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Long Noncoding RNA XIST Regulates miR-137–EZH2 Axis to Promote Tumor Metastasis in Colorectal Cancer

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We aimed to investigate the significant role of long noncoding RNA X inactive specific transcript (XIST) in regulating tumor metastasis in colorectal cancer (CRC), as well as its possible mechanism. Expression of lncRNA XIST in CRC tissues and CRC cells was detected. CRC cells were transfected with pc-XIST, blank control si-XIST, or si-control, and then the effects of lncRNA XIST on CRC cell migration and invasion were investigated, along with the interaction between lncRNA XIST and miR-137. lncRNA XIST was upregulated in CRC tissues. Compared with HT29 cells that had low metastatic potential, XIST was markedly more highly expressed in LoVo cells that had a higher metastatic potential. Overexpression of XIST promoted the migratory and invasive potential of HT29 cells, while knockdown of XIST inhibited the migratory and invasive potential of LoVo cells. Moreover, epithelial–mesenchymal transition (EMT) markers, including E-cadherin, N-cadherin, and vimentin, exhibited corresponding expression changes. In addition, miR-137 was inhibited by XIST, and inhibition of miR-137 could reverse the effects of knockdown of XIST on the migratory and invasive potential of LoVo cells. Furthermore, enhancer of zeste homolog 2 (EZH2) was confirmed as a target of miR-137. Our data reveal that lncRNA XIST may promote tumor metastasis in CRC possibly through regulating the miR-137–EZH2 axis. lncRNA XIST may serve as a prognostic indicator for CRC progression.

Key words: Colorectal cancer (CRC); miR-137; Enhancer of zeste homolog 2 (EZH2); Metastasis; Long noncoding RNA (lncRNA) X inactive specific transcript (XIST); Epithelial–mesenchymal transition (EMT)

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer mortality in the US[1]. The 5-year survival rate of CRC patients is approximately 65%[2]. Poor prognosis is widely found in CRC patients with advanced stages[3]. Like many other cancer types, numerous oncogenes and tumor suppressors have been found to be involved in the progression of CRC[4]. Therefore, the growing incidence and poor outcome of CRC drive us to unravel the pathological mechanisms underlying CRC progression, as well as to discover the effective diagnostic and prognostic biomarkers for this disease.

Accumulating evidence has confirmed that long noncoding RNAs (lncRNAs) are widely involved in multiple cellular processes, such as cell growth, differentiation, and apoptosis[5,6]. Moreover, lncRNAs are frequently aberrantly expressed in several cancers to mediate tumor progression and metastasis, and functional lncRNAs have been used for cancer diagnosis and prognosis[7–9]. Several lncRNAs have been identified to be associated with tumor progression and poor prognosis in CRC, including metastasis associated with lung adenocarcinoma transcript 1 (MALAT1)[10], homeobox transcript antisense intergenic RNA (HOTAIR)[11], taurine-upregulated gene 1 (TUG1)[12], and zinc finger antisense 1 (ZFAS1)[13]. Recently, lncRNA X inactive specific transcript (XIST) has been found upregulated and plays a key role in regulating the malignant behaviors of tumor cells in many cancers, including gastric cancer[14], non-small cell lung cancer[15], nasopharyngeal carcinoma[16], and bladder cancer[17]. Notably, lncRNA XIST is recently found to play an oncogenic role in human CRC through targeting miR-132-3p[18]. However, there is a lack of adequate knowledge on the regulatory mechanism of lncRNA XIST in CRC progression and metastasis.

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In this study, we detected the expression of lncRNA XIST in CRC tissues and CRC cells with different metastatic potential. Then we explored the effects of lncRNA XIST on CRC cell migration and invasion in vitro. Last, the interaction between lncRNA XIST and miR-137 along with a possible mechanism was further elucidated. Our findings will provide a new insight for our better understanding of CRC pathogenesis and treatment.

MATERIALS AND METHODS

Tissue Sampling

This study was approved by the ethic committee of our hospital. Twenty CRC patients who underwent surgical resections at our hospital were recruited. Primary CRC and matched adjacent normal colonic epithelial tissues were isolated from these patients during surgery and then stored at −80°C after being snap frozen in liquid nitrogen. All patients enrolled in this study provided informed consent.

