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Hypermethylation of *miR-203* in endometrial carcinomas

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Abstract

Objectives—Aberrant expression of *SOX4* in endometrial cancer has been identified and partially was contributed to hypermethylation of *miR-129-2*. Other miRNAs are suspected to influence *SOX4* as well. The current study seeks to identify other hypermethylated miRNAs that regulate *SOX4* in endometrial carcinomas.

Methods—Methylation levels of miRNA promoter regions were measured by combined bisulfite restriction analysis (COBRA) and pyrosequencing assays. Gene expression was determined by RT-qPCR. Methylation level of a miRNA locus was corrected with clinicopathologic factors for 252 gynecological specimens.

Results—*In silico* analysis identified 13 miRNA loci bound on the 3'-UTR of *SOX4*. Using COBRA assays, increased methylation of *miR-203*, *miR-219-2*, *miR-596*, and *miR-618* was detected in endometrial cancer cells relative to those seen in a normal cell line and in normal endometrium. Transfection of a *miR-203* mimic decreased *SOX4* gene expression. Hypermethylation of *miR-203* was detected in 52% of type I endometrioid endometrial carcinomas ($n=131$) but was not seen in any of 10 uninvolved normal endometria ($P<0.001$). Methylation status of *miR-203* was significantly associated with microsatellite instability and *MLH1* methylation in endometrial tumors ($P<0.001$). Furthermore, hypermethylation of *miR-203* was found in endometrioid and clear endometrial subtype tumors, but not in cervical squamous cell and ovarian carcinomas.

Conclusions—Hypermethylation of *miR-203* is a frequent event in endometrial carcinomas and is strongly associated with microsatellite instability and *MLH1* methylation status. Thus, *miR-203*

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Conflict of interest statement

The authors have no conflicts of interest to declare.

methylation level might represent a marker for patients with endometrioid and clear endometrial sub-cancers.

Keywords

Endometrial carcinoma; DNA methylation; *SOX4*; *miR-203*

Introduction

The SRY-related high-motility group box 4 gene (*SOX4*) is overexpressed in a variety of cancers, including prostate, lung, bladder, breast, gastric, and endometrial cancers [1, 2]. Its known functions include the regulation of embryonic development and differentiation to determine cell fate, as well as cellular transformation, cell survival and metastasis [1], suggesting an oncogenic role for *SOX4* in some solid malignancies. In order to account for this overexpression, various mechanisms have been explored, such as chromosome amplification and post-transcription by microRNAs (also known as miRNAs) [1]. miRNAs are small noncoding RNAs that have recently been gaining attention in their roles in gene regulation [3]. More than 2500 human mature miRNAs have been identified [4], each believed to have the potential to post-transcriptionally modulate the expression of multiple mRNA targets [3, 4]. miRNAs regulate mRNA expression by forming imperfect pairing at the 3'-end of untranslated regions (3'-UTRs) of a target locus, which inhibits translation and may even promote degradation of the target mRNA.

While it is expected that promoter hypomethylation could upregulate a specific oncogene, hypermethylation-mediated silencing of a miRNA could activate its oncogenic target [2, 5]. This post-transcriptional regulation allows miRNA to control many regular processes of the cell [3], possibly even tumorigenesis. Recent studies have demonstrated that some cancers are associated with downregulation or even complete chromosomal deletion of specific miRNAs. In 2010, frequent deletions of *miR-15* and *miR-16* were first found in cells from patients with chronic lymphocytic leukemia [6]. In this study these deletions appeared to be associated with cell cycle arrest and apoptosis. Downregulation of miRNAs in cancers frequently occurs via DNA methylation. In endometrial and gastric cancers, repression of *miR-129-2* by DNA hypermethylation was found to be correlated with overexpression of *SOX4* [2, 7]. Subsequent demethylation of *miR-129-2* resulted in partial downregulation of *SOX4* expression, in a manner analogous to the downregulation of tumor suppressors by promoter hypermethylation. In this study we investigate the methylation status of other miRNAs and their relationship to *SOX4* in endometrial cancer in search of potential markers of this disease.

