A functional polymorphism in the miR-146a gene and age of familial breast/ovarian cancer diagnosis

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A G to C polymorphism (rs2910164) is located within the sequence of miR-146a precursor, which leads to a change from a G:U pair to a C:U mismatch in its stem region. The predicted miR-146a target genes include BRCA1 and BRCA2, which are key breast and ovarian cancer genes. To examine whether rs2910164 plays any role in breast and/or ovarian cancer, we studied associations between this polymorphism and age of diagnosis in 42 patients with familial breast cancer and 82 patients with familial ovarian cancer. Breast cancer patients who had at least one miR-146a variant allele were diagnosed at an earlier age than with no variant alleles (median age 45 versus 56, P = 0.029) and ovarian cancer patients who had at least one miR-146a variant allele were diagnosed younger than women without any variant allele (median age 45 versus 50, P = 0.014). In further functional analysis, we found that the variant allele displayed increased production of mature miR-146a from the precursor microRNA compared with the common allele. Consistent with the target prediction, in a target in vitro assay, we observed that miR-146a could bind to the 3′ untranslated regions (UTRs) of BRCA1 and BRCA2 messenger RNAs (mRNAs) and potentially modulate their mRNA expression. Intriguingly, the binding capacity between the 3′ UTR of BRCA1 and miR-146a was statistically significantly stronger in variant C allele than those in common G allele (P = 0.046). Taken together, our data suggest that breast/ovarian cancer patients with variant C allele miR-146a may have high levels of mature miR-146 and that these variants predispose them to an earlier age of onset of familial breast and ovarian cancers.

Introduction

MicroRNAs (miRNAs) are endogenous non-coding ~22 nucleotide RNAs, which suppress gene expression in a sequence-specific manner (1). Because of the particular way in which miRNA functions—by targeting a number of functionally important protein-coding genes—it is hypothesized that genetic variations in miRNA genes, if inherited in the germ line, could affect the levels of expression of tumor suppressor genes or oncogenes and, thereby, cancer risk (2). These genetic variations could have important consequences for the cell because of the large number of targets of each miRNA, making it very likely that two or more protein-coding genes from different molecular pathways or interacting pathways may be disturbed. Several lines of evidence support a role for genetic variants in miRNA genes in affecting the processing and/or target selection of human miRNAs and thereby cancer risks (3–5). For example, Calin et al. (3) reported a germ line mutation in the pre-miR-16-1/15a precursor in a chronic lymphocytic leukemia patient with familial chronic lymphocytic leukemia and breast cancer in first-degree relatives. This germ line mutation caused low levels of miRNA expression in vitro and in vivo.

Abbreviations: miRNA, messenger RNA; miRNA, microRNA; PCR, polymerase chain reaction; UTR, untranslated region.

Genotyping

Genotypes were analyzed by direct DNA sequencing. A 192 bp fragment containing the pre-miR-146a region and polymorphism site (rs2910164) was amplified using the following primers: 5′-CCGATGTGTATCCTCAGCTTTG-3′ and 5′-GGCTGAGACTTGCTCTTTG-3′. The polymerase chain reaction (PCR) cycling conditions for the assay were 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s with a final extension step at 72°C for 7 min. The amplified PCR products were sequenced using the dideoxynucleotide chain termination method. Both strands of the amplified PCR products were sequenced with an ABI-PRISM 3730xl Autosequencer (Applied Biosystems, Foster City, CA) in the Roswell Park Cancer Institute Biopolymer Core. For quality control, random duplicate samples (5%) were run for each sequence analysis.

