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Regular paper

Long non-coding RNA MINCR regulates the growth and metastasis of human osteosarcoma cells *via* Wnt/β-catenin signaling pathway

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There is concrete evidence that lncRNA-MINCR is involved in tumorigenesis of a number of human cancers through modulation of Wnt/β-catenin signaling pathway. However, the characterization of regulatory role of lncRNA-MINCR has not been worked out in osteosarcoma yet. The present study was undertaken to explore the role of lncRNA-MINCR in human osteosarcoma. The osteosarcoma tissues and cell lines were found to exhibit significant ($P<0.05$) over-expression of lncRNA-MINCR. Silencing of lncRNA-MINCR in osteosarcoma cells suppressed their cell viability through the induction of apoptosis. The Saos-2 osteosarcoma cells exhibited significant ($P<0.05$) decline in migration and invasion rate together with inhibition of EMT under transcriptional knockdown of lncRNA-MINCR. Western blot analysis revealed that lncRNA-MINCR operated through Wnt/β-catenin signaling pathway to control the growth and metastasis of osteosarcoma cells. *In vivo* mice tumorigenesis was significantly ($P<0.05$) restricted under lncRNA-MINCR repression. The study clearly indicated that lncRNA-MINCR exhibits crucial growth regulatory role in osteosarcoma together with its ability to control the metastasis of cancer cells through Wnt/β-catenin signal.

Key words: osteosarcoma, long non-coding RNA, Wnt/β-catenin pathway, metastasis, malignancy

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum; DMSO, Dimethyl sulfoxide; DAPI, 4',6-diamidino-2-phenylindole; PVDF, Polyvinylidene fluoride or polyvinylidene difluoride

INTRODUCTION

Osteosarcoma is considered as one of the most prevalent health disorders in young children and teenage adolescents (Mirabello *et al.,* 2009). The research data suggests that osteosarcoma counts for about one-fifth of all the malignant bone tumors, and among children and adolescents it is more common with more than 60% prevalence rate of all malignant bone disorders (Mertens & Mandahl, 2015). Among children and teenagers, osteosarcoma accounts for the highest number of cancer-related mortality (Smith *et al.,* 2010). Although the 5-year survival rate for non-metastatic osteosarcoma is quite high, it is lower than 20% for the patients detected with metastatic or recurrent disease (Pakos *et al.,* 2009). The treatment procedures presently used against human osteosarcoma include surgical resection, chemotherapy, nano-technological therapeutic interventions, and gene therapy, to name a few (Botter *et al.,* 2014). However, the utility of these treatment measures is limited for chances of recurrence and disease resistance. Recently, much focus has been laid on unraveling the molecular pathways which govern the growth and development of osteosarcoma. In this regard, the long non-coding RNAs (lncRNAs) have remained the subject of active research for their growth controlling role in human cancer (Arun *et al.,* 2018). LncRNAs are RNA species which do not code for proteins and are greater than 200 nucleotides in length (Ma *et al.,* 2019). The altered expression levels of lncRNAs have been depicted to associate with almost all human cancers (Yan *et al.,* 2015). Recent investigations have shown that a number of lncRNAs are involved in regulating the growth and metastasis of osteosarcoma which include SNHG12, KCNQ1OT1, FLVCR-AS1, and DANCR to name a few (Zhou *et al.,* 2018; Shen *et al.,* 2020; Yang *et al.,* 2020; Pan *et al.,* 2020). However, the molecular characterization of lncRNA-MINCR has not been performed in osteosarcoma. Previously, lncRNA-MINCR was found to promote the growth and metastasis of oral squamous cell carcinoma through activation of Wnt/β-catenin signaling pathway (Lyu *et al.,* 2019). The Wnt/β-catenin pathway is one of the key signals mediating the onset and development of human cancers (Yang *et al.,* 2008). Researchers have stressed upon the clinical utility of this key signaling cascade and suggested its molecular targeting as immunotherapy against human cancer (Wang *et al.,* 2018). With this background, molecular role of lncRNA-MINCR was explored in human osteosarcoma. The results were suggestive of marked over-expression of lncRNA-MINCR in osteosarcoma. Down-regulation of lncRNA-MINCR reduced the cancer cell growth and viability effectively, *in vitro*. Further, the cancer cells were shown to be inducted with apoptotic cell death and exhibited significant decline in migration and invasion potential under lncRNA-MINCR repression. Moreover, the transcriptional knockdown of lncRNA-MINCR was seen to oppose the epithelial-mesenchymal transition (EMT) of osteosarcoma cells and proved to act through Wnt/β-catenin signaling pathway. The *in vivo* tumorigenesis of osteosarcoma was significantly restricted under the lowered expression levels of lncRNA-MINCR. Collectively, the findings of the present study highlighted the prognostic value and therapeutic potential of lncRNA-MINCR in osteosarcoma.

