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LncRNA LINC00467 contributes to osteosarcoma growth and metastasis through regulating HMGA1 by directly targeting miR-217

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Abstract. – OBJECTIVE: Osteosarcoma (OS) is a common primary bone tumor. Despite multiple treatment strategies have made great progress, the overall clinical outcome of OS patients is frustrating. Long non-coding RNA (lncRNA) LINC00467 has been reported in several cancers, while the research of the role of LINC00467 in OS is limited. The aim of this study was to figure out the potential mechanism of LINC00467 in OS.

PATIENTS AND METHODS: The expression levels of LINC00467, microRNA-217 (miR-217) and high mobility group A1 (HMGA1) were quantified by reverse transcription quantitative polymerase chain reaction (qRT-PCR). Cell counting kit-8 (CCK-8), flow cytometry and transwell assay were performed to detect cell proliferation, apoptosis, migration and invasion, respectively. The protein levels of HMGA1, B-cell lymphoma-2 (Bcl-2), Bax, Cleaved Caspase-3 (C-Caspase 3), E-cadherin, N-cadherin and vimentin were measured by Western blot assay. Online software starbase was used to predict the binding sites of miR-217. Luciferase report assay and RNA Binding Protein Immunoprecipitation (RIP) assay were carried out for detecting the interaction between miR-217 and LINC00467 or HMGA1.

RESULTS: The expression of LINC00467 and HMGA1 was increased in OS tissues and cells, while miR-217 expression was reduced. High expression of HMGA1 led to the poor overall survival. Down-regulation of LINC00467 or up-regulation of miR-217 could accelerate cell apoptosis, and slump cell proliferation, migration, invasion and EMT. However, miR-217 under-expression or HMGA1 over-expression could rescue these effects. Moreover, it was indicated that LINC00467 directly targeted miR-217 and HMGA1 was a target of miR-217.

CONCLUSIONS: LINC00467 promoted cell proliferation, migration, invasion and EMT, and suppressed cell apoptosis by up-regulating HMGA1 via targeting miR-217.

Key Words:
Osteosarcoma, LINC00467, MiR-217, HMGA1, Proliferation, Migration, Invasion, EMT.

Introduction

Osteosarcoma (OS), deriving from primitive bone-forming mesenchymal cells, is the most common primary bone malignancy among children and adolescent[1]. OS was always regarded as a cancer with low survival rate and poor prognosis due to the rapid progression and metastasis capacity[2]. The development of OS is accompanied by diversely genetic changes, and researchers have extensively studied the genetic, molecular and protein levels in OS[3,4]. However, the highly complex molecular mechanisms of OS progression still require further exploration to find the novel markers to improve treatment strategies.

Long non-coding RNAs (lncRNAs), ranging from 200 nt to 100 kb, belong to non-coding RNAs (ncRNAs)[5]. LncRNAs in the cytoplasm regulate mRNA stability and translation act as competitive endogenous RNAs (CeRNAs) to regulate the distribution of miRNAs on their targets[6]. LncRNAs represented a novel class of tumor biomarkers in the development of various cancers, including OS[7]. Ectopic expression of lncRNA loc285194 inhibited tumor cell growth both *in vitro* and *in vivo* in OS[8]. Up-regulation of lncRNA zinc finger E-box binding homeobox 1 antisense 1 (ZEB1-AS1) predicted poor prognosis of OS patients and promoted OS cell proliferation and migration[9]. LncRNA differentiation antagonizing nonprotein coding RNA (DANCR) was up-regulated in OS cells and promoted tumor invasion and metastasis[10]. Although these data have manifested the vital roles of lncRNAs in OS, the effect of LINC00467 on the progression of OS has not been elucidated.

MicroRNAs (miRNAs) are short ncRNAs with ~22 nucleotides[11]. MiRNAs inhibit gene expression of post-transcriptional and translational levels by targeting to the 3'-untranslated region (3'UTR) of the target messenger RNA (mRNA)[12].

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Mutations in miRNA-encoding genes or dysregulated expression of miRNAs were important factors in many human diseases, including cancer[13]. MiR-217 was down-regulated and could inhibit tumor cell growth by targeting kirsten rat sarcoma viral oncogene (KRAS) in pancreatic ductal adenocarcinoma[14]. Overexpression of miR-217 weakened the ability of migration and invasion in esophageal squamous cell carcinoma cells[15]. The expression of miR-217 was diminished in epithelial ovarian cancer (EOC) tissues and the ectopic expression of miR-217 inhibited tumor growth both *in vitro* and *in vivo*[16]. Therefore, miR-217 functions as a vital role in the occurrence and growth of cancer. However, the investigation about role of miR-217 in OS was still limited.

High mobility group A1 (HMGA1) is a basic protein that binds to the AT-rich region of deoxyribonucleic acid (DNA) and primarily acts as a specific cofactor for activating genes. HMGA1 has no intrinsic transcriptional activity and drives gene transcription by facilitating the assembly and stability of stereospecific DNA-protein complexes[17,18]. Senigagliesi et al[19] claimed that HMGA proteins were strongly expressed and played essential roles in the process of neoplastic transformation. HMGA1 amplified Wnt signaling and maintained Wnt and other transcriptional networks, suggesting that HMGA1 overexpression contributed to carcinogenesis and progression of tumor through dysregulation[20]. HMGA1 was highly expressed in pancreatic head cancer compared with periampullary cancer and presented as an independent prognostic marker to predict poor outcome[21]. HMGA1 overexpression blocked the apoptosis through a pathway involving in caspase-3 activation, which led to a malignant phenotype of rat and human thyroid cells and caused the programmed cell death of normal thyroid cells[22]. These data provided the available information for finite research of HMGA1 on OS progression at present.

Epithelial-interstitial transformation (EMT) is a basic process that controls the morphogenesis of organisms. During this process, epithelial cells lose their polarity and turn into a mesenchymal phenotype[23], such as loss of E-cadherin and gain of N-cadherin and vimentin expression. E-cadherin is a transmembrane glycoprotein that mediates cell-cell adhesion and maintains a normal polarized epithelial phenotype, which can inhibit tumorigenesis[24]. Besides, the expression of mesenchymal cadherins (such as N-cadherin and vimentin) is another mean by which tumor cells adjust their adhesion function[25]. Here, we determined the expression of E-cadherin, N-cadherin and vimentin to detect the regulatory effect during tumorigenesis.

In our previous study, we measured the expression of LINC00467 and explored its role in OS. We also investigated the interaction between miR-217 and LINC00467 or HMGA1, aiming to provide a novel strategy for the therapy of OS.

