each lncRNA-TF pair was the result of multiple gene enrichment.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay

In order to verify the microarray results, we randomly selected six differentially expressed lncRNAs that were associated with carcinogenesis or chronic inflammation in the common bile duct. We measured the lncRNA expression levels of these genes using qRT-PCR and the SYBR Green PCR Kit (Applied BI) according to the manufacturer's instructions. Expression levels were calculated using the $2^{-\Delta\Delta}$Ct method and normalized to those of β-actin lncRNA.

Statistical analysis

SPSS 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Data were expressed as mean ± standard deviation (SD), and inter-group differences were assessed for significance using the rank sum test. The diagnostic accuracy of certain differentially expressed lncRNAs was assessed in terms of the area under the receiver operating characteristic curve (AUC). Statistical significance was defined as $p < 0.05$.

Results

General information

Patients' demographic and clinical data are summarized in Table I. There was no significant difference between the two groups in body weight, age, or sex distribution.

Differential gene expression between PBM and control subjects

The microarray analysis identified 2915 upregulated mRNAs, 2121 downregulated mRNAs, 173 upregulated lncRNAs, and 316 downregulated lncRNAs in subjects with PBM compared to healthy controls. Principal component analysis of differentially expressed lncRNAs and a heatmap are presented in Figure 1, while the 20 most up- or downregulated lncRNAs are presented in Table II. The most highly upregulated lncRNA was LOC105378608, which showed a 14.39-fold difference from controls.

Functional analysis of differentially expressed genes

The most enriched GO categories associated with the differentially expressed transcripts were extracellular matrix (p = 2.9E-12, up-regulated), extracellular region (p = 0.000427, up-regulated), and kinetochore (p = 2.29E-8, up-regulated) (Figure 2). The most enriched KEGG pathway was protein digestion and absorption, in which 250 differentially expressed genes were involved. Some pathways were associated with cancer, such as pathways in cancer (associated with 11 genes) and the PI3K-Akt signaling pathway (associated with 15 genes, Figure 3).