Cell Culture

Three human CRC cell lines (LoVo, HT29, and SW620) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and cultured in a 37°C humidified incubator of 5% CO_2.

Cell Transfection

Overexpression of lncRNA XIST and miR-137 was achieved by transfection with pcDNA-XIST and miR-137 mimic (GenePharma, Shanghai, P.R. China) using Lipofectamine 2000 (Invitrogen). With the same method, knockdown of lncRNA XIST, enhancer of zeste homolog 2 (EZH2), and miR-137 was completed using siRNAs specially targeting lncRNA XIST and EZH2 (si-XIST and si-EZH2) and miR-137 inhibitor (GenePharma). After transfection, cells were incubated for another 48 h and then harvested for further assays.

MTT Assay

Cells were seeded in 96-well plates at a density of 2,000 cells per well. According to the manufacturer's instructions, 20 µl of MTT solution (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added into each well. After incubation at 37°C for 4 h, 0.2 ml of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used to treat cells for 30 min. Absorbance at 495 nm was then recorded.

Transwell Assay

The invasive and migratory potential of cells in different groups was evaluated using Transwell chambers with 8-µm pores (Corning, Corning, NY, USA). The chambers used for invasion assay were precoated with Matrigel matrix (BD Biosciences, Franklin Lake, NJ, USA). At 24 h after transfection, cells (3.0 10^5) were added to each upper chamber containing serum-free medium, and 500 µl of medium containing 10% FBS was added to the lower chamber. After incubation for another 48 h, noninvaded or nonmigratory cells were removed from the upper chamber with a cotton swab. The invaded or migrated cells on the lower chamber were fixed in methanol and stained with 0.1% crystal violet (Merck, Darmstadt, Germany). Cells in six random fields of each chamber at 100 magnification were counted. Each experiment was conducted in triplicate.

Luciferase Reporter Assay

The *EZH2* 3 -UTR or lncRNA XIST containing miR-137 seed binding sites (*EZH2* 3 -UTR-wt or lncRNA XIST-wt) was subcloned into a psiCHECK-2 vector (Promega, Madison, WI, USA) immediately downstream to the luciferase gene sequence. A psiCHECK-2 construct containing *EZH2* 3 -UTR or lncRNA XIST with a mutant seed sequence of miR-137 (*EZH2* 3 -UTR-mut or lncRNA XIST-mut) was also synthesized. All constructs were confirmed by DNA sequencing. HT29 cells were seeded in 96-well plates and then cotransfected with 100 ng of constructs with miR-137 mimic or mimic control. At 48 h after transfection, luciferase activity of each group was detected using a Dual-Luciferase Reporter Assay system (Promega). *Renilla* luciferase activity was used as an internal control for normalization.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from CRC tissues and cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. Reverse transcription was then completed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was then performed to detect the expression of lncRNA XIST and EZH2 using TaqMan Universal Master Mix II (Applied Biosystems) on the Bio-Rad CFX96 qPCR system. For determining the expression of miR-137, the All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) was performed following the manufacturer's instructions. GAPDH was used as the reference for normalizing the expression of lncRNA XIST and EZH2, while U6 small nuclear RNA was used as the internal control for miR-137. The fold changes were determined with the $2^{-\ CT}$ method. The primers used in this study were shown as follows: lncRNA XIST: 5 -CAGACGTGTGCTCTTC-3 (forward) and 5 -CGATCTGTAAGTCCACCA-3 (reverse); EZH2: 5 -CCCTGACCTCTGTCTTACTTGTGGA-3 (forward)

and 5 -ACGTCAGATGGTGCCAGCAATA-3 (reverse); GAPDH: 5 -CCACATCGCTCAGACACCAT-3 (forward) and 5 -ACCAGGCGCCCAATACG-3 (reverse); miR-137: 5 -TATTGCTTAAGAATACGCGTAG-3 (forward) and 5 -AACTCCAGCAGGACCATGTGAT-3 (reverse); U6: 5 -CTCGCTTCGGCAGCACATATACT-3 (forward) and 5 -CGCTTCACGAATTTGCGTGT-3 (reverse).