MATERIALS AND METHODS

Human tissues and cell lines

The 91 pairs of human osteosarcoma and adjacent normal tissues were procured at The First People's Hospital of Tianmen In Hubei Province, Tianmen, Hubei, China, from osteosarcoma patients just after surgery. Written consents were obtained from the cancer patients prior to any tissue collection and the hospital ethical guidelines for obtaining clinical specimens and their usage for study experimentation were strictly followed. Cryo-preservation was used for maintaining the viability of clinical tissue specimens for future experimental purposes.

Three osteosarcoma cell lines (HOS, MG-63 and Saos-2) and the normal human osteoblast cell line (hFOB1.19) were procured from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cell culturing was carried out using Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories) in supplementation with 10% fetal bovine serum (FBS; Gibco Laboratories), 100 mg/mL of streptomycin, 100 U/mL of penicillin at 37ºC with 5% CO_2 saturation. The Lipofectamine 2000 reagent (Thermo Fisher Scientific) was used for transfecting Saos-2 osteosarcoma cells with RNA-interference construct of lncRNA-MINCR (si-MINCR) or its negative control (NC) as per the manufacturer's instructions.

RNA extraction and qRT-PCR expression analysis

The extraction of total RNA from tissues and cell lines was performed using TRIzol reagent (Thermo Fisher Scientific). Following its quantification, the RNA was reverse-transcribed to cDNA with the help of PrimeScript RT reagent Kit (Takara) as per the manufacturer's protocol. The cDNA was used for carrying out gene expression analysis through qRT-PCR using SYBR Green PCR mix (Takara). The PCR was run on Lightcycler 2.0 (Roche). Human GADPH and β-actin gene was used as an internal control in PCR study. The relative expression of lncRNA-MINCR was inferred through 2^{-ddCT} method. The primers used in the present study are listed in Table 1.

Table 1. List of primers used in the study.

Primer	Direction	Sequence
MINCR	Forward	5′-GAGCCUUGUUUGCCAUUAATT-3′
	Reverse	5′-UUAAUGGCAAACAAGGCUCTT-3′
GADPH	Forward	5′-AAG CCTGCCGGTGACTAA C-3′
	Reverse	5′-GCATCACCCGGAGGAGAA AT-3′

Cell viability assay

The MTT assay was used for analyzing the relative cell proliferations of Saos-2 osteosarcoma cells transfected with si-MINCR with respect to the negative control cells. In brief, the transfected cells were seeded into 96-well plates at a density of 6000 cells per well. The cells were cultured for varying time durations at 37ºC and then 20 μL MTT (5 mg/mL) was added to each well, which was followed by 4 h incubation at 37ºC in a humidified CO_2 incubator. Afterwards, 250 μL DMSO was added to each well to dissolve the formazan crystals. Lastly, the samples were processed for the measurement of optical density (OD) at 570 nm.