miRNA cloning, expression and detection

To study the effect of polymorphism on expression levels of mature miR-146a, we inserted the 192 bp DNA fragment containing precursor of miR-146a with GG or CC genotype into pcDNA3.3 mammalian expression plasmid (Invitrogen, Carlsbad, CA) by using pcDNA3.3-TOPO® TA Cloning® Kit. The insertion and orientation of the fragment were confirmed by sequence analysis. The yielded plasmids were named pcDNA3.3-miR-146G and pcDNA3.3-miR-146C, respectively. The pcDNA3-3-miR-146G or pcDNA3-3-miR-146C was transfected into MCF-7 breast cancer cell line using DOTAP Liposomal Transfection Reagents (Roche, Indianapolis, IN) according to the manufacturer’s recommendations. After 48 h, miRNAs were isolated using mirVana™ miRNA Isolation Kit (Applied Biosystems). The expression levels of mature miRNAs......
miR-146a were measured by Taqman-based microRNA assays (Applied Biosystems) with the use of the ABI StepOne System (Applied Biosystems). The relative amount of each miRNA to tRNA for initiator methionine was described using the equation $2^{-\Delta\Delta Ct}$, where $\Delta Ct = (Ct_{\text{miRNA}} - Ct_{\text{tRNA}})$. Each experiment was triplicated and the mean of the triplicates was used.

**Target in vitro assay**

Luciferase-based target in vitro assay was applied to test whether miR-146a could bind to the 3' untranslated region (UTR) of BRCA1 or BRCA2 messenger RNA (mRNA). The 3' UTR segments of BRCA1 and BRCA2 mRNAs predicted to interact with miR-146a were amplified by PCR from human genomic DNA. A 544 bp fragment of the 3' UTR segment of BRCA1 was amplified using the following primers: 5'-CTGTTGAACTCCCTTGG-3' and 5'-CACCATGACCCGTAAATT-3', and a 698 bp fragment of the 3' UTR segment of BRCA2 was amplified using the following primers: 5'-GC-CGGATCCGGTTGCC-3' and 5'-TTTGGATGACCCACATTGTTGA-3'. Both PCR fragments were inserted into pMIR-REPORT™ miRNA Expression Reporter Vector (Applied Biosystems), using the Pmel site immediately downstream from the stop codon of the luciferase gene. The insertion and orientation of the fragment were confirmed by sequence analysis. The yielded plasmids were named pMIR-REPORT™-BRCA1 and pMIR-REPORT™-BRCA2, respectively. Then, pMIR-REPORT™-BRCA1/2 was transfected into the MCF-7 breast cancer cell line with or without pcDNA3.3-miR-146a using DOTAP Liposomal Transfection Reagents according to the manufacturer's recommendations. Both G and C alleles were used in the analyses. Luciferase activities were measured by using luciferase assays (Promega, Madison, WI) 48 h after transfection. The levels of interaction between BRCA1/2 and miR-146a were determined by the difference of luciferase activities between cells cotransfected with pMIR-REPORT™-BRCA1/2 and pcDNA3.3-miR-146a and cells transfected with pMIR-REPORT™-BRCA1/2 alone. Each experiment was triplicated and the mean of the triplicates was used.

**Statistical analysis**

The non-parametric Wilcoxon rank-sum test was used to compare age at diagnosis of breast or ovarian cancer between miR-146a genotype strata. Linear regression analysis was performed to further investigate the relationship between age at diagnosis of breast or ovarian cancer and miR-146a genotypes by adjusting for gender, BRCA1/2 status and ethnicity simultaneously. Student's t-test was used to compare mean expression levels for significant differences between the G and C alleles of miR-146a in miRNA expression assay and compare the mean luciferase activities between cells cotransfected with pMIR-REPORT™-BRCA1/2 and pcDNA3.3-miR-146a and cells transfected with pMIR-REPORT™-BRCA1/2 alone. A P value <0.05 was considered statistically significant, and all statistical tests were two sided. All analyses were performed using STATA software (Version 9.0, STATA, College Station, TX).

