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Study on the relationship between miR-520g and the development of breast cancer

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Abstract. – OBJECTIVE: Breast cancer (BC) is one of the most common malignant tumors occurred in women. There is no sensitive and specific marker for early diagnosis, treatment and prognosis of breast cancer. It is suggested that miRNA may be a potential tumor marker for breast cancer. Mir-520g is considered to be associated with many tumors. This study aims to test the expression of mir-520g in peripheral blood of BC patients and healthy control. We also explored the relationship between mir-520g and several prognostic factors in breast cancer patients.

PATIENTS AND METHODS: The peripheral blood of 86 cases with breast cancer (including 18 cases with stage 0, 24 cases of phase I, 20 cases of stage II, 24 cases of stage III) and 26 cases of healthy subjects were collected. The miR-520g level was measured by real-time quantitative PCR (RT qPCR) method. The correlation between plasma miR-520g level and the clinical stage, molecular subtype, receptors' expression and other factors related to the prognosis of the patients were examined.

RESULTS: Plasma mir-520g expression levels were significantly higher in BC patients with lymph node metastatic and low differentiation degree grade ($p = 0.033$ and 0.016), and plasma miR-520g expression was significantly higher in breast cancer patients with mammary gland invasion ($p < 0.01$) and low expressed p53 ($p = 0.0039$).

CONCLUSIONS: Highly expressed mir-520g is associated with lymph node metastasis and low differentiation of breast cancer, and also is associated with mammary gland invasion in breast cancer. This study suggests that mir-520g may be associated with some important prognostic factors in breast cancer patients, and may have a potential value for breast cancer marker.

Key Words:
Breast cancer, miR-520g, Correlation analysis, Prognostic factors, Clinical stage, Metastasis.

Introduction

As one of the most common malignant tumors occurred in women, the incidence of breast cancer (BC) is gradually increased world widely. According to epidemiology analysis, every year, more than one million patients were diagnosed with breast cancer, and over 30,000 people died of breast cancer each year[1-4]. In recent years, the incidence and mortality of breast cancer in China is increasing year by year, which seriously endanger the health of women[5]. The research of breast cancer has developed rapidly in the past 30 years, and the individual treatment has been developed as a model based on the guidance of evidence-based medicine, appropriate comprehensive treatment could be performed according to the different clinical stages, molecular subtypes and receptors' expression[6-8]. One of the important criteria for individual treatment is to avoid excessive treatment, which all dependent on the prognosis of BC patients with simple, economic and dynamic evaluation.

The current prognostic factors in BC patients include clinical stage, molecular subtype (including Luminal A/B, HER2 over expression, and others), tumor size, lymph node (LN) and distant metastasis, tumor pathological grading, mammary gland invasion and key receptors' expression such as HER2, EGFR, VEGFR, ER and others. In addition, the use of peripheral blood or tumor tissue specimens for multiple gene loci analysis of gene chip test methods were also used for prognosis, such as the 21-gene signature (Oncotype DX)[9] and the European common 70-gene detection MammaPrint[10]. However, the Dx Oncotype gene chip is only suitable for evaluating the prognosis of patients with ER (+), LN (-), and patients to be treated with endocrine therapy; the latest research suggests that the results of the MammaPrint test are only consistent in 73% of the NPI score, and the inconsistent outcome occurred in 1/4 patients[11]. In addition, the multiple gene loci analysis is difficult to be widely used due to the high price.

Peripheral blood markers are simple and economic[12]. The clinical value of miRNA drawn

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more and more attention. Mir-520g has been found to be closely related to the development of many tumors, such as hepatocellular carcinoma[13], brain tumors[14], and breast cancer[15]. In this study, we used RT-qPCR method to detect plasma mir-520g levels, and used Mann Whitney test, Kruskal Wallis test to analyze the correlation between plasma mir-520g levels with multiple individual prognostic factors, and to evaluate the potential value of mir-520g as tumor markers for breast cancer.

Patients and Methods

Patients

In this study, 86 cases of Chinese female breast cancer patients who were diagnosed by pathology were recruited. Their TNM pathological staging of breast cancer was diagnosed according to National Comprehensive Cancer Network (NCCN) clinical practice guidelines for breast cancer[16]. These patients all received surgery and no other treatment was performed. Personal information including age of operation, their pathological type, pathological grade, tumor size (T), lymph node metastasis (N), distant metastasis (M), receptors' status (PR, HER2, p53, EGFR, ER, etc.) and mammary gland invasion status, etc. were collected for analysis. 26 healthy subjects with similar age who received medical test were selected as control.

