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Original research paper

Post-transcriptional suppression of SP1 by miR-375 regulates the growth and epithelial-to-mesenchymal transition of human osteosarcoma cells

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ABSTRACT

Osteosarcoma is one of the lethal disorders which predominantly affect children and adolescents. Though osteosarcoma patients' overall survival has increased significantly, the clinical outcomes aren't so admirable. The molecular irregularities emerging during the adolescent stage with active bone growth have been hypothesized as key driving factors of osteosarcoma. Of the various genetic alterations, micro-RNA (miR) dysregulation is emerging as one of the highly frequent anomalies facing human cancers including osteosarcoma. MicroRNA-375 (miR-375) has been implicated in exhibiting significant down-regulation ($p < 0.05$) in osteosarcoma by previous studies. Nevertheless, an in-depth understanding of its molecular role in osteosarcoma and its mechanism of action has not been evaluated to date. The present study showed that osteosarcoma tissues and cell lines express significantly lower ($p < 0.05$) transcript levels of miR-375 and the latter employs tumor-suppressive function against osteosarcoma. The tumor-suppressive effects of miR-375 were shown to be mediated *via* the SP1post-transcriptional targeting mediated G2/M phase cell cycle arrest. Moreover, miR-375 inhibits the migration, invasion and epithelial to mesenchymal (EMT) of osteosarcoma cells. Overall, the study highlights the prognostic value and therapeutic potential of miR-375 in osteosarcoma.

Keywords: osteosarcoma, micro-RNA, miR-375, Sp1 transcription factor, cell cycle arrest

Osteosarcoma is one of the rare human cancers with an overall annual incidence of < 4 per million of the human population at the global level (1). However, it is recognized as one of the dominant adolescent malignant tumors resulting from the neoplastic proliferation of osteoid-producing bone cells (2, 3). The relatively higher probability of the development of

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osteosarcoma in children and adolescents has been stated to result from the irregularities in bone growth, being most rapid at these stages (4). With the introduction and subsequent advancement in adjuvant chemotherapeutic interventions, the overall 5-year survival rate of osteosarcoma has increased sufficiently from around 20 % during most of the twentieth century to more than 65 % of present times (1, 5, 6). Nevertheless, researchers have hypothesized that novel therapeutic measures are urgently needed against metastatic osteosarcoma. Therefore, an in-depth osteosarcoma molecular pathogenesis must be understood with the exploration of key regulatory nodes and associated molecular irregularities (7).

Micro-RNAs (miRs), the class of short regulatory RNAs constituting a significant proportion of the human genome, are involved in the post-transcriptional regulatory mechanics of around 60 % of human protein-coding genes (8, 9). The miRs regulate various biological and physiological processes and mediate the development of several human pathological conditions, including tumorogenesis (10). MiRs are emerging as one the crucial therapeutic molecular factors controlling the growth of human cancers *via* regulation of its important hallmarks (11, 12). There is growing support that miRs might be useful in diagnosing and treating human osteosarcoma (13). MicroRNA-375 (miR-375) has been elucidated to exhibit frequent dysregulation in human cancers. It has been shown that miR-375 is significantly up-regulated in breast cancer (14). On the other hand, gastric cancer is linked with marked down-regulation of miR-375 and the latter employs a tumor-suppressive regulatory function *via* the post-transcriptional targeting of JAK2 (15). Osteosarcoma has also been shown to exhibit miR-375 down-regulation (16, 17, 18). However, the molecular function of miR-375 in osteosarcoma is yet to be fully elucidated.

Earlier, SP1 was identified as a target gene for miR-375 in cervical cancer (19, 20). In the present study, we attempted to reveal the mechanisms of miR-375 underlying the biological behavior of osteosarcoma. We investigated the biological functions of proliferation and invasion/migration in osteosarcoma cells and identified the target gene to explore the possible molecular mechanism involving osteosarcoma development, in the hope of identifying a new predictor for prognosis or a new target for diagnosis and therapy.

EXPERIMENTAL

Chemicals and reagents

Cell lines (HOS, MG-63, Saos-2, SW1353 and U2OS and hFOB1.19 were purchased from the Chinese Academy of Sciences (Beijing, China). DMEM, FBS, TRIzol reagent and Lipofectamine 2000 transfection reagent were procured from Invitrogen (USA). Penicillin/streptomycin and Cell Counting Kit-8 assay were obtained from Sigma-Aldrich (USA). MiR-375 mimics/miR-NC, miR-375 inhibitor/Inhibitor-NC and si-SP1/si-NC were purchased from GenePharma (China). Vector (pcDNA3.1-SP1) was obtained from RiboBio (China). pMirGlo luciferase reporter vector was obtained from Promega. RevertAid First Strand cDNA synthesis kit, RIPA lysis buffer and SYBR Green PCR Master-mix were obtained from Thermo Fisher Scientific (USA). Protease and phosphatase inhibitors were obtained from Roche Applied Science (USA). The BCA assay kit was obtained from the Beyotime Institute of Biotechnology (China). PVDF membrane was purchased from Millipore (Germany).