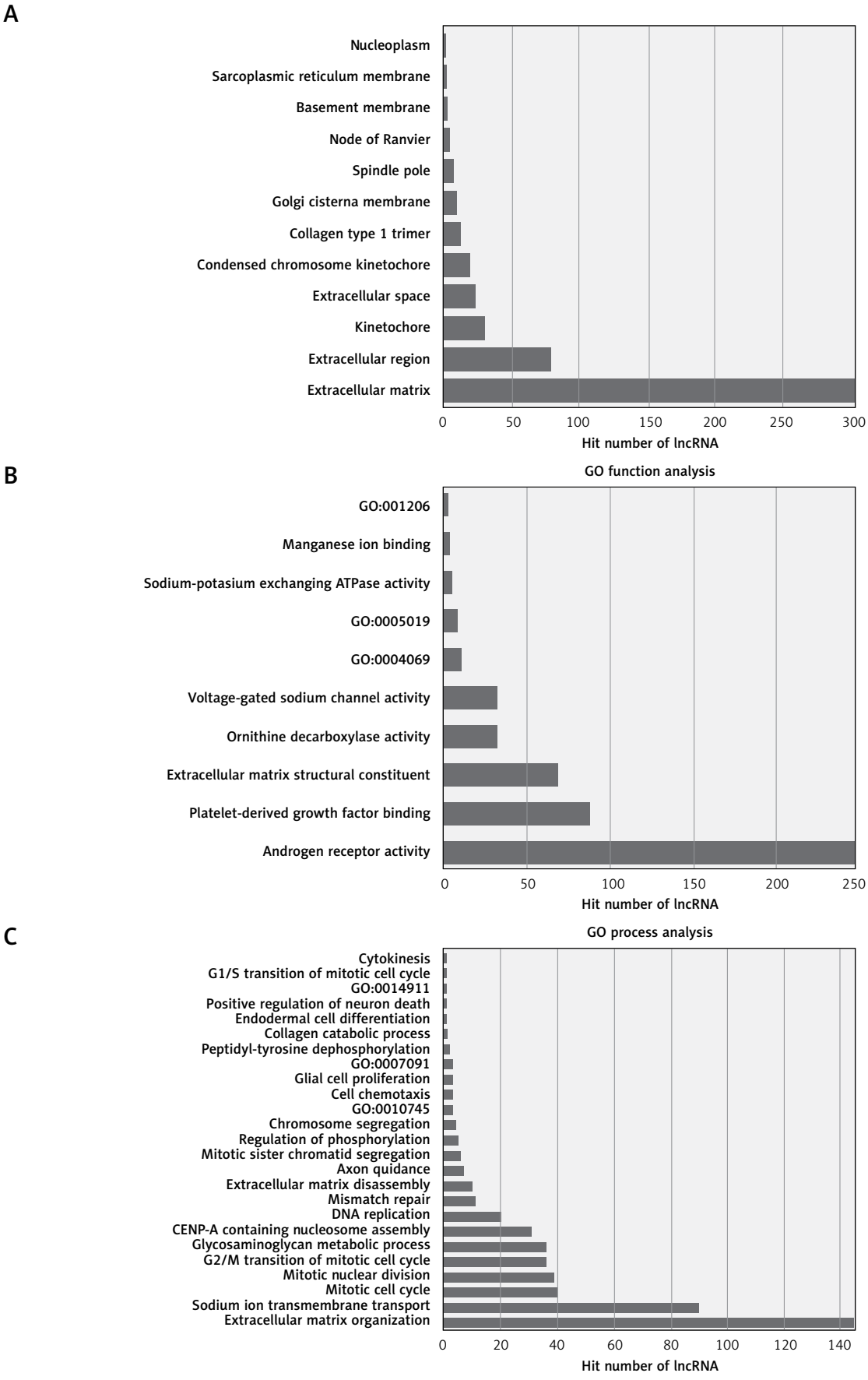

Figure 2. Functional analysis of differentially expressed genes based on Gene Ontology (GO) enrichment analysis

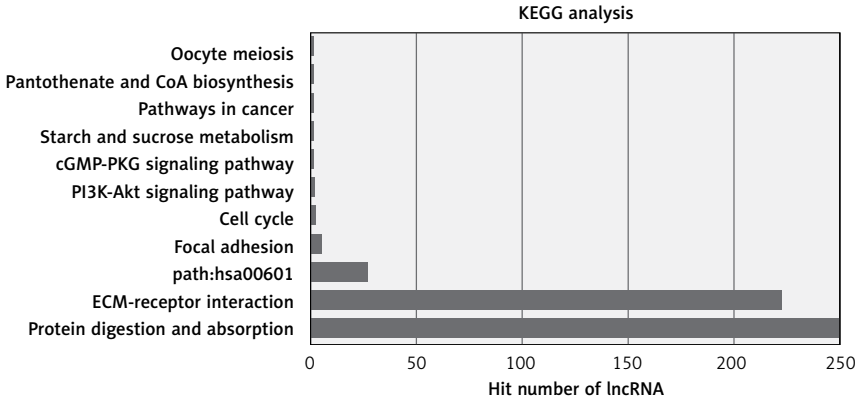

Figure 3. Analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment in differentially expressed lncRNAs

