

Differential expression profiling of microRNAs in para-carcinoma, carcinoma and relapse human pancreatic cancer

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Received: 21 March 2014 / Accepted: 13 October 2014 / Published online: 12 November 2014
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Abstract

Purpose To explore the altered different expression of miRNAs and the mechanisms underlying the relapse and metastasis of pancreatic cancer.

Materials and methods The most differentially expressed miRNAs were analyzed by gene ontology (GO) term analysis, Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis and protein interaction analysis. The potentially regulated target genes of the most differentially expressed miRNAs were also analyzed further by GO term analysis and KEGG pathway analysis, and quantitated by qRT-PCR.

Results In total, we found 12 miRNAs displayed at least a 30-fold increase or decrease in expression of carcinoma and relapse vs. para-carcinoma human pancreatic cancer (C/R vs. P). In addition, our study found that pancreatic cancer was related to pathways in cancer, including

Jak-STAT signaling pathway, MAPK signaling pathway and PPAR signaling pathway.

Conclusions The differential expressed miRNAs and their predicted target genes that involved in Jak-STAT signaling pathway, MAPK signaling pathway and PPAR signaling pathway indicating their potential roles in pancreatic carcinogenesis and progress.

Keywords Pancreatic cancer · miRNA · Metastasis · Relapse · Microarray

Introduction

Pancreatic cancer is one of the most fatal cancers in the world and is currently the fourth leading cause of cancer death [1]. An estimated 43,920 (22,090 male and 21,830 female) new cases and 37,390 (18,850 male and 18,540 female) deaths occurred in the United States in 2012 [2]. In addition, because of its metastatic potential and invasive

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nature, which usually results in unsuccessful surgical resection, the vast majority of patients will die from this cancer [3]. Although in recent years, great progress has been made by the research in the molecular pathogenesis of pancreatic cancer, the clinical diagnosis and treatment of pancreatic cancer is still critical.

MicroRNA (miRNA) is a non-encoding small RNA existing widely in animals, plants, and viruses [4]. These tiny ~22-nt RNAs can induce mRNA degradation or translational repression, or both through specific base-pairing with mRNAs [5]. It binds to specific mRNA 3'-untranslated region (3'-UTR) and thereby suppresses or inhibits the transcription of target genes [6].

miRNAs play important roles in the regulation of multiple critical biological processes including cell apoptosis, proliferation, and tumor genesis [7]. The expression of miRNA is significantly differential between tumor and normal tissue, suggesting the critical role of miRNAs in tumor genesis [8]. Expression of miRNAs was found to alter in pancreatic cancer, suggesting their roles in pancreatic carcinogenesis and diagnosis [9]. The better understanding of the molecular mechanism of miRNAs in pancreatic cancer will be beneficial for the development of novel approaches for early detection and monitoring of pancreatic cancer.

In this study, we aim to comprehensively identify the miRNA expression profile in pancreatic cancer and explore the molecular pathway of pancreatic cancer disorder. We used microarray analysis to identify differentially expressed miRNAs among para-carcinoma, carcinoma and relapse human pancreatic cancer and predicted the potentially regulated target genes of the most differentially expressed miRNA, and then further analyzed by GO term analysis, KEGG pathway analysis and protein interaction analysis, which may clarify the mechanism of pancreatic cancer disorders.

Materials and methods

Tissue samples

The pancreatic samples in this study were obtained from China-Japan Union Hospital and preserved in liquid nitrogen. Match para-tumor, tumor and relapse pancreatic tissues from a patient were donated by consenting patients undergoing surgical cancer resection at China-Japan Union Hospital.

RNA extraction

Total RNA was isolated using TRIzol (Invitrogen) according to manufacturer's instructions. RNA quality and

quantity was measured using nanodrop spectrophotometer (ND-1000, Nanodrop Technologies) and RNA Integrity was determined by gel electrophoresis.

Microarray

For miRNA microarray analysis, the miRCURY Hy3/Hy5 Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's guideline for miRNA labeling. One microgram of each sample was 3'-end labeled with Hy3 fluorescent label, using T4 RNA ligase by the following procedure: RNA in 2.0 μ l of water was combined with 1.0 μ l of Calf Intestine Phosphatase buffer (CIP buffer) and CIP (Exiqon). The mixture was incubated for 30 min at 37 °C, and was terminated by incubation for 5 min at 95 °C. Then 3.0 μ l of labeling buffer, 1.5 μ l of fluorescent label (Hy3), 2.0 μ l of Dimethyl sulfoxide (DMSO), 2.0 μ l of labeling enzyme were added into the mixture. The labeling reaction was incubated for 1 h at 16 °C, and terminated by incubation for 15 min at 65 °C.

After stopping the labeling procedure, the Hy3-labeled samples were hybridized on the miRCURY LNA Array (v.16.0) (Exiqon) according to array manual. The total 25 μ l mixture from Hy3-labeled samples with 25 μ l hybridization buffer was first denatured for 2 min at 95 °C, incubated on ice for 2 min and then hybridized to the microarray for 16–20 h at 56 °C in a 12-Bay Hybridization Systems (Hybridization System–Nimblegen Systems, Inc., Madison, WI, USA), which provides an active mixing action and constant incubation temperature to improve hybridization uniformity and enhance signal. Following hybridization, the slides were achieved, washed several times using Wash buffer kit (Exiqon), and finally dried by centrifugation for 5 min at 400 rpm. Then the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA).

miRNA microarray data analysis

Scanned images of miRNA microarray were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs with intensities ≥ 50 in all samples were chosen for calculating normalization factor. Expressed data were normalized using the Median normalization. After normalization, differentially expressed miRNAs were identified through Fold-Change filtering.

The Scatter Plot was used for assessing the miRNA expression variation between arrays. The values of X and Y axes in the Scatter Plot are the normalized signal values of the samples.

Hierarchical clustering

Hierarchical clustering was performed using MEV software (v4.6, TIGR). The heat map diagram shows the result of the two-way hierarchical clustering of miRNAs and samples. Each row represents a miRNA and each column represents a sample. The miRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. Cluster analysis arranges samples and miRNAs into groups based on their expression levels, which allows us to hypothesize about the relationships between miRNAs and samples.

