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Upregulated exosomal miR-221/222 promotes cervical cancer via repressing methyl-CpG-binding domain protein 2

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Abstract. – OBJECTIVE: Methyl-CpG-binding domain protein 2, a target gene of miR-221and miR-222, plays a crucial role in a large body of human cancers. In this study, we aim to explore the mechanism by which miR-221/222 promotes cervical cancer.

MATERIALS AND METHODS: We have analyzed mRNA expression of MeCP2 and MBD2 in cervical cancer tissues by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and examined the expression of miR-221/222 in C33A, HeLa, and CaSki cells by Western blot.

RESULTS: We found that the expression levels of MBD2 and MeCP2 were significantly reduced in cervical cancer samples as detected by the analysis of MeCP2 in matched tumor-normal samples of patients with cervical cancer, indicating a reduction in a significant percentage of patients. At the same time, we found that miR-221 and miR-222, which targeted MBD2, were upregulated in cervical cancer samples. To further elucidate the relation between miR-221/222 and MBD2, we used a lot of cell lines such as C33A, HeLa, and CaSki. Surprisingly, we found that the expression levels of MBD2 and MeCP2 were significantly lower in HeLa and CaSki than in C33A, as detected by qRT-PCR. Western blot analysis of MeCP2 of HeLa and CaSki was significantly lower than in C33A. MiR-221/222 was significantly higher in HeLa and CaSki than in C33A by qRT-PCR. The knockdown of miR-221/222 in HeLa and CaSki resulted in the downregulation of miR-221/222 levels, which rescued the expression levels of MBD2 and MeCP2 in HeLa and CaSki. However, transfection of miR-221/222 on C33A could upregulate the expression levels of miR-221/222 and decrease the expression levels of MBD2 and MeCP2.

CONCLUSIONS: Our results demonstrate that the upregulated miR-221/222 promotes cervical cancer by repressing MBD2 and MeCP2.

Key Words:
MiR-221, MiR-222, MBD2, MeCP2, Cervical cancer.

Introduction

Carcinoma of the uterine cervix (i.e., cervical cancer) is a major health problem for women worldwide. Exploration of cancer pathological mechanism and treatment of cancer need to be solved urgently. Dysregulation of CpG-methylation is one of the main features of cancers and tumor suppressor genes can be silenced by hypermethylation[1,2]. Nan et al[3] found that methyl-CpG-binding domain (MBD) proteins could suppress transcription from methylated promoters, which suggested an attractive hypothesis for the silencing of methylated genes in cancer. Jones et al[4] demonstrated that the recruitment of histone deacetylase activity (HDAC) by members of the MBD protein family had linked their repressor activity to known transcriptional co-repressors. These transcriptional co-repressors are associated with the HDAC recruitment. So far repressor activity has been confirmed for MBD1, MBD2, and MBD2-related gene (MeCP2)[3]. Two of these transcriptional repressors, MBD2 and MeCP2, could recruit co-repressors and HDACs to methylated DNA[1,3]. These studies[1-4] claimed that MBD2 and MeCP2 were associated with cancer. It's worth paying attention to study the relation between these two genes and cervical cancer.

MiRNAs (19-22 nucleotides) are small non-coding RNAs and are often found to be differentially regulated in cancer. They are also known to play multifaceted roles in tumorigenesis[5-8]. MiRNAs are known to have both oncogenic and tumor suppression functions[7]. As we found, miR-221/222 could target MBD2[12]. This means miRNAs could regulate the stability of methyl-CpG-binding proteins. MBD2 have

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been linked to gene inactivation thanks to their ability to recruit corepressors and HDAC-activity to methylated gene promoters[1]. What's more, there is a large body of evidence that dysregulation of miRNAs is a hallmark of cancer[2]. It's interesting for us to explore whether upregulation of miR-221/222 in cervical cancer inhibits MBD2 and MeCP2.

In this study, we analyzed the mRNA expression of MeCP2 and MBD2 in cervical cancer tissues. We found that expression levels of MBD2 and MeCP2 were significantly reduced in cervical cancer samples as detected by quantitative Real Time PCR (qRT-PCR). Western blot analysis of MeCP2 in matched tumor-normal samples of patients with cervical cancer indicated a protein reduction in a significant percentage of patients. At the same time, we detected that miR-221 and miR-222, which targeted MBD2, were upregulated in cervical cancer samples. This suggests that the expression changes of MBD2 and MeCP2 were caused by miR-221/222.

