<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>MicroRNA-143 is downregulated in breast cancer and regulates DNA methyltransferases 3A in breast cancer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Ng, EKO; Li, R; Shin, VY; Siu, JMT; Ma, ESK; Kwong, A</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>Tumor Biology, 2014, v. 35 n. 3, p. 2591-2598</td>
</tr>
<tr>
<td><strong>Issued Date</strong></td>
<td>2014</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10722/193223">http://hdl.handle.net/10722/193223</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>The original publication is available at <a href="http://www.springerlink.com">www.springerlink.com</a>; This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.</td>
</tr>
</tbody>
</table>
MicroRNA-143 is downregulated in breast cancer and regulates DNA methyltransferases 3A in breast cancer cells

Enders K. O. Ng · Rufina Li · Vivian Y. Shin · Jennifer M. Siu · Edmond S. K. Ma · Ava Kwong

Received: 23 May 2013 / Accepted: 16 October 2013

© International Society of Oncology and BioMarkers (ISOBM) 2013

Abstract MicroRNAs (miRNAs) are small non-protein-coding RNAs that regulate expression of a wide variety of genes including those involved in cancer development. Here, we investigate the role of miR-143 in breast cancer. In this study, we showed that miR-143 was frequently downregulated in 80 % of breast carcinoma tissues compared to their adjacent noncancerous tissues. Ectopic expression of miR-143 inhibited proliferation and soft agar colony formation of breast cancer cells and also downregulated DNA methyltransferase 3A (DNMT3A) expression on both mRNA and protein levels. Restoration of miR-143 expression in breast cancer cells reduces PTEN hypermethylation and increases TNFRSF10C methylation. DNMT3A was demonstrated to be a direct target of miR-143 by luciferase reporter assay. Furthermore, miR-143 expression was observed to be inversely correlated with DNMT3A mRNA and protein expression in breast cancer tissues. Our findings suggest that miR-143 regulates DNMT3A in breast cancer cells. These findings elucidated a tumor-suppressive role of miR-143 in epigenetic aberration of breast cancer, providing a potential development of miRNA-based treatment for breast cancer.

Keywords miR-143 · DNMT3A · Breast cancer · Tumor suppressor · PTEN

Abbreviations
miRNA microRNA
DNMT DNA methyltransferase
qRT-PCR Quantitative reverse transcription–polymerase chain reaction
3′UTR 3′ Untranslated region
MTT 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

Introduction
Breast cancer is one of the three most commonly diagnosed cancers among women, accounting for about 30 % of patients [1]. In the past decades, despite the dedication of research and resources to the development of biomarkers for diagnosis and prognosis, unpredictable response and development of resistance to adjuvant therapy remain major challenges in breast cancer management. Although mammography diagnosis for breast cancer is the currently used screening tool, the cost incurred and expertise required for mammogram has hampered wide application of this procedure. On the other hand, alternative methods such as ultrasound screening has very operator-dependent sensitivity, and tumor markers such as CA15.3 and carcinoembryonic antigen (CEA) are also nonspecific and has limited sensitivity and specificity [2]. Thus, there is still a pressing need to elucidate novel mechanism of breast cancer development so as to develop a cost-effective and accurate screening method for this cancer.

Recently, the emergence of small non-protein-coding RNAs, microRNAs (miRNAs), playing important roles in...
The downregulation of miR-143 in tumor can lead to the expression of protein-coding mRNA genes [5]. Furthermore, miRNA expression signatures have been used to predict prognosis [6, 7]. Importantly, expression of some miRNAs correlated with the molecular subtypes and with two major features of breast cancer (grade and ER status) [8]. Therefore, miRNA has a great potential to be a novel biomarker for breast cancer and holds promising potential for individualizing patients' treatment regimens [9], although, as yet, there is still limited knowledge on the exact mRNA target of the deregulated miRNA in breast cancer. Research shows that each miRNA could target up to 200 mRNA transcripts, and a single mRNA could have multiple miRNA binding sites [10]. This finding indicates that there is a great demand to further investigate on the RNA targets and understand the functional role of these differentially expressed miRNAs, so as to elucidate their potential as therapeutic agents or targets.

