

The function of MiR-21 expression differences and pathogenesis on familial and triple negative breast Cancer serum

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Abstract: This paper is to detect the expression differences of serum miR-21 in breast cancer, in order to further clarify the function of miR-21 in familial and TNBC pathogenesis of breast cancer. The serum had been collected for healthy check-up females, women with high risk of breast cancer and different types of breast cancer patients. Nematodes were taken as the external reference, real-time fluorescent quantitative PCR detection were taken for expression level of miR-21 in 77 cases of serum. The miR-21 expression level of familial breast cancer group, TNBC group and breast cancer high risk group were significantly higher than that in normal control group and other breast cancer group, $P < 0.01$. Serum miR-21 expression level was associated with lymph node metastasis and Ki67 high expression, $P < 0.01$. Results had proved that serum miR-21 expression quantity increased in familial breast cancer and TNBC and was correlated with lymph node metastasis and Ki67 expression. Serum miR-21 was closed related with TNBC and familial breast cancer. The relative expression quantity of miR-21 in breast cancer serum had no obvious relation with unilateral or bilateral tumor and menstrual situation. Its increased expression might be correlated to the breast cancer hereditary, malignant degree and prognosis judgement and its mechanism required further research.

Keywords: miR-21, familial breast cancer, TNBC, serum, FQ-PCR

INTRODUCTION

The incidence of breast cancer ranked first among the world's women malignant tumor. The incidence of breast cancer in China rose at an annual rate of 3%~4%, and became the main cause of cancer death in women. Breast cancer is a malignant disease with obvious genetic characteristics, which was caused by environmental, genetic factors and other factors (Wright *et al.*, 2010). Chinese familial breast cancer accounts for 20%~25% of all breast Cancer. TNBC with negative of estrogen, progesterone receptor and HER-2 accounts for 15%~20% of all breast cancer, and has higher recurrence rate and worse prognosis than other types of breast cancer. In recent years, more and more research evidence has suggested that micro RNA could be the biomarkers to detect the cancer existence and prognosis judgement. The expression quantity change of miR-21 in cancer patients is the most common (Chen *et al.*, 2014). In 2005, Iorio *et al* firstly found that miR-21 expression quantity in breast cancer tissue was significantly higher than that in normal breast tissue, Wang F *et al* found miR-21 expression in breast cancer tissue and serum both had increased (Iorio *et al.*, 2005; Wang *et al.*, 2010). Therefore, this research chose the specimen from patients with high risk of breast cancer and patients with different kinds of breast cancer, especially, detected the miR-21 expression level of serum in familial breast cancer and TNBC patients, and analyzed its function in familial breast cancer and TNBC.

MATERIAL AND METHOD

Study objective

The research objectives were the outpatients and hospitalized patients of The Second Affiliated Hospital of Zhengzhou University from January of 2013 to May of 2014. All the patients were divided into four groups: Group A was 20 cases of TNBC patients, average was 53 ± 1.33 years old; Group B was 8 cases of familial breast cancer patients, exclusive of TNBC patients, average was 49 ± 1.98 years old; Group B2 was 9 cases of the healthy first-degree relatives of familial breast cancer patients, these people were with high risk of breast cancer, average was 37 ± 4.12 years old; Group C was 20 cases of other breast cancer patients, except TNBC and familial breast cancer patients, average was 56 ± 2.43 years old. Group D was the 20 cases of patients in control group of the same time physical examination, average was 45 ± 3.24 years old, the inclusion criteria was the health female without tumor history and clinical signs. The collected serum was placed in refrigerator at negative 80.

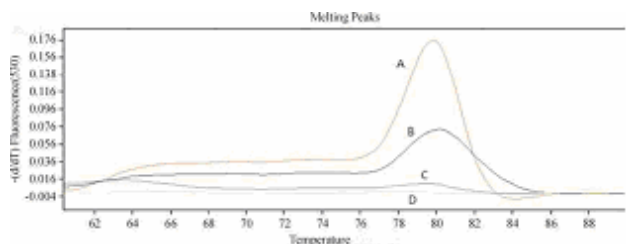
Instruments and equipment applied

Light Cycler 1.5 Instrument was the Germany Roche real-time fluorescence quantitative instrument; ABI Prism 7000 fluorescent quantitative PCR instrument was from ABI Company of USA; High-speed refrigerated centrifuge was from USTC ZONKIA company; α level B2 type bio-safety cabinet was from Singapore ES-CO company; Biophomet sample adding gun was from Germany Eppendorf; Biophotometer Biological spectrophotometer was from Germany Eppendorf.