Patients and Methods

Sample Tissues and Cell Culture

Our research was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. 44 pairs of OS tissues and adjacent normal tissues were obtained from OS patients from Shandong Provincial Hospital Affiliated to Shandong First Medical University. OS patients were diagnosed and classified through pathological examination on the basis of the World Health Organization classification system. OS patients at stage IIB/III were included, and patients with any other primary disease were excluded and patients who accepted local or systemic treatment before surgery were excluded. All samples were frozen in liquid nitrogen after removing and stored at -80°C. Written informed consents were obtained from all the OS patients before surgery.

Human OS cell lines (HOS, MG63, Saos2 and SJSA1) and osteoblast cell line Hfob1.19 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplementing with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin sodium (Gibco, Grand Island, NY, USA) and 100 μg/mL streptomycin sulfate (Gibco, Grand Island, NY, USA) at 37°C in a humidified condition containing 5% CO_2.

Cell Transfection

Small interfering RNA (siRNA) against LINC00467 (si-LINC00467) and siRNA negative control were synthesized from GenePharma (Shanghai, China). MiR-217 mimic (miR-217), miR-217 negative control (miR-NC), miR-217 inhibitor (anti-miR-217) and miR-217 negative control inhibitor (anti-miR-NC) were purchased from GenePharma (Shanghai, China). HMGA1 overexpression plasmid pcDNA-HMGA1 (HMGA1) and

pcDNA empty vector (Vector) were bought from Ribobio (Guangzhou, China). According to the manufacturer's instructions, all cell transfection was performed using Lipofectamine™ 3000. After 48 h transfection, cells were harvested for the following experiments.

RNA Extraction and Reverse Transcription Quantitative Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cells or tissues using TRIzol reagent (TaKaRa, Dalian, China). Complementary deoxyribose nucleic acid (cDNA) was reversely transcribed using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). QRT-PCR was conducted using SYBR Premix Ex Taq II Kit (TaKaRa, Dalian, China) on an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The quantitation of LINC00467 and HMGA1 was normalized by Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using $2^{-\Delta\Delta Ct}$ method.

For mature miR-217 quantitation, cDNA reverse transcription was conducted from total RNAs using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the products were amplified by PCR using TaqMan Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). The specific miRNA primers were purchased from Ribobio (Guangzhou, China). Small nucleolar RNA U6 was used as an internal control. All primers were listed as follows: LINC00467: 5'-GGCAGGGAGGTTTAATAGAC-3' (forward) and 5'-TACCTTCATATTTGCCAGCC-3' (reverse). HMGA1: 5'-GGCCCAAATCGACCATAAAGG-3' (forward) and 5'-GGACAAATCATGGCTACCCCT-3' (reverse). U6: 5'-ACCCTGAGAAATACCCTCACAT-3' (forward) and 5'-GACGACTGAGCCCCTGATG-3' (reverse). GAPDH: 5'-CCGGGAAACTGTGGCGTGATGG-3' (forward) and 5'-AGGTGGAGGAGTGGGTGTCGCTGTT-3' (reverse).

Cell Counting Kit-8 (CCK-8) Assay

Transfected HOS and MG63 cells were seeded in 96-well plates for 24 h at a density of 2.0×10^3 cells per well with five repeats for each group. After incubation, 10 μL CCK-8 solution (Beyotime Biotechnology Co. Shanghai, China) was added into each plate and incubated for another 2 h. Next, the absorbance was measured at 450 nm by microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 24 h, 48 h, and 72 h.

Flow Cytometry

For cell apoptosis, MG63 and HOS cells for 48 h transfection were collected, washed three times with ice-cold phosphate-buffered saline (PBS) and re-suspended in a binding buffer at a concentration of 1×10^6 cells/mL. Then, 150 mL cell suspension was taken, and the cells were stained with annexin V-Fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI) using an apoptosis detecting kit (Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature in the dark. Eventually, the stained cells were detected by FACSCalibur (BD Biosciences, San Jose, CA, USA) and the data were analyzed with CellQuest software.

Western Blot Assay

The transfected cells were captured and lysed by radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio Science & Technology Co., Ltd., Beijing, China). Then, the total protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transformed onto polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA). Next, the membranes were blocked with 5% skimmed milk for 2 h and then incubated with the specific primary antibodies overnight at 4°C. On the second day, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (HRP, 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. Finally, blots were visualized by the enhanced chemiluminescence kit (Millipore, Bradford, MA, USA) on enhanced chemiluminescence (ECL) detection system (Millipore, Bradford, MA, USA). GAPDH acted as internal control. The primary antibodies against B-cell lymphoma-2 (Bcl-2), Bax, Cleaved Caspase-3 (C-Caspase 3), E-cadherin, N-cadherin, vimentin, HMGA1 and GAPDH were all purchased from Abcam (Cambridge, MA, USA).

Transwell Assay

Cell migration and invasion assays were performed using a 24-well transwell plate (8 μm; Corning Incorporated, Corning, NY, USA). For cell migration, transfected cells were resuspended in serum-free DMEM and plated onto the upper chamber. The lower chamber was added with 10% FBS as chemoattractant. For cell invasion, the upper chamber was pre-coated with Matrigel (Corning, Corning, NY, USA). After 24 h incubation, the migrated or invaded cells were fixed with

methanol and stained with 0.1% crystal violet for 20 min. Finally, the cells were numbered using a Leica DC 300F microscope (Leica Microsystems Imaging Solutions Ltd., Wetzlar, Germany) at a magnification of × 100. The results were averaged among three independent experiments.

Separation of Nuclear and Cytoplasmic Fractions

The NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (78835; Thermo Fisher Scientific, Waltham, MA, USA) was used to separate the total cellular fractions into nuclear and cytoplasmic fractions according to the manufacturer's protocol. The expression levels of GAPDH, U6 and LINC00467 in nuclear and cytoplasmic fractions were detected using qRT-PCR, and GAPDH acting as a cytoplasmic control and U6 acting as a nuclear control.

Bioinformatics Analysis

The online software starbase (http://starbase.sysu.edu.cn/) was used for predicting the potential targeting sites among LINC00467, HMGA1 and miR-217.

Luciferase Report Assay and RNA Binding Protein Immunoprecipitation (RIP) Assay

3'UTR sequences of HMGA1 containing wild-type (WT) or mutant (MUT) binding sites of miR-217 were amplified and cloned into pGL3 vector to generate HMGA1-WT or HMGA1-MUT Luciferase reporter vector, respectively. HOS and MG63 cells were co-transfected with pGL3-LINC00467 wild type (LINC00467-WT), pGL3-LINC00467 mutation (LINC00467-MUT), HMGA1-WT or HMGA1-MUT and miR-217 mimics (miR-217) or miR-217 negative control (miR-NC). After 48 h transfection, the relative Luciferase activity was detected with Dual-Luciferase reporter assay Kit (Promega, Madison, WI, USA). The firefly Luciferase activity was normalized to *Renilla* Luciferase activity.