To further elucidate the relations between miR-221/222 and MBD2, we used different cell lines, including C33A, HeLa, and CaSki. Surprisingly, we found that expression levels of MBD2 and MeCP2 were significantly lower in HeLa and CaSki than in C33A as detected by qRT-PCR. Western blot analysis of MeCP2 in HeLa and CaSki showed significantly lower expression at the protein level than in C33A. We suggested that the HeLa and CaSki cell lines were infected by human papillomavirus virus (HPV), which may induce the lower expression of MBD2 and MeCP2 in these two cell lines. These phenomena were consistent with those which we detected in tumor samples. Therefore, we were able to detect the relation between miR-221/222 and their targets MBD2 and MeCP2 in a rapid way using cell lines.

Knockdown of miR-221/222 resulted in the downregulation of miR-221/222 levels, which rescued the expression levels of MBD2 and MeCP2 in HeLa and CaSki. However, transfection of miR-221/222 in C33A could upregulate the expression levels of miR-221/222 and decrease the expression levels of MBD2 and MeCP2. Collectively, our results demonstrate that upregulated miR-221/222 are related to lower expression of MBD2 in patients with cervical cancer. However, the mechanism underlying miR-221/222 that regulates MBD2 and MeCP2 needed for further exploration.

Materials and Methods

Tumor Sample

Fresh tumors from patients with cervical carcinoma (n=6) were obtained between 2016 and 2017 at the Department of Gynecology, the Affiliated Hospital of Weifang Medical University at a molecular diagnostic laboratory after morphological dissection of the tumor by a pathologist. The 6 control samples were obtained from donors.

Western Blot

Western blotting was performed as previously described[9]. The protein was extracted from frozen tumors and the corresponding normal cervical tissue using Radio Immunoprecipitation Assay RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) and sonicated. Cells were harvested at 70-80% confluency in a cocktail lysis buffer. Protein lysates were cleared by centrifugation and the concentration was assessed using a bicinchoninic acid (BCA) kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) The protein was transferred on to nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), blocked with 5% nonfat milk for 1 h and then incubated overnight at 4°C with the following primary antibodies: anti-MeCP2 (1:200; Upstate Biotechnology, Inc.), Anti-actin (1:200; Abcam, Inc., Cambridge, MA, USA). The anti-actin antibody (1: 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to control for equal protein loading. Membranes were washed with 0.1% of Tris-Buffered Saline and Tween (TBST) for 3x10 min and incubated with secondary antibody (1:3000; ECL Rabbit or Mouse IgG, HRP Linked whole antibody) for 1 h at room temperature. Membranes were washed with TBST and detection was performed with an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

RNA Isolation And cDNA Preparation

The RNA was extracted from tumors samples (around 100-180 mg) using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The RNA was isolated from cells and from serum-free conditioned media using the miRNeasy mini or the miRNeasy serum plasma kit RNA isolation kit, respectively, according to the manufacturer's protocol. The RNA concentration was measured

using NanoDrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

A total of 1 ug of RNA from each sample was reversely transcribed using an oligo-d (T) primer and RNase H-MMLV reverse transcriptase according to the protocol of the manufacturer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cDNA was diluted to give a total volume of 200 μl, and 5 μl of this dilution was used for each PCR reaction. The quality of the cDNA was confirmed by the amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, cytosolic protein) and only samples with consistent and strong amplification were included in the final analyses. PCR was performed using the following amplification conditions: 5 min at 95°C, followed by 35 cycles of 10 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Relative quantification of target gene expression was evaluated using the comparative CT method. The PCR primer sequences[10] used for quantitative Real Time PCR were: Human MeCP2, forward primer: ACT CCC CAG AAT ACA CCT TGC TT, reverse primer: TGA GGC CCT GGA GGT CCT; Human MBD-2, forward primer: AGT GAA ATC AGA CCC ACA ACG AA, reverse primer: CAT CTG ATG CAC TAA GTC CTT GTA GC; Human GAPDH, forward primer: GAA GGT GAA GGT CGG AGT C, reverse primer: GAA GAT GGT GAT GGG ATT TC.

