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MicroRNA-15 suppresses viability, migration and invasion of the human MG-63 osteosarcoma cells *via* inhibition of cyclin dependent kinase 6 (CDK6)

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MicroRNA-15a-3p (miR-15) acts as tumor-suppressor in different human cancers including osteosarcoma. Nonetheless, the molecular function of miR-15 in osteosarcoma *via* suppression of cyclin dependent kinase 6 (CDK6) is yet to be studied. The results showed significant downregulation of miR-15 in osteosarcoma tissues and cell lines. Overexpression of miR-15 inhibited the proliferation and colony formation of the MG-63 osteosarcoma cells *via* induction of apoptosis. Moreover, miR-15 inhibited the migration and invasion of MG-63 osteosarcoma cells. The tumor-suppressive functional role of miR-15 was shown to be exerted *via* suppression of CDK6. The expression of CDK6 was upregulated in osteosarcoma and its silencing could exert growth inhibitory effects on human osteosarcoma cells. However, overexpression of CDK6 could nullify the tumor-suppressive effects of miR-15 on the MG-63 osteosarcoma cells. Taken together, miR-15 negatively regulates growth, migration and invasion of osteosarcoma cells by targeting CDK6 at post-transcriptional level. These findings suggest the therapeutic potential of miR-15/CDK6 in human osteosarcoma.

Keywords: Osteosarcoma, micro-RNA, miR-15, CDK6, epithelial to mesenchymal transition

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Abbreviations: CDK6, cyclin dependent kinase 6; EMT, epithelialto mesenchymal transition; qRT-PCR, quantitative real-time polymerase chainreaction; PI, propidium iodide

INTRODUCTION

Osteosarcoma is highly aggressive and lethal type of bone cancer with mesenchymal origin resulting from the osteoid producing cells (Lindsey *et al*., 2017). This malignancy is comparatively more prevalent among the children and young adolescents (Rojas *et al*., 2021). It is the most dominant type of primary bone cancer and accounting for approximately 10% of the solid tumors in young human individuals below 20 years of age (Mirabello *et al*., 2009). Moreover, the incidence of osteosarcoma is slightly higher in males than females (Mustafa *et al*., 2018). Osteosarcoma treatment generally involves surgery combined with chemotherapy and parallel adjuvant therapeutic procedures (Zhang *et al*., 2018). Although, this disease has an overall 5-year survival rate ranging between 60 to 70%, nevertheless in more than 20% cases, osteosarcoma exhibits metastasis to lung tissues and therefore the 5-year survival rate goes down to less than 20% (Shaikh *et al*., 2016; Tsiambas *et al*., 2017). With the advancement of molecular biology, several genetic alterations and chromosomal abnormalities have been shown to be associated with the pathogenesis of osteosarcoma (Czarnecka *et al*., 2020). Characterization of such molecular irregularities might thus be fruitful in devising more efficient therapeutic measures against this deadly disorder.

Small non-coding RNAs, in particular the microRNAs (miRs), have been shown to act as potential prognostic and therapeutic targets in osteosarcoma (Czarnecka *et al*., 2020; Evola *et al*., 2017; Zhao *et al*., 2019). For instance, miR-210-5p has recently been reported for its oncogenic role in osteosarcoma growth and epithelial to mesenchymal transition (EMT) *via* PIK3R5 (Liu *et al*., 2020). In addition, the regulation of NOTCH1 by miR-139 has been shown to be crucial for inhibition of osteosarcoma progression by resveratrol (Xiao *et al*., 2020). MicroRNA-15a-3p (now onwards referred as miR-15), since its identification in chronic lymphocytic leukemia, has been deduced to act as tumor-suppressor in different human cancers like ovarian cancer, breast cancer, pancreatic cancer and non-small cell lung cancer (Calin *et al*., 2002; Bhattacharya *et al*., 2009; Luo *et al*., 2013; Zhang *et al*., 2010; Bandi *et al*., 2009). The negative regulatory role of miR-15 in osteosarcoma growth and proliferation through multiple regulatory targets has also been reflected by a number of studies (Cai *et al*., 2012; Tian *et al*., 2015; Leng *et al*., 2018; Shi *et al*., 2018). Additionally, the oncogenic role of cyclin dependent kinase 6 (CDK6) has been reported in osteosarcoma (Zhu *et al*., 2016; Yuan *et al*., 2017). However, the characterization of miR-15 functional role via cyclin dependent kinase 6 (CDK6) in osteosarcoma is yet to be studied. Against this backdrop, the present study was designed to study the role of miR-15/CDK6 axis in osteosarcoma proliferation and epithelial to mesenchymal transition.