Cell proliferation assay

EdU staining procedure was employed for assessing the proliferation of transfected osteosarcoma cells. Here, the cells transfected with si-MINCR or negative control, si-NC were added into 96-well plate at cellular density of 2×10^3 cells per well. EdU staining assay was performed using EdU assay kit (RiboBio Co. Ltd.) as per the manufacturer's guidelines. DAPI staining solution was used for staining the nuclei of the transfected cancer cells. The fluorescence microscope was used for visualizing the EdU-positive cells.

Colony formation assay

The Saos-2 cancer cells transfected with si-MINCR or si-NC were studied for colony formation through clonogenic assay. Approximately, 250 cells were added into 6-well plates, suspended in DMEM medium with 10% FBS and incubated at 37ºC for 14 days. After 2 weeks, the colonies formed were fixed using methanol for 20 min and then stained with 0.1% crystal violet solution (Beyotime). The colonies were visualized with naked eye and manually counted to estimate the percent colony formation.

Annexin V/PI assay

For studying the cancer cell apoptosis, 10^5 Saos-2 cells transfected with si-MINCR or si-NC were cultured in each well of 12-well plate for 24 h post transfection at 37ºC. Cold PBS buffer was used for washing the cells and then Annexin V-FITC Apoptosis Detection Kit (Thermo Fisher Scientific) was used for apoptosis study using a standard protocol. The cells were treated with Annexin V-FITC and PI, serially. Flow cytometer (BD Biosciences) was used for detecting the apoptotic cells. The experiment carried three replicates.

Western blotting analysis

The lysates were prepared from the transfected cancer cells using RIPA lysis and extraction buffer (Thermo Fisher Scientific) with Complete Protease Inhibitor Cocktail (Roche). Protein lysates were resolved using SDS-PAGE. The protein lysates were loaded into 5% stacking gel and separated on 10% running gel. The gel was blotted to PVDF membrane which was exposed to specific primary antibodies. The membrane was then incubated with secondary antibodies conjugated with horse-radish peroxidase (1:1000 dilution) at room temperature, and finally the protein bands were visualized using Enhanced Chemiluminescence Reagent. Human β-actin, GADPH or tubulin was used as an internal control in blotting studies.

Immunofluorescence assay

Immuno-fluorescence was also used for the protein expression analysis. The Saos-2 cancer cells were transfected with s-MINCR or si-NC at 37ºC and then cultivated on cover slips for 48 h. Then, the cells were given cold PBS wash and were fixed with 4% paraformaldehyde for 20 min. The blocking was performed using 5% BSA (in PBS) for 25 min at room temperature. The cells were incubated with specific primary antibodies at 37ºC for 2 h and then the cells were visualized and photo-

graphed using fluorescent microscopy after treating them with FITC-conjugated secondary antibodies.

Transwell migration and invasion assays

The invasion and migration of osteosarcoma cancer cells were determined using transwell chambers with or without Matrigel (BD Biosciences), respectively. The Saos-2 cancer cells were transfected with si-MINCR or si-NC for 24 h. After transfection, approximately 2×10^5 cells suspended in 200 µl culture medium were added to the upper chamber of each transwell (having 8 µm pore size, Costar). DMEM medium, 600 µl, carrying 10% FBS was added into the lower chamber. An incubation of 37ºC was given for 24 h following which the cells from the upper chamber were carefully swabbed with cotton. Meanwhile, those sticking to the lower surface of intervening membrane were ethanol fixed, stained using 0.1% crystal violet. The stained cells were finally photographed under light microscope (×100 magnification) and the relative percentage of cell migration and invasion was estimated using at least five randomly chosen microscopic fields.

Mice *in vivo* tumor xenograft study

The *in vivo* tumorigenesis study was conducted using 4–5-week-old healthy male BALB/c-nude mice. The animal study was approved by the Institutional Animal Care and Experimental Use Committee. For xenograft tumor assay, mice were injected subcutaneously with si-MINCR or si-NC transfected Saos-2 cancer cells and the tumor volumes were determined every 3 days for 3 weeks. At the end of those 3 weeks, the mice were sacrificed; dissection of tumors was made, and their average weight was recorded.

