Methylation of *miRNA-34a* Promoter: a Preliminary Study in Vietnamese Breast Cancer Patients

Pham Anh Thuy Duong, Thieu Minh Thu, Vo Thi Thuong Lan*

*Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam*

Received 15 July 2016
Revised 25 August 2016; Accepted 09 September 2016

**Abstract:** Methylation of *miR-34a* (*miR*-34a) promoter, a target gene of p53 which induces apoptosis, cell cycle arrest or senescence, was reported in many cancer types, including breast cancer, one of the most common malignancies in women. However there has not been any study on *miR*-34a methylation in Vietnam. Therefore, this research provides the first sight of methylation status of *miR*-34a promoter in breast cancer in Vietnam. By using methyl-specific PCR (MSP) technique, we found that the frequency of *miR*-34a promoter methylation in breast tumors and adjacent tissues was 43.3% (13/30) and 13.3% (4/30), respectively. At the transcriptional level, two target genes of *miR*-34a, *AXL* and *MDM4*, were expressed only in the breast tumor where methylation of *miR*-34a promoter was detected, but not in the adjacent tissue.

**Keywords:** DNA methylation, microRNA, *miR*-34a, breast cancer.

1. Introduction

DNA methylation is one of the most common epigenetic mechanisms, taking place in the mammalian genome. It is a covalent modification that primarily occurs at Carbon-5 position of cytosine within CpG dinucleotides [1]. DNA methylation plays an important role in the regulation of gene expression. It may affect the affinity of transcription factors for their binding sites [2], or recruit repressor proteins such as histone deacetylase (HDAC) or methylation-binding protein (MBP) [3, 4]. Aberrant methylation on CpG islands in the promoter of gene is one of the earliest molecular alterations occurring during carcinogenesis [5]. Therefore, a number of studies have focused extensively on the identification of DNA methylation which acts as a prospective marker for cancer diagnosis and prognosis with high sensitivity and specificity.

MicroRNAs (miRs) are 21–25 nucleotides non-coding RNAs that can post-transcriptionally down-regulate the expression of various target genes. MiRs are significant for cell proliferation, apoptosis, and differentiation during mammalian development [6]. Many miRs are expressed in a tissue- and tumor-specific manner, implying that some miRs are under epigenetic control [7].

There are several examples of DNA methylation processes that influence the activity of miRs in many cancer types. Methylation of *miR-9-1* was reported to associate with the lymph node metastasis in colorectal cancer cells [8]. Methylation of *miR-200c/141* is tightly
associated with the invasive capacity of breast cancer cells [9]. Screening investigations in colorectal cancers identified that the epigenetic silence of miR-34b and miR-34c was due to hypermethylation of CpG islands [10].

MiR-34a is considered as a target of p53 to induce G1 cell cycle arrest, senescence and apoptosis in response to DNA damage [11, 12]. DNA hypermethylation of CpG islands in the promoter region is one of the most common reasons for the silence of miR-34a. Study on the aberrant CpG methylation of miR-34a promoter in multiple types of carcinoma cell lines showed that the frequency of this phenomenon is 79.1% (19/24) in primary prostate, 25% (6/24) in breast, 29.1% (7/24) in lung, 13% (3/23) in colon, 21.4% (3/14) in kidney, 33.3% (2/6) in bladder, and 15.7% (3/19) in pancreatic. Re-expression of miR-34a in prostate and pancreatic carcinoma cell lines induced senescence and cell cycle arrest at least in part by targeting CDK6 [13]. These results showed that miR-34a represents a tumor-suppressor gene which was inactivated by CpG methylation and subsequent transcriptionally silenced in a broad range of tumors.

To the best of our knowledge, there are few researches on methylation of miR-34a promoter in patient samples. In this study, we reported the profile of miR-34a promoter methylation in Vietnamese breast cancer patients with the desire to develop epigenetic markers in diagnosis and treatment of breast cancer.

2. Material and methods

2.1. Materials

Breast tumor and adjacent tissue samples from 30 Vietnamese women diagnosed with breast cancer were supplied by Vietnam National Cancer Hospital. These samples had been characterized by histological methods. All the specimens were frozen in liquid nitrogen immediately after resection and stored at -80°C until processing.