Human tissues, cell lines and transfection

A total of 34 paired clinical tissue specimens corresponding to osteosarcoma and matching normal tissues were obtained from The First Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang, China, after written consent signing by the participants. The specimens were thrice washed with chilled phosphate-buffered saline (PBS), immediately frozen using liquid N_2, and stored at ultra-low temperatures until experimentation. The study was approved by the Institutional Ethics Committee.

Five different human osteosarcoma cell lines (HOS, MG-63, Saos-2, SW1353 and U2OS) as well as the normal human osteoblast cell line (hFOB1.19) were used for the excremental work. The cell lines were propagated in DMEM supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin. For their maintenance, the cell lines were incubated at 37 °C with 5 % CO_2 in a humidified incubator.

MG-63 cancer cells were transfected with miR-375 mimics for inducing miR-375 overexpression while miR-NC served as the negative control. For miR-375 silencing, the MG-63 cells were transfected with miR-375 inhibitor oligos with Inhibitor-NC as the negative control. si-SP1 oligos were transfected into MG-63 cancer cells for performing SP1 knock--down with si-NC as a respective negative control. Lipofectamine 2000 transfection reagent was used for performing the transfection of cancer cells following the manufacturer's instructions. The cells were collected after 48 h of transfection to be used for subsequent experiments.

RNA isolation and qRT-PCR

Extraction of total RNA from the cells and tissues was performed by treating them with TRIzol reagent as per the manufacturer's protocol. RevertAid First Strand cDNA synthesis kit was used for the reverse transcription of RNA isolated. Then, the quantitative real-time polymerase chain reaction (RT-qPCR) was performed on Bio-Rad CFX96 real-time PCR system using SYBR Green PCR Master-mix for analyzing the expression of miR-375, SP1, E-cadherin, α-catenin, fibronectin and vimentin. The U6 snRNA and GADPH were used as an endogenous control for miRNA and mRNA respectively. The primer sequences used were:- miR-375, F: 5′-GGCTCTAGAGGGGACGAAGC-3′ and R: 5′-GGCAAGCTTTTTCCACACCTCAGCCTTG-3′; U6 snRNA, F: 5′-CTCGCTTCGGCAGCACA-3′, and R: 5′-AACGCTTCACGAATTTGCGT-3′, SP1, F: 5′-TTGAAAAAGGAGTTGGTGGC-3′ and R: 5′-TGC TGG TTC TGT AAG TTG GG-3′; E-cadherin, F: 5′-CGACAAAGGACAGCCTATTT-3′ and R: 5′-AGTTGGGAAATGTGAGCAAT-3′; α-catenin, F: 5′-CAACCCTTGTAAACACCAAT-3′ and R: 5′-ACTGAACCTGACCGTACACCTTCTCCAAGAAATTCTCA-3′; fibronectin, F: 5′-CCACCCCCATAAGGCATAGG-3′ and R: 5′-GTAGGGGTCAAAGCACGAGTCATC-3′; vimentin, F: 5′-TGTCCAAATCGATGTGGATGTTTC-3′ and 5′-TTGTACCATTCTTCTGCCTCCTG-3′, GADPH, F: 5′-GTCTCCTCTGACTTCAACAGCG-3′ and R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′. The $2^{-\Delta\Delta Ct}$ method was used for the relative quantitation of miRNA and mRNA expression.

CCK-8 proliferation and clonogenic assays

Cell Counting Kit-8 assay (CCK-8) was performed to examine the proliferation of transfected cells. Briefly, 2.5×10^5 stably transfected cancer cells were inoculated into each well of 96-well plates. After culturing for 0 h, 1 day, 2 days, 3 days or 4 days, the growth

medium was replaced with fresh DMEM carrying 10 % CCK-8 solution. The cells were then incubated for 4 h at 37 °C. Finally, the absorbance was read at 450 nm with the help of a microplate spectrophotometer (PerkinElmer, USA). The absorbance values were plotted to construct the proliferation curves.

