Association scrutiny between the miRNA 148a/152 polymorphisms and risk of breast cancer in Isfahan population

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DOI: 10.22034/HBB.2017.11
Received: May 23, 2017; Accepted: July 2, 2017

ABSTRACT

Breast cancer is 26% of diagnosed cancer in 2008 and it is second cause of cancer related deaths in women. This type of cancer is a complex genetic disease, because microRNAs (miRNAs) are expressional regulators of genes, genetic variations such as single nucleotide polymorphisms in their genes. Therefore, this study was conducted to evaluate the association between single nucleotide polymorphisms (SNPs) in miRNA 148/152 genes and breast cancer risk in Isfahan population. This case control study was performed on 200 samples containing 100 controls and 100 cases. Polymorphism genotyping was performed using polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) and tetra primer amplification refractory mutation system (T-ARMS-PCR).

Our observations indicated no significant association between rs185641358 and rs12940701 in miRNAs and breast cancer risk because their p-values are 0.08 and 0.19, respectively. No association between miRNA148/152 genes polymorphisms and risk of breast cancer was found.

Keywords: Breast cancer, miRNA148a, miRNA152, SNP
INTRODUCTION

Breast cancer is leading cause of cancer related death of women in the world [1]. In the United States, 12.5 % of the women suffering from cancer in their life [2]. In Iran, it is also one cause of malignancy among women [3, 4]. Research on twin have shown that hereditary genetic factors are associated with progression of breast cancer and accounted for more than one third of all cancers [5]. Many genetic loci are associated with breast cancer risk and described 35 % of dangers [5, 6].

Micro RNAs are single stranded RNA molecules that have 20 to 23 nucleotides in length. They have key role in gene expression regulation of many cellular mechanisms such as development and differentiation. MicroRNA maturation is a multistep process that mediated by proteins such as Drosha and DGCR8 in the nucleus which can convert long primary miRNAs (pri-miRNAs) into short pri-miRNAs [7, 8]. Then pri-miRNAs are transported to cytoplasm and processed by Dicer to mature miRNAs [9]. Also miRNAs are caused mRNA degredation or translation inhibition by pairing with the 3’ or 5’ untranslated region as well as coding region. When miRNA binds to 3’UTR of the mRNA, more efficient degradation is achieved [10-12]. One miRNA may regulate nearly 100 mRNAs and over 1000 miRNAs can regulate more than half of the mRNAs encoded by the human genome [13, 14]. In addition, many of the mRNAs have binding sites for miRNAs [15]. The miRNAs play an important role in tumor formation, in that case they can act as oncogenes or guardian of the genome when tumor suppressor genes or oncogenes are targeted, respectively [16]. Furthermore it has been proposed that they can mediate metastasis and chemoresistance [17, 18]. Different miRNA expression levels have also observed in tumors versus normal tissue and between tumor subtypes [19, 20]. SNPs in miRNAs can alter miRNA processing or miRNA operation. Additionally they may provide, eliminate or change their binding site regions. These variations in miRNA genes can cause their dysregulation [21].

This study was conducted to examine the relationship between miRNA148a and miRNA152 polymorphisms with the breast cancer risk. These miRNAs involved in inhibition of cell proliferation, colony formation and angiogenesis in breast cancer cells. They repress PI3K/AKT and MAPK/ERK signaling pathways through targeting IGF-IR and IRS1. Thus both miRNAs suppress expression of IGF-IR and IRS1, thereby inhibit HIF-1a and VEGF. The expressions of these factors have important mechanisms in angiogenesis [22]. Down regulation of miRAN148/152 and up regulation of miRNA221 and miRNA186 showed their active role in breast cancer development [22-25].
In this study, association between single nucleotide polymorphisms (SNPs) rs185641358 of miRNA148a and rs12940701 of miRNA152 with the breast cancer risk were evaluated.

MATERIALS AND METHODS

Study population and DNA extraction
In this study, 100 breast cancer cases were detected and genotyped. The cases have been gathered from Omid Hospital (Isfahan, Iran). Healthy women of Isfahan were matched for age and ethnicity as control. The control women had no cancer in their family history. The average ages for cases and controls were 48 and 48.5 years, respectively. Venous blood samples were collected in tubes containing ethylene diamine tetra acetic acid (EDTA) and kept at -20 °C. Genomic DNA was extracted by Miller's salting out method [26].