2.2. Methods

Genomic DNA isolation and bisulfite conversion. Genomic DNA was extracted from tissue using E.Z.N.A. Tissue DNA Kit (Omega Bio-tek) and stored at -20°C until processing. About 250 ng – 700 ng DNA was treated with sodium bisulfite using EZ DNA Methylation-Gold™ kit (Zymo Research). For the final elution from the column, 20µl elution buffer was used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>β-globin-F</td>
<td>5’-GAAGAGCCAAGGACAGGTAC-3’</td>
</tr>
<tr>
<td>β-globin-R</td>
<td>5’-CAACTTCATCCACGTTTACC-3’</td>
</tr>
<tr>
<td>miR34a-MF</td>
<td>5’-TTTTGGGTAGGCAGCTTTGC-3’</td>
</tr>
<tr>
<td>miR34a-MR</td>
<td>5’-CCCATCCCAGCAGACACGAAA-3’</td>
</tr>
<tr>
<td>miR34a-MR1</td>
<td>5’-GCCGGCGCTTTGCGATTAGC</td>
</tr>
<tr>
<td>miR34a-UF</td>
<td>5’-GCCGGCGCTTTGCGATTAGC</td>
</tr>
<tr>
<td>miR34a-UR</td>
<td>5’-CGAACCACCATTCGCACGGAAC</td>
</tr>
<tr>
<td>AXL-F</td>
<td>5’-AGACCCTAAGGATTCCTGAG</td>
</tr>
<tr>
<td>AXL-R</td>
<td>5’-TCAGGATGATGCTACTGCAC</td>
</tr>
<tr>
<td>MDM4-F</td>
<td>5’-AAGGAGTGGCCGGCTTCTCC-3’</td>
</tr>
<tr>
<td>MDM4-R</td>
<td>5’-CATTTCGGCTTCTGTCGTAGCC-3’</td>
</tr>
</tbody>
</table>
Polymerase chain reaction (PCR). The presence of β-globin gene was tested by PCR using specific primers β-globin-F/β-globin-R (Table 1). Reactions were performed in 15 µl volume containing 1x GoTaq Green Mastermix (Promega), 0.1µM each primer, and 4 – 6 ng of bisulfite-treated DNA.

Methylation specific PCR (MSP). The MSP was performed in a total volume of 15µl using 0.25U Jump Taq polymerase (Sigma), 0.3 µM each primer, and 7 – 11 ng of bisulfite-treated DNA per reaction. Primer sequences used for MSP were described in Table 1.

RNA isolation and Reverse transcript-PCR (RT-PCR). RNA from specimens was isolated by using SV Total RNA Isolation system Z3100 kit (Promega). cDNA was generated from 1 µg total RNA per sample, using hexamer and AMV-Reverse Transcriptase (Promega), following instruction of manufacturer. Two µl of cDNA mixture were used for PCR with GoTaq Green Mastermix (Promega). The primer sequences were shown in Table 1.

Electrophoresis. Amplified fragments were separated by electrophoresis on 8% polyacrylamide gels and visualized by staining with ethidium bromide.

3. Results

3.1. Bisulfite conversion

Genomic DNA isolated from specimens was treated with sodium bisulfite to convert unmethylated-cytosine to uracil but not methylated-cytosine. The efficiency of this process affects the precision of MSP in which specific primers for converted DNA and unconverted DNA were designed separately. If unmethylated DNA was not completely altered, false positive of methylation status could not be avoided. To define whether the chemical conversion was complete or not, a pair of primers was designed to amplify a fragment of β-globin gene without conversion of cytosine. If DNA were perfectly converted, the result of PCR with β-globin primers would be negative.

As the statement of manufacturer Zymo Research, 2 µg of DNA treated with EZ DNA Methylation-Gold™ kit in 2.5 hours could be completely converted. However, β-globin fragment was still amplified from bisulfite-treated DNA (Fig. 1A), even when 500 ng – 700 ng input DNA was used and followed the manufacturing protocol. The process of conversion depends on several factors such as amount of input DNA, concentration of sodium bisulfite, and incubation time. In order to raise the efficiency of conversion, input DNA was used less and incubation time was longer. It was shown that 250 ng DNA was fully converted after 4 hours of incubation with sodium bisulfite (Fig. 1B). This procedure was applied for all DNA samples.

