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

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Abstract: Methylation of *miRNA-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

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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 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^{TM} kit (Zymo Research). For the final elution from the column, 20μl elution buffer was used.

Table 1. Sequence of primers

Primer	Sequence
β-globin-F	5'-GAAGAGCCAAGGACAGGTAC-3'
β-globin-R	5'-CAACTTCATCCACGTTCACC-3'
miR34a-MF	5'-TTTTGGGTAGGCGCGTTTCGC-3'
miR34a-MR	5'- CCAATCCCGCCGAACACGAAA-3'
miR34a-MR1	5'- GCCCCCGCCTAAACTAACG-3'
miR34a-UF	5'-GGCGCGCTTTGCGATTTAGC GCGGCGTG-3'
miR34a-UR	5'- CGAAACCAATCCCGCCGAAC ACGAAATCCC-3'
AXL-F	5'-AGACCCTAAGGATTCCTGTAG CTGC-3'
AXL-R	5'-TCAGGATGATGCTACTGCAC AGAG-3'
MDM4-F	5'-AACGAGTGGCCCGTCCTTCC-3'
MDM4-R	5'- CATTTCGGCTCCTGTCGTTAGACC-3'

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 Transciptase (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.
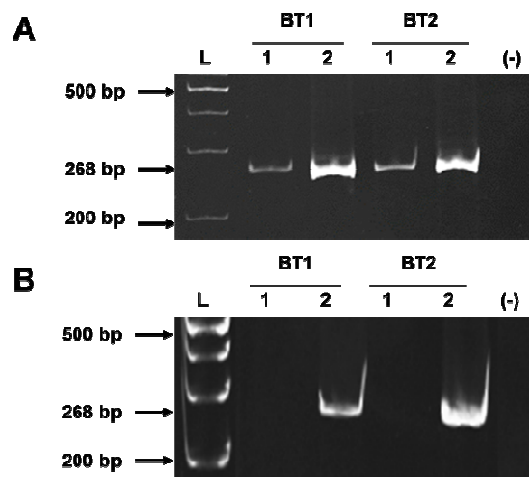

Figure 1. Evaluation of bisulfite conversion efficiency by PCR with β-globin primers. (A) 500 – 700 ng input DNA, 2.5 hours of incubation (followed instruction of manufacturer). (B) 250 ng input DNA, 4 hours of incubation. BT1, BT2 – breast tumor sample 1 and 2. L – 100 bp ladder. 1 – DNA after conversion.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

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.
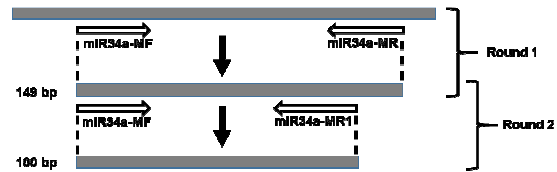

Figure 2. miR-34a methylation-specific primer map.

Table 2. Optimized conditions for the MSP

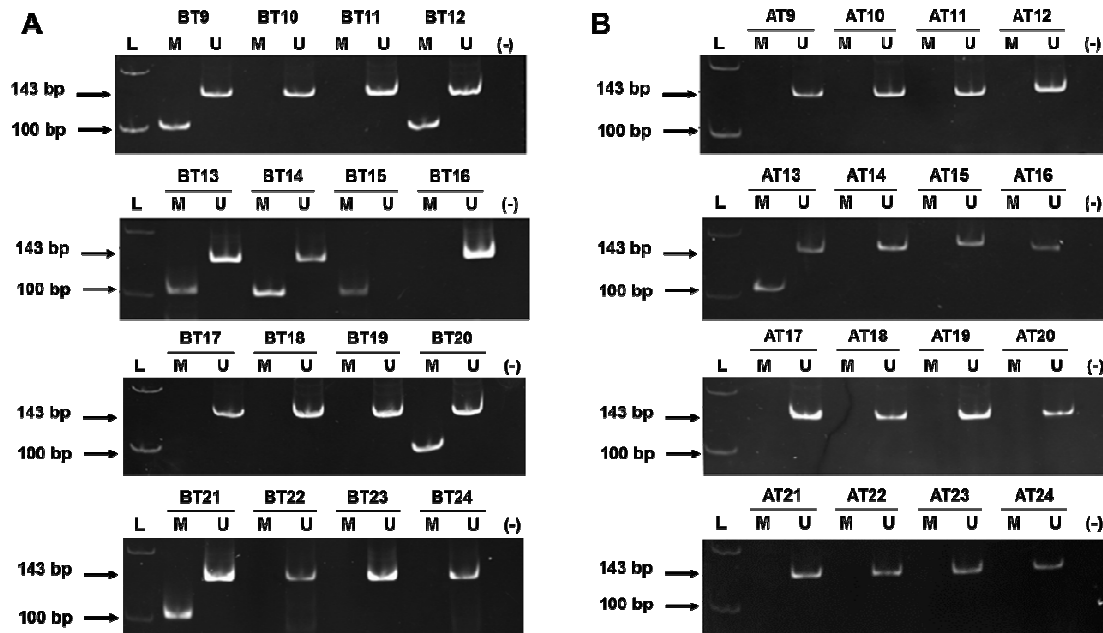| Pair of primers	Thermal cycle	Template
miR34a-MF/MR	Denature:94° C 30 s Anneal: 60° C 10 s Synthesize: 72° C 10 s	7 – 11 ng of bisulfite-treated DNA
miR34a-MF/MR1	Denature:94° C 30 s Anneal: 62° C 10 s Synthesize: 72° C 10 s	1 μl of round 1 product
miR34a-UF/UR	Denature:94° C 30 s Anneal: 65° C 10 s Synthesize: 72° C 10 s	7 – 11 ng of bisulfite-treated DNA

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.

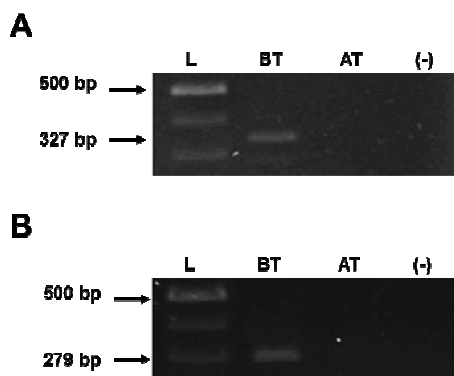*3.3. Expression of AXL and MDM4, the target genes of miR-34a, at the transcriptional level*

Figure 4. 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.

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

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Tóm tắt: Hiện tượng methyl hóa promoter của *miRNA-34a* (*miR-34a*), một gen đích của protein p53 làm ảnh hưởng tới các quá trình chết theo chương trình, 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 tình trạng methyl hóa promoter *miR-34a* ở bệnh nhân ung thư vú Việt Nam. Bằng kĩ thuật PCR đặc hiệu methyl (MSP), chúng tôi nhận thấy tỉ lệ methyl hóa promoter *miR-34a* ở mẫu u ung thư vú là 43.3% (13/30) và ở mẫu mô liền kề là 13.3% (4/30). Tìm hiểu ở mức độ phiên mã, 2 gen đích của *miR-34a* là *AXL* và *MDM4* được thấy biểu hiện ở mẫu u có promoter *miR-34a* bị methyl hóa mà không phải ở mẫu mô liền kề (không phát hiện sự methyl hóa promoter *miR-34a*).

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