In this study, we investigated the functional role of miR-143 in breast cancer. MiR-143, located on chromosome 5q33, is a miRNA found to be deregulated in colon cancer [11] and bladder cancer [12]. It is previously demonstrated that miR-143 targets on DNA methyltransferase 3A (DNMT3A) mRNA [11]. DNMT3A is the member of the methyltransferase family. DNMT3A and 3B are responsible for de novo methylation in the genome [13], while DNMT1 is responsible for maintaining methylation in the genome. The expression level of DNMT3A is high in early embryonic stage and downregulated in differentiated cells, maintaining high expression of DNMT3A in embryonic cells will inhibit cell differentiation [13].

Until now, the role of DNMT3A in cancer is less studied than DNMT3B. There are reports showing that DNMT3A deficiency promotes tumor growth and progression [13]. The downregulation of miR-143 in tumor can lead to the overexpression of DNMT3A, which in turn causes hypermethylation and silencing of the tumor suppressor genes and contributes to tumorigenesis.

One of the most researched tumor suppressor genes is the phosphatase and tensin homolog (PTEN) which acts as a negative regulator of PI3K/AKT signaling pathway [14]. A high proportion of human cancers have a mutated form of PTEN or abnormal PTEN expression, and this attributed to 40 % of breast cancer [15]. Mutation and inactivation of PTEN gene lead to hyperactivation of PI3K/AKT pathway, which causes cell cycle deregulation and suppression of apoptosis [16]. Evidences showed that breast cancer patients with defective PTEN have poor prognosis and high grade tumor [15].

We aimed to show that miR-143 and DNMT3A are both deregulated in breast cancer and prove that overexpression of DNMT3A has caused a change in methylation status of PTEN and TNFRSF10C which contributed to tumorigenesis. These results help to understand the molecular mechanism of how miR-143 promotes cancer progression.

Materials and methods

Cell lines and tissue samples

Five human breast cancer cell lines including MCF-7, MD-MB-231, MD-MB-468, T47D, and SK-BR-3 and two colon cancer cell lines HT-29 and SW480 (American Type Culture Collection, Manassas, VA) were cultured at 37 °C in 10 % CO₂ atmosphere and maintained routinely in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum and 2 mM of L-glutamine (Invitrogen, Carlsbad, CA). A total of 20 pairs of primary breast tumors and noncancerous tissue counterparts were collected. All samples were collected from patients who underwent surgical resection of tumors. Informed consent has been obtained from each patient. This project was approved by the Institutional Review Board of the University of Hong Kong.

Real-time quantitative PCR

Total RNA containing small RNA was extracted from tissues and cell lines by TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. SYBR Green real-time qPCR assay for miRNA expression was used as previously described [11, 17]. In brief, 100 ng of total RNA containing miRNA was polyadenylated and reverse-transcribed to cDNA by using miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Real-time qPCR was performed using miScript SYBR Green PCR Kit (Qiagen) in ABI PRISM 7900 HT System (Applied Biosystems, Foster City, CA). The miR-143-specific forward primer sequence was 5′-TGAGATGAAGCACTGTAGCTC-3′ and was designed based on the miRNA sequences obtained from the miRBase database. Human U6 snRNA was used for normalization. For DNMT3A mRNA qPCR, total RNA was reverse-transcribed to cDNA by using miScript Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. Gene-specific primers for DNMT3A gene were used as previously described [17]. The mRNA expression was normalized to β-actin. ΔCt was calculated by subtracting the Ct values of U6 or β-actin from the Ct values of the gene of interest. ΔΔCt was then calculated by subtracting the ΔCt of the control from the ΔCt of cancer sample. Fold change of gene was calculated by the equation 2^ΔΔCt.
Ectopic miR-143 expression

Ectopic expression of miR-143 in breast cancer cells (MD-MB-231 and MCF7) was achieved by transfection with mature miR-143 mimic (Qiagen). Cells were plated in culture dishes or 6/96-well plates for 24 h and transfected with 1 nM of mimic with HiPerFect Transfection Reagent (Qiagen) for 24 h. Precursor control (Ambion, Austin, TX) was used as negative control. Cells were then subjected to further assays or for RNA/protein extraction.