Statistical analysis

Unless stated otherwise, all statistical analyses were performed with the help of SPSS 19.0 statistical software. The data were presented as mean ± standard deviation (S.D.). Student's *t*-test and chi-square test were used for analyzing the statistical differences which were considered to be significant at $P<0.05$.

RESULTS

LncRNA-MINCR is up-regulated in osteosarcoma to enhance cell proliferation

Following RNA isolation and its reverse transcription, the transcript levels of lncRNA-MINCR were analyzed from the osteosarcoma tissue samples and compared with those from the adjacent normal tissues. The cancer tissues were seen to exhibit significant ($P<0.05$) up-regulation of lncRNA-MINCR in comparison to normal tissues (Fig. 1A). The over-expression of lncRNA-MINCR was also found in the osteosarcoma cancer cell lines (HOS, MG-63 and Saos-2) as compared to normal osteoblast cell line, hFOB1.19 (Fig. 1B). To understand the role of lncRNA-MINCR in osteosarcoma, its transcriptional knockdown was carried out through transfection of Saos-2 cancer cells with si-MINCR constructs (Fig. 1C). Saos-2 cells were used because of comparatively higher lncRNA-MINCR expression among all three osteosarcoma cell lines (Fig. 1B). MTT assay was performed to understand the effect of lncRNA-MINCR silencing on Saos-2 cancer cell proliferation. It was shown

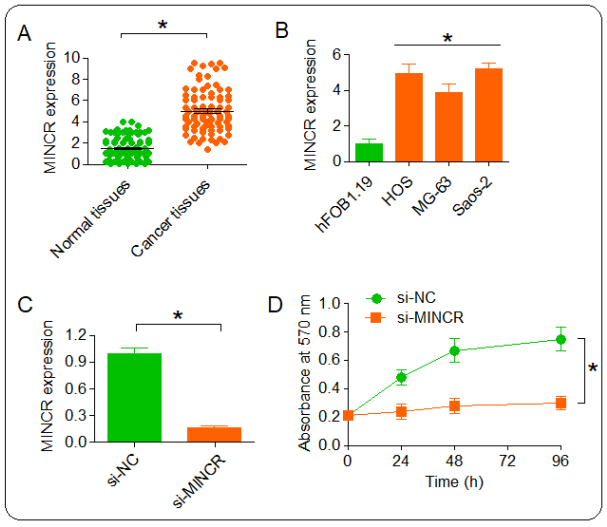

Figure 1. LncRNA-MINCR controls the growth of osteosarcoma cells.
(**A**) Expression analysis of lncRNA-MINCR form osteosarcoma and normal adjacent tissues (**B**) relative expression of lncRNA-MINCR in osteosarcoma cell lines (HOS, MG-63 and Saos-2) with reference to normal human osteoblast cell line (hFOB1.19) (**C**) expression analysis of lncRNA-MINCR from Saos-2 cells transfected with si-MINCR or si-NC (**D**) MTT assay for estimation of proliferation of Saos-2 cells transfected with si-MINCR or si-NC. The experiments were performed in triplicate and expressed as mean ± S.D. (*$P<0.05$)

that osteosarcoma cells proliferated at much lower rates under transcriptional knockdown of lncRNA-MINCR in comparison to the negative control cells (Fig. 1D). The results are thus indicative that lncRNA-MINCR controls the growth and proliferation of osteosarcoma cells, and its up-regulation might be responsible for enhancing their proliferative potential.