**Results**

Because inherited cancers are more likely to be diagnosed at a younger age, we examined the relationships between genetic polymorphism in miR-146a and the age of cancer diagnosis (Table I). The frequency of the variant C allele was 0.286 in breast cancer patients and 0.238 in ovarian cancer patients. In the breast cancer study, there was a gene dosage effect on age at diagnosis, with a median age at diagnosis of 56 for patients with GG genotypes, 46 years for those with GC genotypes and 43 for patients with CC genotypes. Compared with patients with GG genotypes, those with GC or CC genotypes had marginally significantly younger age at diagnosis after adjusting for BRCA1/2 status, gender and ethnicity. When we combined GC and CC groups together, we found that those with at least one C allele had significantly younger age at diagnosis compared with those with GG genotype ($P = 0.029$) after adjusting for BRCA1/2 status, gender and ethnicity. Similar relationships were observed for ovarian cancer, with a median age at diagnosis of 50 years for women with GG genotypes, 41 for those with GC genotypes and 43 for women with CC genotypes. When we combined GC and CC groups together, we found that patients with at least one C allele had significantly younger age at diagnosis compared with those with GG genotype ($P = 0.014$). Furthermore, significant trends of decreasing age at cancer diagnosis with increasing number of C variant alleles were observed in both breast and ovarian cancer patients ($P = 0.012$ for breast cancer and $P = 0.016$ for ovarian cancer).

This genetic variant is located in the middle of the stem hairpin and creates a base pairing mismatch (supplementary Figure 1 is available at Carcinogenesis Online). The optimal free energy was decreased from −42.40 Kcal/mol for G to −39.60 Kcal/mol for C alleles, suggesting a less stable secondary structure for the C variant allele compared with the G allele. It is hypothesized that genetic variants in pre-miRNAs might change the conformation of the secondary structure and thereby alter the expression of mature miRNAs. When we cloned the precursor of miR-146a containing G or C allele into pcDNA3.3, transfected the constructs into MCF-7 breast cancer cell lines and measured expression levels of mature miR-146a by Taqman-based miRNA assays, we found that the expression levels of mature miR-146a in C allele were significantly higher than those in G allele ($P = 0.013$) (Figure 1). The expression levels of mature miR-146a were 60% higher in C allele than G allele, suggesting that this polymorphism in miR-146a could alter the mature miRNA expression.

Using the probability of interaction by target accessibility prediction algorithm, BRCA1 and BRCA2 were predicted as targets of miR-146a. The predicted binding between miR-146a and the 3' UTRs of BRCA1 and BRCA2 are depicted in supplementary Figure 2 (available at Carcinogenesis Online). To experimentally confirm the interaction between miR-146a and BRCA1/2, we applied target in vitro assay to test the interaction between miR-146a and the 3' UTRs of BRCA1 and BRCA2 mRNAs. We cloned fragments of 3' UTR segments of BRCA1 or BRCA2 into pMIR-REPORT™ miRNA Expression Reporter Vector (Applied Biosystems). Then, we transfected pMIR-REPORT™-BRCA1 and pMIR-REPORT™-BRCA2 into MCF-7 cell lines with or without pcDNA3.3-miR-146a. Both G and

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**Table I.** Genetic variant in miR-146a and age of cancer diagnosis

