Microarray expression profile analysis of long non-coding RNAs in pancreatic ductal adenocarcinoma

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Abstract. Long non-coding RNA (lncRNA) is a variety of the human transcriptome that does not code for proteins and plays an important role in the development and progression of multiple solid malignant tumors. However, the roles of lncRNAs in the development of pancreatic ductal adenocarcinoma (PDAC) remain unknown. In this study, we investigated the expression patterns of lncRNAs in three PDAC tumor samples (T) relative to those of matched adjacent non-tumor tissues (N) via a microarray with 30,586 lncRNA probes and 26,109 mRNA probes. The lncRNA microarray revealed 27,279 lncRNAs in PDAC samples, of which 2,331 were significantly upregulated (P<0.05; T/N>2.0) and 1,641 were downregulated (P<0.05; N/T>2.0) compared with matched adjacent non-tumor samples. In addition, 19,995 mRNAs were detected, of which 1,676 were significantly upregulated (P<0.05; T/N>2.0) and 1,981 were downregulated (P<0.05; N/T>2.0). Pathway analysis indicated that 41 pathways corresponded to upregulated transcripts and 25 pathways corresponded to downregulated transcripts (P-value cut-off is 0.05). Gene ontology (GO) analysis showed that the highest enriched GOs targeted by upregulated and downregulated transcripts were tissue homeostasis. The validation results from quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis and microarray analysis were consistent. Furthermore, the expression level of long intergenic non-coding RNA HOTAIRM1 was upregulated in 12 PDAC tissues samples compared with matched adjacent non-tumor samples by qRT-PCR. The results showed that the lncRNA and mRNA expression profiles differed significantly between the PDAC tissues and their adjacent non-tumor tissues, and the revelation of an association between HOTAIRM1 expression and PDAC is especially noteworthy. These findings may provide new potential molecular markers for diagnosis and treatment of PDAC.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC), a common digestive system cancer, is highly malignant and has a poor disease outcome. Despite the progress in the understanding of the molecular and genetic basis of this disease, the 5-year survival rate has remained low and usually does not exceed 5%. Only 20-25% of the patients present with potentially resectable disease, and surgery represents the only chance for a cure (1,2). PDAC is considered as a systemic disease because of the high rate of relapse after curative surgery in patients with resectable disease at diagnosis. Enormous efforts have been made to identify the special molecular markers for PDAC, which show vast application prospect as targets for the disease treatment. The research fields of molecular markers for PDAC include proteins, such as K-Ras, p16, and SMAD4 (3-5); miRNAs, such as miR-210 and miR-221 (6-8); and the recently research hotspot the lncRNAs.

Long non-coding RNAs (lncRNAs) refer to a group of RNAs that are usually more than 200 nucleotides and are not involved in protein generation (9). Recent studies have begun to associate subsets of lncRNAs to specific regulatory mechanisms of important biological processes, including cell proliferation, survival, differentiation, and chromatin remodeling both in *cis* and in *trans* (10-19). Many functional lncRNAs have been shown to play key roles in organ development and cancer. Some lncRNAs act as tumor suppressor;

others participate in cellular replicative immortality, or even regulate angiogenesis and metastasis (20,21). Previous studies have reported several lncRNA, such as HOTAIR, MALAT1 and PVT1 (22-24), which revealed the significance of lncRNAs in the regulation of multiple biological processes at different levels that may served as molecular markers for several cancer. However, the roles of lncRNAs in the progression of PDAC remain not well identified.

To evaluate the expression profile and identify the special lncRNAs in PDAC, we interrogated the differentially expression profiles of lncRNAs and mRNAs between 3 PDAC samples and their matched adjacent non-tumor samples via microarray. Gene ontology (GO) analysis, pathway analysis and network analysis was done for further investigation. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to validate several random upregulated and downregulated lncRNAs in the 3 PDAC tissues. Further, HOTAIRM1, one of thousands of deregulated lncRNAs we identified, was further evaluated in 12 pairs of matched tumor /non-tumor (T/N) tissues via qRT-PCR. This study uncovers the aberrant expression of lncRNAs in PDAC tissues, and may contribute to understanding of the mechanism of PDAC progression and provide new potential molecular markers for diagnosis and treatment of PDAC.

Materials and methods

Patients and tissue samples. A total of twelve PDAC tissue samples and their matched adjacent non-tumor samples were obtained with informed consent from PDAC patients at Department of Surgery, Sichuan Provincial People's Hospital. The diagnosis of all patients was confirmed based on the WHO classification and staged according to the tumor node metastasis classification and were reviewed by two pathologists. Clinical parameters were recorded for each sample, included age, gender, location of tumor, vascular permeation, TNM stage and differentiation. Samples were taken during surgery, immediately frozen in liquid nitrogen, and stored at -80˚C for further analysis. Paired tumor and non-tumor tissues from three PDAC patients were used for the microarray assay. Twelve paired PDAC tissues (not including the 3 paired tissues used for microarray) were used for the qRT-PCR validation assay. The analysis of human tissues were approved by the Human Research Ethics Committee of Sichuan Provincial People's Hospital, and all PDAC patients gave written informed consent for the use of clinical samples for medical research.

RNA isolation. Total RNA was isolated from the 15 PDAC tissues and paired non-tumor tissues using TRIzol reagent (Invitrogen, CA, USA), and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop, DE, USA). The integrity of RNA was assessed by standard denaturing agarose gel electrophoresis, and the purity was estimated by the ratio of absorbance at 260-280 nm.

Microarray. Arraystar Human LncRNA Microarray V3.0 is designed for the global profiling of human lncRNAs and protein-coding transcripts, which is updated from the previous Microarray V2.0. Approximately 30,586 lncRNAs and 26,109 coding transcripts can be detected by our third-generation lncRNA microarray. The lncRNAs are carefully constructed using the most highly respected public transcriptome databases (including Refseq, UCSC known genes, and Gencode), as well as landmark publications. Each transcript is represented by a specific exon or splice junction probe, which can identify individual transcript accurately. Positive probes for housekeeping genes and negative probes are also printed onto the array for hybridization quality control.