Male patients, patients with bilateral breast cancer and patients who received resection or biopsy of the tumor in other hospital before treatment were excluded.

The study has been approved and registered by the hospital Ethics Committee of Logistics University of the Chinese People's Armed Police Force in January 2014, the Ethics Committee approved relating screening, treatment, and data collection of these patients, all subjects signed written informed consent form. All works were undertaken following the provisions of the Declaration of Helsinki.

Sample Collection and Separation:

Blood samples were collected before the treatment (Surgery, chemotherapy, radiotherapy, etc.). A total of 4 ml of blood was drawn from each patient, then separated into 2 vacutainer containing anticoagulant (BD Biosciences, Franklin Lakes, NJ, USA), samples were centrifuged at 3000 rpm for 10 min, the supernatant plasma was drawn carefully to 1.5 ml Eppendorf tube without RNA enzyme. The RNA with length < 200 nt was extract by miRNeasy Mini Kit (QIAGEN Hamburg GmbH, Hamburg, Germany).

RT-PCR for miR-520g

After serum total microRNA was extracted, reverse transcription polymerase chain reaction (RT-PCR) method was used to measure relative expression of miR-520g. The RT-PCR reaction was performed with TOYOBO Reverse Transcription Kit (Toyobo, Tokyo, Japan). miR-16 was selected as internal reference gene. The forward miR-520g RT primer was GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACACTC, and forward primer was GCGGTCTCTAGAGGGAAGCAC; The U6 forward primer (5'-3'): CTCGCTTCGGCAGCACA, reverse primer AACGCTTCACGAATTTGCGT. Reverse transcription was performed with the following conditions: 30°C for 10 min, 42°C for 60 min, 99°C for 5 min and 4°C for 5 min.

The CFX96 Touch™ Real-Time PCR detection system (BioRad, Hercules, CA, USA) was used for quantitative measurement with the following conditions: 94°C for 2 min, 94°C for 30 s (50 cycle), 60°C for 40 s. The relative expression of miR-520g was calculated by Schmittgen's methods: $F = 2^{-\Delta ct}$, $^{\Delta}ct$ = ct miR-520g – ctmiRNA-U6. CT means the number of cycles experienced by the fluorescent signals reached the threshold inside the reactor.

Statistical Analysis

The relative expression of miR-520g was expressed as mean ± SD and compared with different pathological data. SPSS 19.0 (IBM, Chicago, IL, USA) was used for statistical analysis, Mann-Whitney test was used for two-group sample comparison, and Kruskal Wallis test was used for multi-group sample comparison. $p < 0.05$ was considered as statistical difference.

Results

Demographic Data

A total of 86 female BC patients were selected successfully, 18 case of them were at stage 0, 24 of them were at stage I, 20 of them were at stage II and 24 of them were at stage III. 26 cases of healthy subjects were selected as control.

The average age of BC patients was 51.09 ± 10.16 year, their relative miR-520g expression was 45.24 ± 12.86; the average age of healthy subjects was 52.24 ± 9.76 year, their relative miR-520g expression was 26.54 ± 6.82, the relative miR-520g expression between BC patients and healthy subjects were significantly different ($p < 0.01$).

Highly Expressed miR-520g is Not Correlated with T Stage But is Related with N stage

We analyzed the relative expression of miR-520g in BC patients with different TNM stage; results were listed in Table I, as we can see, highly expressed miR-520g is not correlated with T stage, but miR-520g expression is correlated with lymph node metastasis, lymph node metastasis-patients (N1, N2 and N3) have markedly high expression ($p < 0.05$) when compared with patients with no lymph node metastasis (N0).

miR-520g Expressed Highly in Low Differenced BC Tumor

The expressions of miR-520g were analyzed according to tumor differentiation degree (Table II), results showed the lower the differentiation degree is, the higher the miR-520g expression is. The difference between low, middle and high differentiation- tumors are significant ($p < 0.05$).

miR-520g Expressions with or Without Mammary Gland Invasion

As we can see from Table III, the miR-520g expressions in mammary gland invasion patients and patients without mammary gland invasion are significantly different, it's expression in patients with mammary gland invasion are significantly higher than patients without mammary gland invasion ($p < 0.01$).