Cell proliferation assay

Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTT) assay (Promega Corporation, Madison, WI). MB-231 (2×10^5) and MCF-7 (5×10^5) cells were seeded in a 96-well plate for 24 h, transfected with 1 nM miR-143 mimic (Qiagen) and HiPerFect Transfection Reagent (Qiagen) for 24 h and further grown in normal medium for 3 days. Thereafter, cells were incubated in 0.1 mg/ml MTT at 37 °C for 3 h and lysed in dimethyl sulfoxide (DMSO) at room temperature for 30 min. The absorbance in each well was measured at 580 nm by a microplate reader.

Anchorage-independent colony formation assay

Soft agar plates were prepared in 24-well plates with a bottom layer of 0.6 % Noble agar in serum-free DMEM. Cells were trypsinized, and 500 cells were seeded onto the bottom layer after being mixed with 0.3 % Noble agar in DMEM supplemented with 10 % fetal calf serum. Plates were incubated at a 37 °C incubator for 3 weeks. The number of colonies was counted after stained with 0.05 % crystal violet for 1 h and washed extensively with phosphate-buffered saline (PBS).

Western blot analysis

Cells were lysed in Laemmli’s lysis buffer, resolved in SDS-PAGE minigel, and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). Membranes were probed with 1:1, 000 diluted primary antibodies against DNMT3A (Cell Signaling) at room temperature for 2 h, washed extensively with 0.1 % Tween-20 in PBS, and incubated with secondary antibodies conjugated with horse-radish peroxidase (1:10,000 dilution). The signals were visualized with enhanced chemiluminescence (Amersham Life Science Inc., Buckinghamshire, UK).

Luciferase activity assay

DNMT3A 3’UTR containing an intact miR-143 recognition sequence was amplified, and the PCR product (199 bp) was subcloned into pGL3 basic vector (Promega, Madison, WI) immediately downstream of luciferase gene, as described previously [11]. A pGL3 construct containing DNMT3A 3’ UTR with point mutations in seed sequence was also synthesized using Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Cells were co-transfected with 800 ng of pGL3 constructs with or without miR-143 precursor for 24 h. Each sample was co-transfected with 0.05 μg pRL-CMV plasmid expressing Renilla luciferase to monitor the transfection efficiency (Promega, Madison, WI). Luciferase activity assay was performed 24 h after transfection using Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was normalized with Renilla luciferase activity.

Methylation-sensitive PCR

Genomic DNA from cell lines with or without miR-143-mimic transfection used for methylation analysis was extracted by DNeasy Mini Kit (Qiagen) according to the user manual. Methyl-Profiler DNA Methylation qPCR Primer Assays (SA Biosciences) was used to determine the methylation status of the promoter in different genes. In brief, 250 μg of genomic DNA was used for enzyme digestion by using a Methyl-Profiler Enzyme Kit. For each sample, mock digestion (Mo), methylation-sensitive digestion (Ms), methylation-dependent digestion (Md), and double digestion (Msd) was performed by adding different combinations of enzyme according to the manufacturer's protocol and was placed in 37 °C heating block for 6 h, followed by heat inactivation at 65 °C for 20 min. SYBR Green-based qPCR was performed with a panel of 26 breast cancer methylated gene promoters (MeAH-011C, SA Biosciences), on PRISM 7900 HT. Ct was obtained after qPCR, and the relative amount of methylation was calculated by first determining the relative amount of DNA resistance to enzyme digestion (Cr): 2^\Delta Ct(Msd−Mo). Then the degree of methylation of each gene promoter can be calculated as follows:

\[ \Delta Ct(Ms−Mo)/\Delta Ct(Ms−Mo) \]

Statistical analysis

Paired t test was used in the expression comparison of miR-143 between paired breast tumor and adjacent noncancerous tissues. Two-sided Student's t test was used to analyze MTT assay, anchorage-independent soft agar assay, and luciferase reporter assay. Data are expressed as the mean ± SD from at
Results

MiR-143 is downregulated in breast tumor and human breast cancer cell lines

MiR-143 has been reported to be downregulated in other cancers like bladder cancer and colon cancer. To examine the expression levels of miR-143 in breast cancer, 20 pairs of breast tumor with adjacent normal tissue and five breast cancer cell lines were quantified by real-time PCR. The expression level of miR-143 in all the five breast cancer cell lines (MCF7, MB-231, MB-468, T47D, and SK-BR-3) was lower than that of noncancerous breast tissue (Fig. 1a). For patient samples, low expression of miR-143 was found in tumor compared with the adjacent normal tissues ($P < 0.05$, Wilcoxon test; Fig. 1b).