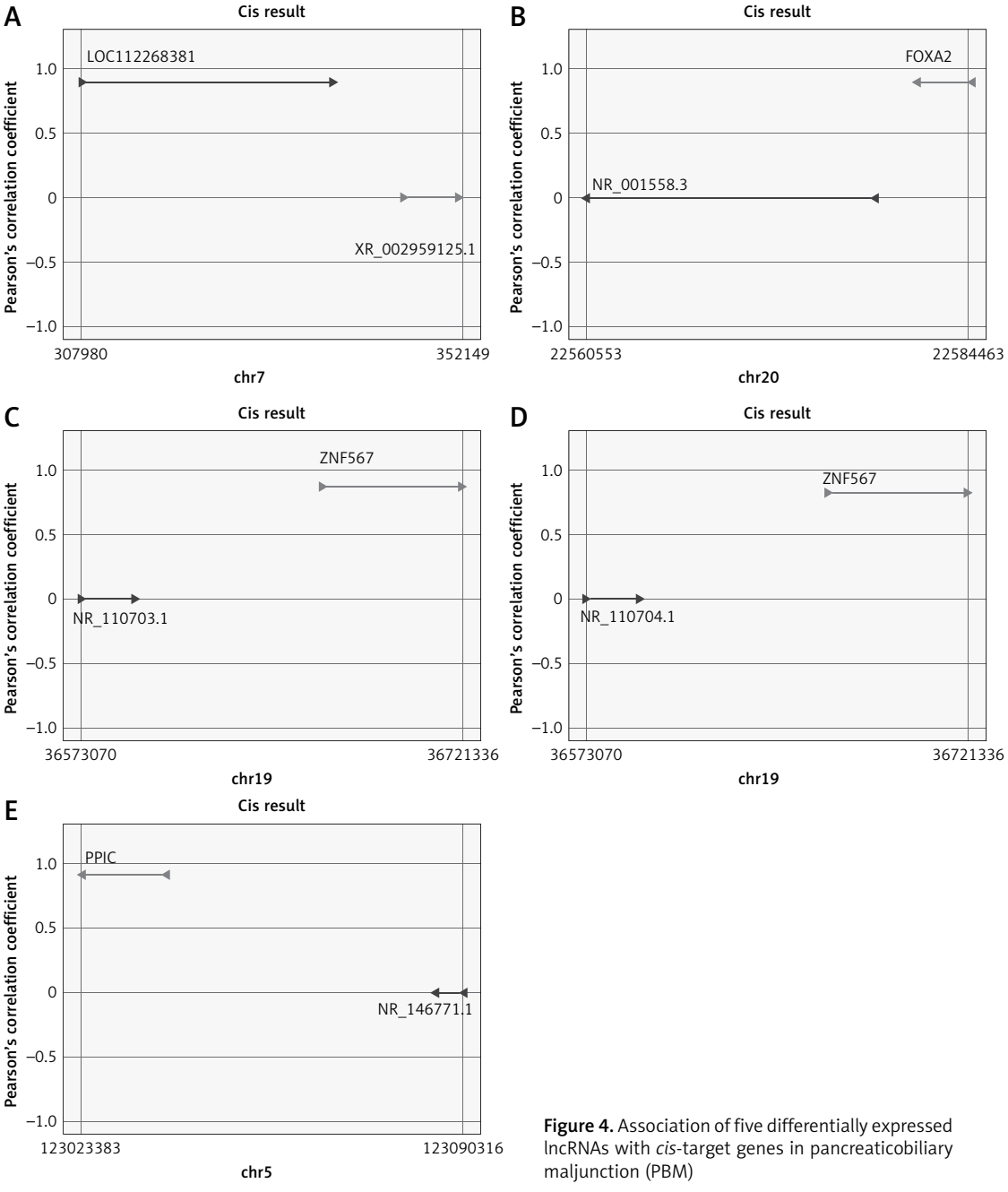

Figure 4. Association of five differentially expressed lncRNAs with *cis*-target genes in pancreaticobiliary maljunction (PBM)

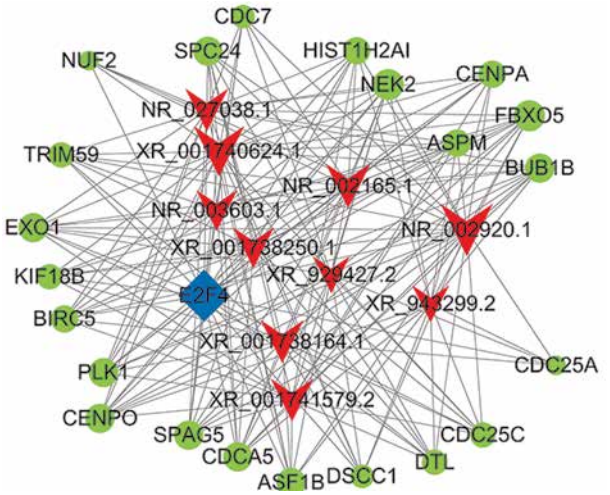

Figure 5. Prediction of *trans*-target for differentially expressed lncRNAs in PBM

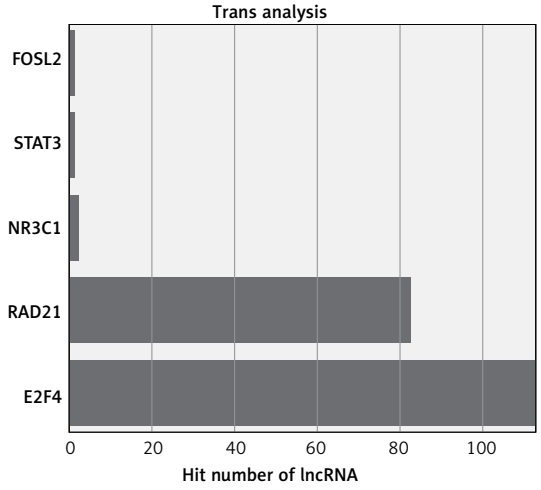

Figure 6. Functional distribution of differentially expressed lncRNAs based on transcription factor analysis

