Ecotropic viral integration site 1 regulates the progression of acute myeloid leukemia via MS4A3-mediated TGFβ/EMT signaling pathway

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Abstract. Acute myeloid leukemia (AML) is a type of malignant tumor that is caused by malignant clone hematopoietic stem cells. The ecotropic viral integration site 1 (Evi1) is a zinc finger transcription factor, which is highly expressed in AML, and its expression level has been associated with poor prognosis of AML. Previous studies have indicated that Evi1 may regulate cell proliferation, differentiation and apoptosis by inhibiting the membrane-spanning-4-domains subfamily-A member-3 (MS4A3) gene in AML. The aim of the present study was to investigate the role of Evi1 in the progression of AML. The results revealed that Evi1 was overexpressed in leukemia cells compared with normal T lymphocytes. MicroRNAs (miR)-133 and -431 that target Evi1 were investigated, and it was observed that there was a low expression of miR-431 in AML. The transfection of miR-431 was able to decrease the promoter methylation levels of the Evi1 gene in AML cells. The transfection of miR-431 also suppressed the migration and invasion of AML cells. The present study revealed that the transfection of miR-431 mimic was able to downregulate MS4A3 expression in AML cells. Furthermore, the expression levels of transforming growth factor β (TGFβ) and epithelial-to-mesenchymal transition (EMT) markers fibronectin, α-smooth muscle actin, and vimentin were downregulated following the transfection of miR-431 in AML cells. The overexpression of MS4A3 was also able to suppress miR-431-mediated inhibition of the expression of TGFβ and EMT markers in AML cells. The addition of TGFβ inhibited the downregulation of EMT markers by transfection of miR-431 in AML cells. The transfection of miR-431 suppressed the migration and invasion of AML cells, which was also abolished by the addition of TGFβ. In conclusion, the results of the present study indicated that Evi1 may be a potential molecular target of leukemia therapy via MS4A3-mediated TGFβ/EMT signaling pathway.

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Introduction

Acute myeloid leukemia (AML) is a type of bone marrow leukocyte (white leukocyte) caused by abnormal proliferation of leukemia (1). AML is characterized by the rapid proliferation of abnormal cells in the bone marrow that affects the generation of normal blood cells (2). Clinical analyses indicate that AML may be induced by a variety of factors, including virus, ionizing radiation, chemical substances and genetic factors (3,4). Fever, infirmity and other complications are the characteristics of patients with AML (4). A previous study analyzed therapy and efficacy post-remission of AML (5). Mechanism analyses have suggested that cellular molecular signaling pathways are involved in the progression of AML in patients (6,7). Therefore, understanding the potential mechanisms of AML is essential for the development of therapy for AML.

The ecotropic viral integration site 1 (Evi1) is a zinc finger transcription factor, which is highly expressed in numerous human tumors (8,9). A report indicated that the overexpression of the EVI1 oncogene is associated typically with aggressive myeloid leukemia (10). Jazaeri *et al* (11) reported that Evi1 and EVI1s (Delta324) may be regarded as potential therapeutic targets in ovarian cancer. In addition, the transcription factor Evi1 may regulate cellular proliferation, differentiation and apoptosis, and its overexpression may contribute to an aggressive course of disease via transcriptional repression of membrane-spanning-4-domains subfamily-A member-3 (MS4A3) in AML and other malignancies (12). Furthermore, the results indicated that Evi1 is a transcriptional suppressor of

the microRNA (miRNA)-143 gene, and miRNA-143 mediates its action via the K-Ras axis in human colon cancer (13). Additionally, Evi1 targets DeltaNp63 and upregulates the cyclin-dependent kinase inhibitor p21, independent of p53, which may delay cell cycle progression and cell proliferation in colon cancer cells (14). However, the potential underlying mechanism mediated by Evi1 in AML requires further investigation.

In the present study, Evi1 as a target of miR-431 was analyzed and a low expression of miR-431 was detected in AML cells. It was previously reported by the present authors that Evi1 is a potential molecular target for leukemia therapy via the TGFβ-induced EMT signaling pathway. Therefore, the present study focused on the molecular mechanism of AML regulation by Evi1 via miR-431, which further mediate the TGFβ-induced EMT signaling pathway in leukemia. It was indicated that miR-431 may suppress the invasion and proliferation of AML cells via the Evi1-mediated MS4A3/TGFβ/EMT signaling pathway.

Materials and methods

Cell culture. HuT-78, HTL-90 (http://www.cnki.com.cn/Article/CJFDTotal-HNLG6S1.025.htm) and normal T lymphocyte cells (15) were obtained from Department of Anatomy, Southern Medical University (Guangzhou, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5 μg/m blasticidin and supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were cultured in a 37˚C humidified atmosphere of 5% CO_2.

