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Expression of miR-520d in breast cancer

Shahram Savad[1], Parvin Mehdipor[2], Hoda Shirdast[3], Ladan Nekoohesh[4]
Leili Nekoohesh[4], Reza Shirkoohi[5], Vahid Nikoui[6], Mohammad Miryounesi[7]
Mohammad Hossein Modaresi[2,*]

ABSTRACT

Background: Breast cancer is the most common cancer in women. Non-coding RNAs especially miRNAs have important regulatory roles in cancer. MiRNAs are 21-24 nucleotides which have different levels of expression between tumors and normal tissues. In this study, we have analyzed expression level of miR-520d in three different groups of breast cancer.

Methods: Fifty nine samples were divided into different groups according to their immunohistochemistry (IHC) classification: estrogen receptor (ER) positive and/or progesterone receptor (PR) positive group (as group I); human epidermal growth factor receptor 2 (HER2) positive group (as group II); and Triple negative group (as group III). After small RNA extraction from tissues, cDNAs were synthesized and Real time RTPCR carried out using DNA binding dye. Expression levels were analyzed by LinRegPCR and REST software.

Results: MiR-520d under- expressed in all of three different groups. The expression ratio in groups I ,II, and III were 0.193, 0.167, 0.21, respectively, but only the result from group II was significant (P=0.017). According to the different clinic-pathological status of breast cancer, miR-520d under-expressed significantly not only in patients with metastatic lymph node (P=0.019) but also in patients which have cancer at stage III (P=0.036).

Conclusion: In this study, we found that miR-520d possibly acts as a tumor suppressor. It may be useful for diagnosis of tumor from normal tissue. In addition, miR-520d significantly under-expressed in HER-2 positive group of breast cancers. Therefore, it may be useful as an additional diagnostic test in this group of breast tumors along with other biomarkers.

Keywords: ***Breast cancer, miR-520d, real time RT-PCR, SYBR green I Dye.***

1. Medical Geneticist, Alborz University of Medical Sciences, Alborz, Iran.
2. Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran.
3. Department of Medical Microbiology, Qazvin University of Medical Sciences, Qazvin, Iran.
4. General Practitioner.
5. Department of Genetics and Genomics, Cancer Research Center, Tehran, Iran.
6. Department of Pharmacology, Tehran University of Medical Sciences, Tehran, Iran.
7. Genomic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

***Corresponding Author:**
Mohammad Hossein Modaresi M.D., Ph.D., Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Pour Sina St., Qods St., Keshavarz Blvd., Tehran, Iran.
Tel: +98- 21- 88953005
E-mail: modaresi@tums.ac.ir

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Introduction

Several mutations are found within the genome of cancerous tissue cells, and thus, cancers are considered genetic diseases. Yet, only in a small percentage of families, single gene mutation cancer is passed down from one generation to the next. Most cancers are affected by the environment.[1] Breast cancer is the most common type of cancer in women. Prevalence of breast cancer includes one out of every 8-12 women in different communities.[2] Nearly 30% of all single-gene breast cancers are caused by mutation in breast cancer genes (BRCA), with subsequent dominant inheritance pattern of breast cancer. However, due to incomplete penetrance of these genes, the individual may not develop breast cancer despite having defective allele.[3] In the past, researchers mostly focused on protein encoding genes such as oncogenes, tumor suppressors, and also on genes with products involved in genome repair.[4] Products of cell surface receptor encoding genes are highly important due to determining treatment response in breast cancer. Tumors with positive human epidermal growth factor receptor 2 (HER2) respond well to treatment with Herceptin, and tumors with positive estrogen receptors (ER+) and progesterone receptors (PR+) respond to hormonal therapy.[5 & 6] In the past two decades, special attention has been paid to the regulatory role of non-encoding small RNA genes.[7] Aided by RNA polymerase II, microRNAs, usually inside other intron genes, are transcribed from their gene sequence. After processing, mature microRNAs are created with about 21-24 nucleotide sequence. MicroRNAs can play their regulatory role of inhibition at translation level in two ways. If they are not complementary to full sequence of target RNA, they inhibit translation by binding to it, and if they are complementary, they will break it.[8 & 9] Presently, it has been found that profile of microRNA expression varies in normal and tumor tissues, and these tissues can be distinguished with some of these profiles.[10] By examining two microRNAs (miR-342 and miR-205), triple negative breast tumors can be distinguished from other types.[11] This study was conducted to examine expression of miR-520d in order to find a potential biomarker to determine receptor status of breast tumor.