Western Blot Assay

Different transfected cells were lysed in RIPA buffer (Beyotime, Jiangsu, P.R. China) with 1% PMSF (Thermo Fisher Scientific Inc., Waltham, MA, USA) for extracting total protein. Equal amounts of protein extracts were then loaded onto an SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were then probed with 1:1,000 diluted primary antibodies to E-cadherin, N-cadherin, vimentin, EZH2, and GAPDH (Abcam, Cambridge, UK) at 4°C overnight and subsequently incubated with HRP-conjugated secondary antibody (1:5,000). The protein blots were visualized using ECL Substrates (Millipore), and GAPDH was applied as an endogenous protein for normalization.

Statistical Analysis

All experiments were repeated three times independently, and the obtained data were expressed as mean±SD. The statistically significant difference was estimated by Student's *t*-test (for two groups) or one-way ANOVA (for three groups or more) by means of SPSS 13.0 software (SPSS, Chicago, IL, USA). There was statistical significance with a value of $p<0.05$.

RESULTS

lncRNA XIST Is Upregulated in CRC Tissues and Cells

As shown in Figure 1A, lncRNA XIST was significantly upregulated in CRC tissues relative to that in adjacent normal colonic epithelial tissues. Furthermore, the expression of lncRNA XIST in CRC cells with different metastatic potential was detected. The results showed that lncRNA XIST expression was highest in LoVo cells that had the highest metastatic potential among the three cell lines, while it was lowest in HT29 cells that had the lowest metastatic potential (Fig. 1B). These data indicate that lncRNA XIST is associated with the development and progression of CRC, especially tumor metastasis.

lncRNA XIST Promotes CRC Cell Migration and Invasion Possibly via Regulating EMT

To investigate the role of lncRNA XIST in CRC, HT29 cells were transfected with pc-XIST and blank control, while LoVo cells were transfected with si-XIST and si-control. At 48 h after transfection, lncRNA XIST expression was significantly upregulated in pc-XIST-transfected HT29 cells and markedly downregulated in si-XIST-transfected LoVo cells compared to those in their corresponding control transfected cells ($p<0.001$) (Fig. 2A). MTT assay showed that HT29 cell viability was significantly increased after overexpression of lncRNA XIST at 48 and 72 h after transfection, whereas LoVo cell viability was markedly decreased after knockdown of lncRNA XIST ($p<0.05$) (Fig. 2B). Transwell assay showed that overexpression of lncRNA XIST obviously promoted the migratory and invasive potential of HT29 cells, while knockdown of lncRNA XIST inhibited the migratory and invasive potential of LoVo cells ($p<0.05$) (Fig. 2C and D). Moreover, epithelial–mesenchymal transition (EMT) markers, including E-cadherin, N-cadherin, and vimentin, exhibited corresponding expression changes ($p<0.05$) (Fig. 2E): E-cadherin was significantly downregulated in pc-XIST-transfected HT29 cells, while N-cadherin and vimentin were markedly upregulated; opposite expression changes of these EMT markers were found in si-XIST-transfected LoVo cells. These data indicate that lncRNA XIST may promote CRC cell migration and invasion via affecting EMT.

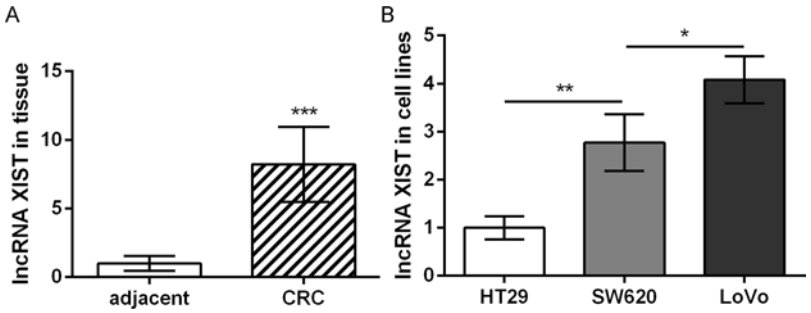

Figure 1. The expression of long noncoding RNA (lncRNA) X inactive specific transcript (XIST) in colorectal cancer (CRC) tissues (A) and cells with different metastatic potential (B). Data were expressed as mean±SD. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to corresponding control.