Cell proliferation assay. All miR-431 mimics were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.), including pmiR-431 pvector (control). The proliferation of pvector or pmiR-431-transfected HuT-78 cells was detected using Cell Counting Kit-8 (CCK-8) kit according to the manufacturer's protocols. Briefly, HuT-78 cells were cultured in 48-well plates at the density of $1x10^4$ cells/well and then cultured for 24 h. Finally, 10 μl CCK-8 solution was added to each well and incubated for 2 h at 37˚C. The results were measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 570 nm.

Methylation assay. gDNA was extracted using phenol-chloroform. A total of 2 μg DNA in HuT-78 cells was treated with bisulfite using the EpiTect Bisulfite kit (Qiagen, Inc., Valencia, CA, USA), and polymerase chain reaction (PCR) was performed using PCR cloning kit (Zero Blunt™ PCR Cloning Kit, Invitrogen™; Thermo Sisher Scientific, Inc.) according to the manufacturer's protocol. Primer sequences were as follows: Forwards, 5'-AGGTTTTAGAGTAGGATTGGAAATGT-3' and reverse, 5'-ACCCCCTCTCCCAAAACTA-3'. The PCR conditions were set as following: An initial denaturation at 95˚C for 60 sec, followed by 45 cycles of 95˚C for 15 sec, 58.5˚C for 60 sec, 72˚C for 1 min. Pyrosequencing was conducted according to the manufacturer's protocols of the PyroMark PCR kit (Qiagen, Inc.).

Reverse transcription-quantitative (RT-q)PCR. HuT-78, HTL-90 and normal T lymphocyte cells were cultured, and total RNA was isolated from HuT-78, HTL-90 and normal T lymphocytes using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) and transcribed into cDNA using Super Script VILO cDNA Synthesis kit (Life Technologies; Thermo Fisher Scientific, Inc.). The forward and reverse primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The sequences of the primers were as follows: Evi1 forward, 5'-CACGGATCCGAGGCGCCATGTCAGAAC-3' and reverse, 5'-CTGACTCGAGGGATTAGGGCTTCCTCTTGG-3'; β-actin forward, 5'-CGGAGTCAACGGATTTGGTC-3' and reverse, 5'-AGCCTTCTCCATGGTCGTGA-3'. The changes in relative mRNA expression were calculated using the $2^{-\Delta\Delta Cq}$ method (16). The qPCR thermocycling conditions were as follows: 95˚C for 5 min, then 35 cycles of 95˚C for 20 sec, 56˚C for 20 sec and 72˚C for 20 sec, and a final extension at 72˚C for 5 min. The results were expressed as the n-fold way compared with the housekeeping gene (β-actin).

Boyden chamber migration and invasion assays. Serum-free HuT-78 cells were seeded ($1x10^5$ cells/well) in 500 μl DMEM medium. For the invasion assay, HuT-78 cells were suspended at a density of $1x10^5$ in 500 μl serum-free DMEM. The cells were seeded in the upper Matrigel invasion chamber (BD Biosciences, Franklin Lakes, NY, USA) according to the manufacturer's protocols. For the migration assay, the cells were subjected to a control insert (BD Biosciences) instead of a Matrigel invasion chamber. The number of tumor cells that migrated and invaded was counted in ≥3 randomly stained fields for every membrane under a light microscope at x40 magnification.

Transfection of miR-431. HuT-78 cells ($1x10^6$) were transfected with 100 pmol pmiR-431 (Applied Biosystems; Thermo Fisher Scientific, Inc.) with pvector as control (Applied Biosystems; Thermo Fisher Scientific, Inc.) by using a Cell Line Nucleofector kit L (Lonza Group, Ltd., Basel, Switzerland). After 72 h transfection, cells were used for further experiments. HuT-78 cells transfected with miR-431 and then were treated by TGFβ (2 mg/ml) for 12 h at 37˚C for further analysis.

Construction of lentivirus for MS4A3 overexpression. S4A3 was cloned into a lentivirus plasmid using the Lentivirus vector system (System Biosciences, LLC, Pallo Alto, CA, USA) with vector as control. Plasmids were named pMS4A3 and pvector (control), respectively. All DNA sequences were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). pvector or pMS4A3 plasmid was transfected into HuT-78 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells that were transfected with pvector or pMS4A3 were used for further analysis.