Method and Methods

In this case-control study, 59 breast tumor samples, and 59 healthy tissue samples (adjacent to tumor) were received from patients referred to the Cancer Institute of Tehran University of Medical Sciences between 2009 and 2011. Selected samples had not undergone surgery, chemotherapy, or radiotherapy. Written consent was obtained from all patients prior to commencement of the study. This study was approved by the ethics committee of the Cancer Institute. Samples were divided into the following three groups:

Group 1: Samples with positive tumor receptors of estrogen, progesterone, or both

Group 2: Samples with positive HER2

Group 3: Samples with negative estrogen, progesterone, or HER2 receptors

Analysis of Immunohistochemistry of breast cancer samples: All samples were examined for receptor status using commercial antibodies. Estrogen and progesterone receptors were assessed with ID5 and PGR-1A6 antibodies, respectively (Dako Denmark). HER2 was assessed with GB11 antibody (Dako Denmark). In assessment of HER 2, cases that scored zero or one were considered negative12.

Extraction of microRNAs: To extract small RNAs, Mirvana Paris (Qiagen, USA) kit was used. Small RNAs with less than 200 open pair length (microRNAs existed among them) were extracted. Next, concentration of small RNAs was measured using NanoDrop (Thermoscientific, USA) and then, they were maintained at -80°C.

Synthesis of cDNA from small RNAs: To produce cDNA, Miscript Reverse Transcription (QIAGEN, USA) kit was used. According to the protocol, first poly A was added to 3' end of all extracted small RNAs by poly A polymerase. Then, reverse transcription was performed using Oligo dT VN primer (Qiagen, USA) (containing a specific 5' region for reverse primer connection). With the aid of two primers, Real Time PCR (RT-PCR) was performed. Direct primer is similar to miR-520d sequence, and reverse primer complements the unique region of oligo dT VN 5' primer. For every duplicate sample reaction, 10 ng per microliter of cDNA was used in Rottor Gene Q (Qiagen, USA) in real time. Also, for expression normalization, House Keeping Gene, RNU6B is used.

Optical data were entered into LinReg PCR-11 software, and for every sample, crossing point (CP) and mean PCR efficiency[13] were found. Finally, using the following formula, expression ratio of miR-520d in tumor sample over normal sample adjacent to tumor was determined[14] using Rest-2009 software. Pair-wise fixed reallocation randomization test was used in this software.

Results

Expression of miR-520d was examined using RT-PCR method and SUBR Green I staining. Single peak curve was obtained in melting miR-520d and RNU6B, which is indicative of single specific PCR product only (**figure 1**). By placement of PCR product on gel, only one specific band was observed in every reaction involving miR-520d and RNU6B primers, which confirms uniqueness of PCR results in the study samples. miR-520d has a higher CP in tumor samples than in adjacent healthy samples (internal CP control in tumor samples compared to adjacent healthy samples). This means that miR-520d has a lower expression in tumor than in healthy tissue (**figure 2**). Examination showed reduced miR-520d expression in all three study samples such that expression ratio of tumor sample to normal tissue was found 0.193 in group 1, 0.167 in group 2, and 0.21 in group 3. However, the reduction in expression was only significant in group 2 (positive HER2 group) (P=0.017) (**figure 3**). Expression

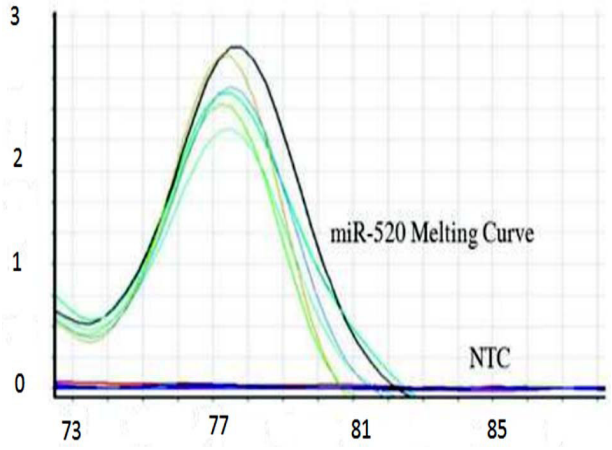

Figure 1: miR-520d melting curve in tumor, horizontal axis: temperature in °C, vertical axis: fluorescent level. NTC= Non Template Control, without DNA

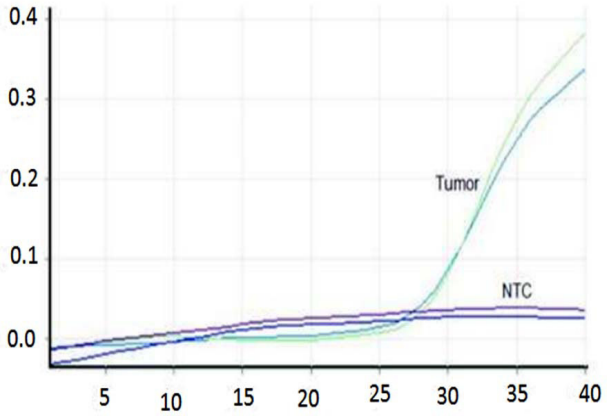

Figure 2: miR-520d growing curve in positive HER2 tumor (top curve). Horizontal axis: number of growing cycles, vertical axis: fluorescent level. NTC=Non Template Control, without DNA.