miR-520g Expressions in Patients with Different Estrogen Receptor (ER) Levels

We compared the miR-520g expressions in patients with different estrogen receptor (ER) levels, results showed the miR-520g expressions have no difference with ER levels ($p > 0.05$, Table IV).

miR-520g Expressions in Patients with Different Progesterone Receptor (PR) Levels

We compared the miR-520g expressions in patients with different estrogen receptor (PR) levels, results showed the miR-520g expressions have no difference with PR levels ($p > 0.05$, Table V).

miR-520g Expressions Have No Difference in Human Epidermal Growth Factor Receptor-2 (Her2) Levels

We compared the miR-520g expressions in patients with different Her2 levels, results showed the miR-520g expressions have no difference with Her2 levels ($p > 0.05$, Table VI).

Highly Expressed miR-520g is Related with Low Expression of p53

In addition, we compared the miR-520g expressions in patients with p53 (+) or (-), results showed the relative expression of miR-520g in patients with p53 (+) were statistically lower than patients with p53 (-) (Table VII).

Expression of Mir-520g was Not Significantly Related with ki67 Grading

We also compared miR-520g expression with different Ki67 grading (0%-15%, 16%-30% and > 30%), results showed miR-520g expressions have no difference in patients with different Ki67grading (data not show).

Table I. Relative miR-520g expression in BC patients with different TNM stage.

Stage		Case number	miR-520g expression	*p*-value
T stage	0	18	34.14 ± 8.37	0.142
	1	42	44.56 ± 13.24	
	2	22	48.77 ± 15.64	
	3	2	54.72 ± 17.41	
	4	2	64.36 ± 29.50	
N stage	0	44	35.36 ± 12.32	0.033
	1	18	45.2 ± 14.57	
	2	12	57.34 ± 19.21	
	3	12	68.46 ± 24.22	
M stage	0	86	45.24 ± 12.86	0

Table II. Comparison of miR-520g expression in tumor differentiations.

		Case number	miR-520g expression	*p*-value
Differentiation degree	High	10	30.27 ± 11.22	0.016
	Middle	36	38.48 ± 13.54	
	Low	36	53.36 ± 17.51	
	Unclear	4	52.42 ± 17.86	

Table III. Comparison of miR-520g expression in patients with or without mammary gland invasion.

		Case number	miR-520g expression	*p*-value
Mammary gland invasion	No	72	41.79 ± 14.01	< 0.01
	Yes	14	62.94 ± 19.86	

Table IV. miR-520g expressions in patients with different estrogen receptor (ER) levels.

		Case number	miR-520g expression	*p*-value
Estrogen receptor (ER) levels	ER (-)	32	42.62 ± 14.54	0.825
	ER (+)	4	38.65 ± 12.68	
	ER (++)	14	46.26 ± 15.66	
	ER (+++)	30	45.42 ± 14.87	
	ER (++++)	6	48.65 ± 16.43	

Table V. miR-520g expressions in patients with different progesterone receptor (PR) levels.

		Case number	miR-520g expression	*p*-value
Progesterone receptor (PR) levels	PR (-)	42	39.84 ± 14.21	0.529
	PR (+)	10	42.66 ± 15.20	
	PR (++)	12	48.64 ± 14.88	
	PR (+++)	13	51.35 ± 17.68	
	PR (++++)	9	55.24 ± 18.62	

Table VI. miR-520g expressions in patients with different human epidermal growth factor receptor-2 (Her2) levels.

		Case number	miR-520g expression	*p*-value
Her2 levels	Her2 (-)	27	38.47 ± 11.45	0.651
	Her2 (+)	24	43.96 ± 13.66	
	Her2 (++)	22	46.64 ± 15.34	
	Her2 (+++)	13	50.87 ± 18.97	

Table VII. miR-520g expressions in patients with p53 (+) or (-).

		Case number	miR-520g expression	*p*-value
p53	p53 (-)	66	49.04 ± 16.86	0.0039
	p53 (+)	20	34.16 ± 11.25	

Discussion

The prognosis factors of breast cancer include their clinical stage, molecular subtype (including Luminal A/B, overexpression of Her2 and their basal type, etc.), tumor size, lymph node (LN) metastasis, distant metastasis, tumor grade, ER, Her2, and other key receptor's expressions[17-20]. Currently, prognostic evaluation method in breast cancer patients generally include the following: the prognostic score method based on clinical staging and surgical pathology information, such as the Nottingham Prognostic Index (NPI), USC / VNPI prognostic index; the gene chip inspection method underlying the multiple loci expression spectrum analysis use of peripheral blood and tumor tissue, such as the commonly used the 21-gene signature (Oncotype DX) and 70-gene detection MammaPrint, which is commonly used in Europe [21].