RIP assay was performed using the Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, HOS and MG63 cells were collected, washed with PBS twice and lysed with RIP lysis buffer. Then, cell extract was incubated with RIP buffer containing magnetic beads conjugated to human anti-AGO2 antibody (Millipore, Billerica, MA, USA) and IgG control (Millipore, Billerica, MA, USA). The co-precipitated RNA was purified and quantified by qRT-PCR.

Statistical Analysis

Statistical analyses were performed using Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS, Chicago, IL, USA). Data were accepted from at least 3 independent experiments and presented as mean ± standard deviation (SD). Difference between two groups was analyzed by Student's *t*-test. Comparison among the multiple groups was analyzed using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Correlation analyses were performed by Spearman rank correlation. p-value <0.05 was regarded as statistically significant.

Results

LINC00467 Was Up-Regulated In OS Tissues and Cells

We first examined the expression of LINC00467 in tissues and cell lines. As shown in Figure 1A, the level of LINC00467 was remarkably augmented in OS tissues compared with the adjacent normal tissues. Meanwhile, LINC00467 was significantly higher in OS cell lines (HOS, MG63, Saos2 and SJSA1) than that in normal cell line hFOB1.19 (Figure 1B). In addition, overall survival by Kaplan-Meier survival analysis showed that OS patients with low LINC00467 expression owned a higher survival rate rather than that with high LINC00467 expression (Figure 1C), suggesting the inhibition effect of LINC00467 on OS patients. The statistics of OS clinicopathological factors were recorded in Table I. All data suggested that LINC00467 may act as an oncogene in OS progression.

LINC00467 Knockdown Could Take the Edge Off Ability Of Cell Proliferation, Migration And Invasion but Enhance Cell Apoptosis

CCK-8 assay, transwell assay and flow cytometry were carried out to explore the role of LINC00467 in OS cells. First, the interference efficiency of LINC0067 was validated by using qRT-PCR, and the result showed that LINC00467 was the lowest in HOS and MG63 cells transfected with si-LINC00467#1 compared to other groups (Figure 2A). Therefore, si-LINC00467#1 group was chosen for the following detection. From Figure 2B and 2C, we concluded that cell proliferation was

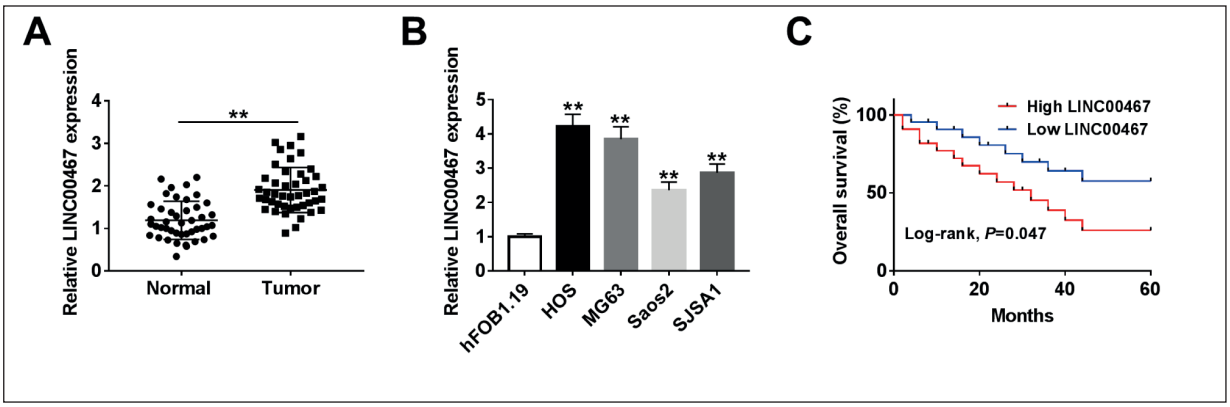

Figure 1. LINC00467 was up-regulated in OS tissues and cells, and high LINC00467 expression was associated with low overall survival. **A**, The expression of LINC00467 in tumor tissues was higher than that in normal tissues. **B**, The expression of LINC00467 was increased in OS cell lines (HOS, MG63, Saos2 and SJSA1) compared with normal osteoblast cell line (Hfob1.19). C, The overall survival of OS patients with high LINC00467 expression (n=22) was lower than patients with low LINC00467 expression (n=22). $^{**}p<0.01$.

inhibited in HOS and MG63 cells after transfection with si-LINC00467#1. Then, flow cytometry exhibited that the number of apoptotic cells was rapidly increased in HOS and MG63 cells transfected with si-LINC00467#1 (Figure 2D). Moreover, we detected the expression of cell apoptosis-related proteins by Western blot. As shown in Figure 2E, Bax and C-Caspase 3 expression was markedly enhanced in HOS and MG63 cells transfected with si-LINC00467#1, while Bcl-2 expression was weakened. As for cell migration and invasion, transwell assay clarified that the number of migration and invasion cells was significantly decreased by LINC00467 deletion in HOS and MG63 cells (Figure 2F and 2G). Likewise, several EMT-related proteins were assessed by Western blot to verify this result. The expression of E-cadherin was reinforced, whereas N-cadherin and vimentin expression were strongly diminished by LINC00467 downregulation (Figure 2H). These data showed that LINC00467 was associated with tumor growth and metastasis in OS.

Table I. Clinicopathological factors and LINC00467 expression in patients with osteosarcoma.

Parameters	Case	LINC00467 expression		*p*-value
		High(n=22)	Low(n=22)	
Age at diagnosis				
≤18	24	13	11	0.540
>18	20	9	11	
Gender				
Female	21	9	12	0.332
Male	23	13	10	
Tumor size (cm)				
<8	20	6	14	0.018*
≥8	24	16	8	
TNM stage				
I + IIA	21	7	14	0.022*
IIB + III	23	15	8	
Distant metastasis				
No	31	12	19	0.016*
Yes	13	10	3	
Anatomic location				
Tibia/femur	37	20	17	0.357
Elsewhere	7	2	5	

*$p<0.05$, [a]Chi-square test.