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>n</th>
<th>Median age at diagnosis (range)</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Breast cancer patients</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>GG</td>
<td>22</td>
<td>56 (32–79)</td>
<td>Reference</td>
</tr>
<tr>
<td>GC</td>
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<td>46 (36–68)</td>
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<tr>
<td>CC</td>
<td>4</td>
<td>43 (30–57)</td>
<td>0.095</td>
</tr>
<tr>
<td>GC + CC</td>
<td>20</td>
<td>45 (30–68)</td>
<td>0.029</td>
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<tr>
<td>Ovarian cancer patients</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>50</td>
<td>50 (26–83)</td>
<td>Reference</td>
</tr>
<tr>
<td>GC</td>
<td>25</td>
<td>41 (21–69)</td>
<td>0.026</td>
</tr>
<tr>
<td>CC</td>
<td>7</td>
<td>43 (25–52)</td>
<td>0.16</td>
</tr>
<tr>
<td>GC + CC</td>
<td>32</td>
<td>45 (21–69)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Real-time quantitative PCR for mature miR-146a expression in the cells transfected with pcDNA3.3-miR-146G or pcDNA3.3-miR-146C. Data are means (±SDs) from three independent experiments. Data are normalized with reference tRNA, as mentioned in the text. P value was calculated from a two-sided, one-sample t-test.
C alleles of miR-146a were used in the analyses. After 48 h, we observed significant reduction of luciferase activity in the cells transfected with both pcDNA3.3-miR-146a and pMIR-REPORT™BRCA1 or pMIR-REPORT™BRCA2 compared with the cells transfected with pMIR-REPORT™BRCA1 or pMIR-REPORT™BRCA2 alone, indicating that miR-146a could bind to the 3' UTRs of BRCA1 and BRCA2 mRNAs in vitro (Figure 2). Compared with the cells transfected with pMIR-REPORT™BRCA1 alone, the cells cotransfected with pMIR-REPORT™BRCA1 and pcDNA3.3-miR-146aG had 60.9% decreased luciferase activities (mean luciferase activities: 3743 versus 9571; \( P = 0.001 \)). Compared with the cells transfected with pMIR-REPORT™BRCA1 alone, the cells cotransfected with pMIR-REPORT™BRCA1 and pcDNA3.3-miR-146aC had 71.6% decreased luciferase activities (mean luciferase activities: 2719 versus 9571; \( P = 0.001 \)). The binding capacity between miR-146a and the 3' UTR of BRCA1 was statistically significantly higher in variant C allele than those in common G allele (mean luciferase activities: 2719 versus 3743; \( P = 0.046 \)). In BRCA2-binding analysis, compared with the cells transfected with pMIR-REPORT™BRCA2 alone, the cells cotransfected with pMIR-REPORT™BRCA2 and pcDNA3.3-miR-146aG had 54% decreased luciferase activities (mean luciferase activities: 4846 versus 10 541; \( P = 0.002 \)). Compared with the cells transfected with pMIR-REPORT™BRCA2 alone, the cells cotransfected with pMIR-REPORT™BRCA2 and pcDNA3.3-miR-146aC had 54% decreased luciferase activities (mean luciferase activities: 3649 versus 10 541; \( P = 0.001 \)). However, the binding capacity between miR-146a and the 3' UTR of BRCA2 was not significantly different between G and C alleles (mean luciferase activities: 4846 versus 3649; \( P = 0.145 \)). In control experiment, the luciferase activities were not statistically different between cells transfected with pMIR-REPORT™reporter control vector with or without pcDNA3.3-miR-146aG (\( P = 0.289 \)) or pcDNA3.3-miR-146aC (\( P = 0.108 \)).

Discussion

It has been estimated that miRNAs can regulate at least 50% of human genes, including tumor suppressor genes such as BRCA1, BRCA2, p53 and PTEN. Because of the particular way in which miRNA functions, by targeting a number of functionally important protein-encoding genes, it is appealing to propose that genetic variations in miRNA genes and/or their responsive elements in their target mRNA might represent a newly described mechanism of cancer predisposition. The results from this study fully support this hypothesis. We found that the genetic polymorphism in the miR-146a gene (rs2910164) was associated with young age of familial breast/ovarian cancer diagnosis. This G to C change in the precursor of miR-146a resulted in elevated expression of mature miR-146a. In further analysis, we found that the link between rs2910164 and young age of breast/ovarian cancer diagnosis might be through regulation of BRCA1/2 mRNA expression.

Overexpression of miR-146a has been reported as a signature in breast, pancreatic and prostate cancers (6). In a microarray-based miRNA expression analysis, miR-146a was significantly upregulated in breast carcinoma tissues compared with normal breast tissues (6). miR-146 overexpression has also been linked to papillary thyroid carcinoma (9). Upregulation of miR-146a corresponded to dramatic loss of KIT transcript and Kit protein, a key tumor suppressor gene in the thyroid carcinogenesis pathway. In addition, miR-146a has been reported to regulate tumor necrosis factor receptor-associated factor 6 and interleukin-1 receptor-associated kinase 1 (10). Both tumor necrosis factor receptor-associated factor 6 and interleukin-1 receptor-associated kinase 1 are key adapter molecules downstream of the Toll-like and cytokine receptors in the signaling pathway that plays a crucial role in cell growth and immune recognition. It might be interesting to look at the links between rs2910164 and the invasive biological features of breast and ovarian cancers. Unfortunately, the clinical data are not available for these patients.