The effect of miR-143 on cell growth and DNMT3A expression

Low expression levels of miR-143 in breast cancer cells suggest that miR-143 has a role in breast cancer carcinogenesis. To prove this, enforced expression of miR-143 on cell growth in MB-231 and T47D breast cancer cells was tested by MTT assay and colony formation assay. After transfection with miR-143, both MB-231 and T47D showed a significant decrease in growth rate (22 % decrease for MB-231 and 30 % decrease for T47D; Fig. 2a, b). Colony formation assay was performed to determine the degree of invasiveness in different cell lines after miR-143 mimic transfection. Figure 2c showed the overexpression of miR-143 after transfection with miR-143 mimic when compared to the precursor. The increased miR-143 expression significantly reduced anchorage-dependent growth in both cell lines as shown in Fig. 2c ($P < 0.05$; Mann–Whitney test), confirming that miR-143 also affects the malignant transformation phenotypes. These results suggested that miR-143 has a role in suppressing tumor cell growth. We then examined the correlation between miR-143 and DNMT3A. Our results indicated that restored expression of miR-143 leads to decreased expressions of DNMT3A mRNA (Fig. 2d) and protein (Fig. 2e) in both cell lines, which suggest a potential regulatory role of miR-143 on DNMT3A.

Direct interaction between DNMT3A and miR-143

To confirm that DNMT3A is the direct target of miR-143, luciferase assay was performed. In short, wild-type (WT) or mutated (MUT) 3′UTR of DNMT3A (11) was subcloned into downstream of the firefly luciferase reporter and co-transfected with miR-143 precursor or precursor control into both MB-231 and T47D breast cancer cell lines. In the presence of miR-143, the relative luciferase activity of breast cancer cell lines with WT construct was significantly reduced (Fig. 3b; $P < 0.05$ for MB-231, $P < 0.01$ for T47D; Mann–Whitney test). While no significant suppressive effect by miR-143 was found in cells transfected with the MUT construct, this suggested a direct and specific interaction of miR-143 on DNMT3A 3′UTR in breast cancer cells.

Expression relationship between miR-143 and DNMT3A in breast tumor tissue

To confirm the relationship between miR-143 and DNMT3A, we assessed the expression of miR-143 and DNMT3A protein in breast tumor tissues from ten patients. As shown in Fig. 4a, there is no correlation between miR-143 and DNMT3A mRNA expression. However, DNMT3A protein levels were inversely correlated with miR-143 ($r = -0.61$, $P < 0.05$; Spearman’s correlation; Fig. 4b).

Methylation status of PTEN

To examine the effect of DNMT3A downregulation on methylation status of PTEN gene, we performed the methylation-sensitive PCR. As shown in Fig. 5, ectopic expression of miR-143 in MB-231 reduced hypermethylated DNA on PTEN gene promoter from 50 to 2.3 %, while that of unmethylated DNA raised from 50 to 97.7 %. In addition, hypermethylated TNFRSF10C reduced from 50 to 25 %, whereas unmethylated TNFRSF10C reduced from 50 to 0.8 %, and that of intermediate methylated DNA increased from 0 to 73.8 %.
Discussion

Since the discovery of miRNAs, the differential expression pattern of miRNAs in various cancers has been reported; however, the functional roles of individual miRNAs towards cellular transformation and tumorigenesis continue to be actively studied. Increasing evidence showed that miRNAs might be involved in tumorigenesis by regulating oncogenes or tumor suppressor genes. A recent report showed that miRNA and epigenetic methylations are interconnected and contributed to tumorigenesis [18, 19]. In this study, we showed that miRNA can affect methylation through altering methyltransferase synthesis, which in turn affects tumor malignancy.
It has been reported previously that miR-143 is deregulated in colorectal cancer [20], prostate cancer [21], B cell lymphoma [22], etc. In this study, we demonstrated that miR-143 is downregulated not only in breast cancer cell lines but also in primary breast tumors. The frequent downregulation of miR-143 suggests a tumor-suppressive role in breast cancer. We verified this by the enforced expression of miR-143 in breast cancer cells, resulting in a suppression of malignant transformation.