Cell Transfection And Cell Culture

C33A, HeLa, and CaSki cells were purchased from the National Infrastructure of Cell Line Resource (China). Cells used in this study were authenticated and checked for mycoplasma contamination. Locked nucleic acid oligonucleotide microRNA inhibitor (antagomiR) hsa-miR-221-3p and hsa-miR-222-3p and scrambled control miR (Exiqon) were transfected into HeLa and CaSki using Lipofectamine RNAimax (Life Technologies, Inc., Carlsbad, CA, USA). Similarly, miR-221 and miR-222 were transfected into C33A. All cells were trypsinized for 5 min using 0.25% trypsin (wt/vol), and replaced onto glass coverslips in fresh Dulbecco's Modified Eagle's Medium (DMEM): F12 medium (Life Technologies Inc., Carlsbad, CA, USA) containing 1 mM nonessential amino acids (Gibco; Rockville, MD, USA), 1 mM sodium pyruvate (Gibco; Rockville, MD, USA), and 10% fetal bovine serum (FBS; (HyClone, Inc., South-Logan, UT, USA). Cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to standard protocols. The cells were cultured after transfection for 5-6 h at 37°C in a humidified atmosphere of 5% CO_2.

Statistical Analysis

Data were expressed as mean ± SEM. Performing normal distribution test on multiple sets of data, the result of p-value > 0.1 represents that data obeys the normal distribution. Statistical differences were evaluated by Student's t-test and One-way ANOVA. Otherwise, non-parametric tests were used. Data were considered statistically significant when $p<0.05$. Data were considered statistically highly significant when $p<0.01$. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA).

Results

Expression of MBD2 And MeCP2, Targets of MiR-221/222, were Reduced in Cervical Cancer Tissues

We first demonstrated that the expression of MBD2 and MeCP2, i.e., the target of miR-221/222, was reduced in patients with cervical cancer (Figure 1). QRT-PCR was performed to analyze mRNA expressions of MBD2 and MeCP2 in normal samples and tumor samples. The data showed that the expressions of these two genes were decreased in patients with cervical cancer (Figure 1A). Furthermore, Western blot showed that the expression of MeCP2 at the protein level was reduced in cervical cancer tissues compared to normal tissues (Figure 1B). We wondered if the expression levels of miR-221/222 could be changed, causing the expression alteration of MBD2 and MeCP2. QPCR analysis of miR-221/222 showed that the expression of miR-221/222 was upregulated in patients with cervical cancer (Figure 1C).

Expression of MBD2 And MeCP2, Targets of miR-221/222, were Reduced in HeLa And CaSki Cell Lines Compared with C33A Cell Line

We detected the expression of miR-221/222, MBD2, and MeCP2 in different cell lines. Surprisingly, we detected a similar phenomenon in the cell lines relative to tissues (Figure 2). The mRNA expressions of MBD2 and MeCP2 were

