

# miR-4668-5p is up-regulated in serum of patients with pancreatic cancer

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## Research

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# Abstract

**Background** Pancreatic cancer is the most common of gastrointestinal tumor, with a high malignancy and poor prognosis, and early and effective diagnosis is the key for prolonging patient's overall survival, especially for fluid biopsy. **Methods** In this study, expression profile array was downloaded from GEO database, and differentially expressed miRNAs in serum were screened from patients with pancreatic cancer and healthy individuals, then co-expressed serum miRNAs in pancreatic cancer patients was analyzed by R software with weighted correlation network analysis (WGCNA) package. Finally, key differentially expressed miRNA was verified in serum of 16 patients with pancreatic cancer and 10 healthy individuals by quantitative real-time PCR (qRT-PCR). **Results** Results found that there were eleven differentially expressed miRNAs (miR-155-5p, miR-4668-5p, miR-3613-3p, miR-3201, miR-548ac, miR-486-5p, miR-548a-3p, miR-8084, miR-455-3p, miR-6068, and miR-1246) between pancreatic cancer and healthy individuals. WGCNA results further found that miR-4668-5p has a higher associated modules compared to other differentially expressed miRNAs. Then, the expression level of miR-4668-5p was further verified, and results found that serum miR-4668-5p expression level was significantly higher in patients with pancreatic cancer than that of healthy individuals. **Conclusion** Our results firstly substantiated that miR-4668-5p was significantly up-regulated in serum of patients with pancreatic cancer, which may be a potential biomarker for pancreatic cancer diagnosis.

## Background

With the development of socioeconomic and population ageing, pancreatic cancer is becoming the most common malignant tumor in China, and usually with a poor prognosis<sup>1</sup>. The most profound issue surrounding poor prognosis is widely considered as lack of early diagnostic method and effective treatment<sup>2</sup>.

In order to improve patients' prognosis, a majority of methods associated with diagnosis, therapy, and risk stratification are constantly exploring. Number of novel molecular biomarkers, associated with pancreatic cancer, was increasing gradually, including mutation status and abnormal expression of genes<sup>3</sup>. Recently, DNA methylation<sup>4</sup>, cell-free DNA<sup>5</sup>, exosomes<sup>6</sup> are becoming research hotpot. But there are very limited markers can be really used in clinic.

As we known, it is arduous to discover a novel biomarkers with high sensitivity and specificity<sup>7</sup>. Consequently, it is indispensable to explore more novel biomarkers associated with occurrence and development of pancreatic cancer. Here, a combination analysis of expression profile array and WGCNA was performed, which aimed to find and identify key molecular biomarkers related to pancreatic cancer diagnosis.

## Materials And Methods

**Download and analysis of miRNAs expression profile.**

miRNAs expression profile array(GSE85589) was downloaded from GEO database(<https://www.ncbi.nlm.nih.gov/gds/>), and serum miRNAs expression data was obtained from 88 patients with pancreatic cancer and 19 healthy control. Differentially expressed miRNAs were screened by R software. Compared with healthy control, those miRNAs that absolute value of  $\log_2\text{FoldChange(FC)}$   $\geq 1$  and  $\text{FDR} \leq 0.05$  were considered as differentially expressed miRNAs.

### **Weighted correlation network analysis(WGCNA) .**

Serum miRNAs expression data from 88 patients with pancreatic cancer was obtained from GSE85589, WGCNA analysis was performed according to previous report<sup>8</sup>.

### **cDNA synthesis and quantitative real-time PCR .**

Serum(2 ml) was collected from 10 healthy control and 16 pancreatic cancer patients from our hospital, and total RNA was extracted referring to previous report<sup>9</sup>. First-Strand cDNA synthesis kit (Agilent Technologies Inc.)was used for cDNA synthesis, and SYBR<sup>®</sup> Green PCR master mix (Applied Biosystems, NY, USA) was used to qRT-PCR. U6 as an internal control, and 1  $\mu\text{l}$  of a universal reverse primer (Agilent Technologies Inc., CA, USA) were used in this experiment. The relative miRNAs levels were determined in terms of their FC between patients with pancreatic cancer and healthy controls using  $2^{-\Delta\Delta\text{Ct}}$ , and this experiment was performed in triplicate<sup>10</sup>. Primer sequence as follow:

miR-4668-5p:(F) AGGGAAAAAAAAAAGGATTTGTC;

U6:(F) CGCTTCGGCAGCACATATACTAA;

U6:(R) TATGGAACGCTTCACGAATTTGC.