MATERIALS AND METHODS

Human tissues

35 pairs of osteosarcoma and normal adjacent osteoid clinical tissue specimens were collected from the Cangzhou People's Hospital, Cangzhou, Hebei, China. The study procedures involving human tissues were approved by the Institutional Ethics Committee. The tissue collection was made prior to application of chemotherapy and all the participants were informed in advance and written consents were obtained from them. Sterilized cold phosphate-buffered saline (PBS) was used for washing the tissues collected which were then frozen using liq-

uid N_2 and –80°C temperature conditions were used for their long-term storage.

Cell lines

Four osteosarcoma cell lines (HOS, 143B, MG-63 and U2OS) as well as the normal human foetal osteoblastic cell line (hFOB 1.19) were procured from ATCC (American Type Culture Collection, Manassas, VA). RPMI 1640 (Sigma-Aldrich, MO, USA) carrying 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 1% penicillin/streptomycin (Sigma-Aldrich) as supplementation was used for propagation and maintenance of cell lines at 37°C with 5% CO_2 in humidified CO_2 incubators.

Cell transfection

The MG-63 osteosarcoma cell line was transfected with miR-15 mimics (25 nM) for miR-15 overexpression with miR-NC as negative control. For the silencing of CDK6, small interfering RNA targeting CDK6 (si-CDK6) was used while si-NC served as a negative control. The miR-15 mimics/miR-NC and si-CDK6/si-NC were ordered from GenePharma (Shanghai, China). The overexpression vector construct of CDK6 (pcDNA-CDK6) was synthesized by RiboBio, Gunagzhou China. The pcDNA3.1 empty vector served as a corresponding negative control. Cell line transfection was performed with the help of Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

Expression analysis

Total RNA from tissue and cell lines was extracted using the RNeasy Mini Kit (Qiagen, Hiden, Germany) following the manufacturer's procedure. RevertAid First starnd cDNA synthesis kit (Thermo Fisher Scientific) was used for the generation of cDNA from isolated RNA. The transcript levels of miR-15 and CDK6 were analyzed with quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR was performed using Power SYBRTM Green PCR Master Mix (Thermo Fisher Scientific). The cycling conditions were: 3 min at 95°C, 45 cycles of 30 sec at 95°C and 60 sec at 60°C. GAPDH, snRNA U6 and Rn18s were used for normalizing the expression levels of target genes which were quantified with $2^{-\Delta\Delta Ct}$ method. The sequences of the primers used in the present study are mentioned in Table 1.

Table 1. Nucleotide sequences of the primers used in this study

Gene	Direction	Sequence (5′ to 3′)
miR-15	Forward	CGGCTAGCAGCACATAAT
	Reverse	GTGCAGGGTCCGAGGT-
CDK6	Forward	GCACAGTGTCACGAACAG
	Reverse	CCTCGGAGAAGCTGAAAC
Vimentin	Forward	TGTCCAAATCGATGTGGATGTTTC
	Reverse	TTGTACCATTCTTCTGCCTCCTG
E-Cadherin	Forward	AGAGGGTCACCGCGTCTATG
	Reverse	CTCACAGGTGCTTTGCAGTT
N-Cadherin	Forward	TGAAACGGCGGGATAAAGAG
	Reverse	GGCTCCACAGTATCTGGTTG
Snail	Forward	TGCCCTCAAGATGCACATCCGA
	Reverse	GGGACAGGAGAAGGGCTTCTC
U6	Forward	ACG AATTGC GTG TCATCCT
	Reverse	ACGAATTTG CGT GTCATC CT
GAPDH	Forward	CAACTTTGGTATCGTGGA
	Reverse	CCATCACGCCACAGTTTC
Rn18s	Forward	GTAACCCGTTGAACCCCATT
	Reverse	CCATCCAATCGGTAGTAGC