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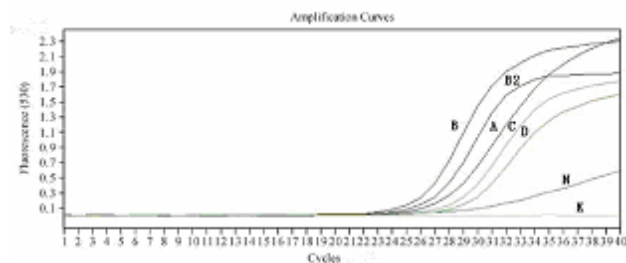
Main reagen

Trizol reagen was from Invitrogen of USA; Isopropyl alcohol, chloroform and anhydrous ethanol were from Guangzhou Dahui Company; HmiR-21 Hairpin-it™ Real-Time PCR Kit and Cel-miR-39 Hairpin-it™ Real-Time PCR Kit were from Shanghai Gene Pharma Co., Ltd; RNA enzyme inhibitors were from Thermo Fermentas of Canada.



A: miR-21; B: miR-39; C: miR-21 negative control; D: miR-39 negative control.

Fig. 1: Melting curves for miR-21 and miR-39



Triple negative breast cancer; B: Familial breast cancer; B2: High risk for breast cancer C: Other breast cancer; D: Healthy control; N: Negative control; K: Blank control

Fig. 2: The amplification curves of fluorescence quantitative PCR for serum miR-21 in different groups.

Method

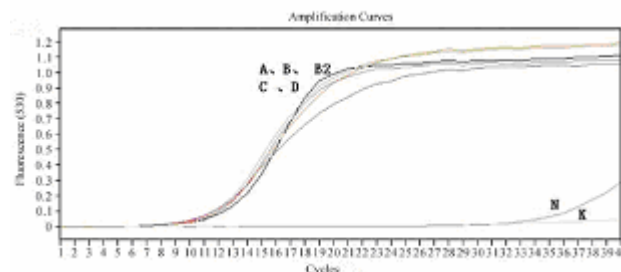
Sample preparation

4mL peripheral venous blood was took from objective by thromboplastic vocuum blood collector tube. Samples had be performed centrifugal 3 000 r/min for 10 minutes after blood clotted, 1.5mL of upper serum were taken into EP tube and placed into refrigerator at negative 80.

Extraction of total RNA

Serum samples melted on ice. 250µL of sample was taken and added into 1mL Trizol for blending. Then placed at room temperature for 5 minutes, then added 4µL of mimics. Then 200µL of chloroform was added, and then massively oscillated in vortex generator for 15 seconds, then added into centrifuge at 4□ 12 000 r/ min for 15 minutes. The upper liquid, around 600µL was sucked out, then 500µL isopropyl alcohol was added and reversed for blending, then placed at room temperature for 10 minutes. After the centrifuge 12 000 r/min for 10 minutes at 4□, the upper liquid were removed. The 1mL of precooled volume fraction was 750µL andydrous ethanol and 250µL DEPC treated water, then went for washing RNA

precipitation for few times. Then for centrifugal 7 500 r/min for 5 minutes at 4°, then the upper liquid was removed. Then cap was open, at room temperature, dried the RNA precipitation 25 minutes. 30 µL DEPC treated water were added and repeatedly blending with RNA precipitation in order to dissolute well. Total RNA concentration was detected, the total RNA purity ranged from 1.8 to 2.0, the concentration was 25~50 mg/L.

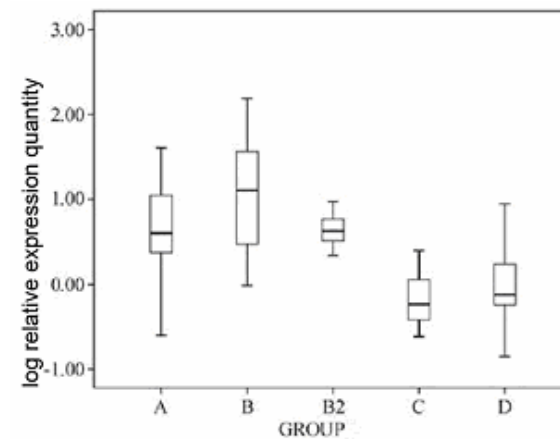


A: Triple negative breast cancer; B: Familial breast cancer; B2: high risk for breast cancer C: Other breast cancer; D: Healthy control; N: Negative control; K: Blank control

Fig. 3: The amplification curves of fluorescence quantitative PCR for exogenous internal reference miR-39 in difference groups.