Western blotting. HuT-78 cells were collected and lysed in radioimmunoprecipitation assay buffer (M-PER reagent for the cells and T-PER reagent for the tissues, Thermo Fisher Scientific, Inc.) followed by homogenization at 4˚C for 10 min. Protein concentration was measured by a BCA protein assay kit (Thermo Scientific, Inc.). A total of (20 μg) protein was electrophoresed on 12.5% polyacrylamide gradient gels and then transferred to nitrocellulose membranes. Rabbit anti-human antibodies used in the immunoblotting assays were: Evi1

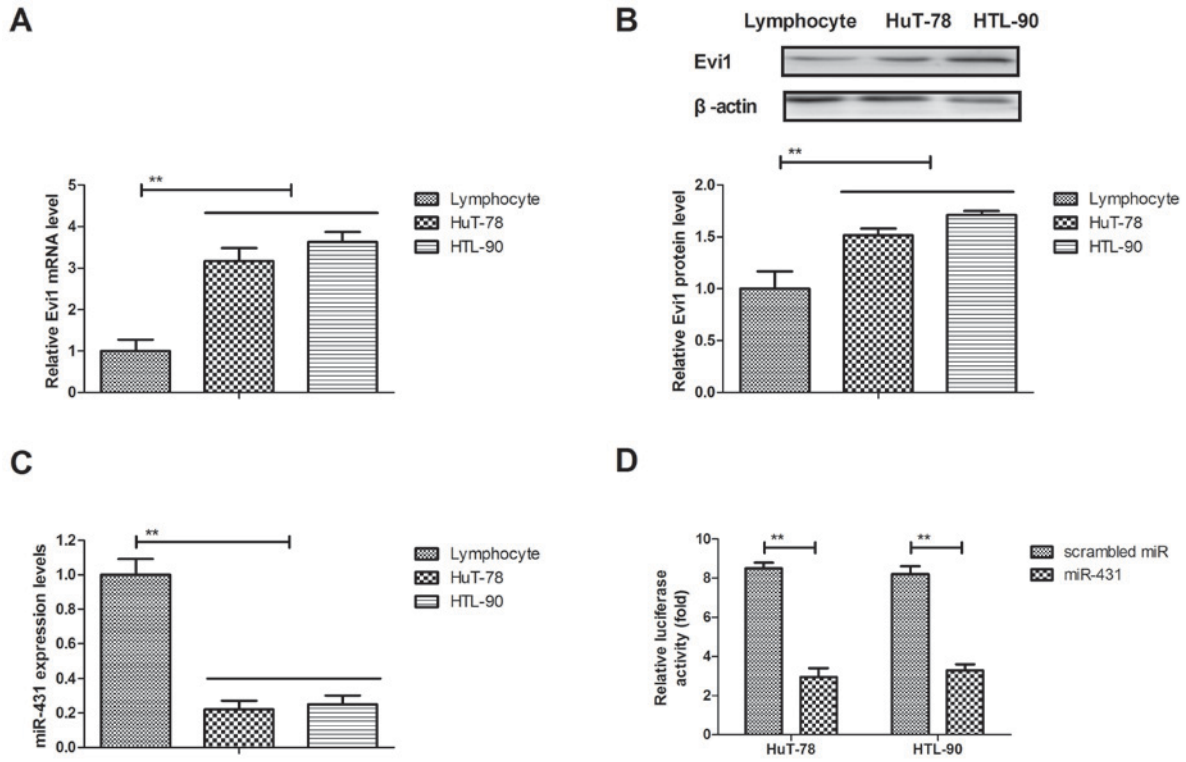

Figure 1. Analysis of Evi1 and miR-431 expression in acute myeloid leukemia cells. (A) mRNA and (B) protein expression levels of Evi1 were upregulated in HuT-78 and HTL-90 compared with normal T lymphocytes. (C) miR-431 expression levels were downregulated in HuT-78 and HTL-90 cells compared with normal T lymphocytes. (D) Evi1 is the target gene of miR-431 in HuT-78 and HTL-90 as determined by dual-luciferase reporter assay with scrambled miR as control. **P<0.01. Evi1, ecotropic viral integration site 1; miR, microRNA.

(1:500; catalog no. ab28457; Abcam, Cambridge, UK), MS4A3 (1:500; catalog no. ab173761; Abcam), TGFβ (1:200; catalog no. ab92486; Abcam), fibronectin (FIB; 1:500; catalog no. ab6328; Abcam), α-smooth muscle actin (α-SMA; 1:500; catalog no. ab7817; Abcam), vimentin (VIM; 1:500; catalog no. ab92547; Abcam) and β-actin (1:500; catalog no. ab8226; Abcam). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibodies (cat. no. 166-2408-MSDS, Bio-Rad Laboratories, Inc.) were used at a 1:5,000 dilution and detected using a Western Blotting Luminol reagent (Roche Diagnostics, Basel, Switzerland). Densitometric quantification of the protein expression was performed using Quantity-One software (version 1.02; Bio-Rad Laboratories, Inc.).