RNA labeling and array hybridization. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY™ Eukaryotic mRNA Isolation kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3' bias utilizing a random priming method (Arraystar Flash RNA Labeling kit, Arraystar). Each labeled cRNA (1 μg) was fragmented by adding 5 μl 10X blocking agent and 1 μl of 25X fragmentation buffer, then heated the mixture at 60˚C for 30 min, finally 25 μl 2X GE hybridization buffer was added to dilute the labeled cRNA. Hybridization solution (50 μl) was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated for 17 h at 65˚C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed and scanned with using the Agilent DNA Microarray Scanner (part number G2505C).

Data analysis. Data analysis were performed by KangChen Biotech (Shanghai, China). Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed with using the Gene Spring GX v12.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs of ≥3 out of 6 samples have flags in Present or Marginal (All Targets Value) were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs with statistical significance between the two groups were identified through P-value/FDR filtering. Differentially expressed lncRNAs and mRNAs between the two samples were identified through fold-change filtering. Hierarchical clustering and combined analysis were performed using in-house scripts.

Quantitative real-time PCR (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen) and then reverse transcribed using PrimeScript® RT Reagent kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian, China) according to the manufacturer's instructions. The expression levels of seven upregulated and seven downregulated lncRNAs in the 3 patients included in the microarray study were measured by qRT-PCR using SYBR Green assays (Takara). The expression levels of HOTAIRM1 in twelve PDAC specimens and their paired adjacent non-cancerous tissues were also measured by qRT-PCR. The lncRNA expression differences between the matched cancer and non-cancerous samples were analyzed using Student's paired t-test with the IBM SPSS Statistics

Table I. Clinical parameter of 15 PDAC patients.

Sample nos.	Age (years)	Gender	Location of tumor	Vascular permeation	TNM stage	Differentiation
1	46	Male	Head	Present	T2N1M0	Poorly
2	53	Female	Body and tail	Absent	T3N1M0	Moderately
3	67	Female	Head	Absent	T2N0M0	Poorly
4	72	Male	Head	Absent	T3N1M0	Moderately
5	61	Male	Body and tail	Present	T1N0M0	Moderately
6	42	Female	Body and tail	Absent	T2N1M0	Poorly
7	55	Male	Head	Absent	T1N0M0	Well
8	56	Male	Head	Absent	T2N0M0	Well
9	75	Male	Head	Absent	T4N1M0	Moderately
10	52	Female	Head	Absent	T2N0M0	Poorly
11	60	Male	Head	Absent	T1N1M0	Moderately
12	65	Male	Body and tail	Absent	T3N1M0	Well
13	71	Female	Head	Present	T2N1M0	Poorly
14	52	Male	Body and tail	Absent	T1N0M0	Moderately
15	49	Male	Body and tail	Present	T3N1M0	Poorly

version 20.0 (IBM Corp., New York, NY, USA). A probability value of $P<0.05$ was considered statistically significant.

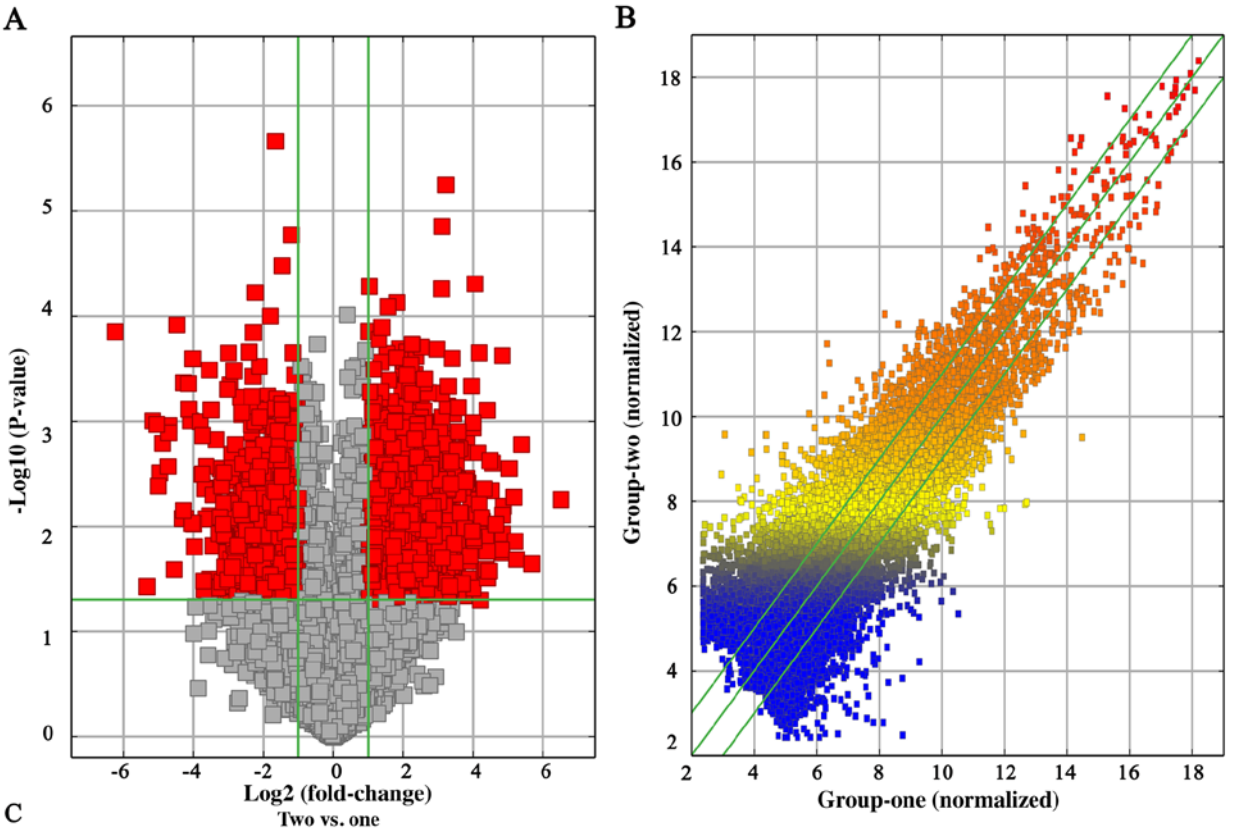
Results

Differentially expressed lncRNAs in PDAC. The clinical parameters of all patients are shown in Table I. The lncRNA expression profile data from the microarray analysis contained a total of 21,558 lncRNAs that were expressed in PDAC tissue samples. To determine the relationships among the specimens, hierarchical clustering analysis was used to group the specimens according to their expression levels (data not shown). Volcano Plots and the scatterplot of lncRNA expression profile are useful for assessing the variation or reproducibility (Fig. 1A and B). We identified hundreds of significantly differentiated lncRNAs (fold change ≥2.0, P≤0.05) between 3 human PDAC tissue samples and the matched adjacent non-tumor samples. In total, there were 2,331 upregulated lncRNAs and 1,641 downregulated lncRNAs found in the 3 PDAC patients (Fig. 1C). Upregulated lncRNAs were more common than downregulated lncRNAs in our microarray data. Among these lncRNAs, ASHGA5P050875 (fold change, 91.4095293) was the most upregulated lncRNA, and ASHGA5P044551 (fold change, 75.9252755) was the most downregulated lncRNA.