Materials and Methods

Gynecological specimens

A total of 252 tissue specimens were obtained, either from Washington University in St. Louis described in a previous report [2], or through the Cooperative Human Tissue Network (CHTN). The Washington University cohort contained 131 tumors of type I endometrioid endometrial carcinomas (EEC). The CHTN cohort included 10 cancer-free normal, 23 EEC

each paired with an adjacent normal, 17 EEC, 37 non-endometrioid endometrial carcinomas (NEEC), 24 ovarian tumors, and 10 cervical squamous cell carcinomas. Patient characteristics are summarized in Supplementary Table 1. All participants consented to both the molecular analyses and any follow-up studies, and the protocols were approved by the Human Studies Committees at Washington University in St. Louis, the Ohio State University, and Medical College of Wisconsin. Tumor specimens and adjacent normal tissues were collected from primary endometrial carcinomas at the time of hysterectomy. Normal control tissues were procured from women undergoing hysterectomy for non-cancer-related causes. All specimens were evaluated by at least one pathologist, who confirmed the diagnoses from hematoxylin- and eosin-stained tissue sections. The presence of microsatellite instability (MSI) and *MLH1* methylation status was determined and reported previously [2]. Standard methods were used to extract DNA from tumors, corresponding non-neoplastic tissues, and normal controls.

Cell culture

Human endometrial cancer cell lines, AN3CA, HEC1A, Ishikawa, KLE, RL95-2 and SK-UT-1B, and a normal endometrial cell line, E6/E7, were used in this study [2]. For epigenetic studies, these cells were treated with 5-aza-2'-deoxycytidine (DAC, 5 μ M, Sigma-Aldrich, St. Louis, MO) for 48 h and/or trichostatin A (TSA, 0.5 μ M, Sigma-Aldrich) for 24 h. For transfection studies, Ishikawa cells were transfected with mature miRNA, and miRNA negative control #1 (Life Technologies, Grand Island, NY), using the Cell Line Nucleofector Kit (Lonza, Walkersville, MD) according to manufacturer's instructions. DNA and RNA from treated and untreated cells were isolated as described previously [2].

Reverse transcription and quantitative PCR (RT-qPCR)

Total RNA (1 μ g) underwent reverse transcription using Superscript III reverse transcriptase (Life Technologies). PCR was performed as described previously [2]. *SOX4* primers were published previously (Table S2) [2], and TaqMan microRNA assay kits were obtained from Life Technologies. The relative expression of a gene in cells was determined by comparing the threshold cycle (Ct) of the gene against the Ct of housekeeping genes, *GAPDH* or *U48*.

Combined bisulfite restriction analysis (COBRA)

Genomic DNA (500 ng) was treated with sodium bisulfite using the EZ DNA Methylation kit from Zymo Research (Irvine, CA), following the manufacturer's recommended protocol. COBRA was used to evaluate promoter methylation of miRNAs. Target sequences were amplified by PCR, and the products were digested with methylation-sensitive restriction enzymes, such as *AciI* (New England Biolabs, Ipswich, MA) to identify methylated alleles. Primer sequences are denoted in Table S3. Digested and non-digested PCR products were resolved on 2% agarose gels stained with ethidium bromide. Smaller DNA fragments digested by *AciI* were scored as "methylated" in a given sample.

Pyrosequencing

Pyrosequencing was performed using the PyroMark MD system (Qiagen, Valencia, CA) according to the manufacturer's protocol. The oligonucleotide primers were purchased from

Life Technologies, and used for the amplified region of *miR-203* as follows: the forward primer, GTTTGGAGTTAGAGTTATAGTTAGG; the reverse biotinylated primer, CCCAACAACACTTAACTCTC; and the pyrosequencing primer, GATTAATTTAGGGGAGTTTA. Specific primers to detect methylation levels of *miR-219-2*, *miR-335*, *miR-596* and *miR-618* are denoted in Table S4. Methylation was quantified using the provided software (Qiagen).