CCK-8 proliferation assay

Cell counting kit-8 (CCK-8, Sigma-Aldrich) was used for the determination of cell viability. In brief, the transiently transfected MG-63 cancer cells were placed at a density of 10^3 cells per well into a 96-well plate. After culturing the cells for 0, 12, 24, 48, 72 or 96 h at 37°C, 10 μL of CCK-8 solution was added to each well and cells were again incubated at 37°C for 3 h. Finally, the absorbance was obtained for each well at 450 nm with the help of a micro-plate spectrophotometer.

Colony formation assay

For the analysis of colony formation, 5×10^3 transfected MG-63 cells were plated into each well of a 6-well plate. The cells were cultured for 16 days at 37°C till colony formation. At this stage, PBS was used for washing the colonies which were then fixed with 70% ethanol and stained with 0.25% crystal violet for 35 min. Finally, the colonies were photographed and counted under microscope.

Annexin V/PI staining assay

A total of 3×10^4 transfected MG-63 cells/well were plated into 96-well plates and were cultured for 24 h at 37°C. The cells were subsequently stained with propidium iodide (PI) and Annexin Vfluroescein isothiocyanate (FITC) kit (Multisciences (Lianke) Biotech Co., Ltd.) and analyzed by flow cytometry. The percentage of live cells, apoptotic cells and dead cells were analyzed using FlowJo software (version 10; FlowJo LLC).

Migration and invasion assays

Transwell chambers (Corning, NY, USA) pre-coated without and with Matrigel (bd Biosciences, Franklin Lakes, NJ, USA) were employed for the analysis of migration and invasion of transfected MG-63 osteosarcoma cells, respectively. Briefly, 2×10^5 transfected cells were seeded into an upper chamber of a 24-well Transwell insert plate. Lower chamber received 750 μL of serum-free culture medium only. After 24 h incubation at 37°C, cells from the upper chamber were swabbed away while those migrating/invading into the lower chamber by passing through the membrane were PBS washed, methanol fixed, crystal violet stained and imagined and counted under an inverted light microscope (Olympus, Tokyo, Japan).

Western blotting

The extraction of total proteins from the transfected MG-63 cells was performed with the help of RIPA lysis buffer (Thermo Fisher Scientific) containing protease inhibitors cocktail (KeyGen Biotech). The protein concentrations were measured with a BCA Protein Assay kit (Beyotime Biotechnology). Around 40 μg protein from each sample were subjected to electrophoresis on

10-12% sodium dodecyl sulfate-polyacrylamide (SGS-PAGE) gels. Proteins separated were then transferred to PVDF membranes. Skimmed milk (5%) was used for blocking the membranes for 1 h at room temperature. The PVDF membranes were treated with primary antibodies against CDK6 (1:1000, Cell Signaling Technology, Danvers, MA, USA). This was followed by the incubation of PVDF membranes with secondary antibodies conjugated with horseradish peroxidase. The specific protein bands were visualized with an Pierce™ ECL kit (Thermo Fisher Scientific). β-actin was used as a reference protein.

MiR target prediction and dual luciferase reporter assay

Potential targets of miR-15 were predicted with TargetScan7.0 (http://www.targetscan.org) online software. The synthetic luciferase reporter plasmids of 3'-UTR segment of CDK6 gene with wild type (WT, pGL3-CDK6-WT) or mutant miR-15 binding site (MUT, pGL3-CDK6-MUT) were obtained from GenePharma (Shanghai, China). Briefly, MG-63 cells were seeded into 24-well plates with a density of 1.5×10^5/well and co-transfected with pGL3-CDK6-WT/MUT (5 ng) luciferase and miR-15 mimics or miR-NC (10 nmol final concentration) into MG-63 osteosarcoma cells using Lipofectamine 2000 transfection reagent and cells were harvested in logarithmic growth phase after 48 h of transfection. Finally, the dual-luciferase reporter system (Promega, Madison, WI, USA) was used for the analysis of luciferase activity of the transfected cells. The host-cell luciferase activities were normalized to *Renilla luciferase activity.*