Further analysis proceeded by classifying and stratifying the lncRNAs into subgroups. Subgroups such as antisense lncRNAs, enhancer lncRNAs and lincRNAs are thought to participate in numerous diseases such as cancers. We found 69 antisense RNAs, 82 enhancer RNAs and 147 lincRNAs were upregulated in PDAC samples, respectively, and 50 antisense RNAs, 70 enhancer RNAs and 236 lincRNAs were downregulated in the adjacent non-tumor samples, respectively (data not shown). The changes of lncRNAs subgroup between the PDAC samples and adjacent non-tumor samples play an important role in the regulation of PDAC tumor progression and we will focus on subgroup lncRNAs and their related mRNA in PDAC samples in our further study.

Differentially expressed mRNAs in PDAC. The mRNA expression profile data from the microarray analysis contained a total of 14,609 mRNAs that were expressed in the PDAC tissue samples. Volcano plots and the scatterplot of lncRNA expression profile are useful for assessing the variation or reproducibility (Fig. 2A and B). Among them, 1,676 mRNAs were significantly upregulated and 1,981 mRNAs downregulated (fold change ≥2.0, P≤0.05) in the PDAC samples (Fig. 2C). The most significantly deregulated mRNAs were ASHGA5P012017 (upregulated, fold change, 89.8272773) and ASHGA5P033632 (downregulated, fold change, 463.3570246).

GO analysis. Gene Ontology (GO) analysis was performed to determine the transcripts with terms under the biological process, cellular component, and molecular function ontology in this study. Fisher's exact test was applied to find if there were more overlap between the differentially expressed list and the GO annotation list than would be expected by chance. The P-values were used to estimate the significance of GO terms enrichment in the differentially expressed lncRNAs and mRNAs; the lower the P-value, the more significant the GO term (P-values ≤0.05 is recommended). We found that the highest enriched GO terms for the upregulated transcripts were purine nucleoside catabolic process (Fig. 3A; GO:0006152 under biological process, P=8.072E-06), cytoplasm (Fig. 3B; GO:0005737 under cellular component; P=1.158E-09), and protein binding (Fig. 3C; GO:0005515 under molecular function; P=1.014E-09). The most highly enriched GO terms targeted by the downregulated transcripts were establishment of localization (Fig. 3D, GO:0051234 under biological process; P=1.968E-05), cytoplasmic part (Fig. 3E, GO:0044444 under