It has been observed that the disturbance of the secondary structure of miRNA precursor by sequence variations may affect the maturation process of miRNA (4,11). For example, the expression of mature miR-125a is decreased when a G:C match is replaced by a U:C mismatch in the stem region (4). Furthermore, introduction of an artificial mutation to the stems of miR-30 and miR-21 precursors revealed that large bulges in the stem regions were detrimental to the production of miRNA (11). In contrast to these observations, we found the G to C variation causes a change from a G:U pair to a C:U mismatch in its stem region of miR-146a precursor and results in increased production of mature miR-146a. The discrepancy between our study and other studies is intriguing. The molecular mechanism of how genetic variants might affect the mature miRNA expression remains to be determined. Some other factors might affect the relationship between genetic variations and mature miRNA expression, such as, the location of the variants in the stem–loop structure, the strength of the binding between nucleotides, etc. Obviously, further studies on the biological mechanisms are warranted.

Germ line mutations in the currently known high-risk breast or ovarian cancer genes (such as BRCA1/2) are common in familial breast and ovarian cancers, but they can explain, at best, 20–25% of the overall excess familial risk in breast cancer and half of the familial aggregation of ovarian cancer, suggesting the presence of other unidentified predisposition genes which confer susceptibility. Traditional approaches to identification of novel genetic predisposition genes focus on protein-encoding genes. The genetic susceptibility by non-protein-encoding genes, such as miRNAs, is often overlooked. miR-146a has been predicted to regulate the key breast/ovarian cancer genes BRCA1 and BRCA2. Consistent with the prediction, in our
target in vitro assay, we observed that miR-146a could directly bind to the 3’ UTR of BRCA1/2 and thereby potentially regulate the expression of BRCA1/2 mRNAs. More interestingly, we observed that the binding capacity between miR-146a and the 3’ UTR of BRCA1 was significantly different between common and variant alleles. The binding capacity was stronger in cell lines transfected with variant C allele compared with those transfected with G allele. The results are consistent with our observation that the variant C allele exhibited higher levels of mature miR-146a than the common G allele. Putting all these pieces of evidence together, our data suggest a model how miR-146a might play a role in familial breast and ovarian cancer through regulation of BRCA1 mRNA expression, and a functional genetic variant in miR-146a can alter expression of mature miR-146 and thereby affect the age of breast and ovarian cancer diagnosis. Surprisingly, we did not observe the significant allelic discrepancy for BRCA2 binding. The binding capacity of miR-146a is stronger for BRCA1 than BRCA2, regardless of the common or variant alleles. It might be easier to detect the difference if the binding capacity is strong. This might be one of the reasons we did not see the allelic discrepancy for BRCA2 binding. However, we cannot rule out other possible factors.

Selection of patients with familial breast and ovarian cancer in this study is appropriate because our research question is to seek the association between miR-146a polymorphism and age of onset. The age effect usually is more evident in familial cases than sporadic cases. However, our case selection limits our ability to test the same questions in sporadic cases. Due to the lack of a control population in this study, the contribution of this genetic variant to familial breast/ovarian cancer risks cannot be assessed. Considering the significant effect of this variant on age of familial cancer onset, we might assume that it will have an impact on the risks of familial breast/ovarian cancers. However, the assumption needs to be determined. Furthermore, it would be interesting to investigate whether the effect on age of cancer diagnosis and cancer risks differs between familial and sporadic breast and ovarian cancers. Patients with sporadic cancers are generally older than those with familial cancers. Whether or not the variant affects age of onset in sporadic cancer remains to be determined. So far, there are no data available. There might be a concern that the variant affects age of onset in sporadic cancer through regulatory effects of this variant. We did not observe the significant allelic discrepancy for BRCA2 binding, regardless of the common or variant alleles. It might be easier to detect the difference if the binding capacity is strong. This might be one of the reasons we did not see the allelic discrepancy for BRCA2 binding. However, we cannot rule out other possible factors.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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