Luciferase reporter assay. Evi1 3'untranslated region (UTR) construct in a PIS2 vector (Invitrogen; Thermo Fisher Scientific, Inc.) was used for luciferase reporter assay as described previously (17). Site-directed mutagenesis of the miR-431 binding site in Evi1 3'UTR was analyzed using PCR amplification with the construction of a mutated 3'-UTR as control. HuT-78 and HTL-90 cells were transfected with Evi1 3'UTR (cytomegalovirus-driven β-gal reporter system, BD Biosciences) and miR-431 mimics or scrambled miR control using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C with untransfected cells as controls. The cell pellets were analyzed using the luciferase reporter assay according to the manufacturer's protocols (Promega Corporation, Madison, WI, USA). The β-gal reporter system was used as an internal control. The miR-431 mimics used in the present study were annealed by the miR-431 mimic 5'-UAAUUAGUGUCAUGUAGUUAGG-3' and 5'-AGACUACAUGAAGCUACCUAAU-3', and the control group was annealed by 5'-GUCACGGAUCGCGGC ACAUTT-3' and 5'-AAUUGCCACGCGUUGAAGATT-3' (MD Bio, Inc. Gaithersburg, MD, USA). The luciferase activities of the miR-431-transfected HuT-78 and HTL-90 cell lines were quantified and subjected to statistical analyses. Results were normalized against the cells transfected with scrambled miR control.

Statistical analysis. All data were expressed as the mean ± standard deviation of triplicate dependent experiments and analyzed by using paired Student t-tests or one-way analysis of variance followed by Tukey's post hoc test. All data were analyzed using SPSS Statistics (version 19.0; SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was also used. *P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of Evi1 and miR-431 expression in AML cells. The expression levels of Evi1 and miR-431 were analyzed in AML cells. The mRNA and protein expression levels of Evi1 were upregulated in HuT-78 and HTL-90 cells compared with (Fig. 1A and B). The results demonstrated that miR-431 expression levels were significantly downregulated in HuT-78 and HTL-90 compared with normal T lymphocytes (Fig. 1C). The dual-luciferase reporter assay indicated that Evi1 may