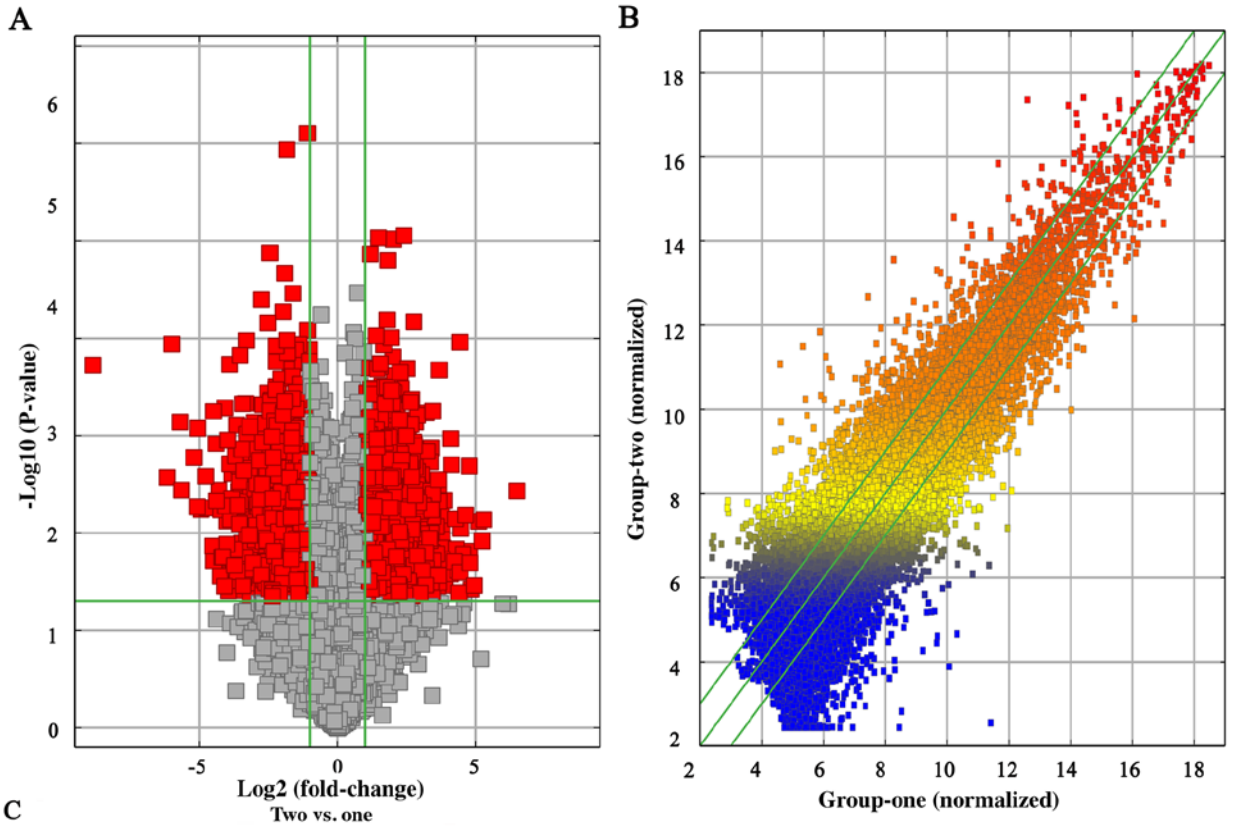
C

Upregulated lncRNAs			Downregulated lncRNAs		
LncRNA seqname	Log_2 fold change(T/N)	P-value	LncRNA seqname	Log_2 fold change(T/N)	P-value
NR_028050	91.4095293	0.005567934	ENST00000441160	75.9252755	0.000141016
ENST00000447424	51.216034	0.022582318	TCONS_00019731	40.4080819	0.037343693
uc002zis.1	41.8248975	0.00166534	NR_045023	35.8997577	0.000991419
ENST00000564464	37.9810627	0.020586736	NR_045028	33.151129	0.001070129
ENST00000563035	36.7143673	0.013827138	uc002hom.1	32.2843834	0.004138791
NR_028308	36.0925624	0.005239717	uc002nbr.3	31.3116436	0.003086069
NR_026860	32.6423134	0.002817089	ENST00000492250	29.6902198	0.001626726
NR_024065	29.0228059	0.006449719	NR_027170	26.488214	0.002693217
uc004aef.3	28.6639587	0.011700039	ENST00000366185	26.2631702	0.001278115
ENST00000507636	28.6279164	0.007792702	NR_027165	26.1238719	0.001097762
ENST00000555934	28.5138142	0.000238531	ENST00000472494	23.477497	0.025761484
ENST00000559977	28.2364119	0.017772394	NR_038940	22.2210271	0.000120804
NR_037856	27.9991637	0.016710478	ENST00000437289	20.0259216	0.0083617
ENST00000427458	25.194325	0.009553842	ENST00000483140	19.6134918	0.000429554
uc003hls.3	23.5852818	0.002516587	uc004dgk.1	19.5784842	0.007077379
ENST00000452320	22.67137	0.004580354	ENST00000413066	18.7889946	0.008380664
ENST00000448636	22.2903469	0.002363901	ENST00000420701	17.7344281	0.000438458
ENST00000567533	21.9287712	0.026622229	NR_045021	17.607198	0.000764117
ENST00000507761	21.3012157	0.030830215	NR_045025	16.9663097	0.000994916
NR_033925	21.1289011	0.0007845	ENST00000472293	16.5440365	0.009320852

Figure 1. (A) Volcano plots of lncRNA expression profile. The vertical lines correspond to 1.5 FC up and down and the horizontal line represents a P-value of 0.05. (B) The scatterplot of lncRNA expression profile, which is useful for assessing the variation (or reproducibility). (C) The top 20 differentially expressed lncRNAs determined by microarray.

cellular component; P=1.394E-09), and protein binding (Fig. 3F, GO:0005515 under molecular function; P=9.546E-09).

Pathway analysis. Pathway analysis indicated that 41 pathways corresponded to the upregulated transcripts (Fig. 4A).



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Upregulated lncRNAs			Downregulated lncRNAs		
mRNA seqname	Log_2 fold change(T/N)	P-value	mRNA seqname	Log_2 fold change(T/N)	P-value
NM_001161728	89.8272773	0.00370309	ENST00000283752	463.3570246	0.000188295
NM_001135865	39.1999316	0.007228058	NM_006761	71.8652785	0.002676106
NM_130464	37.71058	0.012033141	NM_023915	64.1878559	0.000114135
NM_183228	34.3627256	0.007570061	NM_014211	51.8000027	0.000722116
NM_019609	30.4993572	0.03460492	NM_018011	50.1143222	0.003635692
NM_005110	28.4424059	0.037769752	ENST00000372080	36.8932983	0.001672818
NM_212557	27.6440707	0.020302028	NM_000096	33.4949904	0.005368147
NM_001039567	27.2912897	0.015366135	NM_006815	33.2967101	0.000838544
NM_000088	27.1714597	0.002040782	NM_001129828	30.7716315	0.005736589
NM_024697	24.9220171	0.006512799	NM_001130716	27.2999531	0.002596408
ENST00000297307	23.3781203	0.016420116	ENST00000255030	26.5502216	0.005523653
NM_001114395	21.9485861	0.008960227	NM_005672	22.8292739	0.013605653
NM_000169	21.5510327	0.025232162	NM_001145718	22.539428	0.000562348
NM_002381	21.5025144	0.000109238	NM_004645	22.4669379	0.019047715
NM_002429	21.0519188	0.040629544	NM_001006600	20.9951225	0.001202305
NM_001135940	20.1778912	0.023854387	NM_013301	20.7773617	0.004618567
NM_012481	19.4723059	0.014513459	NM_001040100	19.5799368	0.003618937
NM_012261	19.0331982	0.007128184	NM_003400	19.0160782	0.027422151
NM_007281	18.5919201	0.019522239	NM_138720	18.7968138	0.005753083
ENST00000264360	17.2827302	0.001976335	ENST00000264218	18.336285	0.017638669

Figure 2. (A) Volcano plots of mRNA expression profile. The vertical lines correspond to 1.5 FC up and down and the horizontal line represents a P-value of 0.05. (B) The scatterplot of mRNA expression profile, which is useful for assessing the variation (or reproducibility). (C) The top 20 differentially expressed mRNAs determined by microarray.

The most enriched network was 'B cell receptor signaling pathway (human)' (Fisher P=9.08198E-06, Fig. 4C) with 18 transcripts annotated with this term. Twenty-five pathways corresponded to the downregulated transcripts (Fig. 4B) and