Target gene prediction

Target gene prediction software MiRDB (<http://mirdb.org/miRDB/>) and Targetscan (<http://www.targetscan.org>) was used to forecast some potential target genes showing a sequential differential expression profiling. The genes which were identified by these softwares were taken as target genes to minimize the false positivity. All the

predicted target genes were then compared with the differential gene expression profiles of para-carcinoma, carcinoma and relapse human pancreatic cancer in another research we have made [10].

GO analysis (<http://www.geneontology.org/>), pathway analysis (<http://www.genome.jp/kegg/>) and protein interaction (<http://string-db.org/>) analysis were performed in the standard enrichment computation method.

Quantitative RT-PCR (qRT-PCR)

To confirm the microarray results, qRT-PCR was performed from cDNA. The reactions were carried out on an Icyler IQ (BioRad). Each 25 μ l PCR reaction included SYBR Green Supermix (Bio Rad), containing 0.4 mM each of dNTP, 50 U/ml iTaq DNA polymerase, 6 mM Mg²⁺ + SYBR Green I, 20 nM fluorescein, and stabilizers. The efficiency of each primer set was calculated using a standard curve. The comparative cycle threshold method ($2^{-\Delta\Delta C_t}$) was used to quantify gene expression. Fold-change values represent an average of the three separate

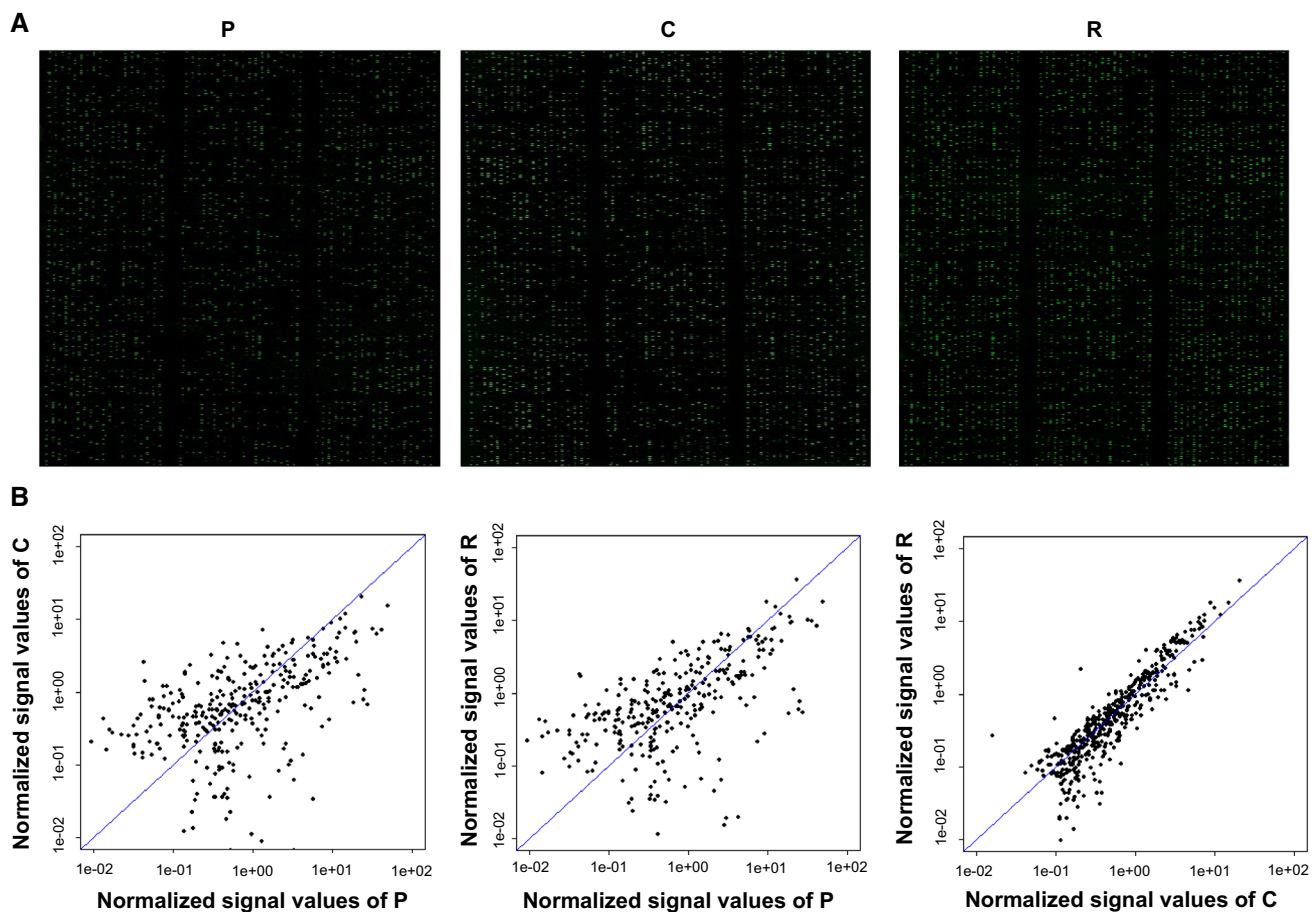


Fig. 1 Microarray analysis. **a** miRNA microarray hybridization fluorescence signal graph in para-carcinoma (P), carcinoma (C) and relapse (R) human pancreatic cancer. **b** The *Scatter Plot* was used for

assessing the gene expression variation between two arrays (C vs. P; R vs. P; C vs. R). The axes of the scatter plot are the normalized signal values of the samples

experiments. β -Actin served as the control in each qRT-PCR reaction. The primers used in this experiment are as the following:

5'-CACCAGCACGTCACAACGCC-3' and 5'-AGACT GCGAGCGTGTGCGAAAG-3' for ZNF24; 5'-GGACACC GTGACCCACGACCT-3' and 5'-ACCACGACCCAGA CAGGCAGAC-3' for BAG5; 5'-TGTGGCAAGGCAGT AGAAT-3' and 5'-GCCACATCATGGCTCGTC-3' for β -Actin.