Statistical analyses

The mRNA expression of endometrial cancer cell lines, and methylation levels of the tumors and adjacent normal endometrial tissues were compared by using the paired two-sample *t*-test. A *P*-value of <0.05 was considered to be significant. The marginal relationship between continuous methylation levels of miRNAs and a relevant categorical clinicopathologic covariate such as MSI was examined using the *t*-test for binary variables or ANOVA for categorical variables. All tests were two-sided, and all statistical analyses were performed using GraphPad Prism 5 software (GraphPad software, La Jolla, CA).

Results

Multiple miRNAs targeted on SOX4 exhibit hypermethylation in endometrial cancer cells

Using *in silico* analysis (miRanda database [4]), we identified 13 miRNA loci embedded in canonical CpG islands (Figs. 1A–B, S1A–B) located in the 5′-flanking regions, which play important roles on regulating gene expression. Among these loci, the expression of seven (*miR-130b*, *miR-132*, *miR-191*, *miR-212*, *miR-335*, *miR-363* and *miR-596*) has been previously reported to be associated with cancer development and/or regulated by DNA methylation [8–13]. All 13 miRNA loci were evaluated by COBRA in six endometrial cancer cell lines (AN3CA, Ishikawa, HEC1A, KLE, RL95-2, and SK-UT-1B, Fig. 1B from left to right), and one (N) pooled sample derived from two noncancerous endometria (Fig. 1B). Methylation levels of *miR-130b/301b*, *miR-191*, *miR-320* and *miR-632* were not found in all of tested endometrial cancer cells. Promoter hypermethylation of *miR-106a-363*, *miR-335*, *miR-203*, *miR-219-1*, *miR-219-2*, *miR-596*, *miR-618*, and *miR-1253* was detected in all or part of six endometrial cancer cell lines, as shown in Fig. 1B. However, because hypermethylation of *miR-106a-363*, *miR-335* and *miR-219-2* was also found in the normal sample (Fig. 1B), these sites were not evaluated further. Hypermethylation of *miR-203* was found in five of six endometrial cancer cell lines, but not in the normal (N) endometrium, and not in the normal endometrial cell line (E6/E7), as analyzed by COBRA (Fig. 1C) and pyrosequencing (Figs. S1B–C). Based upon our COBRA analysis, along with other reports [5, 8, 13], *miR-203*, *miR-219-2*, *miR-335*, *miR-596*, and *miR-618* were selected for further analysis, because these miRNAs were predominantly hypermethylated in cancer cell lines, but not in normal endometrium.

Because the hypermethylation of *miR-203*, *miR-219-2*, *miR-335*, *miR-596*, and *miR-618* was observed in endometrial cancer cell lines (Fig. 1B) and the 5′-end of these loci have canonical CpG islands (Figs. S1B and S2–5, panels A), we examined whether these miRNAs can be reactivated in endometrial cancer cell lines. When these cancer cells were treated with a demethylating agent, DAC (5 μmol/L), a histone deacetylase inhibitor, TSA

(0.5 $\mu\text{mol/L}$), or DAC and TSA in combination, reactivation of *miR-203*, *miR-219-2*, *miR-596* and *miR-618* was observed (Fig. 2). These results suggest that the silencing of *miR-203*, *miR-219-2*, *miR-596*, and *miR-618* in endometrial cancer cells is mediated through promoter hypermethylation.

Methylation *miR-203* is significantly correlated with microsatellite instability (MSI) status and *MLH1* methylation

To determine whether hypermethylation of miRNAs mentioned above is presented in primary endometrial specimens, we performed pyrosequencing or MassARRAY (Supplementary Methods) analysis. The methylation status of *miR-219-2*, *miR-335*, *miR-596*, and *miR-618* was examined. Because these miRNAs were either hypermethylated in both normal samples and tumors (Figs. S2–3), or exhibited low frequency in tumor specimens (Figs. S4–5), these miRNAs were not further evaluated.