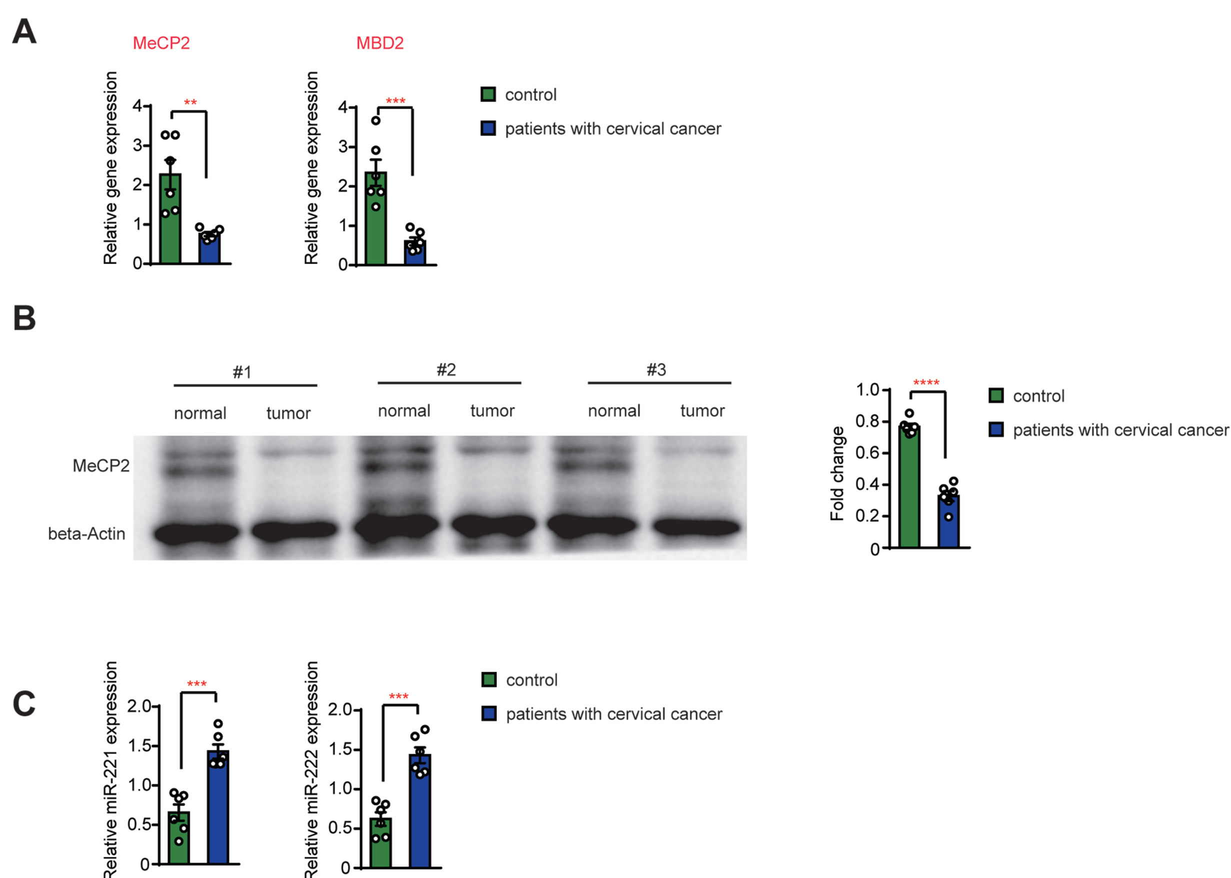

Figure 1. Expressions of MBD2 and MeCP2, targets of miR-221/222, were reduced in cervical cancer tissues. ***A,*** Expression of MBD2 and MeCP2 in a panel of cervical cancer tissues (n=6) and in corresponding normal cervical tissue specimens (n=6). Expression levels were standardized with GAPDH expression. Expression of MeCP2 (p=0.0025) and MBD2 (p=0.0005) was significantly lower in tumor samples than in controls. ***B,*** 30 mg of protein lysates from matched tumor (T) and normal (N) samples from patients with cervical cancer were analyzed for MeCP2 protein expression by Western blot analysis. The blot was then stained with anti-actin antibody to visualize equal protein loading. Most of the analyzed patients showed significantly reduced or no MeCP2 protein expression compared to normal cervical tissue, p<0.0001. ***C,*** Expression of miR-221 and miR-222 in the panel of cervical cancer tissues (n=6) and in corresponding normal cervical tissue specimens (n=6). Expression levels were standardized with GAPDH expression. Expression levels of miR-221 (p=0.0002) and miR-222 (p=0.0001) were significantly higher in tumor samples than in controls.

decreased in HeLa and CaSki compared to C33A (Figure 2A). The protein levels of MeCP2 were also lower in HeLa and CaSki than in C33A (Figure 2B). We suggested that HeLa and CaSki were infected with the human papillomavirus virus (HPV), which may reduce the expression of MBD2 and MeCP2 in these two cell lines.

Further, we measured the expression of miR-221/222 in these three cell lines from their culture media supernatant and cell body. The expression levels of miR-221 and miR-222 were significantly higher in the HeLa and CaSki than in the C33A cell line (Figure 2C). These phenomena were similar to what we detected in tumors. Therefore, we continued to detect the relation between miR-221/222 and their targets of MBD2, and MeCP2 using cell lines.

Knockdown of MiR-221/222 in HeLa Increased MBD2A And MeCP2 Expression

MBD2 and MeCP2 are the targets of miR-221 and miR-222[11,12]. The expression of MBD2 and MeCP2 was downregulated in HeLa, which may be caused by the increased expression of miR-221/222.

The knockdown of miR-221/222 in HeLa was performed using antagomiRs. Knockdown of miR-221/222 in HeLa cells did not have any impact on the cell proliferation (Figure 3A). MiR-

221/222 knockdown was confirmed by qRT-PCR (Figure 3A), and the expression of miR-221/222 was significantly reduced following a knockdown. The knockdown of miR-221/222 in HeLa cells rescued the mRNA expression levels of MeCP2 and MBD2 (Figure 3B). The protein levels of MeCP2 could be rescued in HeLa cells through the hsa-miR-221/222-3p transfection (Figure 3C). Together, these data indicated that miR-221/222 secreted in HeLa culture media is a key component to decrease the expressions of MBD2 and MeCP2.