To detect the miRNA levels, a total of 10 ng of total RNAs were reverse transcribed with TaqMan miRNA reverse transcription kit and TaqMan miRNA Multiplex RT assays (human pool). The reverse transcription products were then preamplified with TaqMan PreAmp kit (Applied Biosystems, Foster City, CA, USA). All steps were performed using a 7900 HT Fast Real-Time PCR System. Results were expressed as Cts. Raw data were analyzed using Data Assist software for TaqMan gene expression assays version 2.0 (Applied Biosystems). The miRNA expression was normalized to the U6 expression level.

Statistical analyses

All experiments were conducted at least three times. The results were expressed as mean \pm SD (standard deviation) and statistical significance between the groups was assessed using one-way analysis of variance (ANOVA). The level of significance was chosen as $p < 0.05$.

Results

Microarray analysis

Microarray experiment was qualified according to quality standards. The experimental system was stable, fluorescent signal intensity was strong and homogenous. In the three chips, 10,798 probes had clearly signals, representing 87.8 % of 12,300 probes (Fig. 1a). The Scatter Plots were used for assessing the miRNA expression variation between three group arrays(C vs. P; R vs. C; P vs. R)

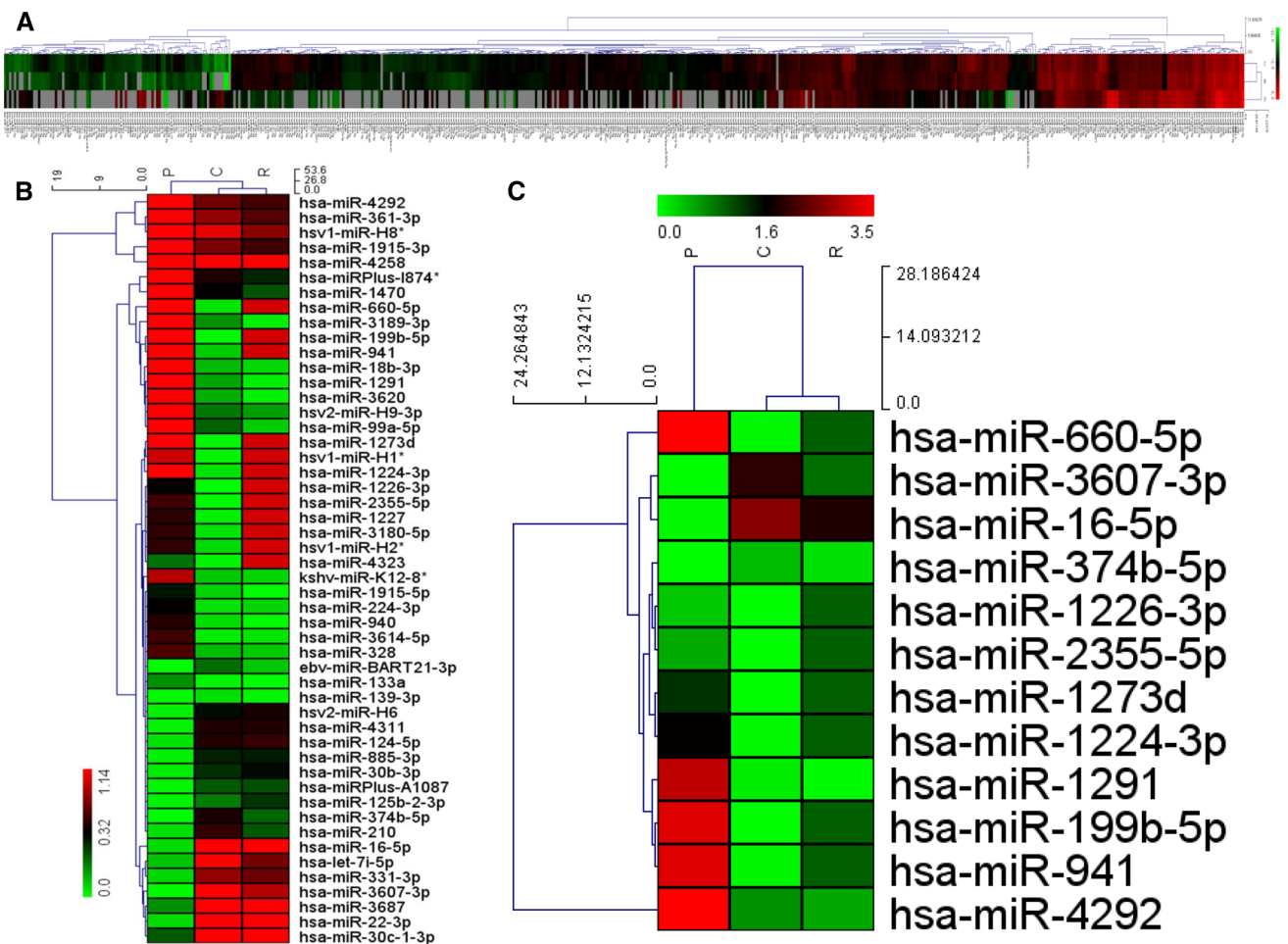


Fig. 2 miRNA transcriptional changes in para-carcinoma, carcinoma and relapse human pancreatic cancer tissues. **a** Result of hierarchical clustering for all miRNAs. **b** Result of hierarchical clustering for a

fold change of ≥ 10 . **c** Result of hierarchical clustering for top 12 differential miRNAs (a fold change of ≥ 30)