The methylation level of *miR-203* is examined in endometrioid endometrial specimens including 131 clinical EEC tumor samples and 10 uninvolved normal endometria. Quantitative methylation level of each CpG site is shown in Fig. 3A. The mean methylation level in all measured CpG sites in each specimen was used to compare differences between the tumor and the normal groups. Extensive methylation of *miR-203* was found in more than 52% of the primary EEC tumors, but was not seen in the normal tissues (cancer free endometrium, $P<0.0001$; Fig. 3B), which has not been reported.

Hypermethylation of *miR-203* was significantly associated with microsatellite instability (MSI) status ($P=0.04$) and *MLH1* gene methylation ($P=0.01$) (Figs. 3C–D). Using linear model analysis, *miR-203* methylation was not associated with age, recurrence, body mass index, tumor grade, or tumor stage (Fig. S6).

Hypermethylation of *miR-203* is found in subtypes of endometrial primary tumors, but not in ovarian and cervical tumors

Since the methylation of *miR-203* has not been extensively examined in primary gynecological tumors, we first determined DNA methylation in 23 paired EEC tissues. Pyrosequencing analysis demonstrated increased methylation levels of *miR-203* in tumors relative to those in their adjacent normal counterparts ($P<0.05$; Fig. 4A). We further found hypermethylation of *miR-203* in EEC and clear cell subtypes of endometrial tumors, but not in other gynecological tumors (Fig. 4B). Interestingly, methylation level of *miR-203* was very low in both cervical squamous cell and ovarian carcinomas (Fig. 4C). The detailed methylation level of each CpG site is shown in Supplementary Fig. S7. These results suggest that among various gynecological tumors, *miR-203* hypermethylation is limited to a few specific subtypes of endometrial carcinomas.

SOX4 is targeted by *miR-203*

To further demonstrate that *miR-203* regulates *SOX4* expression, Ishikawa cancer cells were transiently transfected with *miR-203*, *miR-335* or *miR-618* (0–250 pmol) for 24 h. *miR-335* was chosen as a positive control because it was reported to target *SOX4* in human breast cancer [8]. *miR-618* was selected to prove *in silico* (Figs. 1, S1A) prediction. All of

miR-203, *miR-335* and *miR-618* were found to reduce *SOX4* mRNA expression in transfectants (Figs. 5A–C). In addition, Ishikawa cells transfected with *miR-203* demonstrated suppressed *TP63* mRNA expression (Fig. S8). The *TP63* gene has been reported to be regulated by *miR-203* in various cancers [14–16] as positive transfection control.

Discussion

SOX4 is a member of the SOX family of transcription factors. Its known functions involve the regulation of embryonic development and differentiation to determine cell fate [1]. *SOX4* expression was shown to be elevated in a wide variety of tumors, including those of the endometrium [1, 2], suggesting a fundamental role in tumorigenesis. The functions of *SOX4* in tumor development and progression could be dependent upon cellular content and tumor origin. In many cancers including those of the endometrium, *SOX4* acts as a pro-oncogene and is associated with increased cell proliferation, cell survival, epithelial-to-mesenchymal transition, and metastasis, and with reduced apoptosis [1, 2]. In a subset of cancers such as the bladder and colon, *SOX4* was reported to suppress tumor formation [1] by inhibiting tumor initiation and metastasis.

The *SOX4* gene is located on chromosome 6p22 and has been reported to be amplified in bladder and lung cancers, resulting in increased *SOX4* expression [17, 18]. The expression of this locus can be regulated by signaling pathways, such as the transforming growth factor- β and the WNT pathways, and at the post-translational modifications such as phosphorylation and acetylation on specific residues, or through interaction with specific cofactors such as TCF, syntenin-1 and p53 [1]. In addition, we previously have reported a new cascade regulation of *SOX4* expression by *miR-129-2*, which serves as an upstream regulator. An inverse association between the expression of *miR-129-2* and *SOX4* was found in endometrial cancer. Silencing of *miR-129-2* by an epigenetic event, DNA hypermethylation, resulted in lost expression of this miRNA in endometrial cancer, while also resulting in overexpression of *SOX4* [2].