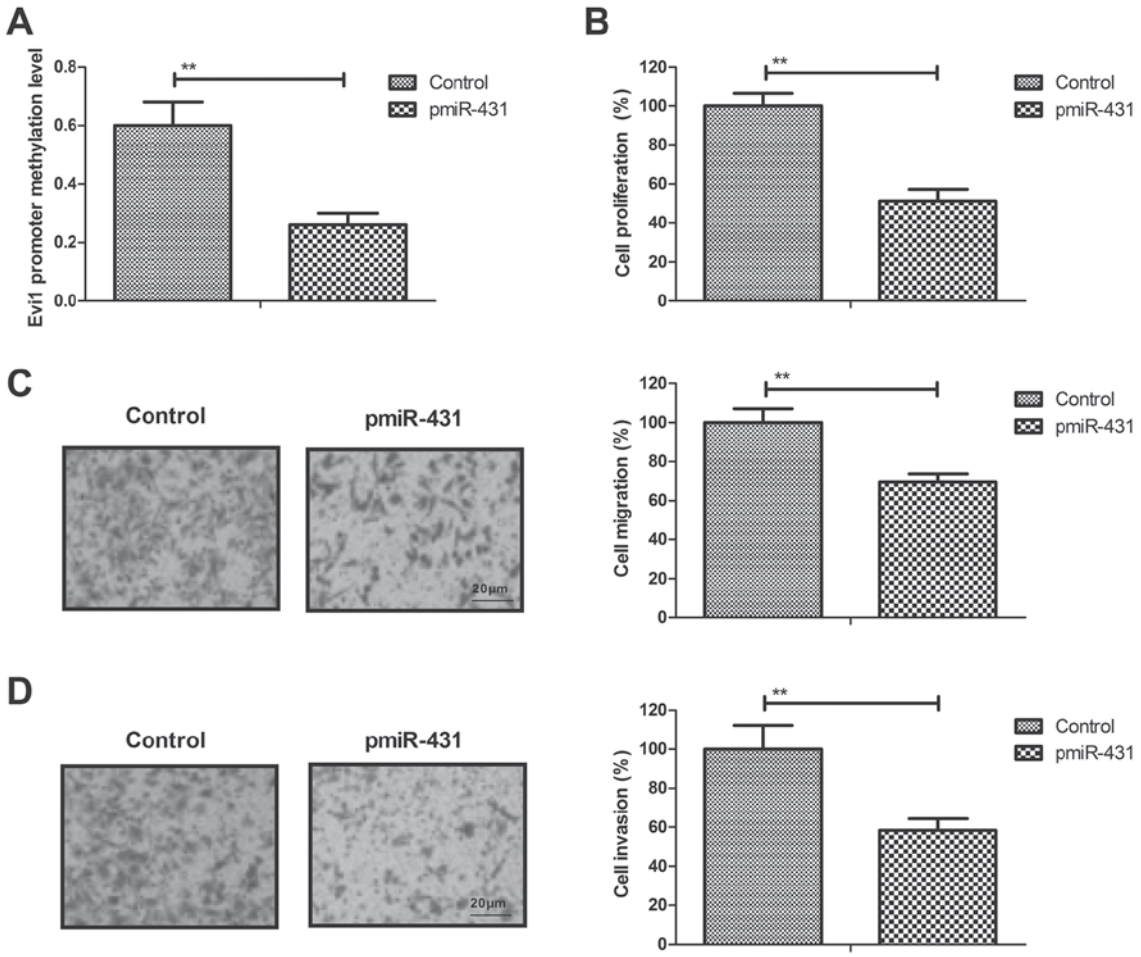

Figure 2. Transfection of miR-431 suppresses the proliferation and invasion of acute myeloid leukemia cells. (A) The transfection of miR-431 mimic decreased the methylation levels of Evi1 gene promoter in HuT-78 cells. (B) The transfection of miR-431 inhibited the proliferation of HuT-78 cells. The transfection of miR-431 significantly suppressed the (C) migration and (D) invasion of HuT-78 cells. Magnification x40. **P<0.01. Evi1, ecotropic viral integration site 1; miR, microRNA.

be the target gene of miR-431 in HuT-78 and HTL-90 cells (Fig. 1D). These results suggested that Evi1 may be upregulated and miR-431 expression may be downregulated in HuT-78 and HTL-90 cells.

Transfection of miR-431 inhibits the proliferation and invasion of AML cells. HuT-78 cell line is a typical and is the most representative AML cells (18). Therefore the role of miR-431 in AML cells was analyzed in the present study. In order to investigate the role of miR-431 in HuT-78 cells, the effects of miR-431 on proliferation and invasion were analyzed in HuT-78 cells. The transfection of miR-431 decreased the promoter methylation levels of the Evi1 gene in HuT-78 cells compared with the control (Fig. 2A). The results revealed that the transfection of miR-431 increased miR-431 expression (data not shown) and inhibited the proliferation of HuT-78 cells compared with the control (Fig. 2B). Furthermore, it was observed that the transfection of miR-431 suppressed the migration and invasion of HuT-78 cells (Fig. 2C and D). These results suggested that the transfection of miR-431 may inhibit the proliferation and invasion of AML cells.

Transfection of miR-431 inhibits MS4A3 and TGFβ/EMT signaling pathway in AML cells. To identify the potential mechanism mediated by miR-431, the effects of miR-431 on the TGFβ/EMT signaling pathway in HuT-78 cells were investigated in the present study. As presented in Fig. 3A, the transfection of miR-431 suppressed the expression levels of MS4A3 in HuT-78 cells. It was demonstrated that the transfection of miR-431 decreased the expression levels of TGFβ and EMT markers (FIB, α-SMA and VIM) in HuT-78 cells (Fig. 3B). These results indicated that the transfection of miR-431 may inhibit MS4A3 and TGFβ/EMT signaling pathways in AML cells.

Overexpression of MS4A3 inhibits miR-431 mediated-inhibition on the expression levels of TGFβ and EMT markers. To investigate the potential molecular mechanism of miR-431 in the TGFβ/EMT signaling pathway, the effects of MS4A3 on the expression levels of TGFβ and EMT markers in HuT-78 cells were analyzed in the present study. It was demonstrated that the overexpression of MS4A3 inhibited the expression levels of TGFβ and EMT markers in AML cells (Fig. 4A). The transfection of miR-431 mimic suppressed the migration and invasion of AML cells, and this effect was also reversed by MS4A3 overexpression in HuT-78 cells (Fig. 4B and C). These results suggested that the overexpression of MS4A3 inhibited miR-431 mediated-inhibition on the expression levels of TGFβ and EMT markers.