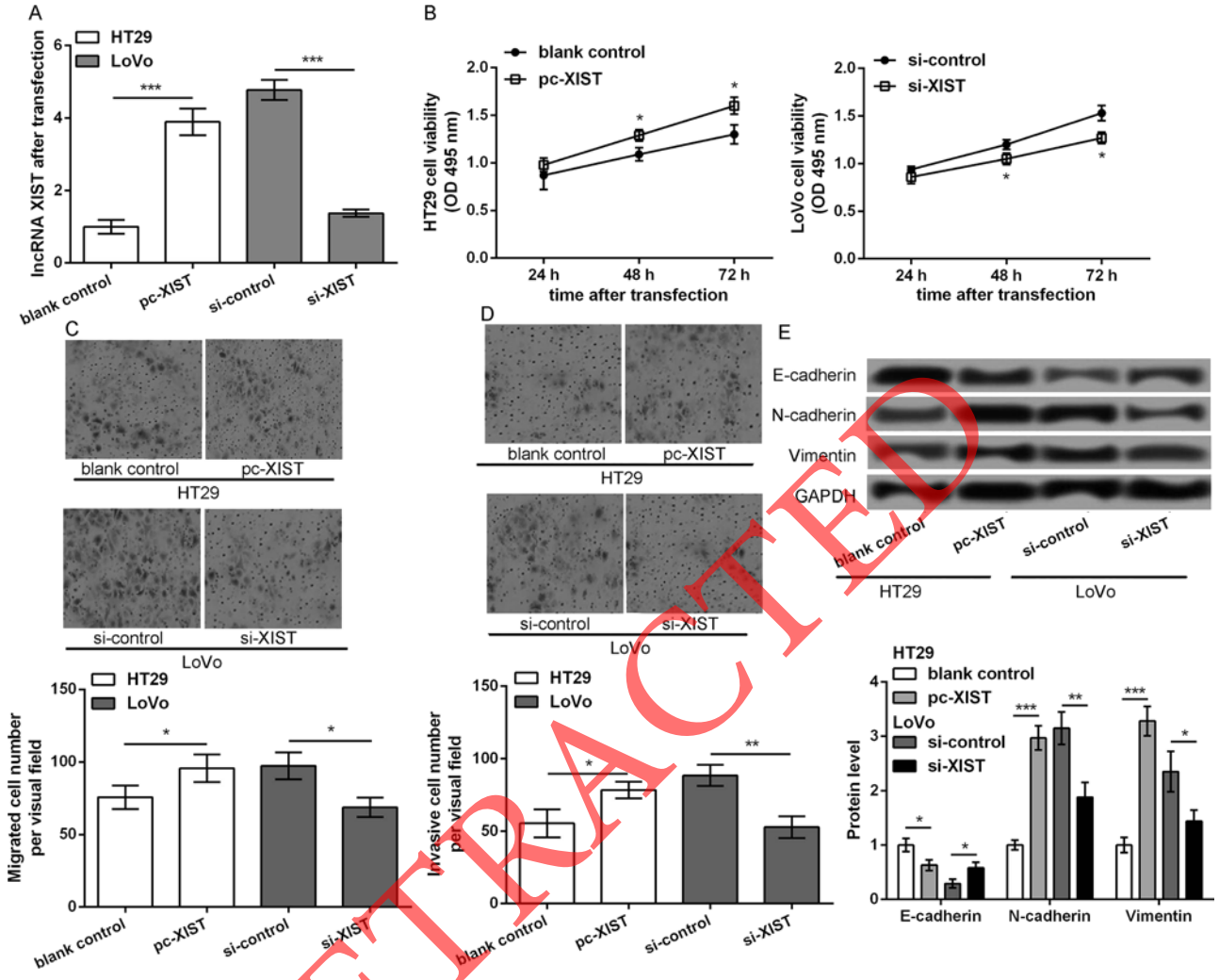

Figure 2. lncRNA XIST promotes CRC cell migration and invasion possible via regulating epithelial–mesenchymal transition (EMT). HT29 cells were transfected with pc-XIST and blank control, while LoVo cells were transfected with si-XIST and si-control. (A) lncRNA XIST expression in the different transfected cells after 48 h of transfection. (B) MTT assay showed cell viability in the different transfected groups. Transwell assays showed (C) cell migration and (D) invasion in the different transfected groups, respectively. (E) The expression of EMT markers, including E-cadherin, N-cadherin, and vimentin, in the different transfected cells. Data were expressed as mean ± SD. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to corresponding control.

miR-137 Is Inhibited by lncRNA XIST and Suppresses CRC Cell Migration and Invasion

To explore the regulatory mechanism of lncRNA XIST, the relationship between lncRNA XIST and miR-137 was investigated. As shown in Figure 3A, the results indicated that there were two binding sites between lncRNA XIST and miR-137, and the relative luciferase activity was significantly lower in transfection with lncRNA XIST-mut + miR-137 mimic ($p<0.05$). miR-137 expression in CRC tissues was significantly lower than that in adjacent normal colonic epithelial tissues ($p<0.001$). Moreover, miR-137 expression in HT29 cells was markedly higher than SW620 and LoVo cells ($p<0.05$) (Fig. 3B and C). LoVo cells were transfected with si-XIST and/or miR-137 inhibitor. qRT-PCR showed that miR-137 expression was significantly upregulated in si-XIST-transfected cells compared with si-control ($p<0.05$) (Fig. 3D). However, compared with the si-XIST + blank control-transfected group, miR-137 expression was significantly lower after cells were cotransfected with si-XIST and miR-137 inhibitor ($p<0.001$) (Fig. 3D). Transwell assay showed that knockdown of lncRNA XIST inhibited LoVo cell migration and invasion, which was significantly reversed by miR-137 inhibitor ($p<0.01$) (Fig. 3E and F). Western blot was also performed to detect the expression of EMT markers. Results showed that the miR-137 inhibitor markedly reversed the increased E-cadherin expression