![Figure 1. Evaluation of bisulfite conversion efficiency by PCR with β-globin primers. (A) 500 – 700 ng input DNA, 2.5 hours of incubation (followed instruction of manufacturer). (B) 250 ng input DNA, 4 hours of incubation. BT1, BT2 – breast tumor sample 1 and 2. L – 100 bp ladder. 1 – DNA after conversion. 2 – DNA before conversion. (-) – negative control.](image-url)

3.2. Methylation status of miR34a promoter in breast tumors and adjacent tissues

The pair of primers named miR34a-MF/MR was used for MSP, detecting methylation of
CpG in *miR-34a* promoter with the appearance of a 149 bp band. Though many PCR conditions were tested, we could not gain a single sharp DNA band. To improve the sensitivity and specificity of MSP, *miR34a-MR1* primer was designed and used for Nested-PCR (Fig. 2). PCR condition was optimized for 2 rounds (Table 2) and a 100 bp band was observed clearly on gel.

We used another pair of primer, *miR34a-UF/UR*, which only binds to C-to-U converted sequence to confirm unmethylation status of *miR-34a* promoter. These primers were used to amplify a 143 bp fragment from bisulfite-treated DNA. Condition for this reaction was shown in Table 2.

**Table 2. Optimized conditions for the MSP**

<table>
<thead>
<tr>
<th>Pair of primers</th>
<th>Thermal cycle</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>miR34a-MF/MR</em></td>
<td>Denature: 94°C 30 s, Annal: 60°C 10 s, Synthesize: 72°C 10 s</td>
<td>7 – 11 ng of bisulfite-treated DNA</td>
</tr>
<tr>
<td><em>miR34a-MF/MR1</em></td>
<td>Denature: 94°C 30 s, Annal: 62°C 10 s, Synthesize: 72°C 10 s</td>
<td>1 µl of round 1 product</td>
</tr>
<tr>
<td><em>miR34a-UF/UR</em></td>
<td>Denature: 94°C 30 s, Annal: 65°C 10 s, Synthesize: 72°C 10 s</td>
<td>7 – 11 ng of bisulfite-treated DNA</td>
</tr>
</tbody>
</table>

Figure 2. *miR-34a* methylation-specific primer map.

Figure 3. Representative result of the MSP products amplified by methyl-specific and unmethyl-specific primers. (A) breast tumor samples. (B) adjacent tissue samples. L – 100 bp ladder. M – methylation specificity. U – unmethylation specificity. (-) – negative control.
Applying optimized conditions for the MSP, 30 pairs of samples (breast tumor and adjacent tissue of same patient) were analyzed for miR-34a promoter methylation. The 100 bp band, specific for methylation, was detected in 13/30 tumor and 4/30 adjacent samples (Fig. 3A). Meanwhile, the 143 bp band, specific for unmethylation, was detected in 29/30 tumor and 30/30 adjacent samples (Fig. 3B). From this result, we found that the frequency of miR-34a promoter methylation in breast tumors and adjacent tissues was 43.3% and 13.3%, respectively.

3.3. Expression of AXL and MDM4, the target genes of miR-34a, at the transcriptional level

![Figure 4](image)

Figure 4. Expression of miR-34a target genes at transcriptional level. (A) AXL. (B) MDM4. L – 100 bp ladder. BT – breast tumor. AT – adjacent tissue. (-) – negative control.

To investigate effects of miR-34a promoter methylation on the expression levels of tumor-related genes, a pair of samples, which was observed methylation in breast tumor but not in adjacent tissue, was chose for further analysis. RNA from these samples was isolated and confirmed non-contamination of genomic DNA. RT-PCR was performed to check the expression of AXL and MDM4 at transcriptional level. The house keeping genes such as GAPDH or β-actin are usually used as internal control for quality of cDNA. However, it is not suitable to use these genes for cancer cells [14, 15]. Therefore, we used same amount of RNA from 2 samples for cDNA synthesis and took 2 µl of RT-PCR product as template for reactions which amplify exons of AXL and MDM4.

The results shown in Fig. 4 indicated that AXL and MDM4 were only expressed in breast tumor where methylation of miR-34a promoter was detected. It was consistent with the explanation that CpG-methylated promoter led to silence miR-34a and resulted in increasing RNA level of 2 target genes AXL and MDM4.

4. Discussion and conclusion

Our study on 30 Vietnamese patients diagnosed with breast cancer has identified methylation of miR-34a promoter in 13 tumor and 4 adjacent samples, with the frequency of methylation in turn is 43.3% and 13.3% respectively. So far as we are aware, there were few publications about miR-34a promoter methylation in breast cancer biopsy samples. Until now, there was only a study found that methylation of miR-34a promoter was observed in 6/10 formalin-fixed, paraffin-embed breast tumor samples [16] and no study analyzed this status in adjacent tissues.