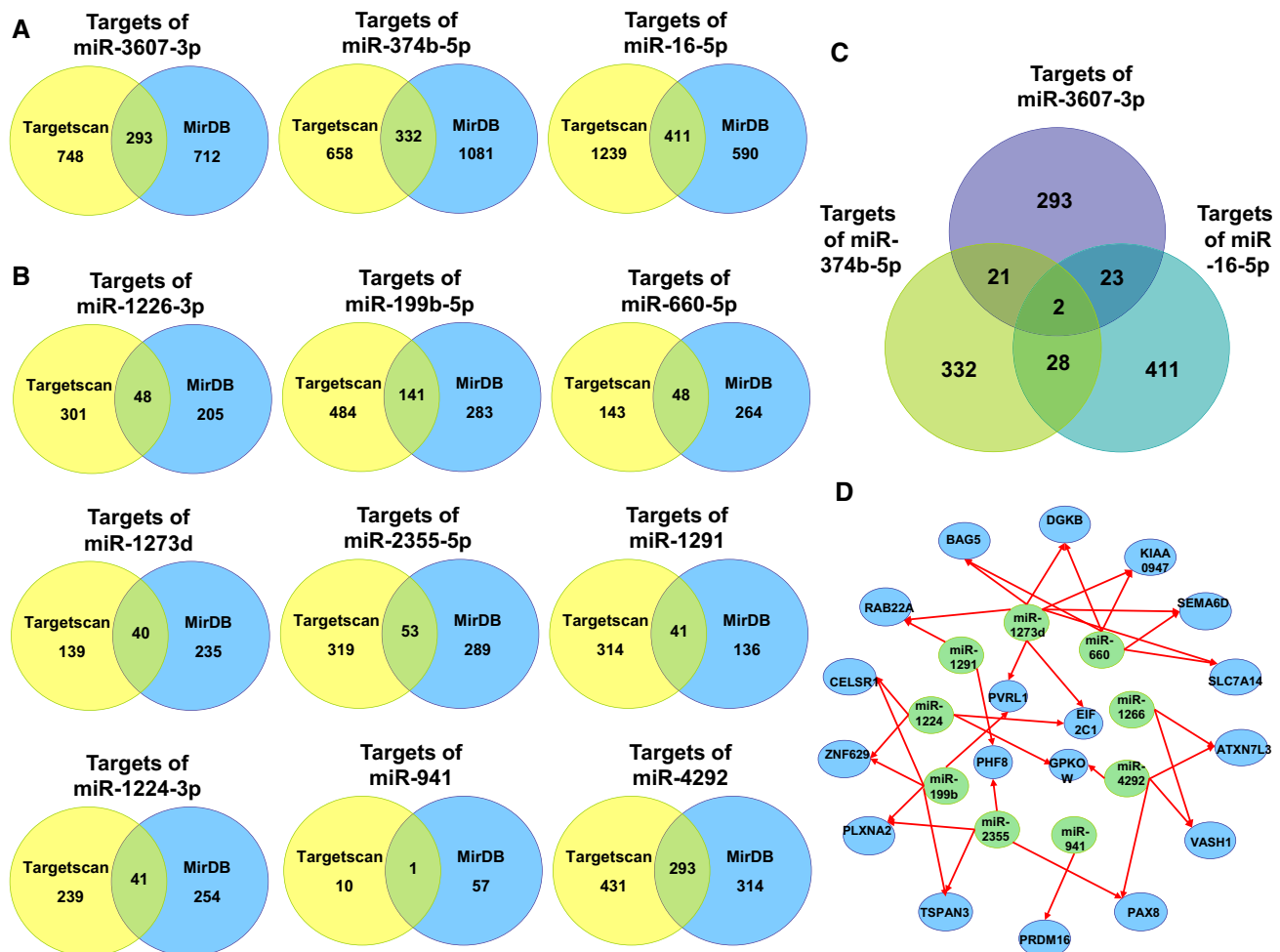


Fig. 3 Target gene prediction for the 12 differential miRNAs. **a** The amounts of predicted targets of three C/R vs. P upregulated miRNAs. **b** The amounts of predicted targets of C/R vs. P nine downregulated miRNAs. **c** Two mutual genes (PLXNA2 and QKI) that were

(Fig. 1b). As shown in Fig. 1b, the miRNA expression between group C and group R was closer.

miRNAs whose Foreground–Background intensities are smaller than 50 in all samples were filtered (Fig. 2a). Of all the miRNAs represented therein, 50 miRNAs displayed at least a 10-fold increase or decrease in expression, of which 18 miRNAs were upregulated, while 32 miRNAs were downregulated in human pancreatic cancer tissue (Fig. 2b). Of all the miRNAs represented in Fig. 2a, 12 miRNAs displayed at least a 30-fold increase or decrease in expression (Fig. 2c).

Target genes prediction

For the 12 miRNAs that displayed at least a 30-fold increase or decrease in expression, miRDB and TargetsScan were used to predict the potentially regulated genes. There are 293 predicted target genes for the C/R vs. P upregulated miR-3607-3p, 332 and 411 genes each for miR-374b-5p and

regulated by all the three upregulated miRNAs. **d** The relationships between the nine downregulated miRNAs and their mutual predicted target genes

miR-16-5p (Fig. 3a). For those nine C/R vs. P downregulated miRNAs, the predicted target genes are present in Fig. 3b. We also found two mutual genes (PLXNA2 and QKI) that were regulated by all the three upregulated miRNAs (Fig. 3c). In Fig. 3d, we show the relationships between the nine downregulated miRNAs and their mutual predicted target genes.

Gene ontology analysis

Compared with the differential gene expression profiles we have made before, the differentially expressed genes associated with pancreatic cancer were 240. We then used gene ontology (GO) to analyze these differentially expressed genes by three domains (Fig. 4).

In biological process, the top 5 GO terms of 1.5-fold C/R vs. P differential genes, C/R vs. P upregulated genes and C/R vs. P downregulated genes are regulation of transcription from RNA polymerase II promoter, regulation