LncRNA-MINCR silencing induced osteosarcoma cell apoptosis to reduce cell viability

The EdU staining assay was performed to investigate the role of lncRNA-MINCR in regulating the proliferation of the osteosarcoma cells. It was noticed that relatively lesser number of Saos-2 osteosarcoma cells were detected with positive EdU staining under down-regulation of lncRNA-MINCR as compared to the negative control transfection (Fig. 2A). It indicates that transcriptional repression of lncRNA-MINCR negatively inhibited the osteosarcoma cancer cell proliferation. Consistently, the colony formation was also significantly ($P<0.05$) inhibited under lncRNA-MINCR down-regulation (Fig. 2B). To unveil the underlying reason for inhibition of osteosarcoma cell growth under lncRNA-MINCR knockdown, flow cytometric study was performed for analyzing the level of cell apoptosis. Results showed that the percentage of both early and late apoptotic cells was significantly ($P<0.05$) higher for Saos-2 cancer cells repressing lncRNA-MINCR as compared to negative control cells (Fig. 2C). The western blotting of apoptosis marker proteins showed that Bax expression increased and that of Bcl-2, decreased (Fig. 2D). Together, the results suggest that lncRNA-MINCR is actively involved in growth and viability regulation of osteosarcoma cells and exercises control on the crucial pathway of apoptotic cell death.

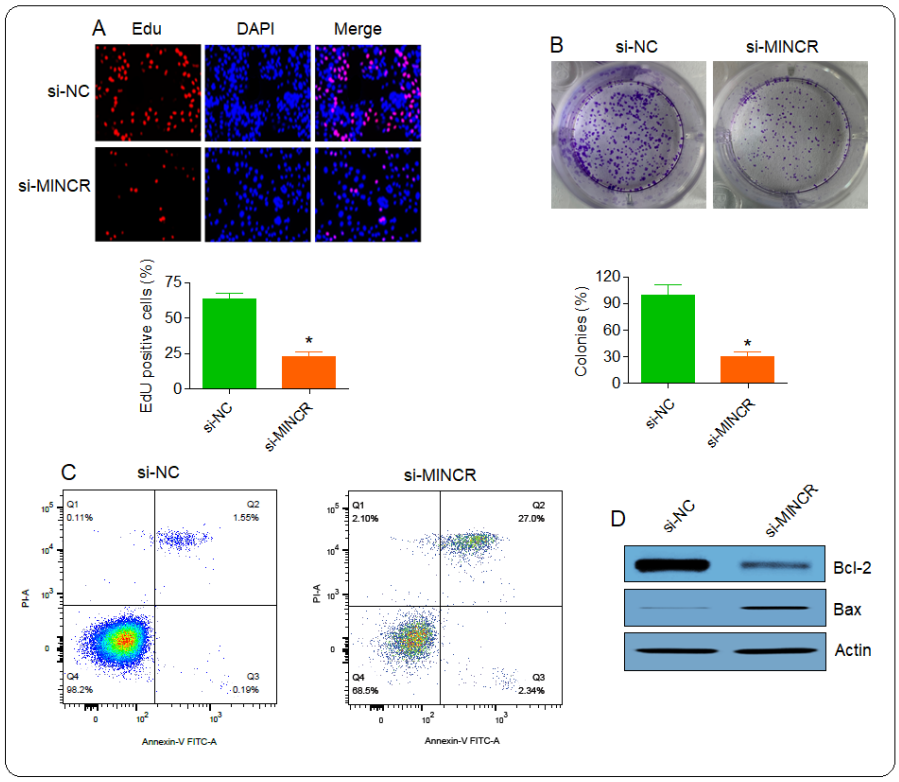

Figure 2. LncRNA-MINCR silencing declines osteosarcoma cell viability through apoptotic induction.
(**A**) EdU assay for the analysis of proliferative viability of Saos-2 cells transfected with si-MINCR or si-NC (**B**) clonogenic assay for the analysis of colony forming potential of Saos-2 cells transfected with si-MINCR or si-NC (**C**) flow cytometric study of apoptosis of Saos-2 cells transfected with si-MINCR or si-NC (**D**) expression analysis of Bax and Bcl-2 proteins from Saos-2 cells transfected with si-MINCR or si-NC. The experiments were performed in triplicate and expressed as mean ± S.D. (**P*<0.05)

LncRNA-MINCR repression in osteosarcoma cells limited migration and invasion and exhibited negative effect on their EMT