Statistical analysis

The analysis of statistical data was performed using the GraphPad Prism 7.0 offline software (San Diego Inc, CA. USA). Results were presented as mean ± standard deviation (SD). Student's t-test (two-tailed) was carried out to make comparisons between two treatment groups. $P<0.05$ was taken to represent a statistically significant difference.

RESULTS

MiR-15 is downregulated in osteosarcoma regulates its proliferation

The results of the qRT-PCR showed that osteosarcoma tissues express significantly ($P=0.03$) lower miR-15 transcript levels than the normal adjacent tissues (Fig. 1A). Further, relative to hFOB 1.19, the normal human foetal osteoblastic cell line, the osteosarcoma cells (HOS, 143B, MG-63 and U2OS) were found to exhibit significantly ($P<0.05$) lower miR-15 transcript levels (Fig. 1B). Among osteosarcoma cell lines, MG-63 cells exhibit the lowest transcript levels of miR-15 and was thus used in further experiments. To proceed with the characterization of miR-15 role in osteosarcoma, MG-63 cells were transfected with miR-15 mimics to induce miR-15 overexpression. The RT-PCR showed more than 7-fold upregulation of miR-15 in miR-15 mimics transfected MG-63 cells (Fig. 1C). The overexpression of miR-15 in MG-63 cancer cells was shown to significantly inhibit ($P=0.02$) their viability relative to the negative control cells at different time intervals (Fig. 1D). The inhibition of viability was found to be due to the induction of apoptosis. The percentage of early and late apoptosis increased from 0.71% and 0.21% in miR-NC