Transfection of MiR-221/222 in C33A Reduced MBD2 And MeCP2 Expression

The expression levels of miR-221/222 were increased in C33A after the transfection of miR-221/222 (Figure 4A). Given miR-221/222 targeted

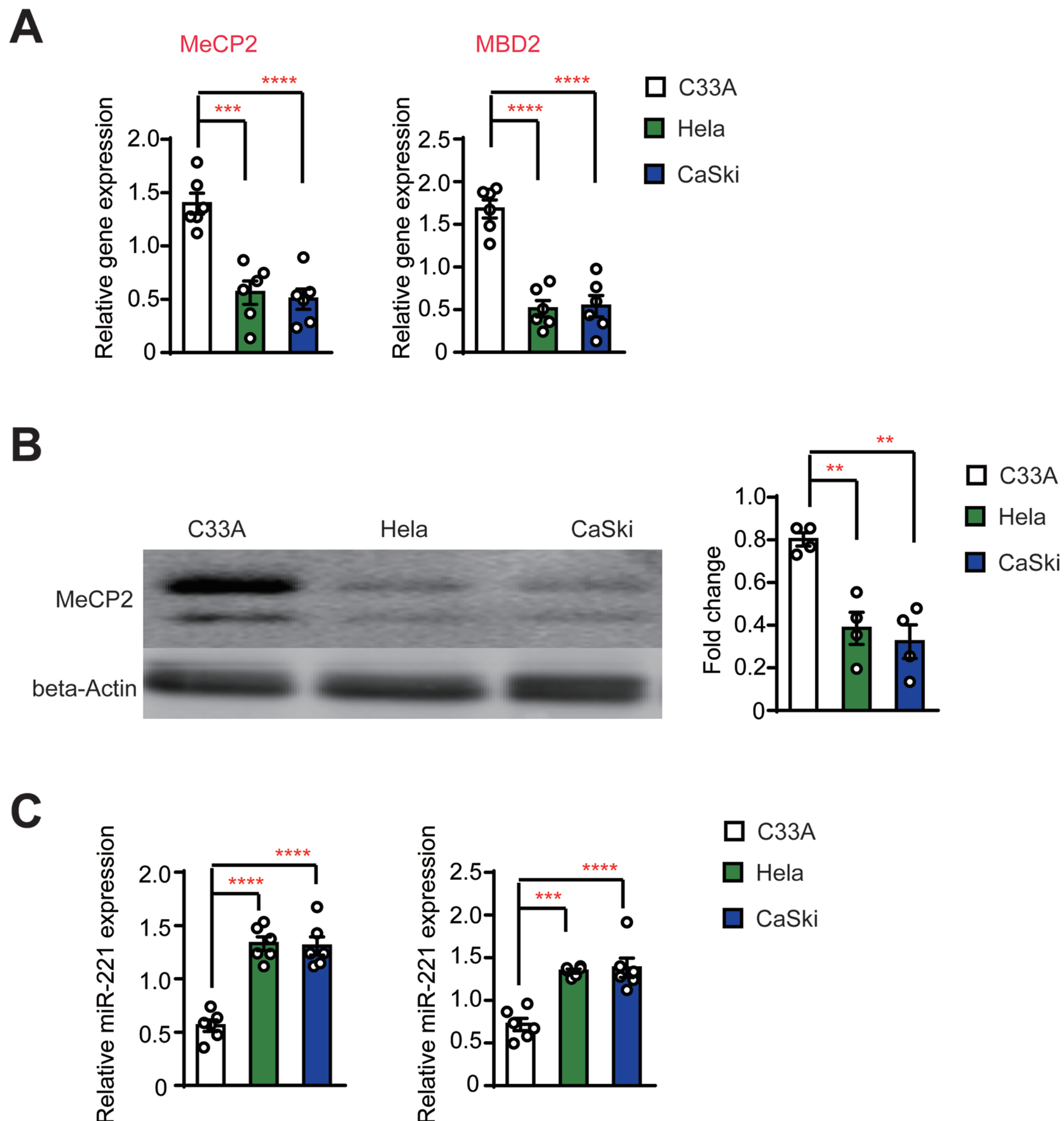

Figure 2. Expressions of MBD2 and MeCP2, targets of miR-221/222, were reduced in HeLa and CaSki cell lines compared with C33A cell line. ***A,*** Expression of MBD2 and MeCP2 in a panel of these different cell lines (n=6). Expression levels were standardized with GAPDH expression. Expression of MeCP2 (HeLa, p=0.0001, CaSki, p<0.0001) and MBD2 (HeLa, p<0.0001, CaSki, p<0.0001) was significantly reduced in HeLa and CaSki than in C33A. ***B,*** 30 ug of protein lysates from three cell lines were analyzed for MeCP2 protein expression by Western blot analysis. The analysis showed that the expression level of MeCP2 in HeLa and CaSki was significantly lower compared to that of C33A, p<0.0001. ***C,*** Expression of miR-221 and miR-222 in the panel of these three cell lines (n=6/each group). Expression levels were standardized with GAPDH expression. Expressions of miR-221 (HeLa, p<0.0001, CaSki, p<0.0001) and miR-222 (HeLa, p=0.0001, CaSki, p<0.0001) were significantly higher in HeLa and CaSki than in C33A.