With the aim of assessing the molecular potential of lncRNA-MINCR in regulating the migration and invasion of osteosarcoma cells, functional knockdown of lncRNA-MINCR was induced in Saos-2 cells. The rate of migration of Saos-2 cancer cells was significantly (P<0.05) inhibited by lncRNA-MINCR repression (Fig. 3A). In a similar fashion, the invasion of cancer cells was also significantly (P<0.05) minimized under lncRNA-MINCR

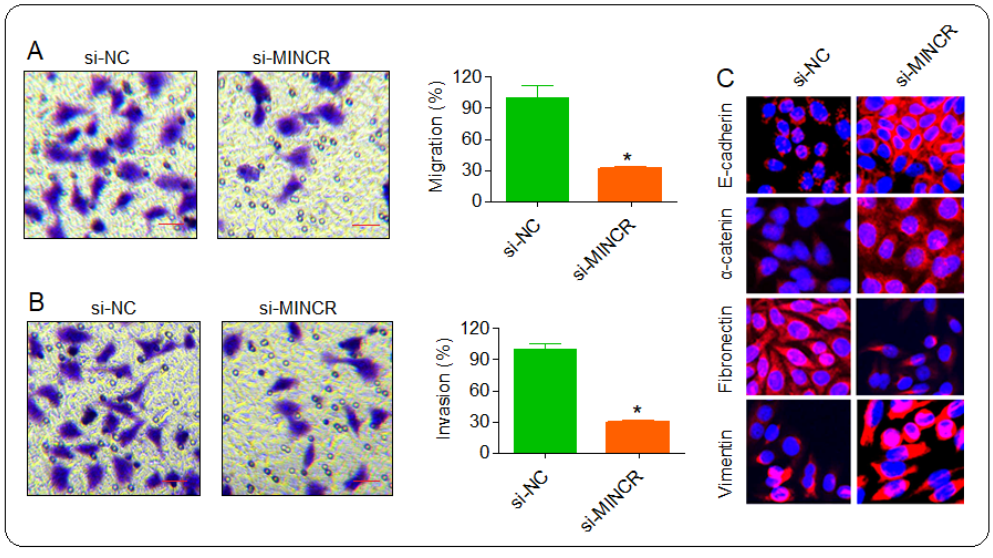

Figure 3. LncRNA-MINCR regulates metastasis of osteosarcoma cells.
(**A**) Transwell assay for analysis of migration of Saos-2 cells transfected with si-MINCR or si-NC (**B**) transwell assay for analysis of invasion of Saos-2 cells transfected with si-MINCR or si-NC (**C**) immune-fluorescence of epithelial (E-cadherin and α-catenin) and mesenchymal (Fibronectin and Vimentin) molecular markers form Saos-2 cells transfected with si-MINCR or si-NC. The experiments were performed in triplicate and expressed as mean ± S.D. (**P*<0.05)

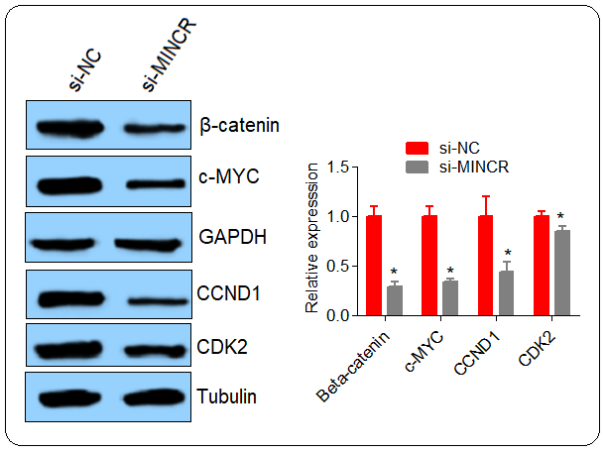

Figure 4. Silencing of lncRNA-MINCR minimized Wnt/β-catenin signal in osteosarcoma cells.
Western blotting of signaling components of Wnt/β-catenin signaling pathway from Saos-2 cells transfected with si-MINCR or si-NC. The experiments were performed in triplicate and expressed as mean ± S.D. (*P<0.05)