SNPs selection and genotyping
SNPs for this study were selected by identifying candidate miRNAs using the target prediction search in the microRNA database. Candidate miRNAs were selected based on strong mirSVR and PhastCons scores of binding to the estrogen receptor alpha, which is one of the main drivers in breast cell proliferation. Candidate miRNAs were examined using the gene database of NCBI to identify presence of SNPs. SNPs genotyping in miRNA148a were performed by T-ARMS-PCR method, which is a quick and simple method for recognition of single nucleotide polymorphism [27-29]. In T-ARMS-PCR technique, polymorphism was detected using 4 primers without any restriction enzymes. Two allelic internal primers for the desired polymorphism and two non-allelic external primers for controls were designed by OLIGO7 software. The PCR primers properties are listed in Table 1.

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Sequence(5’→3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner Forward(A allele)</td>
<td>GGAGTGCTCTCAGAAGCTTTGCCTAAA</td>
<td>732,475</td>
</tr>
<tr>
<td>Outer reverse</td>
<td>CAGGGTAAGATGAAGAGATTTGGG</td>
<td></td>
</tr>
<tr>
<td>Outer forward</td>
<td>CAGGGTAAGATGAAGAGATTTGGG</td>
<td></td>
</tr>
<tr>
<td>Inner reverse(G allele)</td>
<td>AGACAGCAGTTTGGTCGTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Tetra-ARMS-PCR primers for miRNA148a
Fig. 1. Schematic representation of tetra-primer amplification refractory mutation system (T-ARMS-PCR) that used for detection of miRNA148a SNP. Two forward and two reverse specific primers were used to produce three potential products. Product sizes were 475 bp for A allele, 257 bp for G allele, and 732 bp for two outer primers (control band) for miRNA148a.

Ingredients for 25 µL reaction volumes are as follows: 2 µL of 20 ng/µL genomic DNA, 0.8 µL of 10 pmol each inner forward and reverse primers, 1.2 µL of 10 pmol each outer forward and reverse primers, 2.5 µL of 10 XPCR buffer, 0.75 µL of 10 µM dNTPs, 0.3 µL of polymerase (Kawsar Biotech, Iran), 1.75 µL of MgCl₂ (50 Mm). Multiplex PCR was conducted in eppendorf thermal cycler in following conditions: initial denaturation step in 95 °C for 3 min, 35 cycles in 95 °C for 30 sec, 59 °C for 35 sec, and 72 °C for 40 sec followed by a final extension cycle at 72 °C for 10 min, and then cooled to 4 °C.

Finally the products of PCR were separated by electrophoresis on 1 % of agarose gel.

Restriction fragment length polymorphism (PCR–RFLP) was applied to genotyping of miRNA152 SNP. Primers and their size are listed in Table 2.
The PCR–RFLP reaction was performed in a final volume of 25 μL 1 μL of 10 μM forward and reverse primer solutions, 2.5 μL buffer (10X), 0.5 μL of dNTPs (10 μM), 0.25 μL of Taq DNA polymerase (Kawsar Biotech, Iran), 2 μL of DNA (20 ng/μL), and 0.75 μL of 50 mM MgCl₂. The PCR reaction was conducted using eppendorf thermal cycler according to the following protocol 95 °C for 5 min, 35 cycles of 95 °C for 15 sec, 65 °C for 15 sec, 72 °C for 30 sec, 72 °C for 5 min, and cooled at 4 °C. The products were evaluated by gel electrophoresis at 90 V for 30 min on a 2 % of agarose gel, then digestion reactions were performed by adding 2 μL of buffer, and 10 μL of PCR product in a PCR tube and incubated at 37 °C for 16 h and hold at 4°C. Then, results were achieved by gel electrophoresis at 70 V for 60 min on 3% agarose gel.

**RESULTS**

**Polymorphism of miRNA148a**

The studied population composed of 100 verified female breast cancer cases, and 100 healthy women. The clinicopathologic features of cases are listed in Table 3. The study was conducted by tetra amplification refractory mutation system polymerase chain reaction. In Table 4 was summarized the genotype and allele frequencies of the studied polymorphism in miRNA148a and represents the genotyping results as well as statistical analysis for rs185641358 in control and case groups. Fig. 2 illustrates electrophoresis pattern of tetra-ARMS-PCR for miRNA148a. The results obtained from the power marker Software. However the GG genotype was more frequent in the breast cancer population compared to control, also no significant differences between cases and controls were compared using x² test. The odd ratios with 95 % confidence intervals was used to determine the association between the polymorphisms and cancer risk. Hardy Weinberg equilibrium for control population was examined by Power Marker software.
controls for genotypes and alleles were obtained by Chi square analysis. So there is no significant association between this SNP and breast cancer risk.