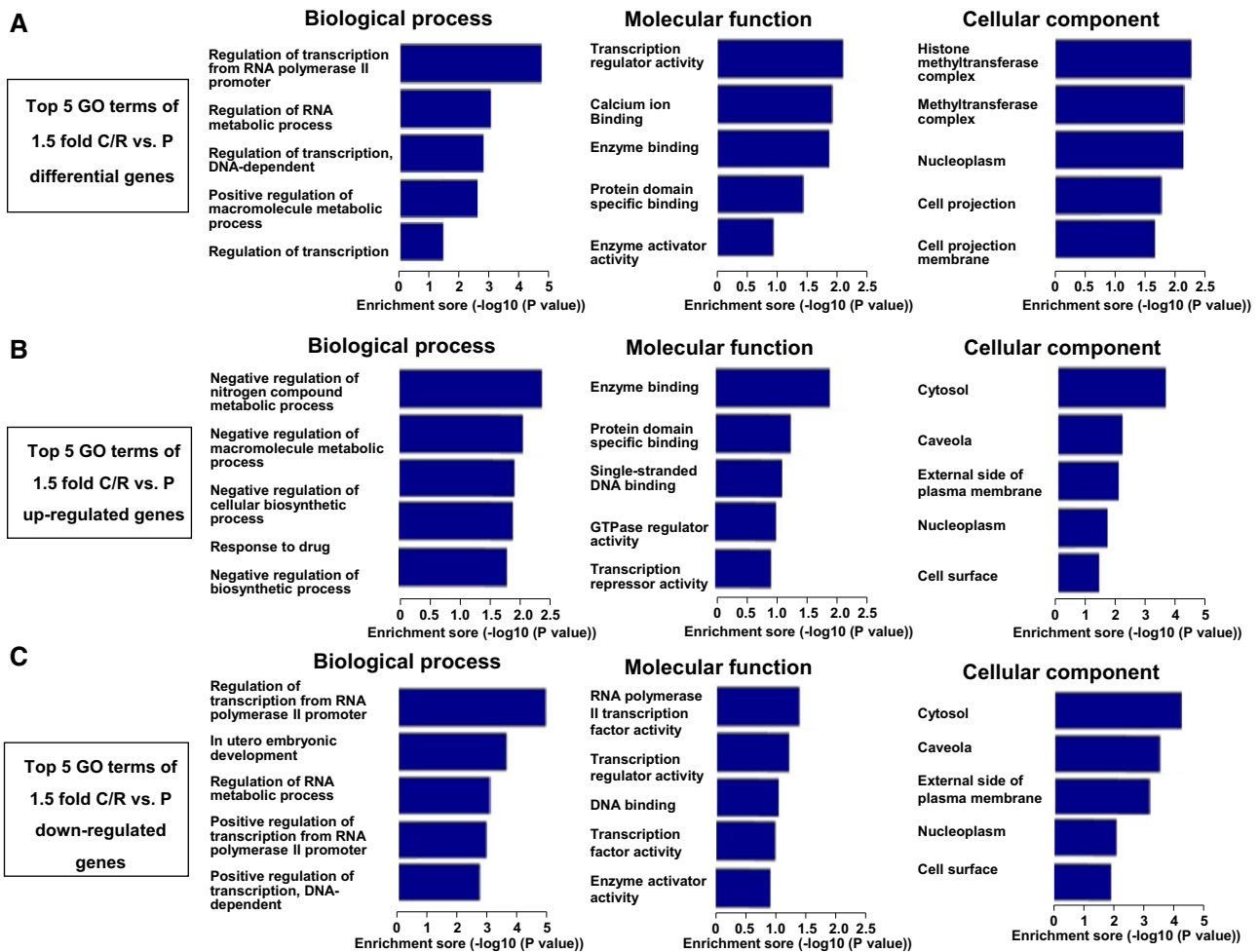


Fig. 4 GO analysis of predicted target differentially expressed genes associated with biological process, molecular function and cellular component. **a** Top 5 GO terms of 1.5-fold C/R vs. P differential genes. **b** Top 5 Go terms of 1.5-fold C/R vs. P upregulated genes. **c** Top 5 Go terms of 1.5-fold C/R vs. P downregulated genes

of RNA metabolic process, regulation of transcription, DNA dependent, positive regulation of macromolecule metabolic process, regulation of transcription; negative regulation of nitrogen compound metabolic process, negative regulation of macromolecule metabolic process, negative regulation of cellular biosynthetic process, response to drug, negative regulation of biosynthetic process; regulation of transcription from RNA polymerase II promoter, in utero embryonic development, regulation of RNA metabolic process, positive regulation of transcription from RNA polymerase II promoter, positive regulation of transcription, DNA dependent.

In molecular function, the top 5 GO terms of 1.5-fold C/R vs. P differential genes, C/R vs. P upregulated genes and C/R vs. P downregulated genes are transcription regulator activity, calcium ion binding, enzyme binding, protein domain specific binding, enzyme activator activity; enzyme binding, protein domain specific binding, single-stranded DNA binding, GTPase regulator activity,

transcription repressor activity; RNA polymerase II transcription factor activity, transcription regulator activity, DNA binding, transcription factor activity, enzyme activator activity.

In cellular component, the top 5 GO terms of 1.5-fold C/R vs. P differential genes, C/R vs. P upregulated genes and C/R vs. P downregulated genes are histone methyltransferase complex, methyltransferase complex, nucleoplasm, cell projection, cell projection membrane; cytosol, caveola, external side of plasma membrane, nucleoplasm, cell surface; cytosol, caveola, external side of plasma membrane, nucleoplasm, cell surface.

Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis

KEGG pathways analysis of gene differential expression patterns in para-carcinoma, carcinoma and relapse human pancreatic cancer tissue revealed many enrichment-related