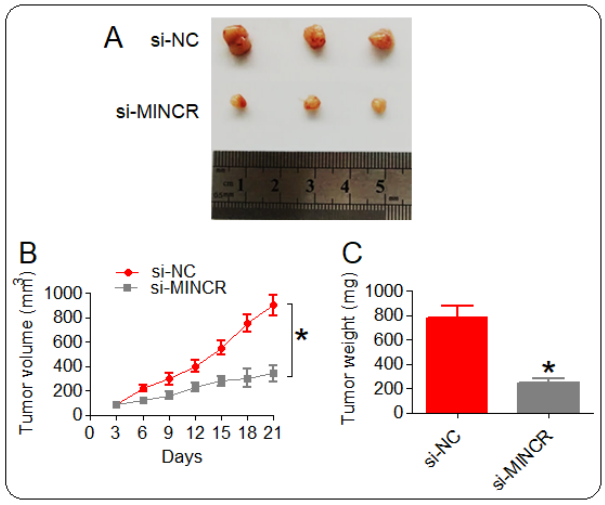

Figure 5. LncRNA-MINCR repression hampered *in vivo* mice xenograft tumorogenesis.
(**A**) Comparison of size of tumors rescued from mice inducted for tumorogenesis with Saos-2 cells transfected with si-MINCR or si-NC (**B**) comparison of average tumor volume under tumorogenesis induction using Saos-2 cells transfected with si-MINCR or si-NC (**C**) comparison of average weight of xenograft tumors rescued from mice inducted for tumorogenesis with Saos-2 cells transfected with si-MINCR or si-NC. The experiments were performed in triplicate and expressed as mean ± S.D. (*P<0.05)

down-regulation (Fig. 3B). The immune-fluorescence of molecular markers of EMT indicated that silencing of lncRNA-MINCR increased the expression of epithelial markers (E-cadherin and α-catenin) while as the expression levels of mesenchymal markers (Vimentin and Fibronectin) were greatly decreased (Fig. 3C). The results thus specify that lncRNA-MINCR has a key role in regulating the metastasis of osteosarcoma cells and its elevated expression might be one of the molecular factors responsible for aggressiveness of osteosarcoma.

Wnt/β-catenin signal mediates lncRNA-MINCR role in osteosarcoma

To specifically find out whether lncRNA-MINCR regulatory control operates through the cellular signaling pathway of Wnt/β-catenin in osteosarcoma, the western blotting of its different signaling components was performed. Wnt/β-catenin signaling pathway was chosen for its key involvement in human cancer progression, and particularly the process of EMT of cancer cells. The results showed that silencing of lncRNA-MINCR reduced the signaling intensity of Wnt/β-catenin pathway by markedly reducing the expression of vital signaling components (Fig. 4). Hence, it was confirmed that lncRNA-MINCR operates through Wnt/β-catenin signaling cascade to exercise its control over the growth and propagation of human osteosarcoma, highlighting its therapeutic utility.

Knockdown of lncRNA-MINCR inhibited *in vivo* mice tumorigenesis

Whether the observed functional role of lncRNA-MINCR in osteosarcoma deduced through *in vitro* experimentation is in agreement with its *in vivo* regulatory effects, the mice xenograft models of osteosarcoma were constituted. Interestingly, it was found that silencing of lncRNA-MINCR markedly reduced the tumor size (Fig. 5A). The *in vivo* tumor size was also found to be significantly minimized under lncRNA-MINCR down-regulation (Fig. 5B). Similarly, the average weight of xenograft mice tumors was also considerably reduced in mice inducted for tumorigenesis with lncRNA-MINCR repression (Fig. 5C). Taken together, the results further demonstrate the key molecular regulatory control of lncRNA-MINCR in osteosarcoma and indicate its therapeutic potential to aid in devising alternate treatment for human osteosarcoma.