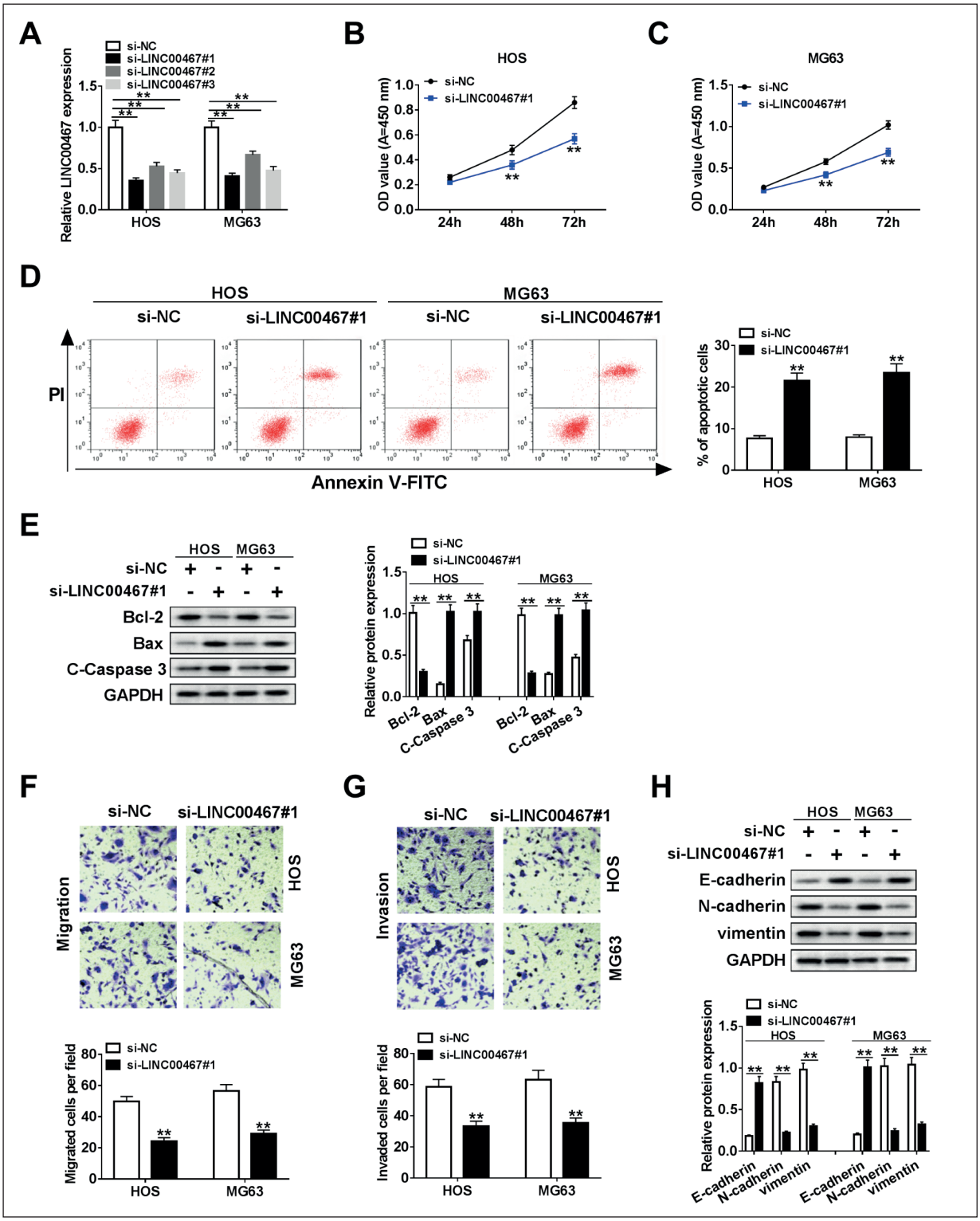

Figure 2. Down-regulated LINC00467 inhibited cell proliferation, migration and invasion, but promoted cell apoptosis in OS cells. **A**, The interference efficiency of LINC00467 was detected by qRT-PCR in OS cells. **B**-**C**, The effect of LINC00467 down-regulation on cell proliferation was examined using CCK-8 assay in HOS and MG63 cells. **D**, Flow cytometry was performed to observe cell apoptosis in HOS and MG63 cells transfected with si-LINC00467#1. **E**, Proteins related to cell apoptosis including Bcl-2, Bax and C-Caspase 3 were quantified by Western blot. **F**-**G**, Transwell assay was carried out to detect cell migration and invasion in HOS and MG63 cells transfected with si-LINC00467#1 (magnification: 100×). **H**, Several proteins (E-cadherin, N-cadherin, vimentin) associated with EMT were measured by Western blot. $^{**}p<0.01$.