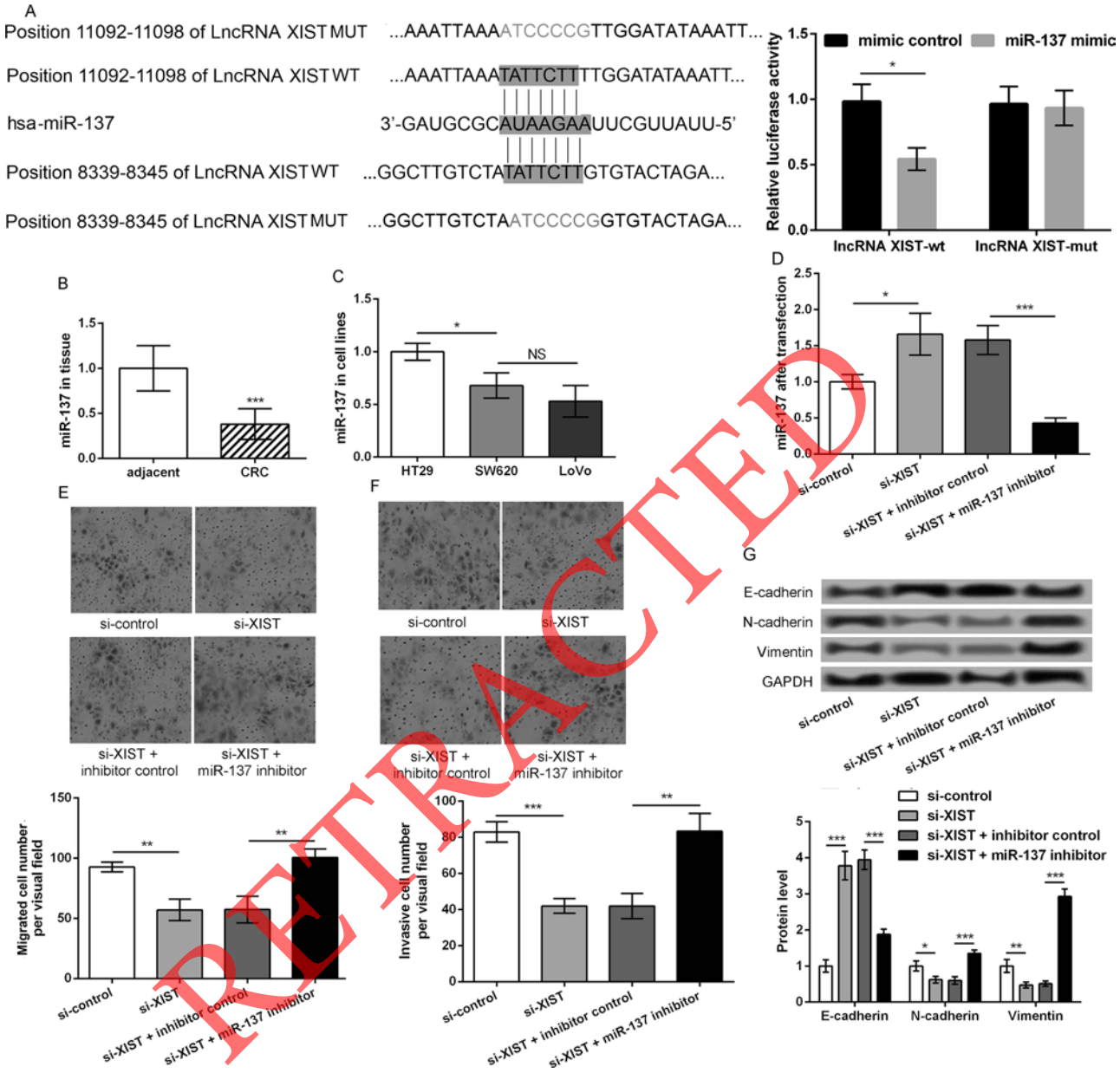

Figure 3. miR-137 is inhibited by lncRNA XIST and suppresses CRC cell migration and invasion. (A) The relationship between lncRNA XIST and miR-137 with sequence and luciferase assay. (B) The expression of miR-137 in CRC tissues and adjacent normal colonic epithelial tissues. (C) The expression of miR-137 in CRC cells with different metastatic potential. (D) LoVo cells were transfected with si-XIST and/or miR-137 inhibitor. Quantitative real-time PCR (qRT-PCR) showed miR-137 expression in the different transfected cells. Transwell assays showed (E) cell migration and (F) invasion in the different transfected groups, respectively. (G) The expression of EMT markers, including E-cadherin, N-cadherin, and vimentin, in the different transfected cells. Data were expressed as mean ± SD. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to corresponding control.

and decreased expression of N-cadherin and vimentin caused by knockdown of lncRNA XIST. In other words, compared with the si-XIST and inhibitor control group, E-cadherin was significantly downregulated in the si-XIST and miR-137 inhibitor group, while N-cadherin and vimentin were markedly upregulated ($p<0.01$) (Fig. 3G). These data indicated that miR-137 may be a regulator to mediate the role of XIST in regulating CRC cell migration and invasion.