### **Statistical analysis.**

IBM SPSS Statistics 21.0 software was used to statistical analysis. miR-4668-5p expression was compared between pancreatic cancer patients and healthy individuals via non-paired *t* test.  $P \leq 0.05$  was considered as statistically significant.

## **Results**

### **Eleven dysregulated miRNAs were screened in serum of patients with pancreatic cancer.**

Serum miRNAs expression levels were compared between patients with pancreatic cancer( $n=88$ ) and healthy controls( $n=19$ )(Fig.1A), and eleven miRNAs were obviously dysregulated in serum of patients with pancreatic cancer, they are miR-155-5p, miR-4668-5p, miR-3613-3p, miR-3201, miR-548ac, miR-486-5p, miR-548a-3p, miR-8084, miR-455-3p, miR-6068, and miR-1246. Among of them, miR-4668-5p, miR-3613-3p, miR-3201, miR-548ac, miR-486-5p, miR-548a-3p, miR-8084, miR-6068, miR-1246 were up-

regulated in serum of patients with pancreatic cancer, and miR-155-5p and miR-455-3p were down-regulated (FDR $\leq$ 0.05; Table 1).

**miR-4668-5p was a key miRNA and associated with a multitude of modules.** WGCNA package provides R functions for weighted correlation network analysis, co-expression of miRNAs in serum of 88 patients were analyzed by R software, and eleven clusters (modules) of highly correlated genes were observed they were darkorange, salmon, darkmagenta, darkgrey, lightgreen, darkgreen, grey60, cyan, midnightblue, greenyellow, and lightcyan. Meanwhile, a heatmap of these miRNAs was shown (Fig.1B-D).As shown in Table 2, differentially expressed miRNAs previous founded were almost included in darkgrey, darkmagenta and lightgreen modules. Then MM value of these miRNAs was calculated, highly correlated modules for these differentially expressed miRNAs were listed in Table 2( $P\leq$ 0.05), and we further observed that number of correlated modules of miR-4668-5p was highest among these miRNAs, including darkmagenta, darkgrey, lightgreen, grey60, and greenyellow. Hence, miR-4668-5p may be a key molecular biomarkers.

**miR-4668-5p was consistently up-regulated in serum of patients with pancreatic cancer.**

According to GEO and WGCNA analysis results, miR-4668-5p was considered as a potential key molecular in pancreatic cancer. Then, expression level of miR-4668-5p was further investigated by database and qRT-PCR assay. In GSE85589, up-regulated miR-4668-5p was observed in serum of patients with pancreatic cancer( $n=88$ ) compared to healthy control( $n=19$ ) ( $P\leq$ 0.0001; Fig.2A). To further identify whether miR-4668-5p was also up-regulated in our serum sample, its expression was detected in patients and healthy individuals by qRT-PCR. And results suggested that miR-4668-5p expression was consistently up-regulated in serum of patients with pancreatic cancer ( $n=16$ ) compared to those healthy control( $n=10$ ) ( $P\leq$  0.05; Fig.2B).

## Discussion

In this study, eleven differentially expressed miRNAs in serum of patients with pancreatic cancer were firstly screened by GEO database. Meanwhile, co-expressed miRNAs in serum of patients with pancreatic cancer was further analyzed by WGCNA package. Among these differentially expressed miRNAs modules correlated with miR-4668-5p was higher than other miRNAs, which may indicate miR-4668-5p was a key molecular biomarkers. Then, up-regulated miR-4668-5p were further investigated in pancreatic cancer patients.