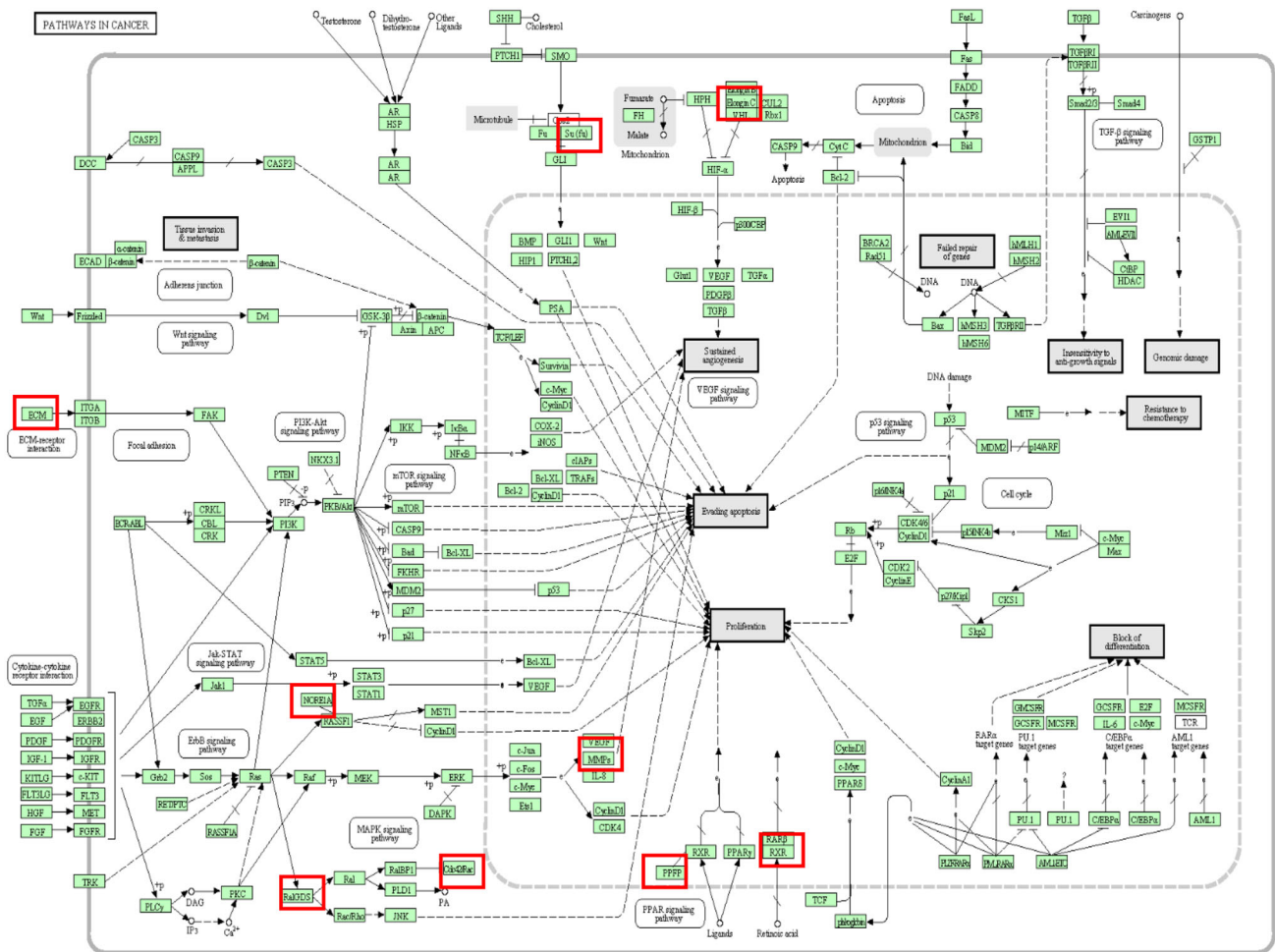


Fig. 5 KEGG pathway analysis of 1.5-fold C/R vs. P predicted genes. Pathways of 1.5-fold C vs. P genes by KEGG

pathways including pathways in para-carcinoma, carcinoma and relapse. Of these, there are nine key genes differentially expressed which included *SUFU*, *TCEB1*, *LAMC1*, *RASSF5*, *MMP9*, *RALGDS*, *CDC42*, *PAX8* and *RXR* in Pathways in cancer (Fig. 5).

Protein interaction analysis

To explore the interaction of the proteins translated by those predicted differentially expressed genes, we did a protein interaction analysis by STRING (<http://string-db.org/>), then we found that many genes had a potential relationship between each other (Fig. 6).

Hierarchical clustering for predicted target genes

Compared with the differential gene expression profiles we have made before, the differentially expressed genes associated with pancreatic cancer were 240 (Fig. 7a). Of all the genes represented therein, 40 genes displayed at least a

2-fold increase or decrease in expression (Fig. 7b). 14 genes expression level (*PTP4A1*, *ABI2*, *CCDC50*, *EIF4E3*, *MAP2K3*, *ZNF24*, *FOXO4*, *UBXN7*, *MINK1*, *CABP4*, *MSX1*, *BAG5*, *CNOT2* and *COTL1*) displayed at least a 3-fold increase or decrease in groups (Fig. 7c).

Validation of microarray data

Among all the 14 differentially expressed genes we have observed, two of the most differential expressed genes (*ZNF24* and *BAG5*) were validated by qPCR along with their relative miRNAs (Fig. 8).

Discussion

Pancreatic cancer is one of the most fatal cancers in the whole world and exhibits aggressive features with short survival rate and high mortality [11]. Therefore, it is very important to understand the molecular mechanisms

