CpG island methylation status of miRNAs in esophageal squamous cell carcinoma

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MicroRNAs (miRNAs) are a large family of small non-coding RNAs and have been shown to be of great importance in a wide variety of biological processes including cell differentiation, proliferation and apoptosis. Hundreds of miRNAs have now been identified in humans and an overwhelming body of evidence now implicates miRNAs in the process of carcinogenesis. Simultaneously, miRNAs undergo the same epigenetic regulatory laws like any other protein-coding gene. An estimated 10% of miRNAs are regulated epigenetically through DNA methylation. In hepatocarcinoma, bladder, lung and gastric cancer, DNA methylation mediated down-regulation of miRNAs by CpG island has been reported by several authors.

The reports about miRNAs in esophageal squamous cell carcinoma (ESCC) showed that miR-203, miR-34a, miR-34b/c, miR-424 and miR-129-2 have different levels of expression in carcinoma and adjacent normal tissues. Previous studies on these miRNAs suggested that they play important roles in carcinogenesis. miR-203 was reported to be downregulated in ESCC and epigenetically silenced in hematopoietic tumor, miR-424 is considered an anti-tumor gene and miR-129-2 is 66.7% (36/54), 40.7% (22/54) and 96.3% (52/54), respectively in ESCC, which are significantly higher than that in the corresponding non-tumor tissues (p < 0.01). Quantitative RT-PCR analysis in clinical samples suggested that CpG island methylation is significantly correlated with their low expression in ESCC. DNA methylation changes have been reported to occur early in carcinogenesis and are potentially good early indicators of carcinoma (Laird, Nat Rev Cancer 2003;3:253–66). The high methylation ratio of miR-129-2 indicated its potential as a methylation biomarker in early diagnosis of ESCC.

Previous studies on esophageal squamous cell carcinoma (ESCC) indicated that it contains much dysregulation of microRNAs (miRNAs). DNA hypermethylation in the miRNA 5’ regulatory region is a mechanism that can account for the downregulation of miRNA in tumors (Esteller, N Engl J Med 2008;358:1148–59). Among those dysregulated miRNAs, miR-203, miR-34b/c, miR-424 and miR-129-2 are embedded in CpG islands, as is the promoter of miR-34a. We investigated their methylation status in ESCC by bisulfite sequencing PCR (BSP) and methylation specific PCR (MSP). The methylation frequency of miR-203 and miR-424 is the same in carcinoma and in the corresponding non-tumor tissues. The methylation ratio of miR-34a, miR-34b/c and miR-129-2 is 66.7% (36/54), 40.7% (22/54) and 96.3% (52/54), respectively in ESCC, which are significantly higher than that in the corresponding non-tumor tissues (p < 0.01). Quantitative RT-PCR analysis in clinical samples suggested that CpG island methylation is significantly correlated with their low expression in ESCC. 5-aza-2’-deoxycytidine (DAC) treatment partly recovered their expression in EC9706 cell line. We conclude that CpG island methylation of miR-34a, miR-34b/c and miR-129-2 are frequent events and important mechanism for their low expression in ESCC. DNA methylation changes have been reported to occur early in carcinogenesis and are potentially good early indicators of carcinoma (Laird, Nat Rev Cancer 2003;3:253–66). The high methylation ratio of miR-129-2 indicated its potential as a methylation biomarker in early diagnosis of ESCC.

Key words: microRNAs, CpG island, methylation, ESCC

Additional Supporting Information may be found in the online version of this article

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methylated miRNAs will be required for the development of a clinically useful screening test. Among those miRNAs we detected, miR-129-2 was indicated as a potential methylation biomarker in early diagnosis of ESCC.

Material and Methods

Patient samples and DNA extraction

ESCC samples were collected from 54 patients who underwent surgery at the Thoracic and Cardiac Surgery of Southwest Hospital in Chongqing from January 2008 to January 2009. All patients had definite pathology diagnosis and none received radiotherapy or chemotherapy before surgery. The demographic characteristics of patients were provided in Supplementary Table 1. All samples were obtained with their informed consent and with institutional review board approval of the Hospital. Corresponding non-tumor tissues were grossly normal tissues 5 cm from esophageal cancer. The specimens were instantly divided into several pieces and stored at −80°C after surgery.

DNA were extracted from 100 mg frozen samples using the Promega DNA Extraction Kit following the manufacturer’s instructions. DNA concentrations were analyzed on GEL DOC 200 GEL Documentation System and set to 500 ng/µl.