In this study, we have extended our search for potential miRNA effectors by scrutinizing the miRBase database [4] and found that the expression of *SOX4* may be regulated by at least 13 putative miRNA loci with upstream canonical CpG islands. Five of these miRNAs (*miR-203*, *miR-335*, *miR-219-2*, *miR-596* and *miR-618*) demonstrated hypermethylation in endometrial cancer cells (Fig. 1). However, only *miR-203*, *miR-335*, and *miR-618* demonstrated a significant increase in hypermethylation relative to non-cancer endometrium (Figs. 3, S2–5). *miR-335* was reported to be lost in primary breast tumors of recurrent patients [8], but its promoter was hypermethylated in both normal endometrium and tumors. Promoter methylation of *miR-618* was less extensive in endometrial tumors and was not further evaluated.

miR-203 has been reported to suppress cancer cell proliferation, invasion, and metastasis [5, 19]. It is downregulated in many cancers, including endometrial carcinosarcoma [20]. Modulation of *miR-203* expression by genetic and epigenetic silencing was found in hematopoietic tumors [5], resulting in enhancing *ABL1* and *BCR-ABL1* oncogene

expression. In endometrial carcinoma, we identified *SOX4* as a potential target of *miR-203*. Hypermethylation of *miR-203* was detected in endometrial cancer cell lines (Fig. 1). When these cancer cells were treated with inhibitors of DNA methyltransferase and histone deacetylase, the expression of *miR-203* was reactivated (Fig. 2), suggesting that this miRNA expression is subjected to epigenetic regulation. Further methylation analysis revealed hypermethylation of *miR-203* in primary endometrioid endometrial tumors (Fig. 5D). This hypermethylation was significantly associated with microsatellite instability and *MLH1* methylation (Fig. 3).

In addition, *miR-203* has been reported to target and regulate the expression of *TP63* (p63) [14–16]. *TP63* is a *TP53* (p53) homologue, and its structure and functions are similar to those of p53, particularly in the DNA-binding domain. Certain specific isoforms of p63 are involved in cellular response to stress, development and tumorigenesis. DeltaNp63 isoform may promote tumorigenesis, but TAp63 is a tumor suppressor in the female germline [21]. In a soft tissue sarcoma, Rhabdomyosarcoma, *miR-203* functions as a tumor suppressor by targeting p63 and by inhibiting cell migration and promoting terminal myogenic differentiation [15]. In the current study, endometrial cancer cells transfected with *miR-203* also showed inhibition of p63 mRNA (delta isoform) expression, which suggests that *miR-203* could regulate endometrial cancer differentiation and migration. This observation needs to be further examined with regard to the role of *miR-203* and p63 in endometrial tumorigenesis.

The hypermethylation status of *miR-203* has not been extensively reported on in cervical, endometrial and ovarian malignancies. Our study adds to the current literature on *miR-203*. In this study, *miR-203* hypermethylation not only is found in endometrioid endometrial carcinomas (EECs), but also was demonstrated in clear cell endometrial tumors. In the cervix, previous reports have demonstrated that the expression of *miR-203* was downregulated in high-grade cervical intraepithelial neoplasia (CIN) and carcinomas [22–24], and in each case this was associated with hypermethylation of this locus. However, we did not detect any *miR-203* methylation in cervical tumors, although it's possible that our sample size is too small to detect significance. Previous report of expression of *miR-203* as upregulated in ovarian cancer [25] is consistent with our finding of very low promoter methylation in this miRNA.