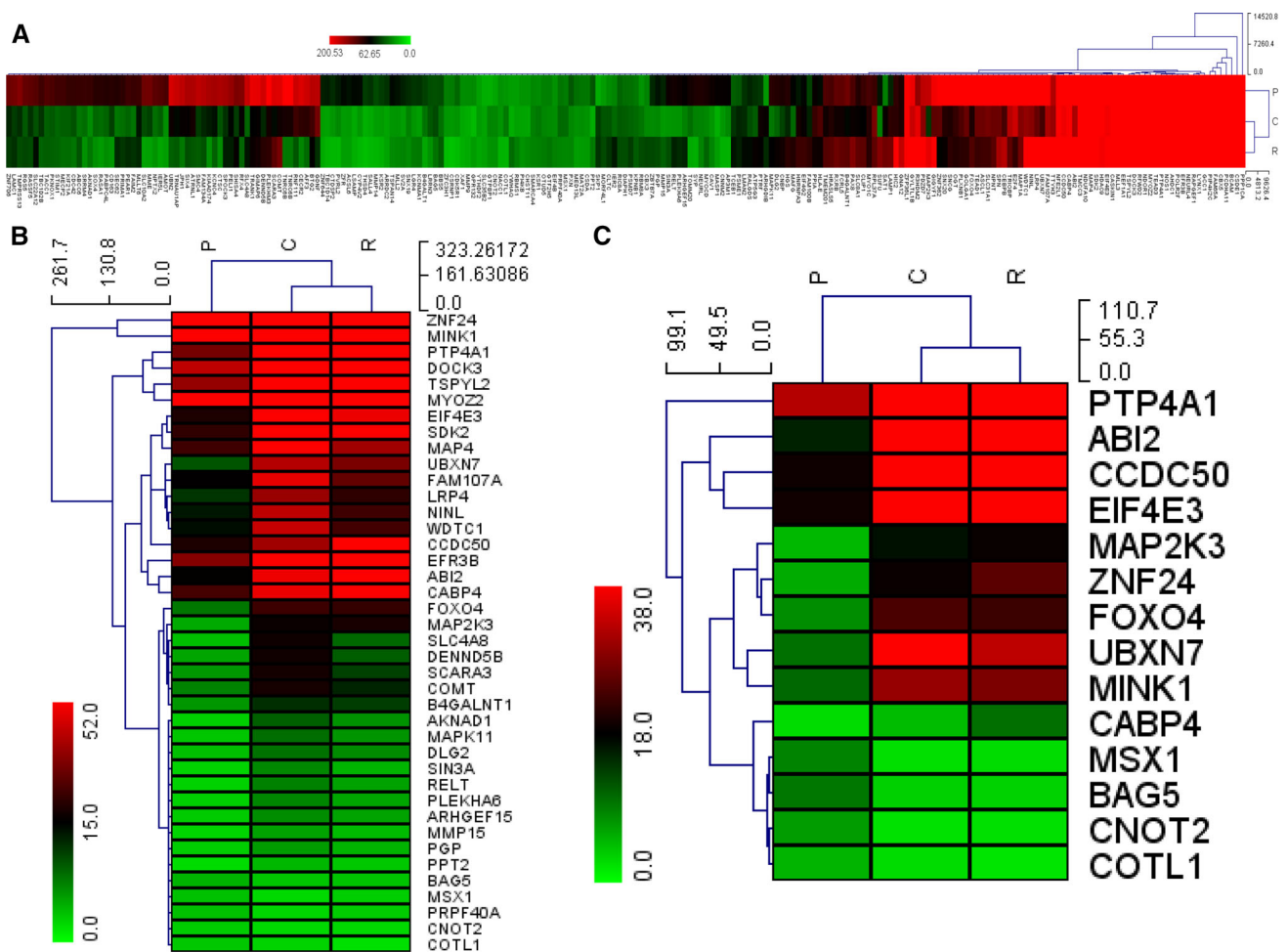


Fig. 7 Predicted target genes transcriptional changes in para-carcinoma, carcinoma and relapse human pancreatic cancer tissues. **a** Result of hierarchical clustering for all the 240 predicted target

miR-374b is downregulated in prostate cancer tissue, and it can be identified as an independent predictor of biochemical recurrence-free survival. Jin et al. [15] found that miR-1226 interacts with the MUC1 mRNA 3'UTR and that miR-1226 downregulates endogenous MUC1 protein levels, indicating that expression of the MUC1 oncoprotein is downregulated by miR-1226 and that miR-1226 thereby functions as a tumor suppressor by promoting the induction of cell death. Fang et al. [16] found that miR-199b-5p inhibited HER2 expression by direct targeting its 3'-untranslated region (3'UTR) in breast cancer cells and that miR-199b-5p could enhance the suppression of trastuzumab on cell migration and clonogenicity suggest that miR-199b-5p may have the potential to be a novel important alternative therapeutic target for HER2-positive breast cancer. Yamasaki et al. [17] suggest that expression of miR-1291 was significantly downregulated in RCC tissues compared with adjacent non-cancerous tissues. Restoration of mature miR-1291 in RCC cell lines (A498 and 786-O) revealed significant inhibition of cell proliferation,

genes. **b** Result of hierarchical clustering for 40 differential genes (a fold change of ≥ 1.5). **c** Result of hierarchical clustering for top 14 differential genes (a fold change of ≥ 30)

migration and invasion, suggesting that miR-1291 functioned as a tumor suppressor. Arola-Arnal et al. [18] investigated miRNA expression in HepG2 cells treated with proanthocyanidins using microarray analysis and Q-PCR, showed that when HepG2 cells were treated with grape seed proanthocyanidin extract (GSPE), cocoa proanthocyanidin extract (CPE) fifteen and six differentially expressed miRNAs, respectively, were identified out of 904 mRNAs. Treatment with GSPE or CPE upregulated miR-1224-3p, miR-197 and miR-532-3p. Zhang et al. [19] found that the overexpression of miR-941 suppressed in vitro cell proliferation, migration and invasion and inhibited the metastasis of hepatocellular carcinoma (HCC) cells in vivo, and that miR-941 was significantly downregulated in HCC tissues and cell lines and was generally hypermethylated in HCC.

Other miRNAs including miR-3607-3p, miR-16-5p, miR-660-5p, miR-1273d, miR-2355-5p and miR-4292 have no reports associated with cancer or pancreatic cancer found so far.