DNA methylation status analysis

Methyl Primer Express Software v1.0 was used to design bisulfite sequencing PCR (BSP) and methylation specific PCR (MSP) primers provided in Supplementary Table 2. Bisulfite modification of 500 ng of genomic DNA was performed by using the EZ DNA Methylation-Gold Kit (Zymo Research Corp, Orange, CA). PCR products were recovered by the Qiagen gel DNA kits and sequenced in Sangon Biotech (Shanghai, China) and Applied Biosystems 3130 Genetic Analyzer in our laboratory. The lengths of the amplification fragments in MSP and BSP for miR-203, miR-34a, miR-34b/c, miR-424 and miR-129-2 are provided in Supplementary Table 2b. Three samples which were proved to be methylated or unmethylated by MSP were verified by BSP. The sequencing results were provided in the Supplementary information (S1).

Quantitative analysis of miRNAs expression by real-time RT-PCR

TaqMan MicroRNA Assay kits were used for miR-34a and miR-34b detection. The 10-µl reverse transcription (RT) reaction mixture was comprised of 200 ng of total RNA samples, 50 nM stem-loop RT primers, 10× RT buffer, 0.25 mM of each deoxyribonucleotide triphosphate (dNTPs), 3.33 U/µl MultiScribe RT and 0.25 U/µl RNase inhibitor (Applied Biosystems, Foster City, CA). The mixtures were incubated in RNA-free PCR tubes for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and subsequently used as templates in PCR. The remainder was stored at −20°C. Real-time PCR was performed on Bio-rad IQ5 Real-time PCR System. The protocol was 3 min at 95°C, 40 cycles of 10 sec at 95°C, 20 sec at 60°C. All-in-One Q-PCR Detection Kit was used in the detection of miR-129. The 10-µl RT reaction mixture was comprised of 200 ng of total RNA samples, 2.5 U/µl PolyA Polymerase, RTase Mix, 5× Reaction Buffer (GeneCopoeia Inc, Germantown, MD). The mixture was incubated for 60 min at 37°C and 5 min at 85°C. The transcript was diluted by ddH2O to 50 ul. Real-time PCR was performed on Bio-Rad IQ5 Real-time PCR System. The mixture included 2× All-in-One Q-PCR Mix, 0.2 µM miRNA Q-PCR Primer, 0.2 µM Universal Adaptor PCR Primer, and 1st strand cDNA. The protocol was 10 min at 95°C, 40 cycles of 10 sec at 95°C, 20 sec at 60°C, 10 sec at 72°C, dwell time of 6 sec.

Cell culture and DAC treatment

The human esophageal cancer cell line EC9706 was purchased from Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China. It was seeded at a concentration of 1.3 × 10^5 cells per 24-well cell culture plate 24 hr prior to treatment with DAC (AZA: 4 µM; Sigma, St Louis, MO). Cells were treated for 4 days.

Statistics analysis

The methylation ratio of miRNAs between carcinomas and non-tumor tissues were assessed using the χ² test or Fisher’s exact test. The Mann–Whitney U-test was used in methylation and expression of miR-34a and miR-34b between the methylated carcinoma group and unmethylated carcinoma group, and the expression level of miR-129 in carcinoma and non-tumor tissues was assessed by paired sample t-test. All p-values presented are two-sided. A p-value < 0.05 was regarded as statistically significant. All statistical tests were performed using the SPSS software package.

Results

The CpG island methylation status of miR-203, miR-34a, miR-34b/c, miR-424, miR-129-2 in carcinoma and non-tumor tissues

Three primers were used to amplify about 886 bp fragments upstream of pre-mir-203 including the region reported by Bueno et al. in hematopoietic tumors. The sequencing result and MSP electrophoretogram showed that miR-203 is unmethylated in 54 carcinoma and 30 non-tumor tissues (Fig. 1a and S1a), which indicated that CpG island methylation might not involve in the regulation of miR-203 in ESCC.