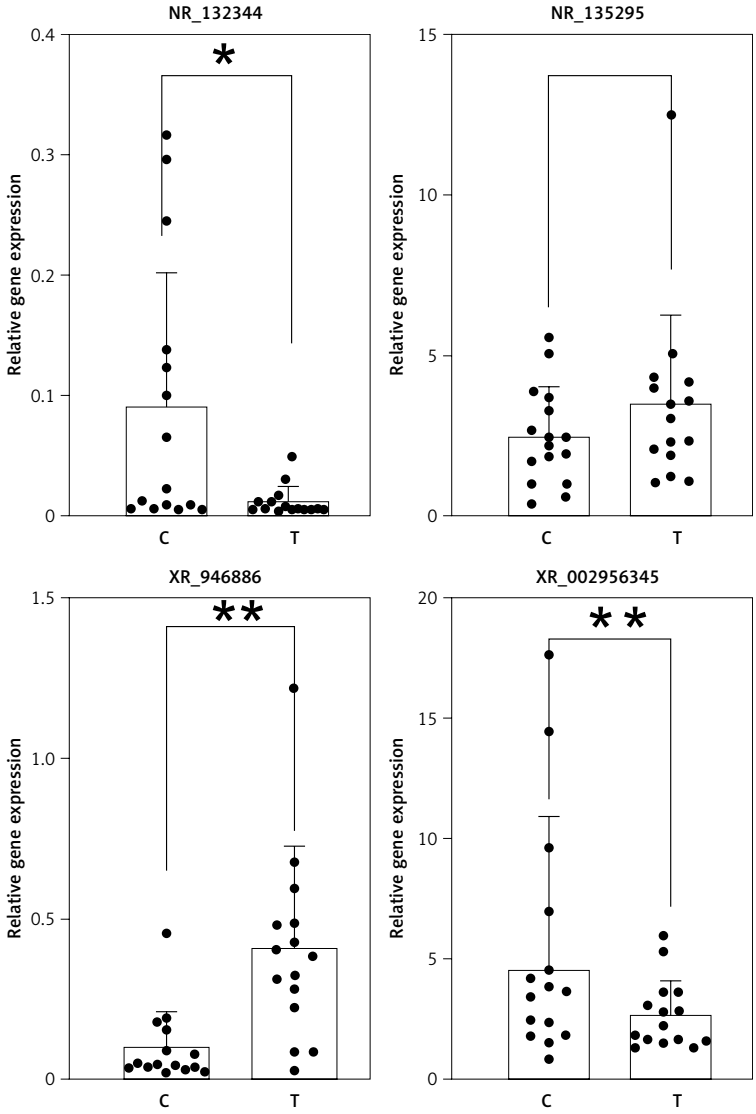

Figure 7. Validation of microarray results by qPCR

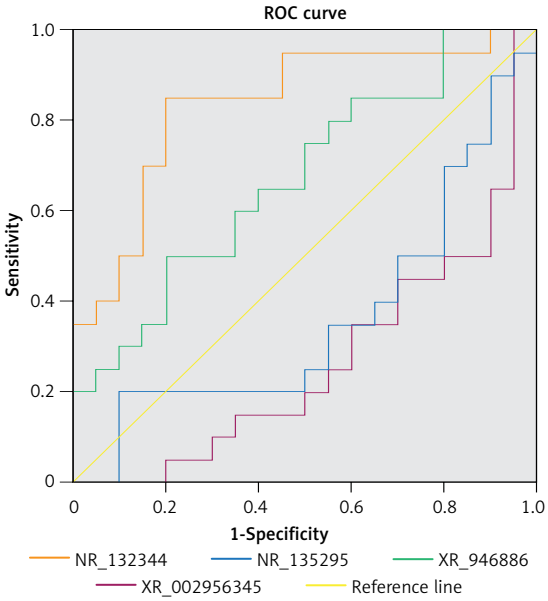

Figure 8. Receiver operating characteristic curves to assess the ability of *NR_110876*, *NR_132344*, *XR_946886*, and *XR_002956345* to predict pancreaticobiliary maljunction

Co-expression analysis and target prediction

The functions of lncRNAs are performed by interacting with their targets. We predicted both *cis*- and *trans*-acting mRNAs that may interact with differentially expressed lncRNAs. The *cis* analysis searched for mRNAs 300 kb up- or downstream of the lncRNAs, and it identified 5 lncRNAs related to those *cis* target genes (Figure 4). The *trans* analysis of lncRNAs was performed by constructing co-expression networks of dysregulated mRNAs and lncRNAs based on Pearson expression correlation (Figure 5). Most of the differentially expressed lncRNAs acted in a *trans* manner. The top 200 predicted relationships with the highest prediction reliability were used to determine which TFs occurred most frequently, and the functions of these TFs were then attributed to the differentially expressed lncRNAs (Figure 6). Among the 964 differentially expressed lncRNAs and the predicted 9358 mRNA targets, more than one mRNAs were predicted to be regulated by one lncRNA and one mRNA corresponded to several lncRNAs.