Table 3. Clinical and pathological characteristics of breast cancer patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age:</td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>73</td>
</tr>
<tr>
<td>&lt;50</td>
<td>27</td>
</tr>
<tr>
<td>Stage:</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>17</td>
</tr>
<tr>
<td>II</td>
<td>41</td>
</tr>
<tr>
<td>III</td>
<td>38</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
</tr>
<tr>
<td>Estrogen receptor:</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>58</td>
</tr>
<tr>
<td>Negative</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 4. Statistical analysis for SNP rs185641358 miRNA148a

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>AA</th>
<th>AG</th>
<th>GG</th>
<th>A</th>
<th>G</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>79</td>
<td>19</td>
<td>2</td>
<td>177</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>87</td>
<td>13</td>
<td>0</td>
<td>187</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.08(non-significant)</td>
<td>0.08(non-significant)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Electrophoresis pattern of tetra-ARMS-PCR for detection of polymorphism in miRNA148a. Genotypes are indicated above the lane. (M =100 bp DNA marker)

Polymorphism of miRNA152

In Fig. 3 was illustrated RFLP results for miRNA152. Table 5 shows summarized results of RFLP genotyping. HWE p values were calculated for controls that p value (HWE p control
= 0.29) were above 0.05 threshold, thus Hardy Weinberg equilibrium about control population is true. $X^2$ analysis of those frequencies illustrated insignificant differences between cases and controls because both genotypes ($p = 0.16$) and alleles ($p = 0.19$) were above the threshold. So this polymorphism was not associated with breast cancer.

Table 5. Statistical analysis for SNP rs12940701 miRNA152

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>C</th>
<th>T</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>43</td>
<td>50</td>
<td>7</td>
<td>136</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>Controls</td>
<td>53</td>
<td>42</td>
<td>5</td>
<td>148</td>
<td>52</td>
<td>0.29</td>
</tr>
<tr>
<td>p-Value</td>
<td>0.16 (non-significant)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.19 (non-significant)</td>
</tr>
</tbody>
</table>

Fig. 3. RFLP results for miRNA152 snp12940701. The first lane is ladder. PCR product has 170 bp size, which is undigested in CC homozygous individuals (1, 3, 5 and 7). Heterozygous samples show three bands at 170 bp, 100 bp, and 70 bp (2 and 6). Samples with only 100 bp and 70 bp bands were homozygous TT (4 and 8).

DISCUSSION

Today, in cancer therapy, researchers pay attention to processes and molecular mechanisms involved in carcinogenesis. To develop of the new treatments, identifying these mechanisms was required. The miRNAs as regulators of gene expression were involved in carcinogenesis of breast cancer [30].

About 50 percent of microRNA genes exist in fragile regions or areas of the genome which are linked to cancer. Transcriptional activation or amplification of miRNA coding gene may lead to regulation of mature miRNA. While deletion of specific chromosomal region or defect of their biosynthesis could reduce their expression [21].

In this study, we examined the genotype frequency of SNPs in two miRNAs using tetra primer ARMS-PCR and RFLP methods and SNPs (rs185641358 and rs12940701). The results were not shown significant association with breast cancer risk. These miRNAs act on genes which induce tumorigenesis. Hence reducing their expression level will lead to a rise of oncogene and cancer cell proliferation [31]. Dysregulation of miRNAs expression presence SNPs in their genes [21].
Several miRNAs are associated with breast cancer. In 2005 Iorio et al. showed presence a specific miRNA signature in breast cancer [32]. The general miRNA expression could be normal in breast cancer tissue.

The miRNA125b, miRNA145, miRNA21 and miRNA155 are important miRNAs with altered expressions [32]. Increased regulation of miRNA21 and miRNA155 suggested their oncogenic roles, whereas reduced expression of the remaining miRNAs introduced them as tumor suppressor genes. It has been shown that the expression of miRNAs may associate with cancer risk.

In cancer cells, oncogenic miRNAs target tumor suppressor genes and overexpression of miRNAs may inhibit the expression of those genes [33, 34]. So miRNA21, miRNA96, and miRNA182. In return tumor suppressor miRNAs for example miRNA205, miRNA27b, miRNA17 are regulated in cancer cells compared to normal cells [32, 35, 36].

This study was examined the effect of targeted SNPs (rs185641358 and rs12940701) in miRNA148a/152 on breast cancer risk. The results illustrated the selected single nucleotide polymorphisms in these miRNAs which were not associated with breast cancer risk.

More ever, there are no association between SNPs in miRNA373, miRNA335 and miRNA149 and breast cancer risk or clinical outcomes [32, 37]. This was suggested any variations in these miRNA expression may be due to other SNPs in different position or another effective DNA copy number variation or DNA methylation in cancer cells.

ACKNOWLEDGEMENTS

This study has been conducted in the genetic laboratory at the University of Isfahan in Iran. We would like to acknowledge all the physicians and nurses of Omid Hospital, especially Dr. Simin Hemmati for her clinical data.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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