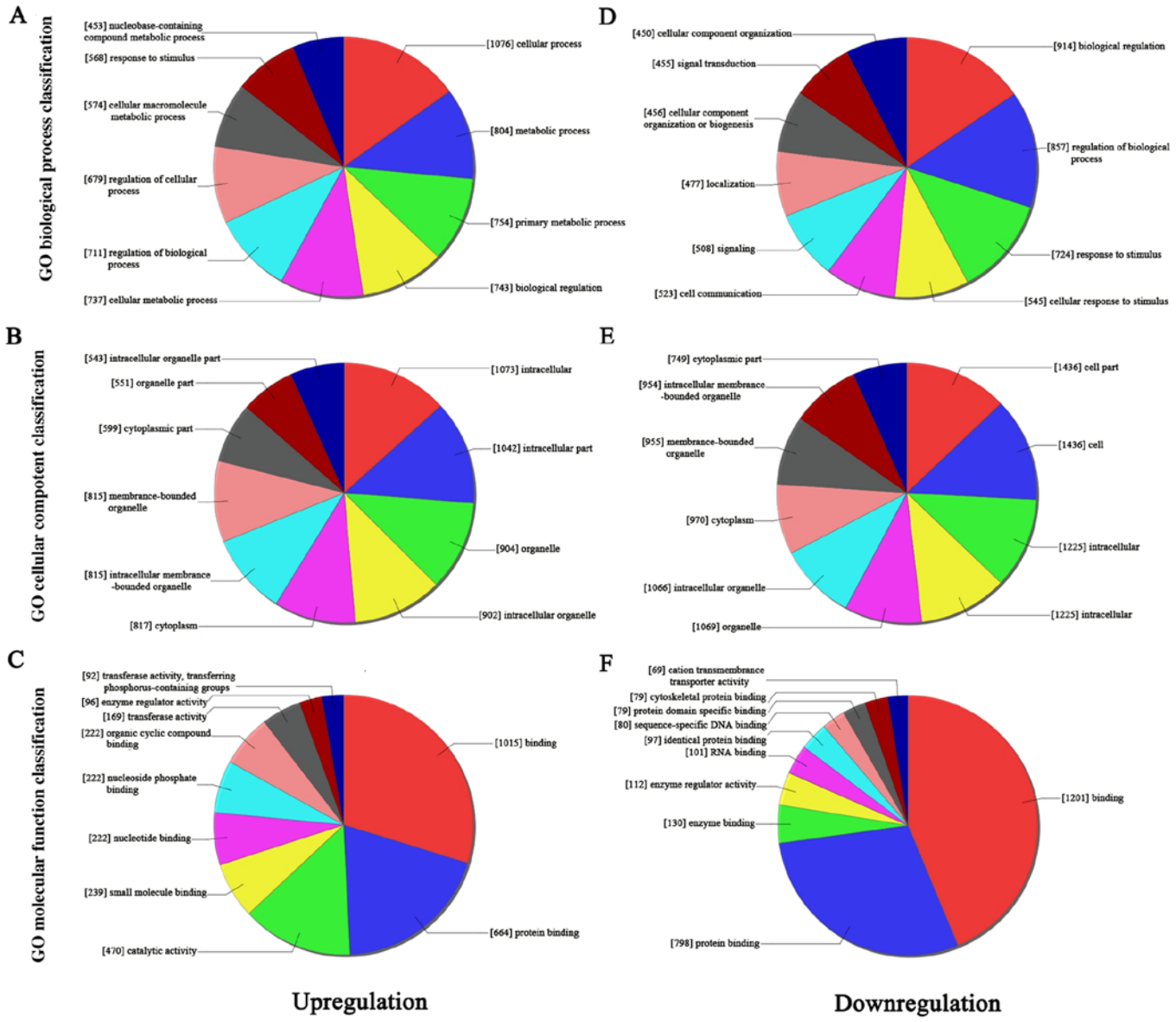

Figure 3. The most highly enriched GO terms for the differentially expressed transcripts. (A-C) Most highly enriched GO terms for the upregulated transcripts. (A) Biological process (BP); (B) Cellular component (CC); (C) Molecular function (MF). (D-F) Most highly enriched GO terms for downregulated transcripts: (D) Biological process (BP); (E) Cellular component (CC); (F) Molecular function (MF).

the most enriched network was 'Pertussis-*Homo sapiens* (human)' (Fisher P=0.0002931875, Fig. 5) with 18 transcripts annotated with this term. P-values ≤0.05 were taken as the cut-off. Among these pathways, the gene category 'Wnt signaling pathway', has been reported to be involved in metastasis of pancreatic carcinogenesis (25), and the gene category 'MAPK signaling pathway' has been shown to participate in the progression of pancreatic cancer though multiple mechanisms (26-28). The gene categories 'FoxO signaling pathway' have been reported to suppress or activate pancreatic cancer progression by different drugs or compound (29,30). The gene categories 'Ubiquitin mediated proteolysis' participate in pancreatic cancer cell growth *in vitro* and *in vivo* (31).

Quantitative real-time PCR validation. We used qRT-PCR to validate the expression levels of the altered lncRNAs in the PDAC patients. We randomly selected ten upregulated lncRNAs and five downregulated lncRNAs among the differentially expressed lncRNAs. We found that ASHGA5P022276, ASHGA5P029774, ASHGA5P028603, ASHGA5P014632, ASHGA5P043753, ASHGA5P036884, ASHGA5P016768, ASHGA5P032173, ASHGA5P051732 and ASHGA5P014130 were upregulated, and ASHGA5P055771, ASHGA5P044524, ASHGA5P039672, ASHGA5P017734 and ASHGA5P018902 were downregulated in the PDAC samples compared with adjacent non-tumor samples. Thus, the results from the qRT-PCR analysis and the microarray data analysis were consistent (P<0.05; Fig. 6A and B).

Moreover, we found a significant increase of the expression level of HOTAIRM1 (fold change, 6.9263288, P=0.00282) in PDAC samples compared with adjacent non-tumor samples via microarray analysis. To examine whether upregulated expression of HOTAIRM1 is pathologically specific, a total of 12 PDAC samples and matched adjacent non-tumor samples