These methods have been applied in clinic, but there are still some problems. The classic NPI score, its information comes mainly from the pathological grade and tumor stage, cannot cover the tumor molecular subtypes of breast cancer, and is difficult to reflect the overall heterogeneity and individual differences; USC/VNPI score is mainly used for the prognosis of in situ catheter cancer (DCIS), and cannot be used for other subsets of patients; peripheral blood markers can be easily used for dynamic measurement at multiple time points, which is very useful for the prognosis of BC.

Mature miRNAs stably existed in the circulation systems and it is generally believed that miRNAs came from the active secretion in the form of capsule[22]. The inherent characteristics of miRNAs have made them an ideal marker for diagnosis of BC[23]. In this study, plasma miR-520g was used to explore its potential value in individual prognostic factors in breast cancer patients. mir-16 has been confirmed have good stability in breast tissue, it is listed in the top 10-15 reference gene, and mir-16 has been widely used as a reference gene in many studies[24,25]. Based on these reasons, we choose mir-16 as the reference miRNA.

In this study, the expression of miR-520g was associated with the lymph node metastasis, mammary gland invasion and differentiation degree of breast cancer patients, which indicated that the high expression of miR-520g is associated with the development of breast cancer, especially in lymph node metastasis. So far, the mechanism of miR-520g in the development of breast cancer is not clear. Zhang et al research[26] indicated the expression of microRNA (miR)-520g is correlated with drug resistance of colon cancer cells, and ectopic expression of miR-520g conferred resistance to 5-fluorouracil (5-FU)- or oxaliplatin-induced apoptosis *in vitro* and reduced the effectiveness of 5-FU in the inhibition of tumor growth in a mouse xenograft model *in vivo*. Wang et al[27] reported the miR-520g is up-regulated in hepatocellular carcinoma patients. In addition, one of the potential target genes of miR-520g, p53, is a tumor suppressor gene that inhibits tumor invasion and metastasis[26,28,29], the loss function of p53 protein can lead to the dysfunction of cell cycle and lead to apoptosis of cells. So far, few study explored the relationship between p53 and miR-520g. Our study suggests that miR-520g may affect the prognosis of patients with breast cancer by promoting tumor cell proliferation and apoptosis.

Present studies show that ER and PR expression have closely relationship with breast cancer prognosis[30-33]. The 2 indexes are referred to guide the important reference for the hormone therapy of breast cancer patients with important clinical value. However, we failed to find the correlation between miR-520g and ER, PR and HER2, the reason might be miR-520g has nothing to do with the involved molecular pathways of ER and PR. Different miRNAs can act on the same target genes, and the same miRNA can target a variety of different genes in breast cancer development, the mechanism of how miR-520g act on and its possible interaction with other genes remains to be further studied.

Ki-67 protein is an alkaline protein with protease properties, it is a nuclear antigen closely related to cell mitosis, and Ki-67 can reflect the state of cell proliferation. Ki-67 is essential for the maintenance of cell cycle, and can be used as a marker to reflect the degree of cell proliferation[34]. Rosa et al[35] found Ki-67 is negatively correlated with transcription and protein expression of ER and PR through RT-PCR and immunohistochemistry study. They indicated that Ki-67 was closely related to lymph node metastasis, and it was suggested that Ki-67 might have a great significance in predicting the prognosis of breast cancer. Recent researches[36,37] demonstrated that expression of Ki-67 in breast cancer might be related with p53. However, we don't observe the relationship between Ki-67 and miR-520g expression, this might cause by our limited sample number, and further study with large samples is still needed.

Conclusions

Our study demonstrated that highly expressed mir-520g is associated with lymph node metastasis and low differentiation of breast cancer, and also is associated with mammary gland invasion in breast cancer. There are still some deficiencies in our research; first of all, the included number are relative small and this affects the credibility of the results. Secondly, the related mechanism is not elicited. Thirdly, the occurrence and progress of tumor involved in multi molecular, the prognosis of breast cancer is caused by a number of abnormal expressions of a number of small RNA, and whether mir-520g alone could serve as a prognostic assessment marker, is need subsequent further research.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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