To further understand the tumor-suppressive role of miR-143, in silico target prediction (PicTar and TargetScan 5) is used for target prediction. Despite a large number of predicted potential targets for miR-143, only a limited amount was verified. There were reports showing that miR-143 acted on extracellular signal-regulated kinase 5 (Erk5) which in turn affects the mitogen-activated protein kinase (MAPK) pathways [21]. MAPK is an important pathway for oncogenesis, as it involves in cell proliferation, differentiation, and migration [23]. Apart from Erk5, DNMT3A is also a predicted target of miR-143.

DNMT3A together with DNMT1 and DNMT3B are catalytically active DNMTs responsible for genome methylation [24]. DNMT1 is a maintenance DNA methyltransferase for retaining methylation pattern, with inefficient de novo methylation ability. DNMT3A and DNMT3B are de novo methyltransferases with different targets [25]. Increasing evidence showed that these DNMTs work together to maintain a normal methylation pattern, and deregulation of either one could promote malignancies [26].

In this study, we showed that there is a correlation between miR-143 and DNMT3A in breast cancer. Enforced expression of miR-143 suppressed tumor transformation and DNMT3A mRNA and protein. Site-directed mutation on the 3'UTR of DNMT3A revealed the presence of specific binding site of miR-143. An inverse correlation of miR-143 and DNMT3A expression in human breast samples further consolidated miR-143 negatively regulated DNMT3A.

Genome-wide hypomethylation is common in cancer genomes which causes genome instability [27]; whereas site-specific hypermethylation in the promoter region of the tumor suppressor gene causing gene silencing is often observed [28]. Deregulation of methyltransferase could be
due to mutations in methyltransferase gene or imbalanced methyltransferase biogenesis. MiRNAs modulate posttranscriptional repression and maintain the balance of gene expression level in the cells [29]. We demonstrated that miR-143 targeted on DNMT3A gene and caused transcriptional repression. Low miR-143 expression increased the expression of DNMT3A enzyme which caused hypermethylation in other tumor-suppressing genes.

PTEN has long been known for its tumor-suppressive property; inactivation of PTEN could lead to various cancers [30]. Homozygous mutation is often found in familial and sporadic cancer. In breast cancer, reduction or complete absence of PTEN protein is found in about 40% of the cases, mostly due to loss of heterozygosity (LOH), rarely somatic mutation [31]. The low mutation rate and high LOH suggested that epigenetic modification is responsible for the lost or reduced expression of PTEN protein. Many reports showed that promoter CpG hypermethylation is the reason for PTEN expression silencing. Methylation of PTEN was also shown to correlate with estrogen and progesterone receptor level which is highly related to the invasiveness of breast cancer, or even drug resistance [16, 32]. Recent research showed that PTEN expression is methylation-dependent and is preferably methylated by DNMT3A [33]. By depleting DNMT3A, PTEN expression could be resumed due to demethylation of the CpG islands in the promoter region. Therefore, depletion of DNMT3A could exhibit antiproliferative effects.

Being the preferential targets of DNMT3A [33], changes in methylation level of PTEN genes depend on the DNMT3A level inside the cells. Our results showed for the first time that in restoration of miR-143, the methylation status of PTEN has been changed. With lowered expression of DNMT3A after miR-143 transfection, percentage of hypermethylated DNA in PTEN promoter drastically reduced, while that of unmethylated DNA increased. This clearly showed that miR-143 indirectly control the PTEN expression level through DNMT3A.

The tumor necrosis factor receptor superfamily member 10C (TNFRSF10C) located on 8p22-p21 encodes a protein in the TNF receptor superfamily [34]. It has an extracellular death domain and a transmembrane domain, but lacks cytoplasmic domain which makes this receptor a phospholipid phosphatase. Trends Cell Biol. 1999;9:125–8.

In conclusion, we found that miR-143 was frequently downregulated in breast cancer, which might be a potential tumor suppressor. The direct targeting of miR-143 on DNMT3A suggested for the first time that miR-143 took part in the regulation of DNA methylation and caused PTEN and TNFRSF10C methylation. These novel findings provided a new insight into the relationship of miRNA and methylation, which may provide a new direction for the development of miRNA-based target treatment.

Conflicts of interest None

References