DISCUSSION

Over the course of recent years, much progress has been made in understanding the biological and physiological aspects of human body at molecular level. Pathological studies are being conducted to look into the molecular progression of different human disorders. The researchers have also stressed upon exploring the molecular events linked with onset, growth and propagation of human cancer (Bose *et al.,* 2020). In this regard, the regulatory nodes controlling the cancer hallmarks, like proliferation and metastasis, have achieved considerable attention with the aim to utilize them for therapeutic purposes (Fares *et al.,* 2020). Apart from the molecular characterization of protein coding genes for their role in cancer progression, the non-coding RNAs, long-coding RNAs (lncRNAs) and micro-RNAs (miRs) have also attained much research attention for their comparatively trifling characterization and involvement in multiple cancer-related signaling pathways (Serviss *et al.,* 2014). The transcriptional repression or up-regulation of lncRNAs has been reported to be linked with human cancers (Yan *et al.,* 2015; Huarte, 2015). Over-expression of MYC-induced long non-coding RNA (lncRNA-MINCR) has been shown to be associated with a number of human cancers like hepatocellular carcinoma and lung cancer (Lian *et al.,* 2019; Wang *et al.,* 2019). Previously, lncRNA-MINCR was found to promote the growth and proliferation of glioma (Li *et al.,* 2020). In the present study, lncRNA-MINCR was shown to be highly expressed in osteosarcoma. The silencing of lncRNA-MINCR was shown to reduce the osteosarcoma cell growth through induction of apoptotic cell death. The regulation of cancer cell apoptosis by lncRNA-MINCR has been already shown and in this study, it was found that silencing

of lncRNA-MINCR inhibited the proliferation of non-small lung cancer cells through the induction of apoptosis and cell cycle arrest (Chen *et al.,* 2019). Invasiveness of cancer cells is one of the key features of cancerous malignancy (Yilmaz *et al.,* 2007). The epithelial-to-mesenchymal transition (EMT) process is one of the crucial events mediating the cancer cell invasion, the phenomenon called metastasis, aggravating the cancer aggression (Vaquero *et al.,* 2017). During EMT, the cancer cells undergo molecular and cytological alterations to become highly motile enabling them to leave their primary sites to invade their surroundings. The results of the present study indicated that lncRNA-MINCR regulates migration and invasion of osteosarcoma cells, as has been reported for its regulatory role in hepatocellular carcinoma (Cao *et al.,* 2018). The lncRNA-MINCR transcriptional silencing was shown to restrict the EMT of osteosarcoma cancer cells. The Wnt/β-catenin signaling pathway is reported to assist in the EMT process of cancer cells and enhances their metastatic potential (Jiang *et al.,* 2007). Herein, we deduced that lncRNA-MINCR operated through Wnt/β-catenin signal to regulate the EMT and therefore the metastasis of osteosarcoma cells. Similar mechanism of action of lncRNA-MINCR has been reported in oral squamous cell carcinoma (Lyu *et al.,* 2019). In sum, the study was an attempt to understand the regulatory control exercised by lncRNA-MINCR in osteosarcoma, and from both *in vitro* and *in vivo* experimentation, it was confirmed to control the osteosarcoma growth and progression through Wnt/β-catenin signaling pathway.

CONCLUSION

Collectively, the results of the current study are suggestive of up-regulation of lncRNA-MINCR in osteosarcoma. The findings that silencing of lncRNA-MINCR inhibited the growth and metastatic potential of osteosarcoma cells and hampered *in vivo* tumorigenesis indicate that lncRNA-MINCR might emerge as a key therapeutic target against human osteosarcoma.

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Authors' contributions

SB, YL and YW designed the protocol of the study. SB, YL, YW, GZ, CL, WX and JC performed the experimental work and collect the data for presented study. SB and YL involve in the statistical analysis. YL supervised the work and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable

Ethics approval and consent to participate

The study was approved by research ethics committee of the institute under approval number FPHT16/2019. Written consent was obtained from the patients before the participation in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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