miRNAs have been found to be dysregulated in tissue-specific manners in various cancers [3, 26]. Many studies have explored the potential usefulness of miRNA expression profiles as biomarkers for cancer diagnosis, prognosis, and response to treatment [26]. miRNAs are likely to be useful as noninvasive biomarkers for both solid tumors and hematologic malignancies. Expression of circulating miRNAs in body fluids as serum, plasma, and saliva has confirmed their potential use as diagnostic and prognostic markers. *miR-129-2* methylation was detectable in hepatocellular carcinoma (HCC) tumors [27], as well as in plasma from HCC patients [28], but not in healthy individuals or patients with liver cirrhosis. This selectivity implies its potential utility as an early diagnostic marker for HCC. We reported that hypermethylation of *miR-129-2* is associated with shorter patient survival time, MSI, and *MLH1* methylation in endometrial tumors [2]. This study suggests *miR-203* as a potential biomarker to discriminate particular subtypes of endometrial cancer from other

gynecological tumors. Such a marker could provide an important diagnostic tool to distinguish specific tumor subtypes if able to be linked with patient outcomes. More work is needed to confirm the use of DNA methylation of circulating miRNAs as a potential biomarker. We are currently examining whether hypermethylation of *miR-203* and/or *miR-129-2* could serve as a biomarker in plasma from endometrial cancer patients before and after hysterectomy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

miR-203 is a novel hypermethylated marker in endometrial cancer. (A) The diagram of predicted miRNA binding sites on *SOX4* 3'-UTR. (B) Summary of the methylation status by COBRA of thirteen miRNA regions in endometrial cancer cell lines (from left to right: AN3CA, Ishikawa, HEC1A, KLE, RL95-2 and SK-UT-1B) and one normal (N) pooled sample derived from two noncancerous endometria as a negative control. m: methylated; and u: unmethylated. (C) Hypermethylation of *miR-203* in endometrial cancer cell lines, as revealed by COBRA analysis. E6/E7: normal endometrial cell line; Sssl, methylated positive control; N: normal endometrium. +, *Acil* restriction enzyme added; and –, without *Acil*.

Fig. 2.

Relative expression levels of *miR-203* (A), *miR-219-2* (B), *miR-596* (C), and *miR-618* (D) in endometrial cancer cells after treatment with DAC and/or TSA. Gene expression was determined by RT-qPCR analysis and compared to untreated controls. *U48* was used as an internal control gene. Error bar: SD; and *: $P < 0.05$ compared with untreated control of the same cell type.

Fig. 3. Methylation of the *miR-203* CpG island and clinicopathologic covariates in primary endometrioid endometrial carcinomas (EECs). (A) Methylation profiles of *miR-203* in 10 normal endometrial tissues and 131 primary tumors following pyrosequencing analysis. (B) Dot plots demonstrating *miR-203* hypermethylation in EEC tumors. (C-D) Dot plots indicating that the methylation level of *miR-203* is correlated with MSI status and *MLH1* methylation. m: *MLH1* methylated; and u: *MLH1* unmethylated.

Fig. 4.

Hypermethylation of *miR-203* in subtypes of primary gynecological tumors. (A) Methylation analysis of 23 paired endometrioid endometrial (EEC) tissues, measured by pyrosequencing analysis. (B) Dot plots demonstrating *miR-203* hypermethylation in another set of primary EEC and clear endometrial tumors, but not in serous, mixed Müllerian (MM), or other mixed (mix) tumors. (C) Dot plots indicating methylation levels of *miR-203* in cervical squamous cell carcinomas (CvSCC) and ovarian carcinomas (OvCa).

Fig. 5.

Down-regulation of *SOX4* mRNA expression by *miR-203* (A), *miR-335* (B) and *miR-618* (C). Cancer cells (Ishikawa) underwent transient transfection with *miR-203*, *miR-335*, or *miR-618* at the indicated concentrations for 24 h. Gene expression was determined by RT-qPCR analysis and compared to untreated controls. *GAPDH* was used as an internal control gene. Error bar, SD; and *, $P < 0.05$ compared with untreated cells. (D) A proposed model of *miR-203* and *SOX4* interactions in normal and cancer cells.