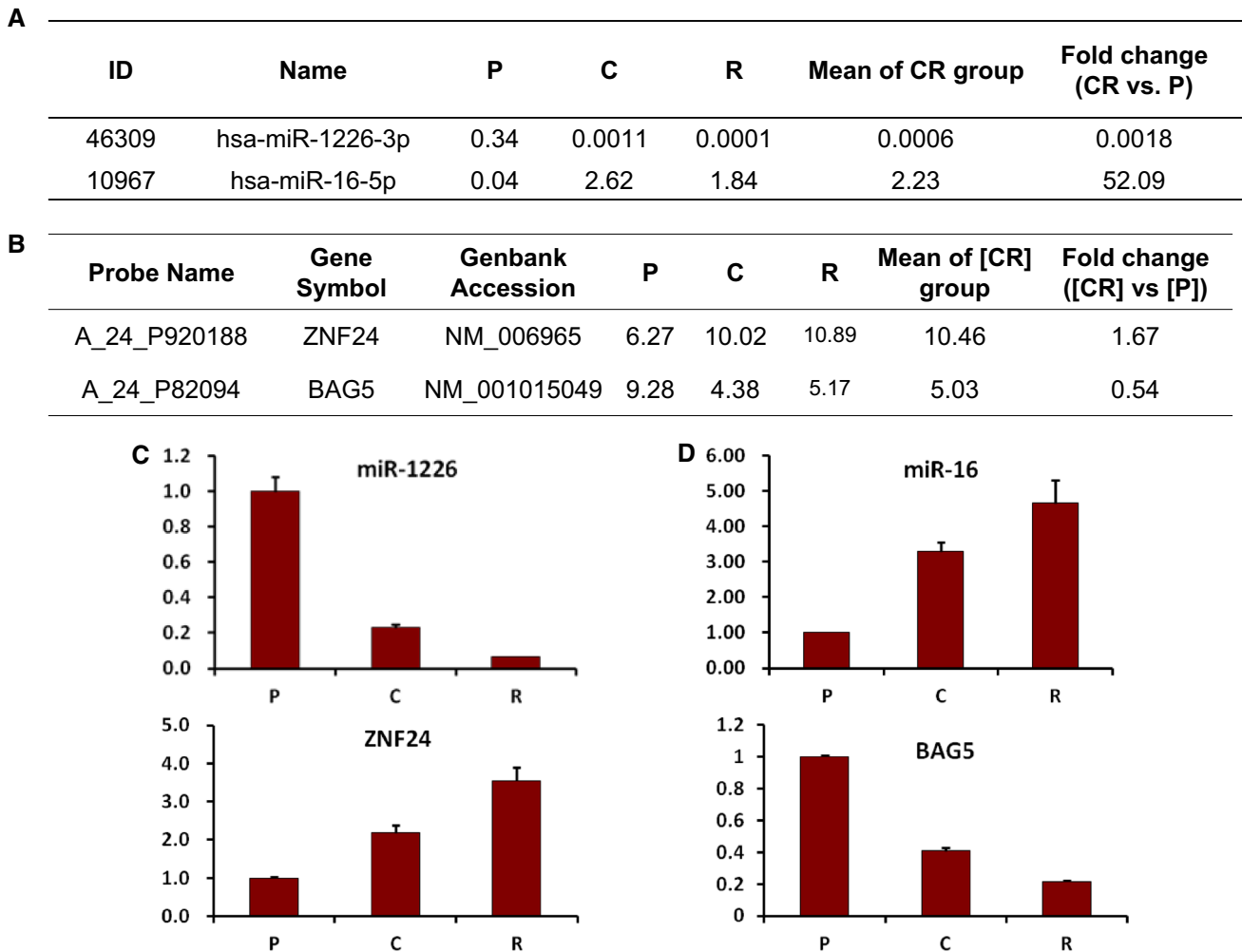


Fig. 8 Validation of microarray data. **a** The miRNA microarray result of two most differential expressed miRNAs (hsa-miR-1226-3p and hsa-miR-16-5p). **b** The gene chip result of the predicted target

genes of hsa-miR-1226-3p (ZNF24) and hsa-miR-16-5p (BAG5). **c** qRT-PCR validation of hsa-miR-1226-3p and ZNF24. **d** qRT-PCR validation of hsa-miR-16-5p and BAG5

In our further research, we found that the most enriched GO terms included nitrogen compound metabolic process, macromolecule metabolic process, cellular biosynthetic process, RNA metabolic process, biosynthetic process and so on. Analysis of KEGG pathways revealed that pathways in cancer have a most biological significance associated with pancreatic tumor formation, including Jak-STAT signaling pathway, MAPK signaling pathway and PPAR signaling pathway. It has been shown that JaK-STAT pathway mediates signaling by cytokine, chemokine and growth factor receptors on cell surface to the nucleus, which is often deregulated in human cancer and the deregulation of Jak-STAT pathway, has a dual role of promoting cell survival/proliferation and immune evasion. In addition, several studies have reported that Jak-STAT pathway activity increases in some tumors, which can be used as the target for cancer treatment.

Nine differentially expressed genes including SUFU, TCEB1, LAMC1, RASSF5, MMP9, RALGDS, CDC42, PAX8 and RXRB were involved in these pathways. Some of these genes have been reported to associate with pancreatic cancer or other cancers. For example, Aavikko et al. [20] combined genome-wide linkage analysis and exome sequencing, and identified in suppressor of fused homolog (*Drosophila*), SUFU, with the meningiomas in the family; their genetic and functional analyses indicates that germline mutations in SUFU predispose to meningiomas, particularly to multiple meningiomas. Agell et al. [21] found that 12-gene expression signature is associated with aggressive histological in prostate cancer; SEC14L1 and TCEB1 genes are potential markers of progression. Nishikawa et al. [22] demonstrated that the migration and invasion in cancer cells would be inhibited by the silencing of LAMC1—an upregulated gene in prostate cancer. Djos et al. [23] suggest that several of the RASSF family genes

(RASSF2, RASSF4, RASSF5, RASSF6, RASSF7, and RASSF10) to various degrees were methylated in neuroblastoma cell lines and primary tumors. Chen et al. [24] found that MDM2 promotes invasion and metastasis in invasive ductal breast carcinoma by inducing matrix metalloproteinase-9, and suggest that high levels of MDM2 are associated with a poorer prognosis in IDC. This might result from increased tumor invasiveness due to enhanced MMP9 expression causing increased extracellular matrix breakdown. Other studies had reported on the association of RALGDS [25], CDC42 [26], PAX8 [27], and RXRB [28] with a variety of tumors.

Taken together, our present study confirmed that carcinogenesis and relapse of pancreatic cancer were involved in multiple-gene interaction. We found that 12 miRNAs displayed at least a 30-fold increase or decrease in expression and 14 genes displayed at least a 3-fold increase or decrease in expression which were closely related to pancreatic cancer. Among all the 12 differentially expressed miRNAs, 6 miRNAs (miR-374b, miR-1226, miR-199b-5p, miR-1291, miR-532-3p and miR-941) have been proved to function as tumor suppressor in pancreatic cancer or other cancer by inhibiting or activating their target gene expression, while the rest of miRNAs have no reports associated with cancer or pancreatic cancer found so far. Moreover, Jak-STAT signaling pathway, MAPK signaling pathway and PPAR signaling pathway were found to be the key pathways in the progress of pancreatic cancer pathogenesis and development. The research of these signal systems and the interactions of genes in pancreatic cancer research will contribute to the early diagnosis and targeted therapy cancer. In addition, the biological function of these differentially expressed miRNAs and genes in the progress of pancreatic cancer pathogenesis and development needs further excavation and research.

Acknowledgments The authors project was supported by the National Nature Scientific Foundation of China (81201535, 81472202, 81302065, 81472501, 81371595) and the research project of Jilin Provincial Science and Technology Department (20130206001YY, 20140414061GH, 20090633).

Conflict of interest The authors have no financial conflicts of interest.

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