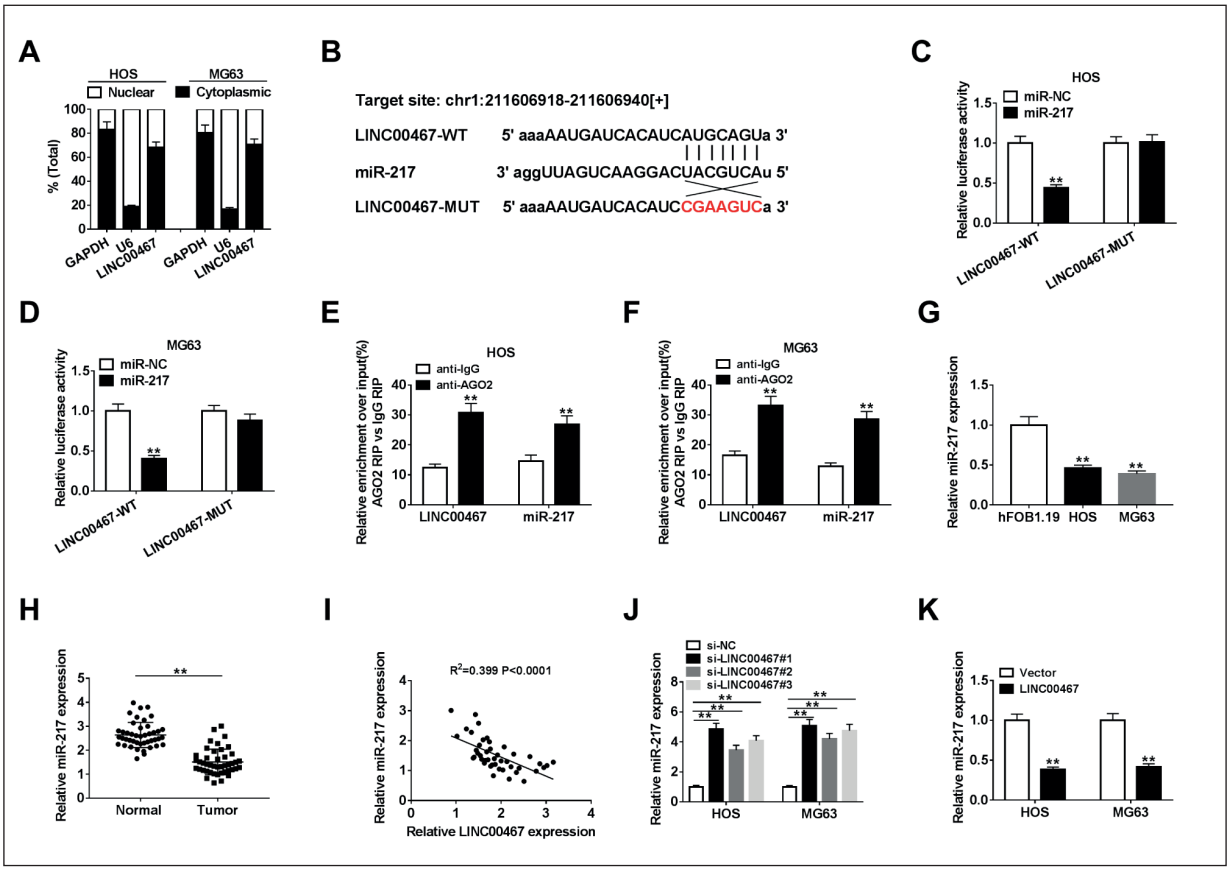

Figure 3. LINC00467 targeted miR-217 directly, and miR-217 was down-regulated in OS. **A**, LINC00467 was mainly expressed in cytoplasm rather than cell nucleus both in HOS and MG63 cells. **B**, Starbase software was used to predict the possible binding sites between LINC00467 and miR-217 online. **C**-**D**, Luciferase report assay verified the interaction of LINC00467 and miR-217 both in HOS (**C**) and MG63 (**D**) cells. E, F, RIP assay was performed to detect the interaction between LINC00467 and miR-217. **G**, miR-217 was down-regulated in HOS and MG63 cells compared with that in hFOB1.19 cells. H, MiR-217 was down-regulated in tumor tissues. **I**, The expression of miR-217 was negatively correlated with the expression of LINC00467. **J**, and **K**, MiR-217 was up-regulated significantly in HOS and MG63 cells transfected with si-LINC00467 and decreased in LINC00467 over-expressed cells. **$p<0.01$.

LINC00467 Directly Targeted MiR-217, and MiR-217 Was Down-Regulated In OS Tissues and Cells

To ascertain whether LINC00467 functioned as a ceRNA, LINC00467 expression was measured in nuclear and cytosolic fractions from HOS and MG63 cells. QRT-PCR assay presented that LINC00467 was mainly assembled in cytoplasm, supplying prerequisite for acting as a ceRNA (Figure 3A). Then, online software starbase predicted that the underlying miRNAs bound to LINC00467. As shown in Figure 3B, some binding sites existed between LINC00467 and miR-217. Moreover, overexpression of miR-217 significantly reduced the Luciferase activity of HOS and MG63 cells in LINC00467-WT group, whereas it showed little impact in LINC00467-MUT group (Figure 3C and 3D). Additionally, RIP assay deemed that LINC00467 and miR-217 were predominantly enriched by anti-AGO2 antibody, respectively (Figure 3E and 3F). Subsequently, qRT-PCR analysis illustrated that miR-217 was significantly down-regulated both in OS cells (Figure 3G) and tissues (Figure 3H) compared with normal cells and tissues. We discovered that miR-217 was negatively correlated with LINC00467 expression (Figure 3I). Furthermore, the expression of miR-217 was rocketed in HOS and MG63 cells transfected with si-LINC00467 (#1, #2 and #3), on the contrary, its expression was collapsed when two cells transfected with pcDNA-LINC00467 (Figure 3J and 3K). These data indicated that miR-217 could bind to LINC00467 and negatively regulated by LINC00467.

HMGA1 Acted as a Target of MiR-217, and It Was Up-Regulated In OS Tissues

Starbase predicted the potential binding sites between miR-217 and HMGA1 (Figure 4A). Luciferase report assay revealed that co-transfection with HMGA1-WT vector and miR-217 mimics substantially reduced the Luciferase reporter activity in HOS and MG63 cells, while the Luciferase activity of cells co-transfected with HMGA1-MUT and negative control showed an inconspicuous difference (Figure 4B and 4C). The mRNA level of HMGA1 was considerably declined in HOS and MG63 cells when miR-217 was up-regulated (Figure 4D), while its mRNA level was swiftly elevated when cells transfected with anti-miR-217 (Figure 4E). Western blot analysis revealed the same results at the protein level (Figure 4F and 4G). Furthermore, the mRNA level of HMGA1 was expectedly elevated in OS tissues (Figure 4H). Correlation analysis found that the expression level of HMGA1 was always negatively correlated with the expression level of miR-217 (Figure 4I). All data suggested that HMGA1 was a target of miR-217.

MiR-217 Affected Cell Proliferation, Apoptosis, Migration and Invasion Through Regulating HMGA1

To explore the influence on OS development of miR-217 targeting HMGA1, we detected the expression level of HMGA1 in OS cells after altering miR-217 expression. Result of qRT-PCR showed that the mRNA level of HMGA1 was plunged in HOS and MG63 transfected with miR-217 mimics, while HMGA1 expression was improved when cells transfected with miR-217 mimics and pcDNA-HMGA1 compared with control (Figure 5A). Then, cell proliferation assay clarified that the inhibition effect of miR-217 overexpression on cell proliferation could be rescued by HMGA1 enrichment in HOS (Figure 5B) and MG63 (Figure 5C) cells. As shown in Figure 5D, the number