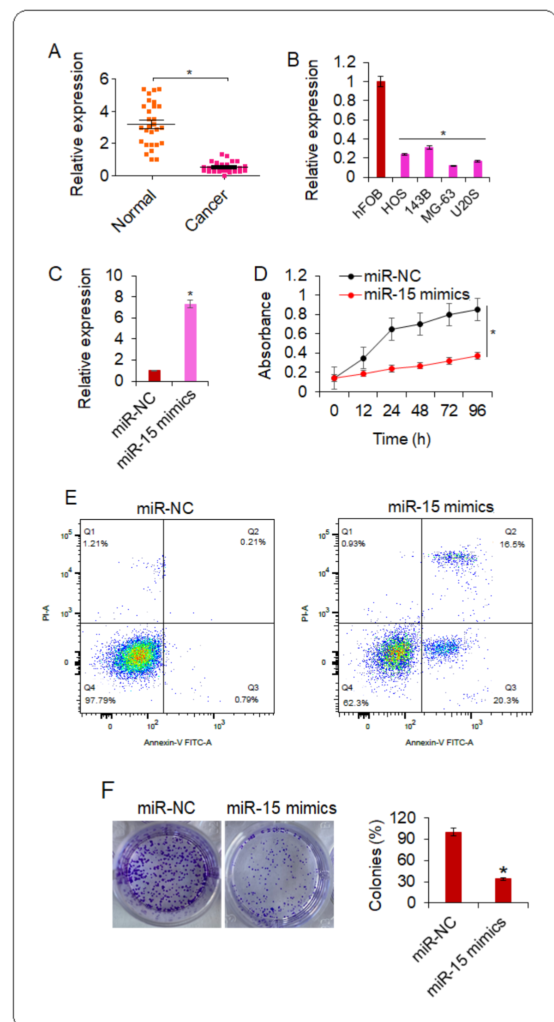

Figure 1. MiR-15 is repressed in osteosarcoma and its overexpression inhibits cell viability.
(**A**) Osteosarcoma tissues express significantly lower miR-15 transcripts as compared to the normal matching tissues (**B**) miR-15 has significantly lower expression levels in HOS, 143B, MG-63 and U2OS osteosarcoma cell lines as compared to normal hFOB 1.19 osteoblast cells (**C**) miR-15 mimics transfection significantly enhanced the expression of miR-15 in MG-63 cancer cells (**D**) miR-15 overexpressing MG-63 cells show markedly lower viability than negative control transfected cells (**E**) Annexin V/PI staining showing the percentage of apoptotic si-NC or miR-15 mimics transfected MG-63 cells (**F**) colony formation assay showing colony formation of miR-NC or miR-15 mimics transfected MG-63 cells. The experiments were performed in triplicates and $*P<0.05$ is indicative of statistically significant difference between the values of two groups.

transfected to 20.3% and 16.5% in miR-15 mimics transfected MG-63 cells (Fig. 1E). In addition, the miR-15 overexpression decreased the colony formation of the MG-63 cancer cells by more than 60% (Fig. 1F). These findings suggest the tumor-suppressive role of miR-15 in osteosarcoma.

MiR-15 inhibits the migration, invasion of MG-63 cells

The effects of miR-15 on the migration and invasion were assessed by transwell assays. The results showed that overexpression of miR-15 lead to a significant ($P=0.03$) decrease in the migration of the MG-63 cells. The migration was inhibited by 60% (Fig. 2A). Similarly, the invasion of the MG-63 cells was inhibited by 65%

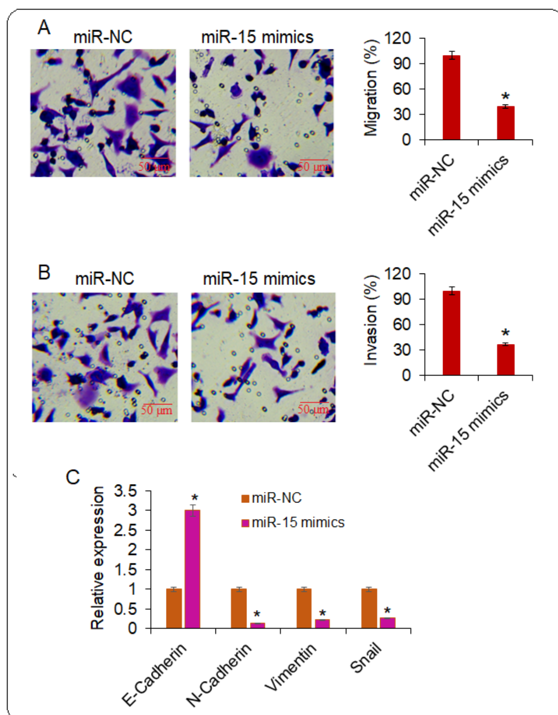

Figure 2. MiR-15 inhibits mobility of osteosarcoma cells.
(**A**) miR-15 overexpressing MG-63 cells show significantly lower migration than negative control transfected cells (**B**) miR-15 overexpressing MG-63 cells show significantly lower invasion than negative control transfected cells (**C**) miR-15 overexpressing MG-63 cells show significantly increased expression of E-cadherin and decreased expression of N-cadherin, Vimentin and Snail. The experiments were performed in triplicates and *$P<0.05$ is indicative of statistically significant difference between the values of two groups..