Recent studies indicated that patients with incidentally diagnosed pancreatic cancer have better prognosis than those with symptoms<sup>11</sup>, which explain the benefits of early diagnosis in improving prognosis. In many of the diagnosis methods, liquid biopsy is a potential non-invasive diagnosis method, and with convenient, economical, and minimally traumatic features<sup>12</sup>. To explore whether there were potential biomarkers existed in serum of patients with pancreatic cancer, non-coding RNA profile was

download from GEO database, and followed by the screening of differentially expressed miRNAs, eleven differentially expressed miRNAs were observed. However, it is indispensable to further identify key miRNA among these differentially expressed miRNAs. Different from other studies, here, a combination of weighted correlation network analysis (WGCNA) and GEO database was used to construct co-expression network and modules, which aimed to increase our understanding of the host-pathogen relationship. WGCNA has been gradually used to construct co-expression network<sup>13</sup>, especially for miRNAs. As an example, hub miRNAs related to prognosis were identified in colorectal cancer (CRC) based on WGCNA analysis<sup>14,15</sup>. Here, WGCNA was used to construct modules and co-expressed network and combined with differentially expressed miRNAs, which indicated miR-4668-5p was a key molecular among these differentially expressed miRNAs.

In fact, dysregulated miR-4668-5p has been observed in blood and tissues of many diseases. For example, miR-4668-5p level was higher in serum of hepatocellular carcinoma patients compared to healthy control<sup>16</sup>. Similarly, dysregulated miR-4668-5p was also observed in gastric cancer tissues<sup>17</sup>, and dedifferentiated liposarcoma tissues<sup>18</sup> compared to healthy controls. Not only in malignant tumor, dysregulated miR-4668-5p was also associated with nervous system diseases, for example AD<sup>10</sup> and mesial temporal lobe epilepsy with hippocampal sclerosis<sup>19</sup>. However, it is limit that studies related to miR-4668-5p in pancreatic cancer. In this study, we observed and confirmed miR-4668-5p was obviously up-regulated in serum of patients with pancreatic cancer. Moreover, a combination analysis of GEO and WGCNA suggested that miR-4668-5p may indicate a key molecular biomarker.

Nevertheless, there are still some limitations in this study. For example, it is better to combine miRNAs expression data with clinical data, such as overall survival, clinical stage, *ect*. Besides, the potential mechanism of miR-4668-5p regulating target genes and its functions should be deeply explored in future. At last, it is necessary to detect miR-4668-5p expression in a large number of samples.

## Conclusions

Our study firstly found that miR-4668-5p was a key differentially expressed miRNAs, and consistently up-regulated in serum of patients with pancreatic cancer compared to healthy control, which may be a potential early diagnosis marker for pancreatic cancer.

## Declarations

### Consent for publication

We confirmed that all subjects participated in this study signed written informed consent for publication their data and relevant information.

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No funding was received.

## Authors' contributions

PL performed the primary bioinformatics analysis, experiments, and wrote the manuscript; ZH and HZ made substantial contributions to data analysis and technical support. JL reviewed and approved this manuscript.

## Ethics approval and consent to participate

The study has been approved by the ethics committee of our hospital, and the patient signed the informed consent form.

## Competing interests

The authors declare that they have no competing interests.

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## Availability of data and materials

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

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## Tables

Table 1 Differentially expressed miRNAs from GEO database.

\*:up-regulation; #:down-regulation;