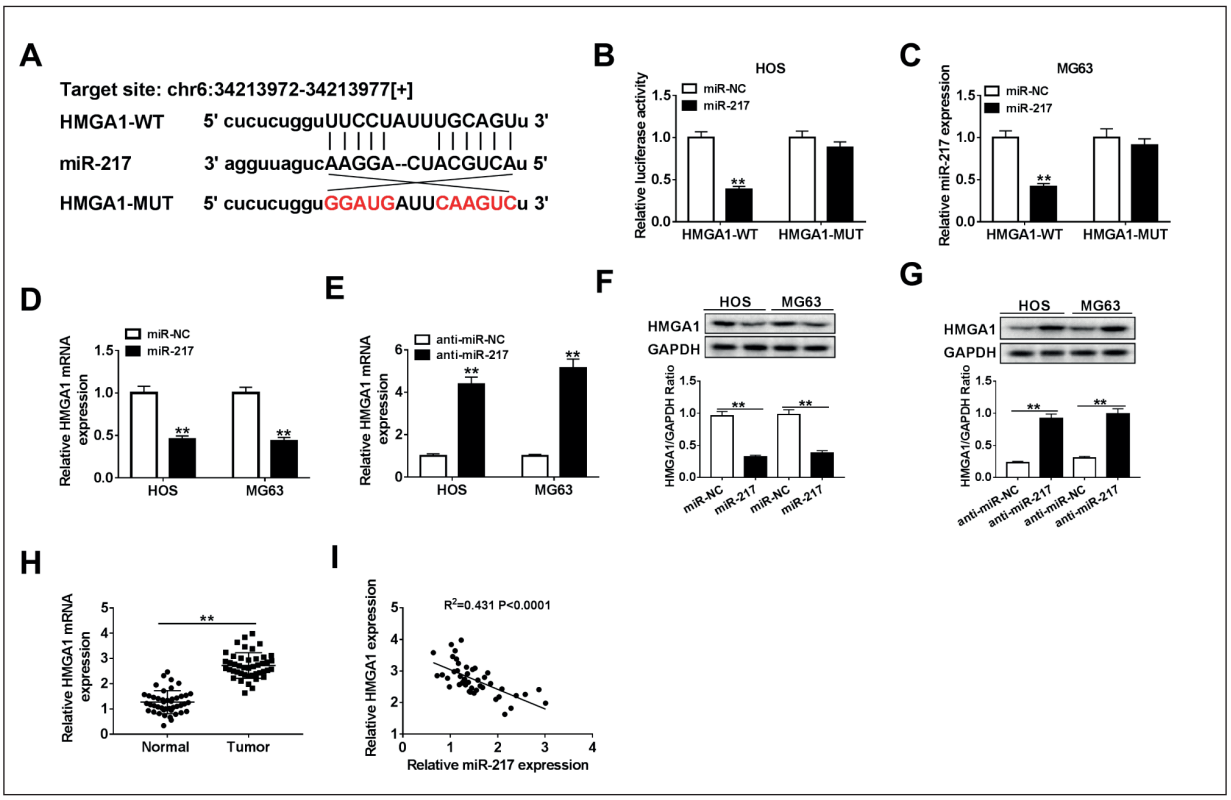

Figure 4. HMGA1 was a target of miR-217, and HMGA1 was up-regulated in OS tissues and cells. **A**, Online software starbase predicted the potential binding sites between miR-217 and HMGA1 3'UTR. **B**, and **C**, The interaction of miR-217 and HMGA1 was determined by luciferase report assay in both HOS and MG63 cells. **D**, and **E**, Relative HMGA1 mRNA expression in HOS and MG63 cells transfected with miR-217 mimics or miR-217 inhibitors was measured by qRT-PCR. **F**, and **G**, HMGA1 protein expression in HOS and MG63 cells transfected with miR-217 mimics or miR-217 inhibitors was measured by Western blot. **H**, Relative HMGA1 mRNA expression in tumor tissues was higher than that in the adjacent normal tissues. **I**, A negative relationship existed in the expression of HMGA1 and miR-217. $^{**}p<0.01$.