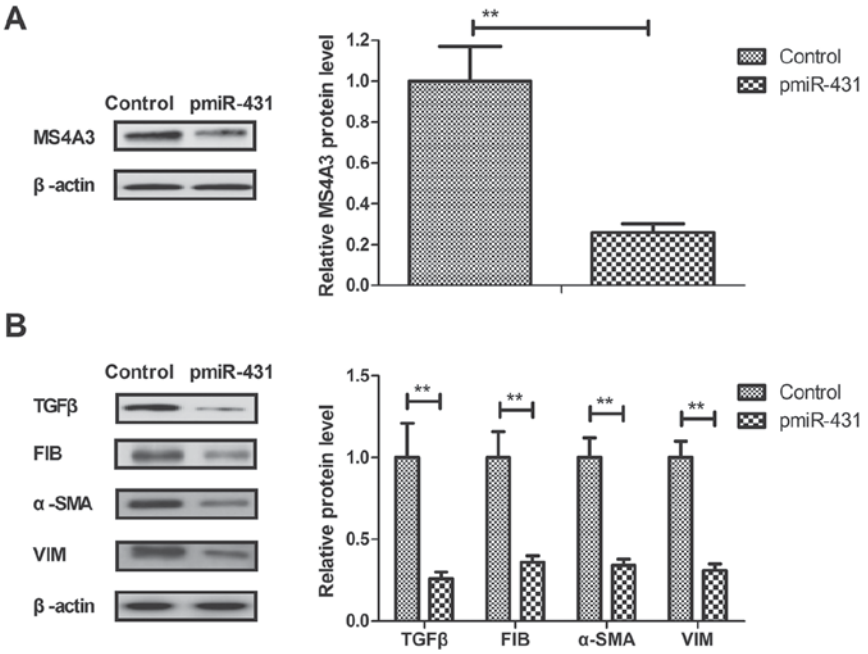

Figure 3. Transfection of miR-431 inhibits MS4A3 and TGFβ and epithelial-to-mesenchymal transition signaling pathway in acute myeloid leukemia cells. (A) The transfection of miR-431 suppressed MS4A3 expression levels in HuT-78 cells. (B) The transfection of miR-431 decreased the expression levels of TGFβ and EMT markers in HuT-78 cells. **P<0.01. α-SMA, α-smooth muscle actin; FIB, fibronectin; miR, microRNA; MS4A3, membrane-spanning-4-domains subfamily-A member-3; transforming growth factor β; VIM, vimentin.

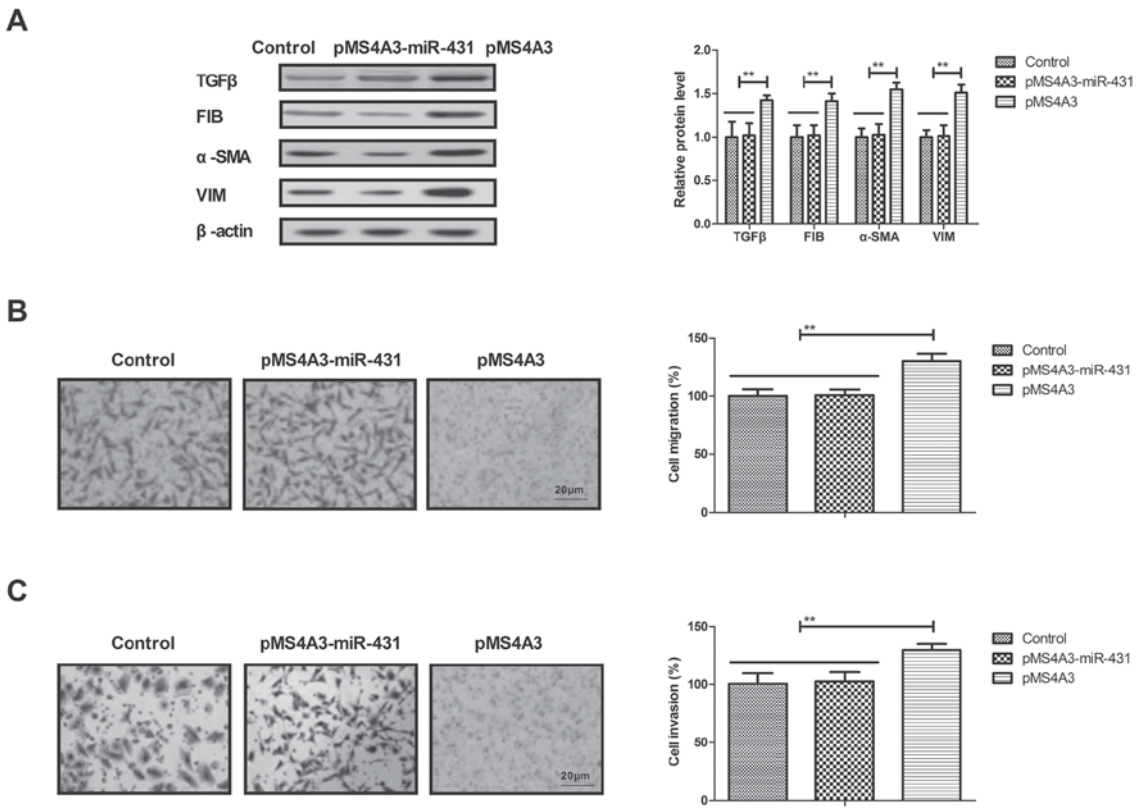

Figure 4. Overexpression of MS4A3 inhibits miR-431-mediated decrease in TGFβ and EMT expression levels. (A) The overexpression of MS4A3 repressed the inhibition of TGFβ and EMT expression that is mediated by miR-431 in AML cells. The transfection of miR-431 suppressed the (B) migration and (C) invasion of AML cells. Magnification x40. **P<0.01. AML, acute myeloid leukemia; α-SMA, α-smooth muscle actin; FIB, fibronectin; miR, microRNA; MS4A3, membrane-spanning-4-domainssubfamily-A member-3; transforming growth factor β; TGFβ, transforming growth factor β; VIM, vimentin.