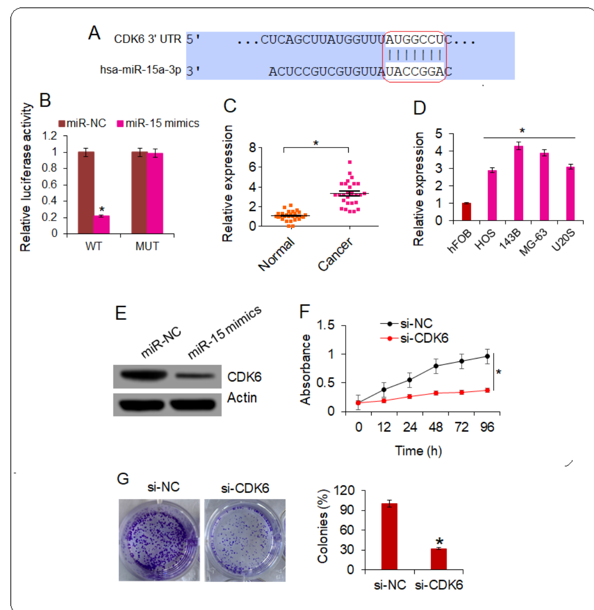

Figure 3. CDK6 acts as the molecular target of miR-15 in osteosarcoma
(**A**) Prediction of miR-15 binding site in 3′-UTR of CDK6 by in silico analysis (**B**) dual luciferase assay confirmed miR-15 binding with 3′-UTR of CDK6 (**C**) osteosarcoma tissues express significantly higher CDK6 transcripts as compared to the normal matching tissues (**D**) CDK6 gene has significantly higher expression levels in HOS, 143B, MG-63 and U2OS osteosarcoma cell lines as compared to normal hFOB osteoblast cells (**E**) overexpression of miR-15 suppresses the expression of CDK6 as depicted by western blotting (**F**) CDK6 downregulating MG-63 cells show markedly lower viability than negative control transfected cells (**G**) silencing of CDK6 inhibits the colony formation of the MG-63 cells. The experiments were performed in triplicates and *$P<0.05$ is indicative of statistically significant difference between the values of two groups.

(Fig. 2B). The qRT-PCR was used to study the effect of miR-15 on the expression of epithelial and mesenchymal markers in MG-63 cancer cells. The results showed that E-cadherin (epithelial marker) levels significantly increased ($P=0.02$) while the expression levels of N-cadherin, Snail and Vimentin (Mesenchymal markers) were significantly decreased ($P=0.02$) by miR-15 overexpression (Fig. 2C). This indicates that overexpression of miR-15 inhibited the migration and invasion of MG-63 osteosarcoma cells, *in vitro.*

MiR-15 exerts its tumor-suppressive effects *via* CDK6

The *in-silico* analysis showed that CDK6 acts as the potential regulatory target of miR-15 and predicted that miR-15 interacts with a specific binding site in 3'-UTR of CDK6 gene (Fig. 3A). To confirm this, the luciferase reporter plasmid of CDK6 3'-UTR with wild type (WT) or mutant (MUT) binding site was co-transfected with miR-15 mimics or miR-NC into MG-63 cells. Dual luciferase reporter assay showed that luciferase activity of host cells was significantly declined ($P=0.02$) only when UTR segment with wild type miR-15 binding site was used, confirming the specific interaction of miR-15 with 3'-UTR of CDK6 (Fig. 3B). Also, the osteosarcoma tissues and cell lines expectedly showed significantly higher ($P<0.05$) CDK6 gene expression relative to the normal tissues and cells (Fig. 3C and 3D). Overexpression of miR-15 suppressed the expression of CDK6 (Fig. 4E). Whether miR-15 exercises its regulatory role in osteosarcoma via suppression of CDK6 protein levels, CDK6 was silenced in MG-63 cancer cells by transfecting them with si-CDK6 while si-NC transfected cells served as a negative control. Downregulation of CDK6 significantly ($P=0.03$) inhibited the proliferation and colony formation of MG-63 cells (Fig. 3F and 3G). Additionally, the effects of CDK6 silencing on the migration and invasion as well as the expression of epithelial to mesenchymal transition markers in MG-63 cells were similar to that of miR-15 overexpression (Fig. 4A–C).

Interestingly, the overexpression of CDK6 mitigated the inhibitory effects miR-15 overexpression on proliferation of MG-63 cells which was also evident from the western blots showing the expression of CDK6 cells (Fig. 5A and 5B). Moreover, CDK6 also rescued the inhibitory effects of miR-15 overexpression on the migration and invasion of MG-63 cells (Fig. 5C–D) suggesting that CDK6 acts as the mediator of the miR-15 functional role in osteosarcoma.