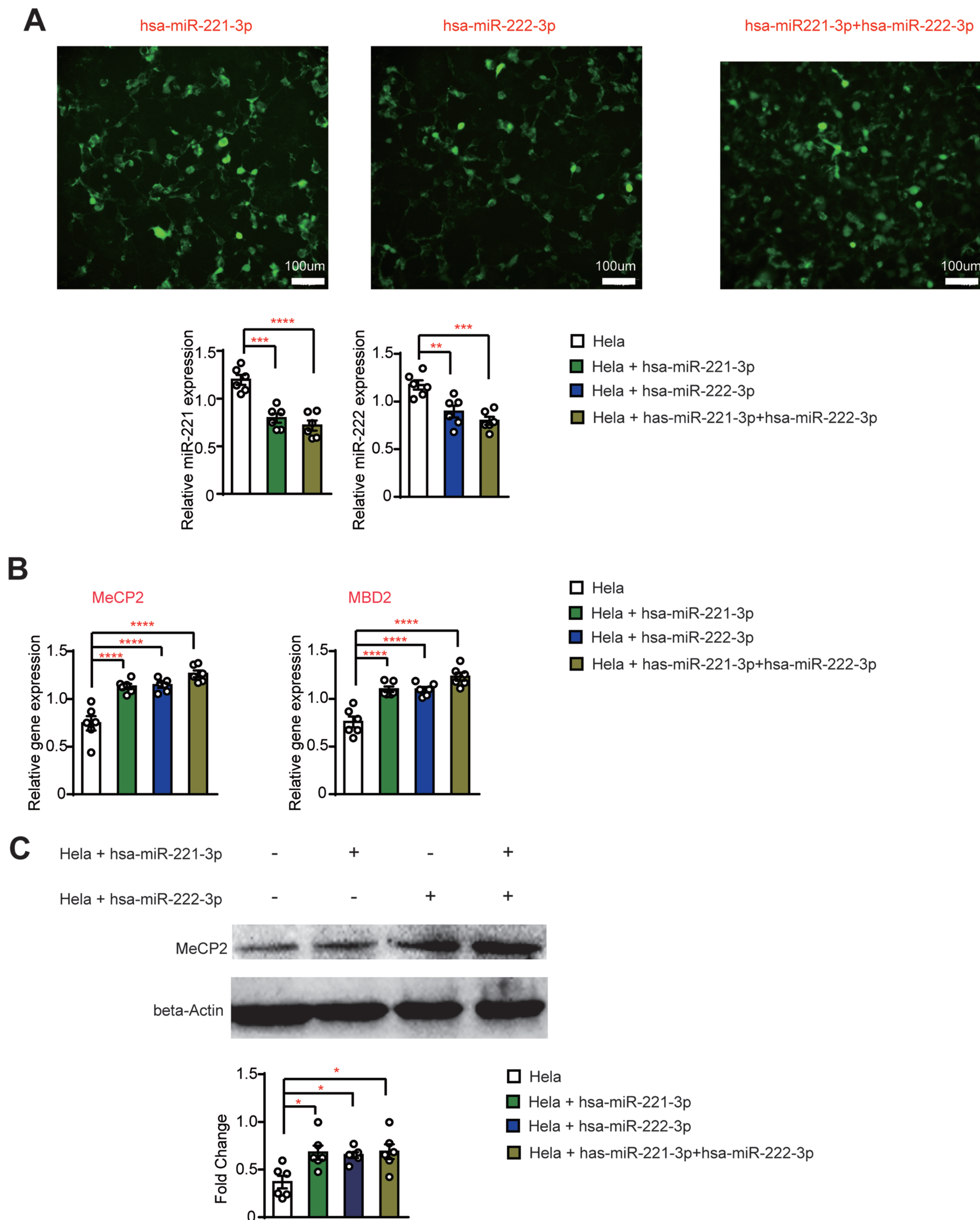

Figure 3. Knockdown of miR221/222 in HeLa increased MBD2 and MeCP2 expression. ***A,*** Expression of miR-221 and miR-222 were detected after transfection with has-miR-221-3p and has-miR-222-3p in HeLa (n=6). Expression levels were standardized with GAPDH expression. Expressions of miR-221 (has-miR-221-3p, p=0.0002, has-miR-221-3p + has-miR-222-3p, p<0.0001) and miR-222 (has-miR-221-3p, p=0.0048, has-miR-221-3p + has-miR-222-3p, p=0.0003) were significantly lower in knockdown groups than in control group. ***B,*** Expression of MBD2 and MeCP2 in a panel of these different cells (n=6). Expression levels were standardized with GAPDH expression. Expressions of MeCP2 (****, p<0.0001) and MBD2 (****, p<0.0001) were significantly increased in knockdown groups than in control group. ***C,*** 30 ug of protein lysates from these four groups (n=6) were analyzed for MeCP2 expression by Western blot analysis. The analysis showed that the expression level of MeCP2 in knockdown groups was significantly lower than that of control group, p<0.05.