miR-137 Targets and Inhibits EZH2

The potential target of miR-137 was further explored to better understand the regulatory mechanism of lncRNA XIST. Using the TargetScan tool, *EZH2* was

predicted to be a potential target of miR-137, and the binding sequence between miR-137 and *EZH2* mRNA 3 -UTR is shown in Figure 4A (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs=ENST00000478654.1&taxid=9606&members=miR-137&showcnc=0&shownc=0&subset=1). Results of the luciferase report assay showed that the miR-137 mimic could obviously inhibit the luciferase activity of *EZH2* 3 -UTR-wt, but not *EZH2* 3 -UTR-mut ($p<0.05$) (Fig. 4B). In addition, the mRNA and protein expression levels of EZH2 in CRC tissues were significantly higher than that in adjacent normal colonic epithelial tissues ($p<0.001$) (Fig. 4C). Moreover, EZH2 expression in LoVo cells was highest, followed by SW620 and HT29 cells ($p<0.05$) (Fig. 4D). Therefore, LoVo cells were transfected with miR-137 mimic and/or pc-XIST. At 48 h after transfection, miR-137 expression was significantly upregulated in the miR-137 mimic group compared with that in the mimic control group, and markedly downregulated in the miR-137 mimic + pc-XIST group relative to that in the miR-137 mimic + blank group ($p<0.001$) (Fig. 4E). The expression of EMT markers in the different transfected groups was detected by Western blot. Results showed the miR-137 mimic markedly increased the expression of E-cadherin and obviously decreased the expression of EZH2, N-cadherin, and vimentin, which were significantly reversed after cotransfection with miR-137 mimic and pc-XIST ($p<0.05$) (Fig. 4F). These data indicated that EZH2 was a target of miR-137 to mediate CRC cell migration and invasion.

DISCUSSION

CRC is a frequently lethal malignant tumor with heterogeneous outcomes, and no treatment options are available for patients with metastatic CRC[19]. Identification of key biomarkers for CRC diagnosis and prognosis is urgent. Given the emerging roles of lncRNAs in cancer diagnosis and prognosis[20], we aimed to explore the key role of lncRNA XIST in tumor metastasis in CRC. Results of this study showed that lncRNA XIST was highly expressed in CRC tissues and cells with higher metastatic potential. The lncRNA XIST may regulate the migratory and invasive potential of CRC cells via modulating EMT. In addition, miR-137 was inhibited by lncRNA XIST, and inhibition of miR-137 could reverse the effects of knockdown of lncRNA XIST on the migratory and invasive potential of LoVo cells. Furthermore, EZH2 was confirmed as a target of miR-137. Consistent with a previous study reported by Song et al.[18], lncRNA XIST was also found to be upregulated in CRC tissues. Moreover, lncRNA XIST was highly expressed in CRC