Reverse transcription reaction

Reaction system overall was 20 µL, including 4µL of 5×buffer, 0.75µL of dNTP (10mmol/L), 1.2µL of miR-21 retrovirus primers (1µmol/L), 0.25µL of RNA enzyme inhibitors (40U/µL), 0.2µL of MMLV reverse transcriptase (200U/µL), 5µL of total RNA sample, 8.6µL of DEPC treated water. Another prepared hole was settled. Reaction condition was respectively at 25° for 30min, 42° for 30min and 85° for 5min. External references Cel-miR-39 reverse transcription reaction system was same as above. In negative control group, total RNA was replaced by saline solution.



A: Triple negative breast cancer; B: Familial breast cancer; B2: high risk for breast cancer; C: Other breast cancer; D: Healthy control.

Fig. 4: The serum miR-21 expression level in different groups.

Real-time fluorescent quantitative PCR

Reaction system overall was 20 μ L, including 2 \times Master Mix 10 μ L, which included MgCl₂, dNTPs, SYBR Green fluorescent dye, 0.32 μ L of RT-PCR primers suit (5mol/ μ L), 0.2 μ L of Taq DNA polymerase (5 \times 10⁶ U/L), 2 μ L of reverse transcription product, 8.6 μ L of DEPC treated water. Reaction was set a prepared hole. Reaction condition was 95° degeneration 12 seconds, and 62° for 40 seconds, total repeated for 40 times. External references Cel-miR-39 reaction system was same as above. The dissolved curve was set to judge whether nonspecific amplification and the appearance of primer dimers. In blank control group, reverse transcription products were replaced by saline solution.

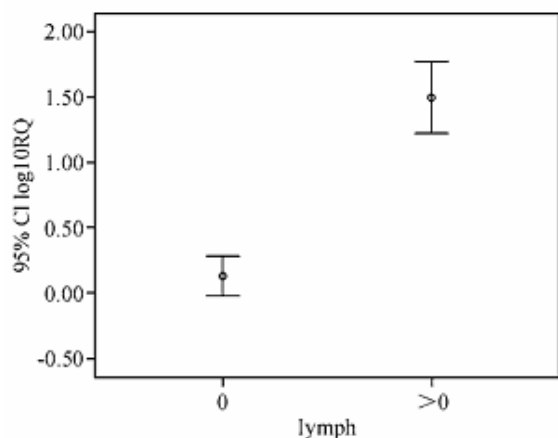


Fig. 5: Association of miR-21 expression with lymph node metastasis

miR-21 expression quantity in serum

PCR amplification results were marked as Ct, Ct means the cycle number fluorescent signal in PCR reaction reacted the set threshold. The RQ of objective gene was marked as $2^{-\Delta\Delta Ct}$ (Rui-shan et al., 2013). $\Delta\Delta Ct$ equals to the minus of the results of sample Ct_{miR-21} minus sample $Ct_{cel-miR-39}$ and the results of control group Ct_{miR-21} minus control group $Ct_{cel-miR-39}$, healthy physical examination specimen serum miR-21 was as the control group.

STATISTICAL ANALYSIS

All data were processed by SPSS17.0 statistical software, RQ $2^{-\Delta\Delta Ct}$ was variance and unevenly variance, the $2^{-\Delta\Delta Ct}$ was processed by data, and performed the simple sample K-S detection, which meets normal distribution ($Z=0.643, P=0.803 > 0.05$). The Levene test results proved the uneven of variance ($P=0.007 < 0.05$). Single factor ANOVA analysis was applied to compare between the sample mean value. Tamhane's T2 method was applied to detect between two samples. The comparison of serum miR-21 expression level between different breast cancer clinical pathological parameter set, Wilcoxon rank sum test, $P < 0.05$ was marked for statistically significance.

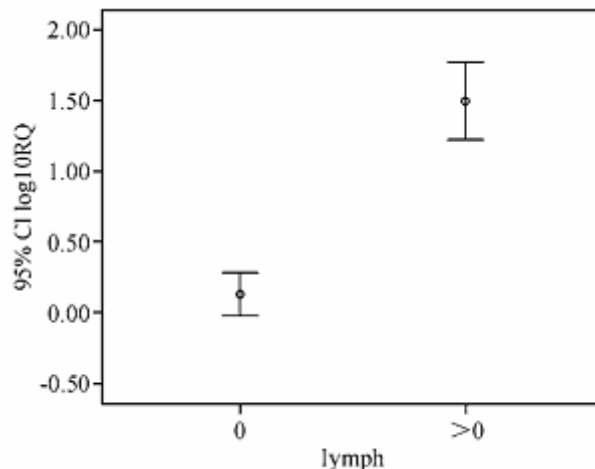


Fig. 6: Association of miR-21 expression with ki67

RESULTS**Dissolution curve of miR-21 and Cel-miR-39**

miR-21 dissolution curve showed unimodal curve at 79°; Cel-miR-39 dissolution curve showed unimodal curve around at 81° and without the existence of primer dimers and impurity peak, which proved PCR reaction parameters fit the research, amplification target product specificity is better, as shown in fig. 1.