DISCUSSION

Molecular irregularities including aberrant expression of miRs have been shown to profoundly affect the tumorigenesis of human osteosarcoma (Czarnecka *et al.*, 2020; Evola *et al.*, 2017; Zhao *et al.*, 2019; Liu *et al.*, 2020). There are growing research evidence that miRs might emerge as prognostic markers and key therapeutic targets against osteosarcoma (Xiao *et al.*, 2020). Different miRs have been shown to act as oncogenes or tumor-suppressors in osteosarcoma to regulate its growth and progression (Jin *et al.*, 2020; Bazavar *et al.*, 2020). MiR-15 known for its tumor-suppressive regulatory function in number of human

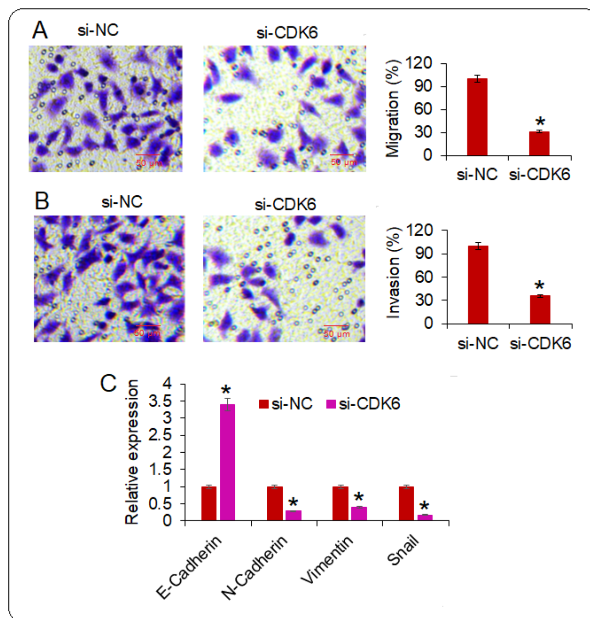

Figure 4. CDK6 silencing reduces mobility of MG-63 osteosarcoma cells

(**A**) CDK6 downregulating MG-63 cells show significantly lower migration than negative control transfected cells (**B**) CDK6 downregulating MG-63 cells show significantly lower invasion than negative control transfected cells (**C**) silencing of CDK6 in MG-63 cells significantly increased the expression of epithelial marker protein (E-cadherin) and significantly decreased the expression levels of mesenchymal marker proteins (N-cadherin, Snail and Vimentin). The experiments were performed in triplicates and *$P<0.05$ is indicative of statistically significant difference between the values of two groups.

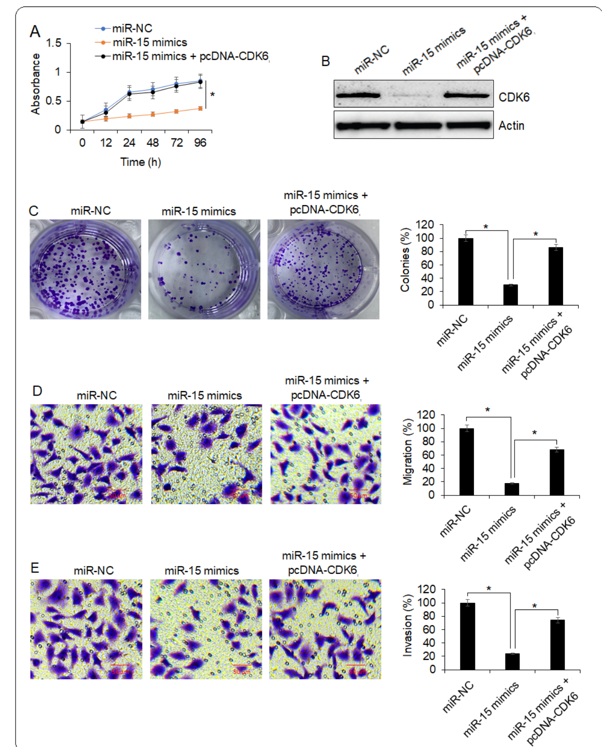

Figure 5. CDK6 recues the tumor-suppressive effects of miR-15 on osteosarcoma cells