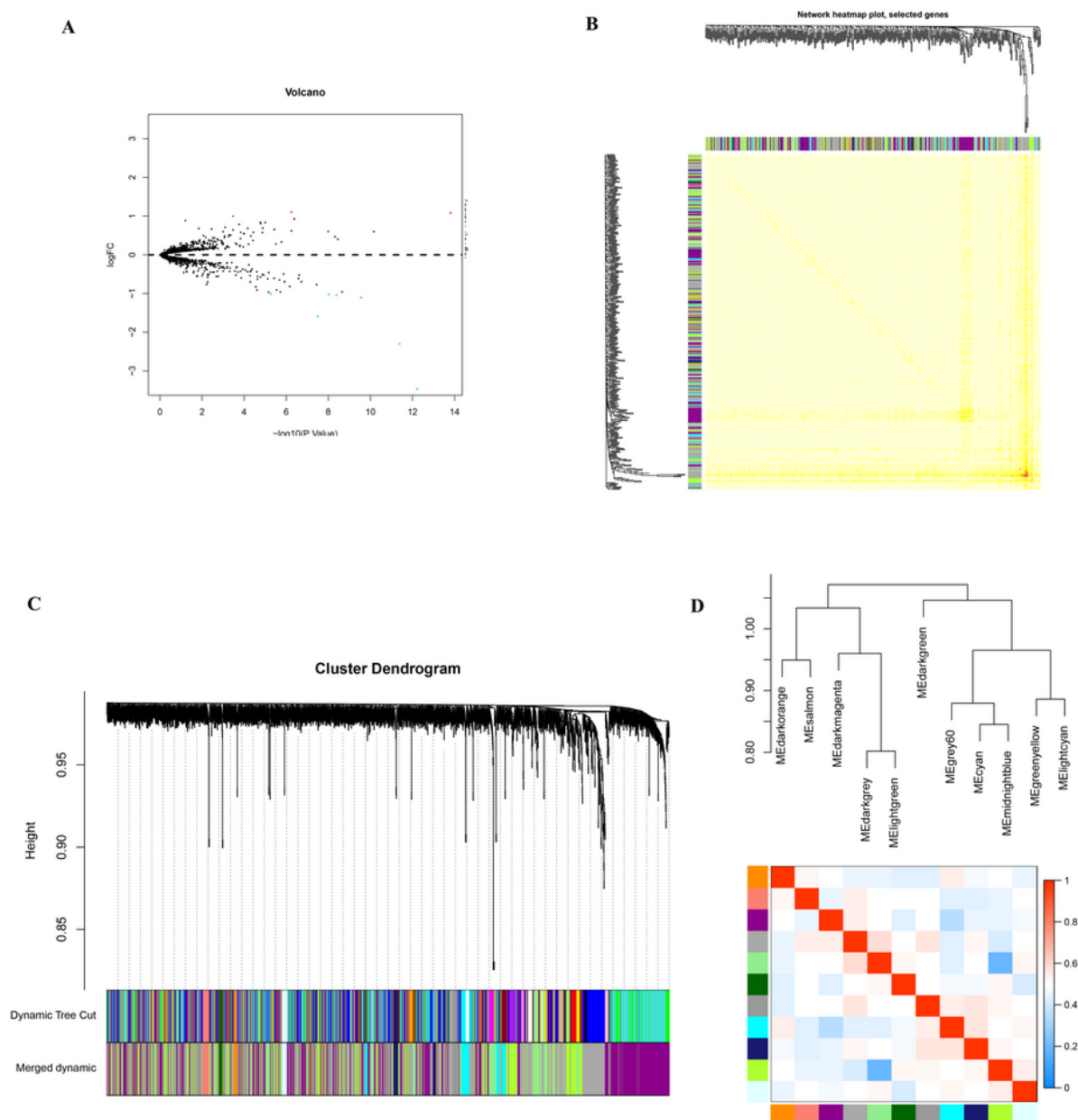
microRNAs	Log <sub>2</sub> FC	AveExpr	t	P.Value	adj.P.Val
miR-155-5p#	1.08	0.85	8.86	1.55E-14	4.00E-11
miR-4668-5p*	3.46	6.48	-8.15	6.05E-13	7.80E-10
miR-3613-3p*	3.63	6.58	-8.07	9.27E-13	7.96E-10
miR-3201*	2.30	2.59	-7.78	4.14E-12	2.67E-09
miR-548ac*	1.10	1.91	-6.94	2.76E-10	1.19E-07
miR-486-5p*	1.04	6.62	-6.39	4.08E-09	1.17E-06
miR-548a-3p*	1.02	2.09	-6.21	9.44E-09	2.18E-06
miR-8084*	1.59	2.14	-5.95	3.19E-08	6.32E-06
miR-455-3p#	1.10	4.71	5.31	5.67E-07	7.39E-05
miR-6068*	1.00	3.02	-4.78	5.44E-06	4.38E-04
miR-1246*	1.00	4.86	3.70	3.39E-04	1.07E-02