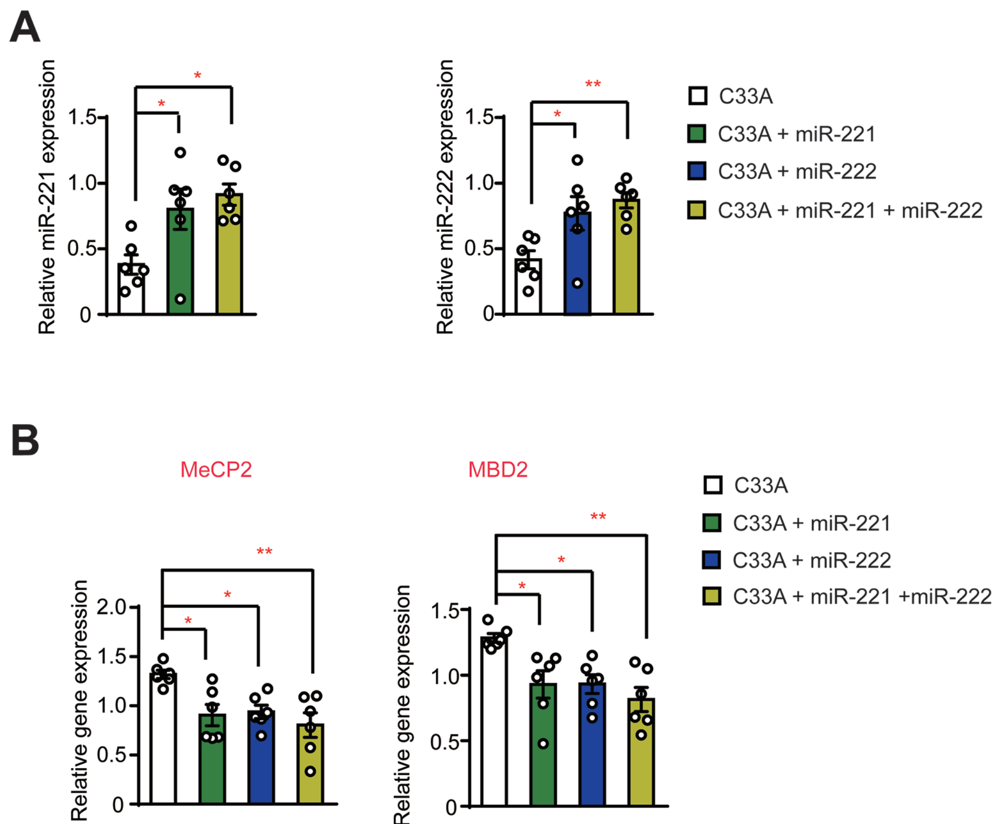

Figure 4. Transfection of miR-221/222 in C33A reduced MBD2, and MeCP2 expression. ***A,*** Expression of miR-221 and miR-222 were detected after transfection with has-miR-221-3p and has-miR-222-3p in HeLa (n=6). Expression levels were standardized with GAPDH expression. Expressions of miR-221 (miR-221, p=0.0457, miR-221+222, p=0.0106) and miR-222 (miR-222, p=0.0435, miR-221+miR-222, p=0.0088) were significantly higher in transfection groups than in control group. ***B,*** Expression of MBD2 and MeCP2 in a panel of these different groups (n=6). Expression levels were standardized with GAPDH expression. Expressions of MeCP2 (miR-221, p=0.0275, miR-222, p=0.0482, miR-221+222, p=0.0044) and MBD2 (miR-221, p=0.0318, miR-222, p=0.0336, miR-221+222, p=0.003) were significantly lower in transfection groups than in control group.

on MBD2 and MeCP2, we detected the expression of MBD2 and MeCP2 in these cells. The expression of these two genes was significantly lower in miR-221/222-transfected cells than in control C33A (Figure 4B). Together, these data indicated that elevation of miR-221/222 is sufficient to decrease the expression of MBD2 and MeCP2.

Discussion

Our study provides strong evidence that miR-221/222 could promote cervical cancer via repressing the expressions of MBD2 and MeCP2. A lot of studies[13] demonstrated that aberrant CpG-methylation participated in the pathogenesis of human cancers. This is the first time we demonstrate that exosomal upregulated miR-221/222 associates with methylation related genes in cervical cancer.

We used qPCR to analyze the expression of MBD2 and MeCP2 in normal human organs, as well as in human cervical tumors. MeCP2 was considered to be an attractive candidate gene to mediate methylation-associated gene silencing in cancer[1]. In our work, we provide strong evidence that MeCP2 mRNA expression is greatly reduced in cervical cancers. This finding strongly argues against a role of MeCP2 in the silencing of methylated genes in cancer. Reduced MeCP2 expression in cervical cancer suggested that MeCP2 function was disturbed. Ng et al[14] found that MBD2 was another MBD family member, which has been demonstrated to be associated with HDAC recruitment and subsequent transcriptional repression[1]. Our study showed that the expression of MBD2 gene is significantly reduced in cervical cancer. Similar findings have previously been reported for a small group of colorectal and gastric carcinomas[15]. This is