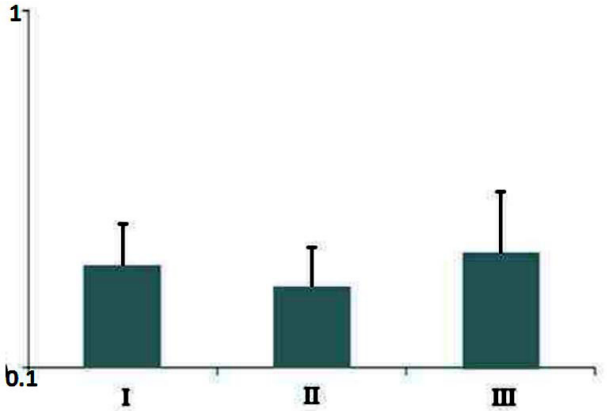

Figure 3: Expression ratio of miR-520d in tumor groups I, II, III to control group
***Significant difference from control group (P<0.05)**

of miR-520d in other clinical and pathological conditions was also studied. All tumors were divided into two groups according to size; small tumors (< 2 cm), and large tumors (≥ 2 cm). Both groups showed reduction in expression, and the ratio was 0.843 in < 2 cm tumors and 0.238 in ≥ 2 cm tumors, but neither was statistically significant (P>0.05). In terms of lymph node metastasis, all samples were divided into two separate groups: 1- more than two nodes, 2- less than two nodes or without metastasis, and ratio of expression in tumor to normal adjacent tissue was obtained 0.256 and 0.761, respectively, and only the first case was significant. Considering the clinical stage of tumor, samples in stage III showed a significant reduction

in expression with expression ratio of 0.281 (P=0.036). While tumors in clinical stages I and II showed insignificant reduction despite reduction in expression (**table 1**).

Discussion

In recent years, researchers have been much interested in studying microRNAs in tumor tissue.[15] This study aimed to examine expression of miR-520d in tumor and normal breast tissue, and eventually, to use this as a biomarker to determine breast tumor receptor status.

A suitable method for examination of expression is Taqman RT-PCR method.16 In this study, double-strand cDNA binding dye (SYBR Green I) was used instead of Taqman Rt-PCR. The latter method is less expensive than Taqman probe, but it may not be specific because it binds to every double-strand cDNA. In the present study, the presence of a single peak melting curve confirmed specificity of the product, which was due to specificity of primers and PCR. Thus, it was shown in this study that inexpensive SYBR Green I method can be used to replace expensive Taqman, and use probe to analyze MiRNA expression.

Analysis in all three groups showed reduced expression, which meant MiRNA can be considered as a potential tumor suppressor. In clinical and pathological terms, reduced expression of miRNA-520d showed similar condition to increased expression of miR-21 in different groups[11] such that expression of these two microRNAs showed an insignificant relationship with tumor size. However, both showed a significant relationship with lymph node metastasis condition, as well as with samples with stage III.

As discussed in the previous study , low expression of miR-250 and miR-342 were proposed as potential biomarkers in early diagnosis of receptor status of cancerous sample in triple negative form.[11] Reduced expression of miR-520d can also be proposed as potential marker in diagnosis of HER2 in breast cancer sample. However, since micro-RNA has a reduced expression in all three groups, it cannot be used alone to diagnose receptor status of breast cancer. Thus, further studies are required to find a miRNA expression panel (besides miR-520d, miR-342, and miR-205) to enable early diagnosis of receptor status of a breast cancer sample.

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Table 1: Expression ratio of miR-520d in classification, given clinical and pathological indicators of tumor

	Clinical and pathological attributes	Sample quantity (% of samples)	Expression ratio of miR-520d	P*
Tumor size (cm)	< 2	25(42.2%)	0.843	0.531
	≥ 2	34(57.6%)	0.238	0.072
Lymph node metastasis	< 2 or no metastasis	38(64.4)	0.761	0.628
	≥2	21(53.6%)	0.256	0.019
Clinical stage of cancer	I,II	46(78%)	0.662	0.153
	III	13(22%)	0.281	0.036

*Pair wise fixed reallocation randomization test, P<0.05 significant level

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