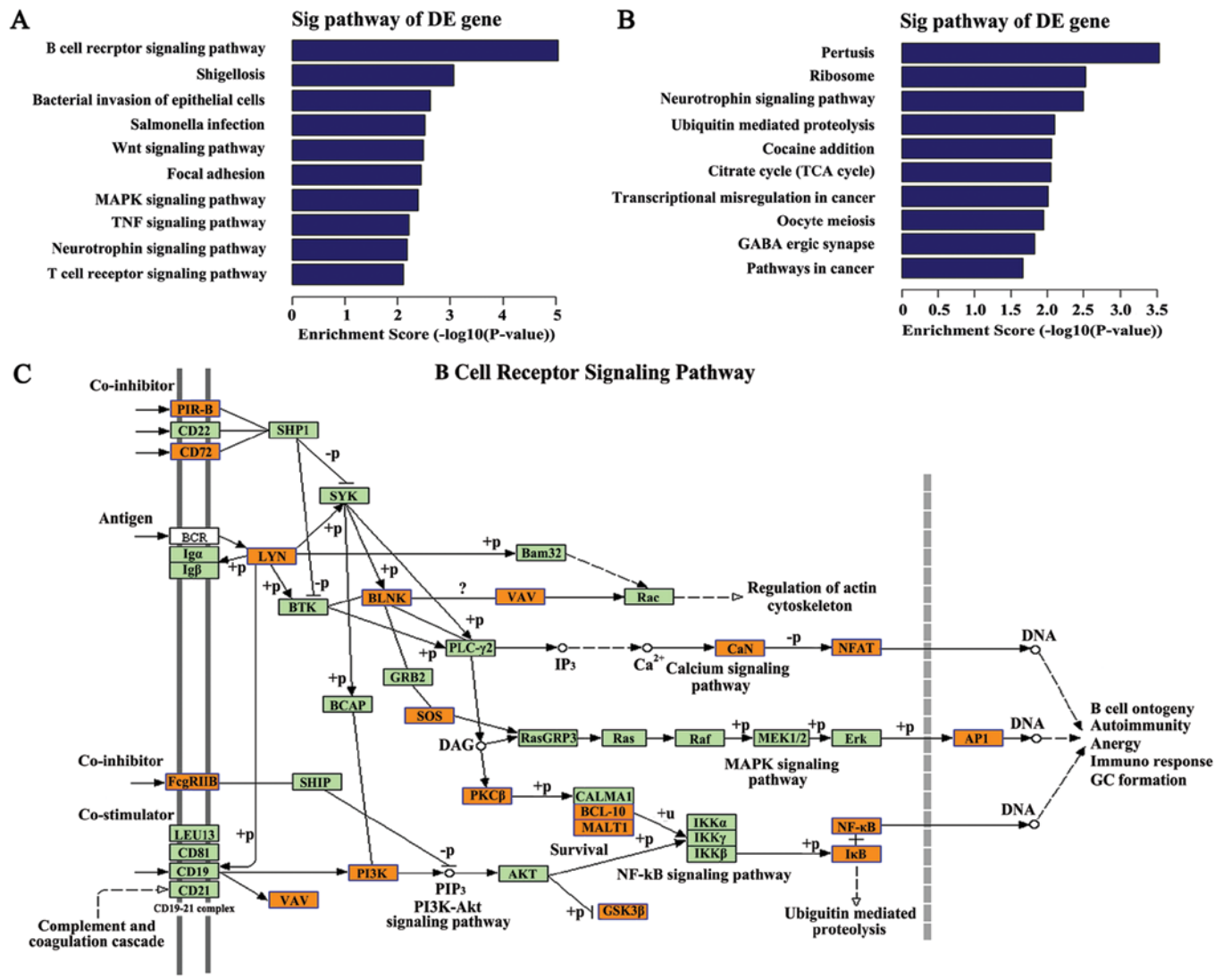

Figure 4. Pathway analysis for the differentially expressed transcripts and schematic diagrams of two gene categories. (A) Pathways corresponding to the upregulated transcripts. (B) Pathways corresponding to downregulated transcripts. (C) Schematic diagram of the gene category 'B cell receptor-signaling pathway'.

were subjected to qRT-PCR. The level of HOTAIRM1 expression was 2.92-8.53-fold higher in PDAC samples than the mean level in matched adjacent non-tumor samples (Fig. 6C). However, the sample size of this study is limited and we will further collect more samples and investigate the function of HOTAIRM1 in PDAC.

Discussion

In this study, we used a microarray to test the lncRNAs expression profiles in PDAC tissues. The lncRNA expression profiling data showed that there were lncRNAs that were differentially expressed between the PDAC tissues and matched adjacent non-tumor tissues. Previous studies showed that dysregulation of lncRNAs expression such as HOTAIR (32,33), HULC (34-36) and GAS5 (37,38), is a potential molecular marker for diagnostic and therapeutic purposes in several human cancers. There are still lncRNAs as potential novel candidate molecular markers for clinical diagnosis and therapy of PDAC that need to be further identified.

Although special lncRNAs as molecular markers in other digestive tumors, such as hepatocellular carcinoma and gastric cancer (39,40) have been reported, there is no direct evidence shown that special lncRNAs are molecular markers for PDAC. Moreover, several lncRNAs have been reported to be significantly correlated with PDAC outcome and are involved in cancer progression. HOTAIR is a negative prognostic factor for breast, colon and liver cancer patient survival, and increased HOTAIR expression in patients has been correlated with enhanced breast and colon cancer metastasis (41-45). Kyounghyun *et al* (46) showed that HOTAIR expression was increased markedly in pancreatic tumors compared to non-tumor tissues, and was associated with more aggressive tumors. MALAT1 (47,48), also known as nuclear-enriched abundant transcript 2 (NEAT2), regulates gene expression and post-transcriptionally modifies primary transcripts and is found to be upregulated in a variety of human cancers of the breast, prostate, colon, liver, and uterus (49). Recently, MALAT1 mRNA level was found significantly higher in PADC tissues and some PC cell lines. A high expression of MALAT1 was detected in PDAC tumors