even more likely to be considered as the high degree of correlation between the loss of MBD2/MeCP2 expression and cervical cancer.

Another interesting point is the upregulated expressions of miR-221/222 among cervical cancer. MiRNAs function to regulate the expression of target genes by either inducing mRNA degradation or inhibiting mRNA translation through imperfect base-pairing with the 3-UTR of target mRNAs[16-18]. Among many miRNAs already identified as regulators of neoplastic transformation, invasion, and metastasis, miR-221/222 have emerged as key miRNAs deregulated in many cancers[11]. Our analysis shows that the expression of miR-221 and miR-222, which targets MBD2, is upregulated in cervical cancer samples. We also found that miR-221/222 could target MBD2[11,12] which means that miRNAs can regulate the stability of methyl-CpG-binding proteins. Given that the function of miR-221 targeted MBD2[11,12], miR-221/222 was supposed to inhibit MBD2 and MeCP2. Further, we found that the expression levels of MBD2 and MeCP2 were significantly lower in HeLa and CaSki than in C33A as detected by qRT-PCR. Western blot analysis of MeCP2 of HeLa and CaSki suggested significantly lower expression than in C33A. We suggested that the HeLa and CaSki cell lines were infected with the human papillomavirus virus (HPV), which may be reduced by the lower expression of MBD2 and MeCP2 on these two cell lines. These phenomena were similar to those which we detected on tumors. The knockdown of miR-221/222 resulted in the downregulation of miR-221/222 levels, which rescued the expression levels of MBD2 and MeCP2 in the HeLa and CaSki cells. In contrast, transfection of miR-221/222 in C33A could upregulate the expression levels of miR-221/222 and decrease the expression levels of MBD2 and MeCP2. Collectively, our results demonstrate that upregulated miR-221/222 is related to the expression of MBD2 and MeCP2 loss in patients with cervical cancer.

Conclusions

Our study shows strong evidence that upregulated exosomal miR-221/222 promotes cervical cancer via suppressing the expression of MBD2 and MeCP2. However, the underlying mechanism by which miR-221/222 regulates MBD2 and MeCP2 needs further exploration.

Conflict of interest

The authors declare no conflicts of interest.

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