Expression difference between groups of serum miR-21

Real-time fluorescent quantitative PCR detection results
Serum miR-21 of each object group and Cel-miR-39 shown 'S' shape response curve, the curve of negative control group showed irregular shape, blank control group showed non-amplification curve, as seen in fig. 2 and fig. 3.

Serum miR-21 expression level comparison of normal control group, breast cancer A, B, B2, C group

ΔCt is applied to mark the expression quantity of sample miR-21. If ΔCt became lower, indicated miR-21 expression quantity became higher in objective. The detection results showed the serum miR-21 expression quantity. miR-21 and miR-39 detected results of all objective serum sample were shown in table 1.

The relative expression of the 5 groups had be compared by single factor ANOVA variance analysis, and had statistical significance, $F=17.11, P=0.00 < 0.01$. Then, the Tamhane's T2 method was applied to compare each group. The miR-21 expression level in TNBC and familial breast cancer group were significantly higher than that in normal control group and other breast cancer groups, ($P < 0.01$). The miR-21 expression level of breast cancer high-risk group serum was significantly higher than that in normal control group and other breast cancer groups, ($P < 0.01$). The difference of TNBC group, familial

breast cancer combined with breast cancer high-risk group had no statistical significance, $P > 0.05$. Normal control group had no statistically significant difference between other breast cancer group, $P > 0.05$, as shown in fig. 4.

Relation between expression of serum miR-21 and clinicopathological parameters of breast cancer

miR-21 relative expression quantity of breast cancer serum did not obvious related with unilateral or bilateral tumor occurrence ($P = 0.679$), age ($P = 0.245$), CA153 ($P = 0.893$) and menstrual status ($P = 0.717$), and there was no statistical significance between each group, each $P > 0.05$. Relative expression quantity of miR-21 in breast cancer serum was positively related with lymph node metastasis ($P = 0.000$) and Ki67 ($P = 0.006$), as seen in table 2, $P < 0.05$ was statistically significant, fig. 5 and fig. 6.

DISCUSSION

miRNA is the endogenous non-coding small RNA, which widely exist in animal, plants and viruses, and could complementary pair with specific target miRNA sequence. The miR-21 regulates the expression of target genes in the transcription or translation and participate in physiological and pathological process of creature. Studies showed that miRNA was closely related with tumor clinical pathological characteristics, and its expression condition could be used in the diagnosis, prognosis judgment and treatment of malignant tumor.

Real-time fluorescent quantitative PCR detected the miRNA expression quantity of human body serum. To eliminate the differences between samples in the process of RNA extraction is critical. Most studies chose internal parameter U6 to achieve this effect. While, it is not known yet whether internal parameter could affect expression quantity in human disease condition, and there were many unknown factors for serum source and expression. This study took synthetic nematodes miR-39 as exogenous internal, as human serum does not contain this sequence and less influence factors, which has been verified (Bing-ming *et al.*, 2013).

The existing studies have demonstrated that miR-21 encoding genes located in 17q23.2 (Fujita *et al.*, 2008) and it expressed abnormal in colon cancer (Schetter *et al.*, 2008), lung cancer (Markou *et al.*, 2008), gastric cancer (Volinia *et al.*, 2006), breast cancer (Yong-rong *et al.*, 2011) and other tumors. The expression level of miR-21 in breast cancer tissue was higher than that in normal breast tissue, and its expression level was correlated with clinical and pathological features (Qian *et al.*, 2009; Wang *et al.*, 2012; Kumar S *et al.*, 2013). And the scholars at home and abroad have focused on miR-21 in human body blood circulation, and proposed that increased expression of serum miR-21 had predictive significance in early diagnosis (Chen *et al.*, 2014), early treatment (Muller *et*

al., 2014), tumor metastasis (Yong-rong *et al.*, 2011), tumor progression (Yan *et al.*, 2008) and so on. Therefore, this research had clinically studied the miR-21 in serum of breast cancer patients.