The disparity between the frequency of miR-34a promoter methylation of tumor and that of adjacent samples was statistical significance (p=0.008 according to Fisher test). However, the other results on 25 pairs of samples we obtained when using 20 ng of template DNA for the MSP (instead of 7 – 11 ng) did not show the difference (data not shown). We concluded that the higher amount of template for the MSP, the higher ability for detecting methylation in both tumor and adjacent samples and hence, the template DNA used for the MSP should be less than 20 ng. Referring to researches using the MSP for methylated DNA analysis and commercial kits, there is no exact value of the amount of bisulfite-treated DNA for MSP. Our results have been questioned about the amount of bisulfite-treated DNA for the MSP so that the outcomes of DNA methylation analysis by using this method could be approached with the correct value in subclinical diagnosis.
Among 30 pairs of samples, 29/30 tumor and 30/30 adjacent samples gave positive result with unmethylation-specific primers mir34a-UF/UR. This is explained that normal cells and cancer cells were mixed together in the specimen, so that methylated and unmethylated DNA could be both perceived. Additionally, methylation of miR-34a promoter could occur in one allele in malignant cells, thus MSP technique could detect unmethylation status of miR-34a promoter.

Analyzing the correlation between miR-34a promoter methylation and histopathological characteristics showed that methylation was detected in 12/24 patients diagnosed with invasive ductal carcinoma, the most common breast cancer type. These samples were at stage-1 and stage-2 of disease. In otherwise, we could not observe methylation in two stage-3 samples. Although there were few samples and most of them belonged to one type of breast cancer, this result initially indicated that the silencing of miR-34a is an important step in formation and development of mammary carcinoma. Thus, methylation at CpG in promoter of miR-34a has potential to be a prospective marker for breast cancer diagnosis and prognosis in Vietnam.

AXL and MDM4 are two target genes under the regulation of miR-34a. Our experimental result displayed that methylation of miR-34a promoter was associated with the increased expression of these genes. It was consistent with previous studies which showed miR-34a reduced mRNA level of AXL and MDM4 [17, 18]. AXL is a key regulator of metastasis, while MDM4 inhibits the activity of p53, a well-studied tumor suppressor protein. Silenced miR-34a by methylation raising the expression of AXL and MDM4 could be the reason of formation, progression and metastasis of breast cancer. Re-expression of miR-34a in cancer cells may inhibit target genes, thereby suppresses the carcinogenesis and metastasis.

Acknowledgments

This study was funded by NAFOSTED as a part of project 106-YS.06-2015.07.

References

Nghiên cứu số bổ hiện tượng methyl hóa promoter gen miR-34a ở bệnh nhân ung thư vú Việt Nam

Phạm Anh Thùy Dương, Thiếu Minh Thu, Võ Thị Thương Lan
Khoa Sinh học, Trường Đại học Khoa học Tự nhiên, DHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam

Tóm tắt: Hiển tượng methyl hóa promoter của miRNA-34a (miR-34a), một gen đích của protein p53 làm ảnh hưởng tới các quá trình chết theo chu kỳ sinh, chu trình tế bào, lão hóa đã được tìm thấy ở nhiều loại ung thư, trong đó có ung thư vú. Đến nay chưa có nghiên cứu nào về hiện tượng methyl hóa miR-34a ở Việt Nam. Vì vậy, nghiên cứu của chúng tôi bước đầu cho thấy tính trạng methyl hóa promoter miR-34a ở bệnh nhân ung thư vú Việt Nam. Bằng kỹ thuật PCR xác định methyl (MSP), chúng tôi nhận thấy tỷ lệ methyl hóa promoter miR-34a ở máu u ung thư vú là 43.3% (13/30) và ở máu mô liên kề là 13.3% (4/30). Tìm hiểu mức độ biến đổi, 2 gen đích cụ của miR-34a là AXL và MDM4 được thấy biểu hiện ở máu u có promoter miR-34a bị methyl hóa mà không phải ở máu mô liên kề (không phát hiện sự methyl hóa promoter miR-34a).

Từ khóa: Methyl hóa ADN, microRNA, miRNA-34a, ung thư vú.