Validation by qRT-PCR

Six up-regulated lncRNAs associated with carcinogenesis or chronic inflammation in the common bile duct were verified by qRT-PCR: *NR_110876*, *NR_132344*, *XR_946886*, *XR_002956345*, *NR_135295*, and *XR_002957935*. The expression differences between patients and controls for *NR_110876*, *NR_132344*, *XR_946886*, and *XR_002956345* were consistent with the microarray results, and the difference was statistically significant for *NR_132344*, *XR_946886*, and *XR_002956345*. However, the qRT-PCR results for *NR_135295* and *XR_002957935* were inconsistent with the microarray results (Figure 7).

To assess the potential of *NR_110876*, *NR_132344*, *XR_946886*, and *XR_002956345* as PBM biomarkers, we examined their ability to predict PBM in our sample. Only *XR_946886* gave a significant AUC (0.837, $p < 0.001$) (Figure 8).

Discussion

Here we identified several lncRNAs that are differentially regulated in PBM, and we explored their potential biological functions.

Congenital dilation of the common bile duct has been shown to involve numerous alterations in the transcriptome, and PBM has been associated with downregulation of several genes in the gallbladder, as well as upregulation of some noncoding RNAs, which may contribute to biliary carcinogenesis [25]. In our previous study [26], we found 876 differentially expressed genes in children with PBM, of which 530 were up-regulated and 346 were down-regulated. Wong *et al.* reported on patients with congenital dilation of the common bile duct, human developmental disorders, cholangiocarcinoma or hepatocellular carcinoma [27]. However, we are unaware of investigations of lncRNAs in PBM.

In the present study, we identified 489 lncRNAs and 5036 mRNAs in bile duct tissues that were differentially expressed in PBM. These differentially expressed lncRNAs may affect many pathways, including those involved in protein digestion and absorption, interactions between extracellular matrix and surface receptors, focal adhesion, PI3K-Akt signaling, carcinogenesis, and inflammation. These predictions are consistent with previous studies [22, 24, 25]. In this way, our study implicates lncRNAs in PBM-associated chronic inflammation and carcinogenesis in the common bile duct.

We explored the potential functions of differentially expressed lncRNAs based on the functions of their target genes. We found that 5 *cis*-target genes were related to differentially expressed lncRNAs, and that most lncRNAs acted in a *trans* manner. For example, we found that one lncRNA (NR_027038.1) was closely associated with E2F4, which is consistent with previous reports linking E2F4 to development and progression of cancers [28, 29].

Of the many lncRNAs that we identified as differentially expressed, validation of a subset using qRT-PCR showed that not all microarray results were accurate. Nevertheless, we were able to validate upregulation of *NR_110876*, *NR_132344*, *XR_946886*, and *XR_002956345*. Among these

lncRNAs, *XR_946886* showed significant diagnostic potential. Thus, this lncRNA seems likely to be involved in the development of PBM complications, and it may serve as a novel biomarker for carcinogenesis or chronic inflammation involving the common bile duct [30].

This is the first study focused on lncRNAs in the common bile duct of PBM. Our findings may provide a basis for developing novel diagnostic and therapeutic targets in PBM. There were some limitations to the present study. The sample was relatively small, and only six lncRNAs identified in microarray experiments were validated using qRT-PCR. Future studies should be conducted with larger samples and more detailed validation and analysis of candidate genes. Future studies should also explore the potential mechanism of *XR_946886* in PBM and its clinical potential as a biomarker.

In conclusion, the present study provided an overall analysis of lncRNAs in the common bile duct in PBM. By further exploring potential functions and pathways in which differentially expressed lncRNAs are involved, we anticipate that lncRNAs such as *XR_946886* will be validated as biomarkers or even potential therapeutic targets for PBM treatment.

Acknowledgments

Lian Zhao and San-Li Shi are co-first authors, they contributed equally to this work.

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Conflict of interest

The authors declare no conflict of interest.

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