Table 2. Models and MM value of these differentially miRNAs

## Figures

Model	miRNAs	MM <i>p</i> 0.05				
Darkgrey	hsa-miR-4668-5p	darkmagenta	darkgrey	lightgreen	grey60	
	hsa-miR-3613-3p	darkmagenta	darkgrey	lightgreen	greenyellow	
	hsa-miR-6068	greenyellow	grey60	lightgreen	darkgrey	
Darkmagenta	hsa-miR-548ac	darkmagenta	darkgrey	cyan		
	hsa-miR-3201	darkmagenta	cyan			
	hsa-miR-548a-3p	darkmagenta	darkgrey			
	hsa-miR-8084	darkmagenta				
Lightgreen	hsa-miR-155-5p	lightgreen				





**Figure 1**

Combined screening of key differentially expressed miRNAs in serum between pancreatic and healthy control by GEO and WGCNA method. (A)Volcano map of differentially expressed miRNAs in serum of patients with pancreatic cancer from GEO database. (B) Heatmap of miRNAs expression in serum of patients with pancreatic cancer from GEO database. (C) Gene dendrogram obtained by average linkage

hierarchical clustering. (D) Hierarchical clustering dendrogram of module eigengenes and heatmap plot of the adjacencies in the eigengene network. green: negative correlation; red: positive correlation.

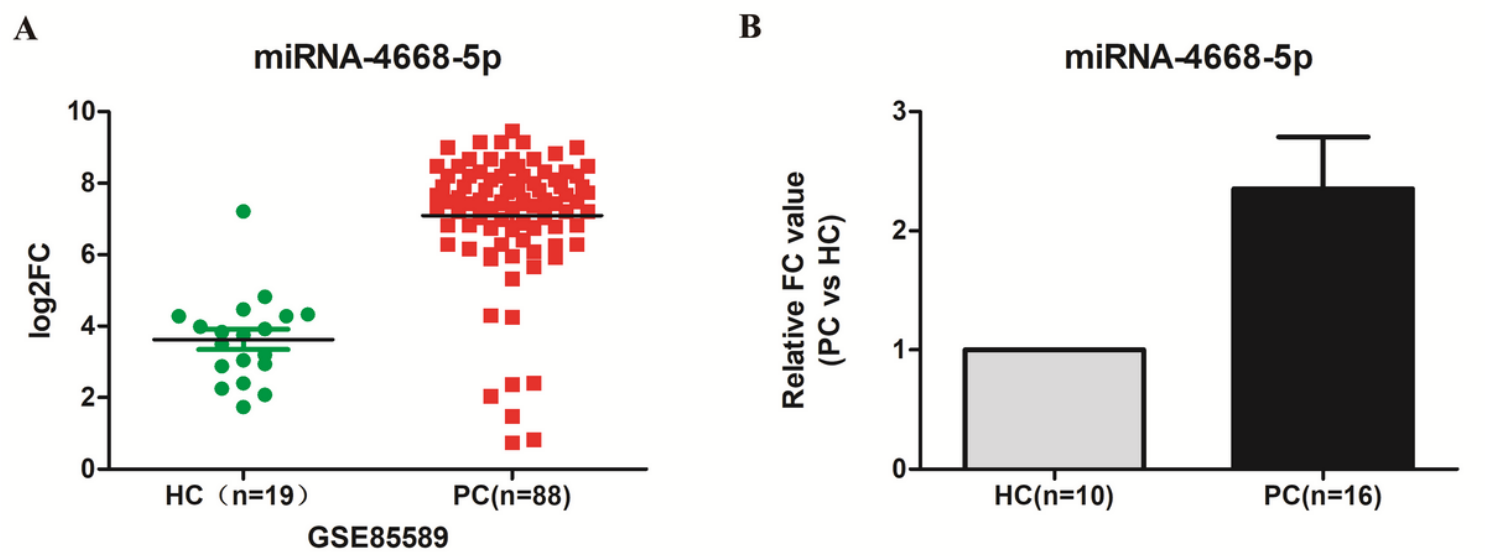


Figure 2

miR-4668-5p was significantly up-regulated in serum of patients with pancreatic cancer. (A) Up-regulated miR-4668-5p was observed in serum of patients with pancreatic cancer compared to healthy control based on GEO database. (B) Up-regulated miR-4668-5p was identified in serum of patients with pancreatic cancer compared to healthy control based on qRT-PCR method. HC: healthy control; PC: pancreatic cancer.