This research mainly detected the miR-21 expression level of serum in normal people, high risk of breast cancer people and breast cancer patients and compared the analysis and difference. Test results showed that the serum miR-21 expression level in familial breast cancer group ranked on the top of all groups; the serum miR-21 expression quantity in familial breast cancer group, TNBC group and high risk breast cancer group were higher than that in other breast cancer group and normal control group, $P < 0.05$. Ze-nian *et al* studied serum miR-21 was highly expressed in breast cancer patients (Ze-nian Wang *et al.*, 2014); Wang B *et al* applied SYBR green real-time fluorescent quantitative quantitative PCR detected miR-21 expression quantity in serum of normal control group and breast cancer group, and researched that miR-21 highly increased in cancer patients serum, $P < 0.001$. The above two research conclusions were consistent with this study results, but there were few difference, which was breast cancer group contained familial breast cancer group and TNBC group. This study had respectively studied the familial breast cancer patients and TNBC patients, so there was no statistical significance in normal control group and other breast cancer group in the conclusion, and the miR-21 expression both increased in familial breast cancer group and TNBC group. Radojicic *et al* studied miR-21 highly expressed in primary TNBC patients, which was consistent with this study (Radojicic *et al.*, 2011).

This study also found that there were 9 cases of healthy first-degree relatives of familial breast cancer patients with increased expression of serum miR-21, and with statistically significance of normal control group, $P < 0.05$. The high expression of miR-21 in serum of breast cancer high-risk people and breast cancer family history could be used to predict high risk of breast cancer, and this conclusion still need to increase the sample quantity for further experiment.

The detection results of miR-21 from 48 cases of breast cancer patients and results of breast cancer clinical pathological parameters had shown the difference between serum miR-21 expression level and lymph node metasis ($P = 0.000$), Ki67 ($P = 0.006$) had statistical significance through statistical analysis. The high expression of miR-21 in breast cancer patient's serum not only related with the occurrence of breast cancer, but also related with lymph node metastasis and Ki67 expression. It demonstrated that miR-21 was correlated with metastasis of breast cancer.

Ki67 antigen is nuclear antigen expressed in proliferated cells, which could reflect tumor proliferation activity

Table 1: Differential expression of serum miR-21 in healthy control group and various of breast cancer group ($\bar{x} \pm s$)¹⁾

Group	N	miR-Ct	Cel-miR-39Ct	$2^{-\Delta\Delta Ct(1)}$	$\log 10^{2^{-\Delta\Delta Ct}}$
A Group	20	25.39±1.59	12.11±0.87	4.00(2.32, 11.71)	0.67±0.57
B Group	8	23.86±1.84	11.77±0.94	12.87(2.63, 56.59)	1.06±0.74
B2 Group	9	25.13±0.80	11.76±0.65	4.26(2.97, 5.98)	0.64±0.19
C Group	20	26.45±1.59	11.02±0.80	0.58(0.38, 1.14)	-0.16±0.30
D Group	20	27.97±0.76	12.46±1.01	0.76(0.54, 1.79)	0.00±0.40

M(P_{25} , P_{75}) is marked, if data was not in a normal distribution.

Table 2: Association of miR-21 expression with clinical and pathological features

Observe Index	Tumor feature		Age		CA153	
	Bilateral breast cancer	Unilateral breast cancer	Less than 45 years old	Not less than 45 years old	Positive	Negative
$C \log 10^{2^{-\Delta\Delta Ct}}$	0.40±0.26	0.62±0.17	0.77±1.01	0.30±0.61	0.35±0.60	0.44±0.78
P value	0.679		0.245		0.893	
Observe Index	Menstrual status		Lymph node metastasis		Ki67	
	Premenopause ¹⁾	Postmenopause ¹⁾	yes	no	≥30%	<30%
$\log 10^{2^{-\Delta\Delta Ct}}$	0.47±0.76	0.24±0.52	1.50±0.36	0.13±0.46	0.86±0.76	0.21±0.59
P value	0.717		0.000		0.006	

(Gong P *et al.*, 2012). The over expression of Ki67 antigen is closed related with the malignant biological behavior and prognosis of breast cancer, and could be identified as a precancerous biological marker for individuals with high risk of breast cancer in crowd. Ki67 and miR-21 was positively correlated. The prediction of miR-21 has certain significance in judging malignant degree of breast cancer, tumor proliferation and high-risk prediction.

Serum miR-21 was closely related with familial breast cancer and TNBC, and its increased expression related with breast cancer hereditary, malignant degree and prognosis, the specific mechanism remained to be discussed. Whether miR-21 in serum could be the certain breast cancer diagnosis, prediction index and BRCA1 mutations relationship needs further study.

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