Evi1 regulates the progression of AML via MS4A3-mediated TGFβ/EMT signaling pathway. The TGFβ/EMT signaling pathway serves an important role in the progression of AML (19,20). Therefore, the role of Evi1 on MS4A3-mediated

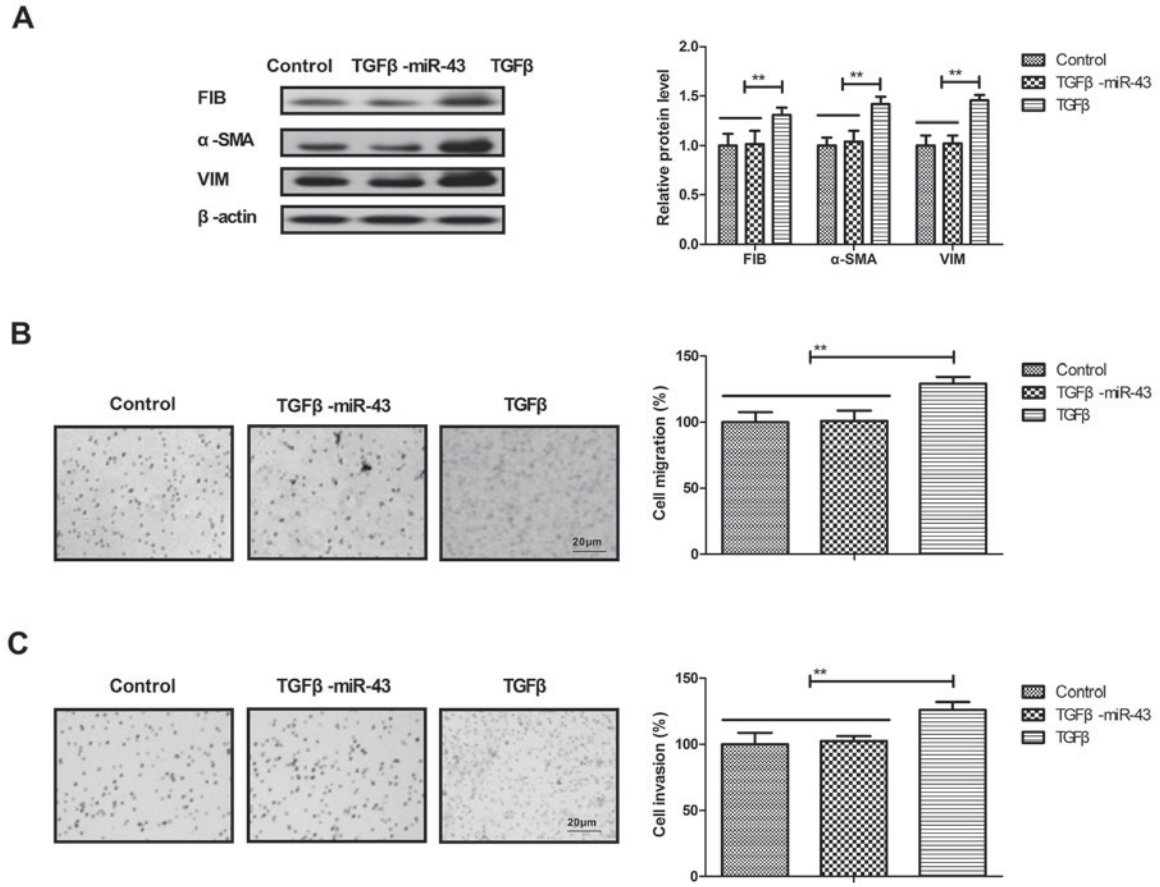

Figure 5. Ecotropic viral integration site 1 regulates the progression of AML via the MS4A3-mediated TGFβ/EMT signaling pathway. (A) The addition of TGFβ eliminated the downregulation of EMT markers that is mediated by miR-431 in AML cells. The addition of TGFβ inhibited MS4A3-induced (B) migration and (C) invasion of AML cells. Magnification x40. **P<0.01. AML, acute myeloid leukemia; α-SMA, α-smooth muscle actin; EMT, epithelial-to-mesenchymal transition; FIB, fibronectin; miR, microRNA; MS4A3, membrane-spanning-4-domains subfamily-A member-3; TGFβ, transforming growth factor β; VIM, vimentin.