The methylation ratio of miR-34a is 66.7% (36/54) in carcinoma and is higher than that in non-tumor tissues(26.7%, 8/30)(p < 0.01)(Fig. 1b and S1b). 40.7% (22/54) of miR-34b/c is methylated in carcinoma and unmethylated in non-tumor tissues (p < 0.01) (Fig. 1c and S1c). The methylation of miR-34a and miR-34b/c might involve in carcinogenesis of ESCC.
There are two CpG islands around miR-424, and one of them is shared by miR-503. BSP results showed that this CpG island is methylated in 54 carcinoma and 30 non-tumor tissues in ESCC. In chromagrams of BSP (S1), all cytosine (C) residues of CpG dinucleotides were methylated and remained unchanged, whereas all the other C residues were unmethylated and converted to thymidine, indicating the “C-U” conversion was complete. In MSP electrophoretogram, “Ca” represented carcinoma samples, “Non-tumor” represented non-tumor tissues, “M” represents the fragment amplified by methylation primers and “U” represents the unmethylated fragment. In right BSP circle graph, black circle represents methylated CpG, and white circle represents unmethylated CpG. (a) Three pairs of BSP primers were designed to detect the CpG island methylation status of miR-203 in ESCC. No methylated CpG sites were found in the sequencing results, and the 131 bp band amplified by unmethylation primer indicated that miR-203 is unmethylated in ESCC. (b) The sample which had 198 bp band of methylation and the other one which had both methylation and unmethylation bands were proved methylation samples by BSP. In carcinoma, the methylation ratio of miR-34a is 66.7%(36/54), and the methylation ratio is 26.7%(8/30) in non-tumor tissues. (c) The methylation ratio of miR-34b/c in carcinoma is 40.7%(22/54), it is unmethylated in non-tumor tissues. (d) The left MSP electrophoretogram represented the methylation status of the CpG island which is about 2,400 bp apart from pre-miR-424, and the results showed that this island is methylated in 1.85%(1/54) carcinoma and unmethylated in non-tumor tissues. The right circle graph indicated that the CpG island shares by miR-424 and miR-503 is methylated in 54 cases of carcinoma and 30 cases of non-tumor tissues. (e) The methylation ratio of miR-129-2 is 96.3%(52/54) in carcinoma, and 10%(3/30) of miR-129-2 is methylated in non-tumor tissues. The quantitative RT-PCR results of miR-34a, miR-34b, miR-129

miR-129-2 is methylated in 96.3% (52/54) of ESCC, and the methylation ratio in non-tumor tissues is 10% (3/30). These two methylation ratios are significantly different ($p < 0.01$) (Fig. 1e and S1e). The methylation status of these miRNAs is summarized in Supplementary Table 3. Among those miRNAs detected, miR-34a, miR-34b/c and miR-129-2 have different methylation ratios between carcinoma and non-tumor tissues. Quantitative RT-PCR was used to evaluate the expression levels of miR-34a, miR-34b in both methylated and unmethylated carcinoma groups, and of miR-129 between paired samples. The $ΔΔCt$ of miR-34a refers to respective RUN6 in the methylated group (18 cases) is lower than that in the unmethylated group (18 cases, $p = 0.018$) (Fig. 2a). The expression level of miR-34b in the methylated carcinoma group is lower than that in the unmethylated carcinoma group ($p = 0.016$) (Fig. 2b).
has two mature miRNAs, miR-129-3p and miR-129-5p, which come from the same transcript. miR-129-3p was chose as our object. The result showed that the expression level of miR-129-3p in carcinoma is lower than that in paired non-tumor tissues ($p = 0.034$) (Fig. 2c). They indicated that methylation of miR-34a, miR-34b/c and miR-129-2 might...
regulate their expression in ESCC. To further confirm this, 5-aza-2' -deoxycytidine was used to treat EC9706 cell line.

**The expression level of miR-34a, 34b, 129 were upregulated after treatment with DAC**

The expression of miR-34a, miR-34b and miR-129 were upregulated in the EC9706 cell line after treatment with 5-aza-2' -deoxycytidine (Fig. 3). This confirmed that CpG island methylation regulates their expression in ESCC.

**Discussion**

A recent study showed that miR-203 is closely related to the differentiation of stratified squamous epithelia.23 Because of the function in decreasing cell proliferation, it was thought to be a tumor suppressor gene by now.8 miR-203 was expressed 2- to 10-fold lower in ESCC than in normal epithelium and was epigenetically downregulated in leukemia and hepatocarcinoma.4,13,14 According to these analyses, we hypothesized that in ESCC, downregulation of miR-203 was caused by hypermethylation of CpG island. However, our results suggested that miR-203 was unmethylated in carcinoma and non-tumor tissues and thus that DNA methylation may not be involved in the regulation of miR-203 in ESCC. Several studies observed the downregulation of miR-203 in esophageal adenocarcinoma and malignant mesothelioma.24,25 Interestingly, the high expression of miR-203 is associated with poorer survival in pancreatic carcinoma.26 In ovarian cancer and colon cancer, miR-203 is upregulated.27,28 Such reports indicated that there are distinct expression styles of miR-203 in different tumor types. Since the experiment examining expression of miR-203 in ESCC was fulfilled by chip,13 more deep study on the regulation of miR-203 is needed.