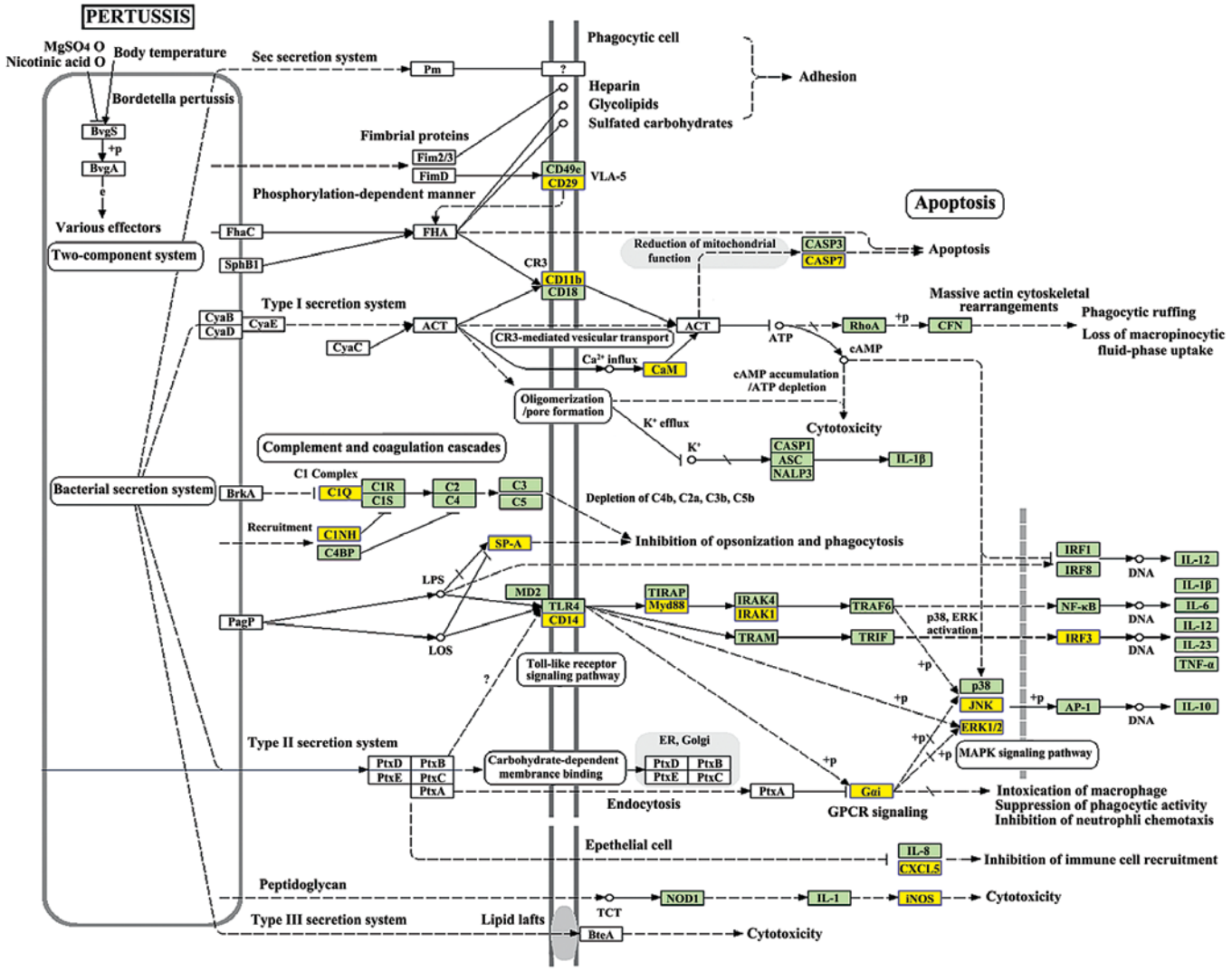

Figure 5. Schematic diagram of the gene category 'Pertussis-*Homo sapiens*".

of larger size, advanced tumor stage and deeper invasion. In addition, the overexpression of MALAT1 was associated with poor prognosis of PDAC patients (50). The HOTTIP lncRNA, located at the 5'-end of the HOXA cluster, was significantly expressed in anatomically distal human fibroblasts (51). Recent study demonstrates that HOTTIP, which is significantly overexpressed in PDAC, plays a significant role in PDAC progression and gemcitabine chemoresistance (52). H19 was characterized as an oncogenic lncRNA in some tumors and upregulated remarkably in primary PDAC tumors that subsequently metastasized, compared to those with non-metastasis. H19 also promoted PDAC cell invasion and migration at least partially by increasing HMGA2-mediated epithelial-mesenchymal transition (EMT) through antagonizing let-7 (53). The previous studies also reported several other lncRNAs related to PDAC, such as HULC, PVTI, MAP3K14, PPP3Cb, DAPKI and LOC285194 (54-57). In the present study, we also examined the expression of some most studied lncRNAs in PDAC, such as MALAT1, HOTTIP, H19, HULC, PVTI, MAP3K14, PPP3Cb, DAPKI and LOC285194 in the combination data set of 3 pairs of microarrays, showing that MALAT1 and HOTTIP were significantly upregulated 16.22- and 23.48-fold in PDAC tissues compared with paired non-tumor tissues respectively; however, other lncRNAs were not significantly differentially expressed between PDAC tissues and paired non-tumor tissues.

The microarray expression profiles revealed 21,558 lncRNAs that were expressed in those samples; 2,331 lncRNAs were significantly upregulated and 1,641 lncRNAs were significantly downregulated in 3 PDAC samples compared with the paired non-tumor tissues. We then randomly selected 14 lncRNAs for validation by qRT-PCR in other 12 PDAC samples and paired non-tumor tissues. Additionally, the results from the qRT-PCR analysis and the microarray data analysis were consistent. In these deregulated lncRNAs, we then analyzed the subgroup lncRNAs, including the antisense lncRNAs, the enhancer lncRNAs and the lincRNAs, and their related mRNA that may play an important role in the regulation mechanism of PDAC progression. Antisense lncRNAs have been recognized to regulate expression of corresponding coding genes at post-transcriptional level (58), and therefore