TGFβ/EMT signaling pathway in HuT-78 cells was analyzed in the present study. It was demonstrated that the addition of TGFβ eliminated the downregulation of EMT markers that is mediated by miR-431 mimics in AML cells (Fig. 5A). The addition of TGFβ promoted the migration and invasion of AML cells compared with the control and reversed the inhibition on migration and invasion of AML cells that is mediated by miR-431 mimic (Fig. 5B and C). These results suggested that Evi1 may regulate the progression of AML via MS4A3-mediated TGFβ/EMT signaling.

Discussion

The overexpression of the Evi1 oncogene gene is associated with typically aggressive myeloid leukemia (21,22). In the present study, a putative target of Evi1 in the progression of AML was analyzed. A previous study reported that Evi1 overexpression was predominantly detected within subtypes of pediatric AML in distinct subtypes of pediatric AML (23). The results of the present study indicated that Evi1 was upregulated and miR-431 expression was downregulated in HuT-78 and HTL-90 cells compared with normal T lymphocytes. The findings also indicated that the transfection of miR-431 inhibited the proliferation and invasion of AML cells, which further suppressed the MS4A3/TGFβ/EMT signaling pathway in AML cells. Notably, the results of the present study indicated that Evi1 may regulate the proliferation and invasion of AML via MS4A3-mediated TGFβ/EMT signaling.

The Evi1 gene may serve as an alternative minimal residual disease marker in AML on the basis of increasing expression levels of MDS1-EVI/EVI 29, 36 and 93 days prior to hematologic manifestation, particularly in samples where other specific markers are lacking (24). Previous studies have reported that high expression of Evi1 may predict the outcome of younger adult patients (<22 years old) with AML, which was associated with distinct cytogenetic abnormalities (23,25). In the present study, it was observed that the inhibition of Evi1 expression mediated by miR-431 transfection significantly inhibited the proliferation and invasion of AML cells. A previous study reported that EVI1 may bind to and down-regulate Serpin family B member 2, which is involved in the Janus kinase-signal transducers and activators of transcription signaling pathway, suggesting that Evi1 is a target gene in the treatment of AML (26). The results of the present study reported that miR-431 may regulate MS4A3-mediated TGFβ and EMT signaling pathway in AML cells.

MS4A3 is a member of a family of four-transmembrane proteins, which acts as a cell surface signaling molecule and an intracellular adapter protein (27). Heller *et al* (12) indicated that Evi1 may promote tumor growth via transcriptional

repression of MS4A3. The results of the present study confirmed previous results and reported that Evi1 regulated MS4A3 via regulation of miR-431 levels. Pan *et al* (28) analyzed the association between the downregulation of miR-431 expression and clinicopathological characteristics in hepatocellular carcinoma tissues. In the present study, the results revealed that miR-431 transfection inhibited the invasion of AML cells, which was abolished by the overexpression of MS4A3.

The TGFβ/EMT signaling pathway serves a crucial role in mediating tumor suppressive effects in human cutaneous melanoma (20). TGFβ-mediated formation of retinoblastoma protein-E2F complexes has been demonstrated to be involved in the proliferation and invasion of human myeloid leukemia cells (29). The results of the present study indicated that the overexpression of MS4A3 inhibited the expression TGFβ and EMT markers, which is mediated by miR-431 mimic in AML cells. Additionally, the transfection of miR-431 mimic suppressed the migration and invasion of AML cells, and this effect was also reversed by MS4A3 overexpression in HuT-78 cells. Notably, the addition of TGFβ abolished the downregulation of EMT markers, which is mediated by transfection of miR-431 mimic. This finding suggests that Evi1 may regulate the progression of AML via the miR-431/EVI1/MS4A3-mediated TGFβ/EMT signaling pathway.

In conclusion, the silencing of Evi1 by miR-431 transfection exerted inhibitory effects on MS4A3-mediated TGFβ/EMT signaling pathway in AML cells. The findings of the present study demonstrated that the oncogenic agent miR-431 may contribute to the inhibition of Evi1-positive AML cells. In addition, the present study revealed that the targeting of Evi1 by miR-431 transfection reversed the downregulation of EMT markers (FIB, α-SMA and VIM) in HuT-78 cells, which attenuated the proliferation and invasion of AML cells. These findings suggested that Evi1 may be a novel therapeutic agent for the treatment of AML.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MJ performed the experiments and wrote the paper. XZ contributes to the data analysis and WH is the designer for this study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

No applicable.

Competing interests

The authors declare that they have no competing interests.

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