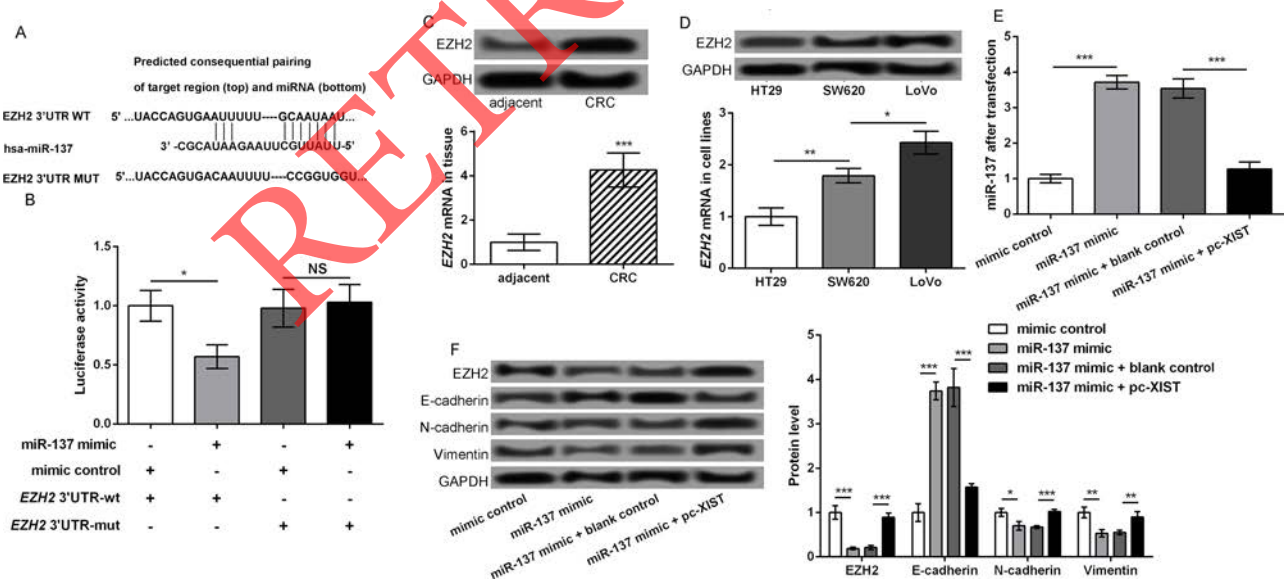

Figure 4. miR-137 targets and inhibits enhancer of zeste homolog 2 (EZH2). (A) The TargetScan tool showed the binding sequence between miR-137 and EZH2 mRNA 3 -UTR. (B) Luciferase report assay showed the miR-137 mimic could obviously inhibit the luciferase activity of EZH2 3 -UTR-wt, but not EZH2 3 -UTR-mut. (C) The mRNA and protein expression levels of EZH2 in CRC tissues and adjacent normal colonic epithelial tissues. (D) The mRNA and protein expression levels of EZH2 in CRC cells with different metastatic potential. (E) LoVo cells were transfected with miR-137 mimic and/or pc-XIST. RT-qPCR showed the miR-137 expression in the different transfected cells. (F) The expression of EMT markers, including E-cadherin, N-cadherin, and vimentin, in the different transfected cells. Data were expressed as mean ± SD. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to corresponding control.

cells with high metastatic potential compared with cells with low metastatic potential. Overexpression of lncRNA XIST promoted the migratory and invasive potential of HT29 cells, while knockdown of lncRNA XIST inhibited the migratory and invasive potential of LoVo cells via regulating the expression of EMT markers. EMT is regarded as an important step in tumor progression and metastasis[21,22]. More importantly, loss of lncRNA XIST is found to induce EMT to mediate tumor progression and metastasis in breast cancer[23]. A recent study shows that lncRNA XIST can modulate EMT and expedite CRC metastasis through competing for miR-200b-3p to modulate ZEB1 expression[24]. Compared with this recent study, we also found that lncRNA XIST can modulate EMT and expedite CRC metastasis, but the regulatory mechanism was different in that lncRNA XIST mediated CRC metastasis via regulating the miR-137–EZH2 axis in our study. These findings imply the key role and complicated regulatory mechanism of lncRNA XIST in CRC progression and metastasis.

Furthermore, miR-137 has been confirmed to regulate tumor growth and metastasis in human hepatocellular carcinoma[25]. Alteration of miR-137 expression contributes to tumor invasion and metastasis in non-small cell lung cancer[26]. miR-137 is also found to have the ability to inhibit EMT and invasion of ovarian cancer cells[27]. Sakaguchi et al. demonstrated that miR-137 can play a key role in regulating the tumorigenicity of colon cancer stem cells[28]. Smith et al. found that miR-137 functioned as a tumor suppressor to repress CRC progression[29]. Notably, lncRNA XIST has been shown to contribute to glioma development via targeting miR-137[30,31]. Furthermore, EZH2 was confirmed as a target of miR-137 in our study. EZH2 is a methyltransferase that can regulate invasion and metastasis in human cancers, including CRC[32]. EZH2 expression is also reported to be significantly correlated with the aggressive behavior and poor prognosis in CRC[33]. Moreover, knockdown of lncRNA XIST is found to suppress gastric cancer progression and metastasis via regulating EZH2 expression[34]. Considering the regulatory relationship between lncRNA XIST and the miR-137–EZH2 axis, we speculate that the miR-137–EZH2 axis is a key mechanism to mediate the key roles of lncRNA XIST in CRC progression and metastasis.

In conclusion, our data reveal that lncRNA XIST may promote tumor metastasis in CRC possibly through regulating the miR-137–EZH2 axis. The lncRNA XIST may serve as a prognostic indicator for CRC progression. Targeting XIST may therefore be a promising therapeutic strategy for CRC. Further studies are still required to verify our findings.

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