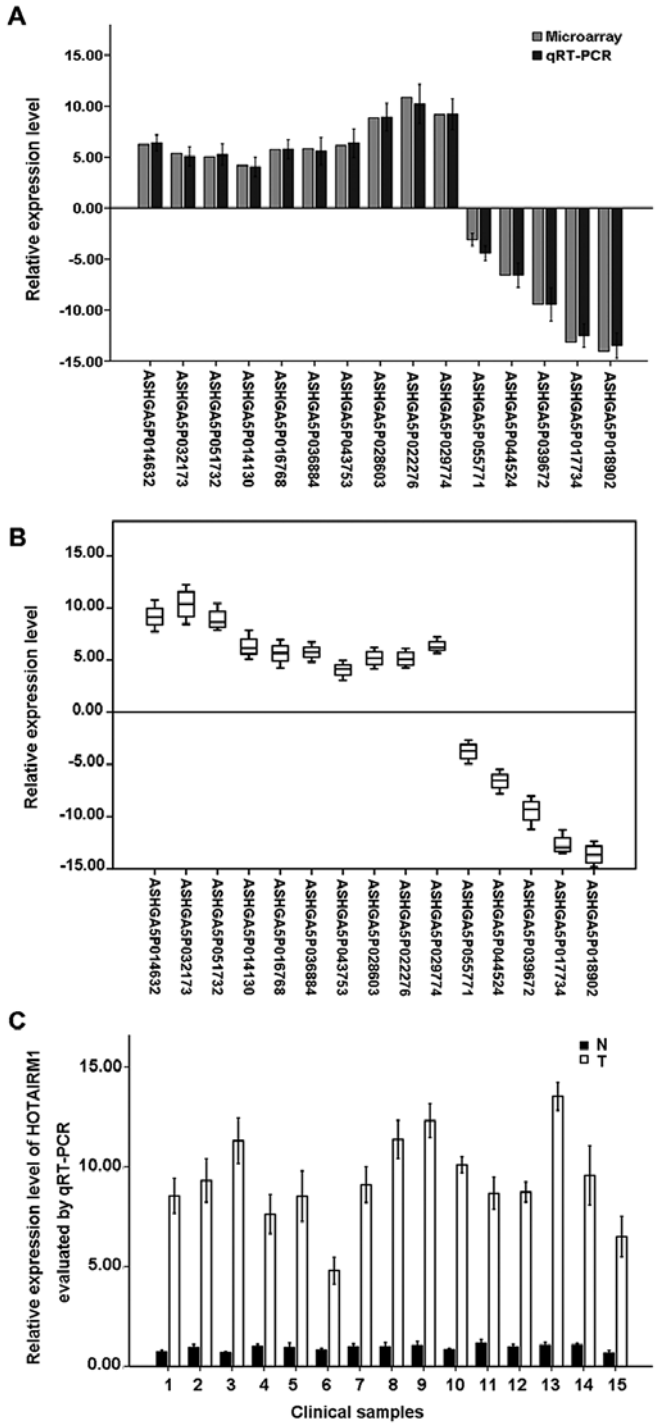

Figure 6. Comparison and distributions of lncRNAs expression levels between the microarray and qRT-PCR results. (A) Comparison of the expression levels of lncRNAs. Fifteen differentially expressed lncRNAs were validated by qRT-PCR. The Y-axis represents the log-transformed median fold changes (T/N) in expression across 15 samples (P<0.05). The qRT-PCR results were consistent with the microarray data. (B) Distributions of lncRNAs expression levels (P<0.05). Fifteen differentially expressed lncRNAs were validated by qRT-PCR in 12 PDAC and paired non-tumor tissue samples. (C) Relative expression levels of HOTAIRM1 evaluated by qRT-PCR in 15 PDAC and paired non-tumor tissue samples.

participate in carcinogenesis by regulation of oncogenes as well as anti-oncogenes. Enhancer RNAs are required for efficient transcriptional enhancement of interacting target genes and also are required for p53-dependent enhancer activity and gene transcription (59-62). LincRNAs play pivotal roles in cancer-related gene regulatory system, and the disorder of their gene expression is thought to promote cancer cell proliferation, invasion and metastasis (63-67). In the present study, our data showed that 69 antisense RNAs, 82 enhancer RNAs and 147 lincRNAs were upregulated in PDAC tissues, and 50 antisense RNAs, 70 enhancer RNAs and 236 lincRNAs were downregulated in adjacent non-tumor tissues. In our additional study, we focused on the function and regulation mechanism of the interesting subgroup lncRNAs in PDAC progression.

We performed GO and pathway analyses to predict the biological functions and potential mechanisms of the differentially expressed lncRNAs in PDAC progression. In this study, we found that the highest enriched GO terms for the upregulated transcripts were purine nucleoside catabolic process, cytoplasm, and protein binding and the most highly enriched GO terms targeted by the downregulated transcripts were establishment of localization, cytoplasmic part, and protein binding. The GO project is a collaborative effort that addresses the need for consistent descriptions of gene products in terms of their 'biology' in a species-independent manner (68). To gain insight into the underlying biology of the differentially expressed transcripts, we performed pathway analysis and found that the upregulated transcripts were associated with 41 pathways; the downregulated transcripts were associated with 25 pathways. Among these pathways, the gene category 'B cell receptor signaling pathway (human)' is involved in the initiation and growth of human pancreatic ductal adenocarcinoma (69). The gene category Wnt signaling pathway, has been reported to be involved in metastasis of pancreatic carcinogenesis (25), and the gene category MAPK signaling pathway has been shown to participate in the progression of pancreatic cancer though multiple mechanisms (26-28,70). The gene categories FoxO signaling pathway have been reported to suppress or activate pancreatic cancer progression by different drugs or compound (29,30). The gene categories 'Ubiquitin mediated proteolysis' participates in pancreatic cancer cell growth *in vitro* and *in vivo* (31). The result of pathway analysis using bioinformatics to find the specific regulation mechanisms in PDAC progression is important for our further studies.

Moreover, we found a significant increase of the expression level of HOTAIRM1 in PDAC samples comparing with the non-tumor tissues via microarray analysis. To examine whether the upregulated expression of HOTAIRM1 is pathologically specific, a total of 12 PDAC samples and paired non-tumor tissues were subjected to qRT-PCR. HOTAIRM1 expression level was higher in PDAC samples than the mean level in paired non-tumor tissues. HOTAIRM1 is a long intergenic non-coding RNA located at the 3'-end of the HOXA cluster, upregulated during myeloid maturation (71). HOTAIRM1 may affect cell fate by regulating cell cycle progression and serving as a link in the coordinated regulation of an extensive gene expression program. Although, the expression of HOTAIRM1 was previously shown to be specific to the myeloid lineage of hematopoietic cells (72), a recent study reported that the HOTAIRM1 was overexpressed in the basal-like subtype of breast cancer (73). Together with our present study in PDAC tissues by microarray and qRT-PCR, the long intergenic non-coding RNA HOTAIRM1 may participate in the development

and progression of several cancers. Thus, further studies are needed to clarify its role in the regulation effect of PDAC.

This study revealed differential expression patterns of lncRNAs in 3 PDAC patients, in which 2,331 upregulated and 1,641 downregulated lncRNAs were found in PDAC tissues relative to paired non-tumor tissues. In addition, the study helped us to understand the potential mechanisms of the carcinogenesis of PDAC preliminarily through 'GO' analysis, signaling pathway analysis and lncRNA classification analysis. Furthermore, this study is the first on the long intergenic non-coding RNA HOTAIRM1 in PDAC, which may be used as a molecular marker in the future to predict response to treatment as well as patient outcome of PDAC.

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