miR-34a and miR-34b/c are direct transcriptional targets of p53. As members of the p53 regulation network, they regulate a number of genes involved in cell cycle, apoptosis and invasion.15 Recently, their epigenetic regulation has come under scrutiny. miR-34a is hypermethylated in non-small-cell lung cancer (64%, 20/31), melanoma (62.5%, 20/32) and prostate carcinoma (79.1%, 19/24).6,29 miR-34b/c is hypermethylated in colorectal cancer (90%, 101/111).30 As to whether there is similar methylation status in ESCC, our results showed that miR-34a is 66.7% methylated in carcinoma and 26.7% methylated in non-tumor tissues, and miR-34b is 40.7% methylated in ESCC and hypomethylated in non-tumor tissues. Lujambio et al. reported that miR-34b/c hypermethylation is involved in metastasis formation.31 In this study, we analyzed the metastasis and miR-34b/c methylation in esophageal carcinoma by χ² test but found no relationship between them (data not shown). In further study on expression regulation of miR-34a and miR-34b/c in ESCC, the effect of methylation should be considered.

miR-424 and miR-503 are derived from a polycistronic precursor mir-424-503. Both of them directly target cell-cycle regulators and induce G1 cell-cycle arrest when overexpressed in...
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The common CpG island is methylated in carcinoma and non-tumor tissues; as for the CpG island about 2400 bp away from pre-miR-424, we only found one methylated case among 54 cases of carcinoma. It seems that these two CpG islands have the same methylation status in carcinoma and non-tumor tissues, so in ESCC, methylation might not have a significant effect on the regulation of miR-424.

miR-129 include miR-129-1 and miR-129-2. They are located, respectively, at 7q32.1 and 11p11.2, and share the same seed sequence “UUUUUGC.” miR-129-2 is located in a CpG island but miR-129-1 is not. miR-129-2 is 96.3% methylated in carcinoma (52/54) and 10% (3/30) methylated in non-tumor tissues, indicating that miR-129-2 has a higher methylation ratio in carcinoma. Following it, we identified its expression level in ESCC. Landgraf et al. showed that the 5’ product (miR-129-5p) of miR-129-1 is the predominant one, whereas both 5’ and 3’ (miR-129-3p) products are significantly expressed from miR-129-2, so we chose to measure the expression level of miR-129-3p in ESCC with quantitative RT-PCR. The result suggested that low level expression of miR-129-3p in carcinoma was consistent with its hyper-methylation. Re-expression of miR-129-3p was seen after treatment of the EC9706 cell line with 5-aza-dC, confirming the role of methylation of the 5’ CpG region in regulating miR-129-3p expression. While this report was different from that of Ogawa et al., the overexpression of miR-129 was observed and identified as a significant and independent prognostic factor in surgically treated ESCC patient, so further more study is needed.

Since there is no difference in the methylation status of miR-203 and miR-424 in carcinoma and non-tumor tissues in ESCC, the effect of methylation of these two miRNAs on their regulation in ESCC can be ruled out. This made us think about which miRNAs we should analyze in methylation study in carcinoma. Researchers have looked for significantly upregulated and 3’ miR-129 products are significantly expressed from miR-129-2, so we chose to measure the expression level of miR-129-3p in ESCC with quantitative RT-PCR. The result suggested that low level expression of miR-129-3p in carcinoma was consistent with its hyper-methylation. Re-expression of miR-129-3p was seen after treatment of the EC9706 cell line with 5-aza-dC, confirming the role of methylation of the 5’ CpG region in regulating miR-129-3p expression. While this report was different from that of Ogawa et al., the overexpression of miR-129 was observed and identified as a significant and independent prognostic factor in surgically treated ESCC patient, so further more study is needed.

In conclusion, we analyzed the methylation status of CpG island in miR-203, miR-34a, miR-34b, miR-424 and miR-129-2 in ESCC. We found that methylation might not be involved in the regulation of miR-203 and miR-424 in ESCC. CpG island methylation of miR-34a, miR-34b and miR-129-2 lead to their downregulation in carcinoma. The high methylation ratio of miR-129-2 made it a potential methylation biomarker in early diagnosis of ESCC.

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