(**A**) Cell viability of miR-NC, miR-15 or miR-15 mimics + pcDNA-CDK6 transfected MG-63 cells (**B**) Expression of CDK6 protein in miR-NC, miR-15 or miR-15 mimics + pcDNA-CDK6 transfected MG-63 cells (**C**) colony formation of miR-NC, miR-15 or miR-15 mimics + pcDNA-CDK6 transfected MG-63 cells (**D**) migration of miR-NC, miR-15 or miR-15 mimics + pcDNA-CDK6 transfected MG-63 cells (**E**) invasion of miR-NC, miR-15 or miR-15 mimics + pcDNA-CDK6 transfected MG-63 cells. The experiments were performed in triplicates and *$P<0.05$ is indicative of statistically significant difference between the values of two groups.

cancers has been elucidated to negatively regulate diverse hall marks of human osteosarcoma (Cai *et al.*, 2012; Tian *et al.*, 2015). The present study aimed at the further exploration of functional aspects and mechanism of action of miR-15 in osteosarcoma. Osteosarcoma tissues and cell lines were shown to express significantly lower transcript levels of miR-15 suggesting its possible regulatory involvement in osteosarcoma tumorigenesis. To confirm the same, miR-15 was overexpressed in osteosarcoma cells. Interestingly, the overexpression of miR-15 in osteosarcoma cells limited their growth and proliferation, *in vitro*, which is consistent with the previous reports (Leng *et al.,* 2018; Shi *et al.,* 2018). Moreover, the miR-15 overexpressing osteosarcoma cells exhibited strikingly lower migration and invasion rates which were suggestive of the anti-metastatic molecular function of miR-15 in osteosarcoma. Similar observations have been made by the contemporary researchers regarding the role of miR-15 in cancer (Tian *et al.,* 2015; Shi *et al.,* 2018). Guo and others (Guo *et al.,* 2014) in 2014 revealed that miR-15 inhibits the epithelial to mesenchymal transition of pancreatic cancer cells. Increase in E-cadherin (epithelial marker) expression levels and decrease in the expression of N-cadherin, Snail and Vimentin proteins (mesenchymal markers) by miR-15 overexpression in osteosarcoma indicates that miR-15 negatively regulates EMT in osteosarcoma, which is further indicative of its anti-metastatic regulatory potential. In order to investigate the mechanism of action of miR-15 in osteosarcoma *in silico* analysis was used to specifically predict the potential regulatory target of miR-15. Although miR-15 has been reported to target CCND1 in osteosarcoma to induce apoptosis and cell cycle arrest (Cai *et al.,* 2012), in the present study we, for the first time, studied cyclin-dependent kinase (CDK6) as a regulatory target of miR-15 in osteosarcoma. The results showed that miR-15 inhibits the expression of CDK6 post-transcriptionally to exert its tumor-suppressive role in osteosarcoma. These findings are in confirmation with previous studies wherein CDK6 has been shown to promote the growth of esophageal squamous cell carcinoma (Baba *et al.,* 2014) and lymphoblastic leukemia (Rodriguez-Otero *et al.,* 2011). The repression of miR-15 transcript levels might be one of the crucial molecular factors responsible for elevation of CDK6 protein levels in osteosarcoma. The resulting overexpression of CDK6 protein might be involved in positively regulating the growth and metastasis of osteosarcoma. Taken together, the results of the present study clarified the regulatory importance of miR-15/CDK6 molecular axis in osteosarcoma and highlighted its therapeutic utility. However, future studies directed at the evaluation of miR-15 in *in vivo* system and evaluating the effects of different drugs on the expression of miR-15 in osteosarcoma are urgently needed.

CONCLUSION

Taken together, the results of the present study are conclusive that miR-15 transcript levels are significantly repressed in osteosarcoma. Overexpression of miR-15 in-

hibits the osteosarcoma cell proliferation, migration and invasion. CDK6 acts as the functional target of miR-15 in osteosarcoma at post-transcriptional level. Therefore miR-15/CDK6 might emerge as a possible therapeutic lead against osteosarcoma in the future.

Declarations

Conflict of interest. The authors declare that there are no conflicts of interest.

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