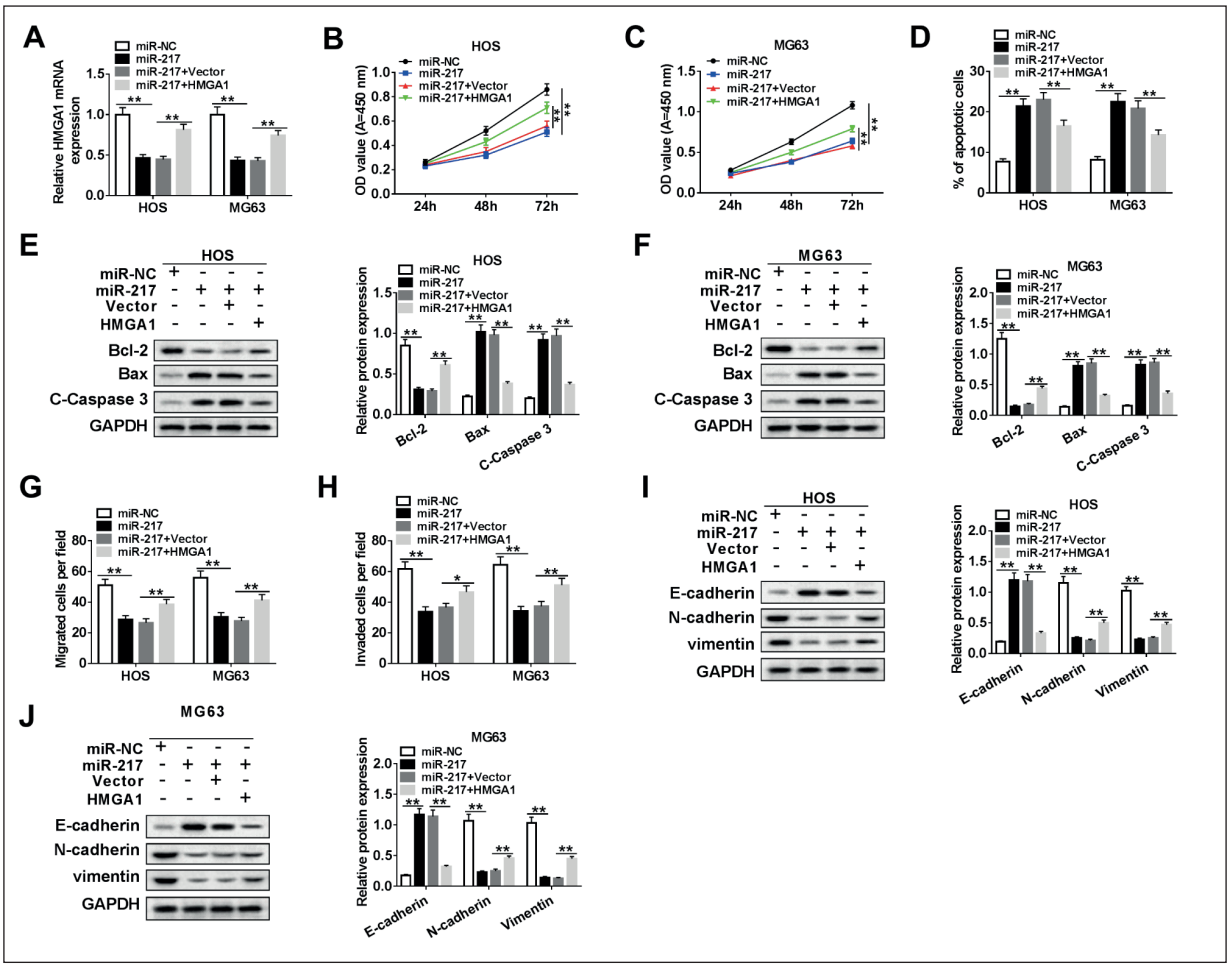

Figure 5. MiR-217 regulated cell proliferation, apoptosis, migration and invasion by targeting HMGA1. **A**, Relative HMGA1 mRNA expression was detected in HOS and MG63 cells transfected with miR-217 mimics, miR-NC, miR-217 + HMGA1 or miR-217 + vector. **B**-**C**, Cell proliferation was determined by CCK-8 assay in HOS and MG63 cells. **D**, Cell apoptosis was measured by flow cytometry in HOS and MG63 cells transfected with miR-217 mimics, miR-NC, miR-217+HMGA1 or miR-217+vector. **E**, and **F**, Bcl-2, Bax and C-Caspase 3 protein expression levels were detected by Western blot both in HOS (**E**) and MG63 (**F**) cells. **G**, **H**, Cell migration and invasion were detected through transwell assay in HOS and MG63 cells. **I**, J, E-cadherin, N-cadherin and vimentin protein expression were checked by Western blot both in HOS (**I**) and MG63 (**J**) cells. $^{**}p<0.01$.

of apoptosis cells was boomed in miR-217 mimics group at 24 h post transfection, which was blocked in miR-217 mimics + pcDNA-HMGA1 group. Besides, we found that down-regulation of Bcl-2 and up-regulation of Bax and C-Caspase 3 resulted from miR-217 mimics in HOS cells could be overturned by HMGA1 upregulation (Figure 5E). The situation in MG63 agreed with the result in HOS cells (Figure 5F). The migrated and invaded cells were decreased by miR-217 mimics, which were abolished by HMGA1 overexpression (Figure 5G and 5H). Synchronously, the expression of several proteins related to EMT was also measured. We found that the effects of miR-217 mimics on the expression of E-cadherin, N-cadherin and vimentin were reversed by pcDNA-HMGA1 (Figure 5I and 5J). These data elucidated that HMGA1 was regulated by miR-217 and thus affected the development of OS.

LINC00467/MiR-217/HMGA1 Axis Regulated the Development and Metastasis of OS

As shown in Figure 6A, qRT-PCR presented that HMGA1 significantly down-regulated by si-LINC00467#1, while HMGA1 expression was elevated in HOS and MG63 transfected with si-LINC00467#1+anti-miR-217 or si-LINC00467#1+HMGA1. Western blot analysis confirmed that transfection with si-LINC00467#1 steeply down-regulated the expression of HMGA1, and HMGA1 expression

strengthened by si-LINC00467#1+anti-miR-217 or si-LINC00467#1+HMGA1 in HOS (Figure 6B) and MG63 (Figure 6C) cells. CCK-8 assay (Figure 6D and 6E) and flow cytometry analysis (Figure 6F) showed that LINC00467 deletion weakened the number of viable cells and increased the proportion of apoptotic cells, while miR-217 inhibitor or HMGA1 overexpression blocked si-LINC00467#1-mediated reduction effect on the number of viable cells and promotion effect on cell apoptosis. In addition, the levels of several proteins related to apoptosis were amply assessed in HOS (Figure 6G) and MG63 (Figure 6H) cells after transfection. In si-LINC00467#1 transfected cells, Bcl-2 expression was decreased, while Bax and C-Caspase3 expression were accelerated. On the contrary, in si-LINC00467#1+anti-miR-217 or si-LINC00467#1+HMGA1 transfected cells, Bcl-2 expression was picked up, while Bax and C-Caspase3 expression was regressed. Transwell assay identified that cell migration (Figure 6I) and invasion (Figure 6J) were inhibited by

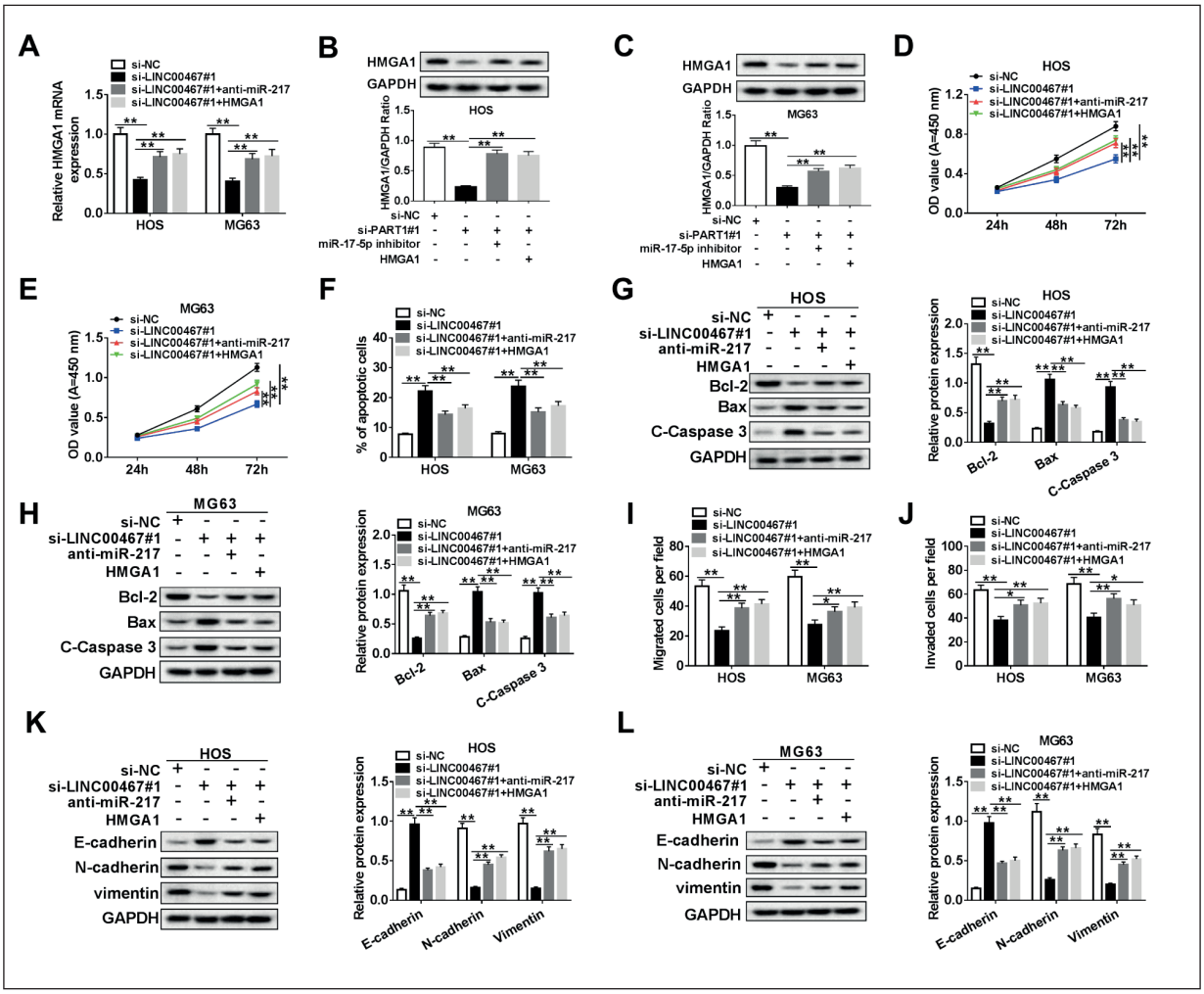

Figure 6. LINC00467 functioned on cell proliferation, apoptosis, migration and invasion by regulating HMGA1 via targeting miR-217. **A**, Relative HMGA1 mRNA expression was measured by qRT-PCR in HOS and MG63 cells transfected with si-NC, si-LINC00467#1, si-LINC00467#1+anti-miR-217 or si-LINC00467#1+HMGA1. **B**-**C**, The protein level of HMGA1 was detected using Western blot both is HOS (**B**) and MG63 (**C**) cells. **D**, **E**, Cell proliferation was observed in HOS and MG63 cells transfected with si-LINC00467#1, si-LINC00467#1+anti-miR-217, si-LINC00467#1+HMGA1 or si-NC. **F**, Flow cytometry was used to determine cell apoptosis in HOS and MG63 cells. **G**, **H**, Bcl-2, Bax and C-Caspase protein expression were measured by Western blot both in HOS and MG63 cells. **I**, **J**, The number of migrated and invaded cells was determined by transwell assay in HOS and MG63 cells transfected with si-LINC00467#1, si-LINC00467#1+anti-miR-217, si-LINC00467#1+HMGA1 or si-NC. **K**, The protein levels of E-cadherin, N-cadherin and vimentin were quantified by Western blot both in HOS and MG63 (**L**) cells, GAPDH acting as control. $^{**}p<0.01$.

LINC00467 deletion, which was recovered in both HOS and MG63 cells after transfecting with si-LINC00467#1 +anti-miR-217 or si-LINC00467#1 + HMGA1. Moreover, si-LINC00467#1 could enhance the expression of E-cadherin and frustrate N-cadherin and vimentin expression, and anti-miR-217 or HMGA1 had the ability to reverse these effects both in HOS (Figure 6K) and MG63 (Figure 6L) cells. These data implied that LINC00467 regulated cell viability, apoptosis, migration and invasion through up-regulating the expression of HMGA1 *via* targeting miR-217.

Discussion

Over the past few years, the chemotherapy regimens for OS diagnosis have effectively improved. However, the survival of OS patients was failed to advance due to the poor outcome with metastatic or recurrent OS[26,27]. Thus, it is urgent to study the pathogenesis and metastatic mechanisms of OS. In this investigation, we observed that lncRNA LINC00467 was highly expressed in OS tissues and cells, which acted as an oncogene in OS progression.

LINC00467 was identified by Human Genome Organisation Gene Nomenclature Committee (HGNC). However, the biological function of LINC00467 is hardly known[28]. Atmadibrata et al[28] showed that knocking-down LINC00467 expression decreased neuroblastoma cell survival. Yang et al[29] stated that LINC00467 was highly expressed in lung adenocarcinoma (LUAD) and promoted LUAD cell proliferation and migration[29]. The present research proved that LINC00467 in OS was enhanced and LINC00467 down-regulation inhibited cell proliferation, migration and invasion for the first time. Bcl-2, an inner mitochondrial membrane protein, can suppress programmed cell death[30]. Bax, a number of the bcl-2 family, can advance cell apoptosis[31]. Caspase-3, downstream of bax/bcl-2 control, plays a key role in the execution of apoptosis[32]. Furthermore, EMT is one of the important mechanisms to regulate tumor metastasis, during which the epithelial cells transform and acquire mesenchymal-like properties[33]. EMT markers include loss of cell-cell adhesion, reorganization of actin cytoskeleton, and increased migration characteristics[34]. It is well known that E-cadherin, N-cadherin and vimentin are important markers of EMT[35]. Prudkin et al[36] found that E-cadherin showed high expression, while N-cadherin and vimentin presented low expression in different pathological types of lung cancer tissues. Surprisingly, the result in this study was in accordance with previous research. These data manifested that LINC00467 may present a vital role in cancer development and progression.

LncRNAs are ceRNAs containing miRNA response elements (MERs), which can inhibit the regulation of miRNAs on downstream genes by competitively binding miRNA sites. The function of LncRNA is closely related to its location. LINC00467 was highly expressed in cytoplasm, providing foundation that LINC00467 functioned as a ceRNA. Bioinformatics analysis, Luciferase report assay and RIP showed that LINC00467 directly targeted miR-217. Several former studies reported that miR-217 was down-regulated in OS and functioned as a potential tumor suppressor[37,38]. Our study exhibited similar results. In addition, miRNAs frequently function roles by binding to the 3'UTRs of mRNAs and arousing mRNA degradation or translational suppression[39]. In OS, certain studies reported that miR-217 regulated the development and metastasis of tumor through genes, such as sirtuin-1 (SIRT1)[40], SET domain-containing protein 8 (SETD8)[41] and zinc-finger enhancer binding protein (ZEB1)[42]. In this study, we found that HMGA1 was a target of miR-217.

The role of HMGA1 in OS has been reported partly. Ren et al[43] suggested that HMGA1 was identified as a direct target of miR-758 in OS cells. Pan et al[44] defined that miR-142-3p could directly bind to HMGA1 3'UTR in OS. Liu et al[45] observed that miR-26a inhibited OS migration and invasion by directly targeting HMGA1. Moreover, this paper showed that HMGA1 was highly expressed in OS cells. Therefore, HMGA1 is a significant target gene in OS, and the study of HMGA1 is essential in OS treatment. The novelty of this study was that LINC00467 regulated the progression of OS by miR-217/HMGA1 axis, which might provide the treatment target of OS patients.

Conclusions

In short, our results revealed that LINC00467 was up-regulated in OS cell lines and clinical samples, which reflected an aggressive OS phenotype and poor survival in OS patients. Importantly, LINC00467 contributed to OS growth and metastasis through enhancing HMGA1 expression by targeting miR-217. Our findings demonstrated that LINC00467/miR-217/HMGA1 axis may be a novel strategy for therapy of OS.

Conflict of Interests

The authors declare that they have no conflict of interests.

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