Approximately, 10^5 stably transfected cancer cells were grown in a 6-well plate in DMEM with 10 % FBS for 2 weeks at 37 °C. Afterwards, colonies formed were washed thrice with cold phosphate-buffered saline (PBS), methanol fixed for 15 min and subsequently stained with 0.1 % crystal violet for 25 min. Photographs of each well were obtained with the help of a camera (Nikon, Japan).

Bio-informatics and dual-luciferase assay

In silico analysis was performed using the online TargetScan database (http://www.targetscan.org/) for making the prediction of possible targets of miR-375. The target prediction was verified with the help of a dual luciferase reporter assay. Briefly, 3′-UTR of SP1 with wild-type (WT) or mutated (MUT) miR-375 binding site was cloned into the pmirGLO luciferase reporter vector. MG-63 cancer cells were co-transfected with luciferase reporter vector with WT or MUT miR-375 binding site harboringSP1 UTR and miR-375 mimics or miR-NC. The quantitative assessment of luciferase activity was made using the Dual-Luciferase Reporter Assay System (Promega Corporation, USA) as per the manufacturer's recommendations.

Western blotting

Total proteins were isolated from the cells using RIPA lysis and extraction buffer containing 1 % protease and phosphatase inhibitors. BCA assay was performed to determine the total protein concentrations. Approximately, 45 μg proteins were resolved on 10 % SDS-PAGE gels which were subsequently blotted to PVDF membranes. Skimmed milk (5 %) was used to block the membranes for 2 h. The incubation of primary antibodies: SP1 (Cell Signaling Technology #5931, dilution 1:1000) and actin (Cell Signaling Technology #4967, dilution 1:1000) were given to the membranes overnight at 4 °C. This was followed by the incubation of PVDF with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology #7074, dilution 1:2000) for 1 h. Finally, the protein bands were detected with the help of a ChemiDoc XRS$^+$ enhanced chemiluminescence detection system (Bio-Rad Laboratories, Inc.).

Cell cycle analysis

For the analysis of the cell cycle, 10^5 transfected MG-63 cells were plated into each well of 12-well plates and incubated at 37 °C. Afterwards, the cells were centrifuged; cell pellets were washed with PBS and suspended using 250 μL solution with 10 μL each of RNase A and PI. The cell suspension was again incubated at 37 °C for 35 minutes in the dark. The FACS caliber (BD Biosciences) was used for cell cycle analysis.

Cell migration and invasion assays

Transwell assays were used for the analysis of migration and invasion of transfected cells using Transwell chambers (8 μm; Millipore Inc.) without or with Matrigel coating (BD

Biosciences, USA). 500 μL of growth medium containing 10^5 transfected cells were placed into the upper chamber of the transwell plate. The underlying chamber was inoculated with serum-free culture medium with 10 % FBS. The cells were cultured for 24 h at 37 °C. Afterwards, the cells penetrating the membrane were fixed with ethanol (70 %) and stained with crystal violet (0.1 %). The cells were imagined using a light microscope (200× magnification) and manually counted to determine the percent cell migration or invasion.

Statistical analysis

The results were given as the mean ± SD. Statistical analysis of the whole data was performed with SPSS 22.0 software (SPSS, USA). The Student *t*-test was used to analyze the results between the treatment groups. Results were considered to vary significantly where $p < 0.05$.

RESULTS AND DISCUSSION

MiR-375 has significant down-regulation in osteosarcoma and its re-expression declines the osteosarcoma cell growth and viability

The RNA was isolated from paired osteosarcoma and matched paired normal tissue samples to analyze the transcript levels of miR-375 using qRT-PCR. The results showed that osteosarcoma tissues express significantly lower ($p < 0.05$) miR-375 transcripts than the matching normal tissues (Fig. 1a). Further, the expression of miR-375 was assessed from the (HOS, MG-63, Saos-2, SW1353 and U2OS) with reference to the normal human osteoblast cell line (hFOB1.19). All five cancer cell lines showed significantly lower ($p < 0.05$) miR-375 expression in comparison to normal osteoblast cells (Fig. 1b). Among cancer cell lines, MG-63 cancer cell line was shown to express the least miR-375 transcripts and was thus used for further study. MiR-375 was transiently over-expressed in MG-63 cancer cells by transfecting them with miR-375 mimics. Using miR-NC transfected cells as the negative control, approximately 6.5 fold up-regulation was observed in miR-375 mimics transfected MG-63 cells (Fig. 1c). The CCK-8 proliferation and clonogenic assays showed that miR-375 over-expression significantly ($p < 0.05$) inhibits the growth and viability of MG-63 cancer cells, *in vitro* (Figs. 1d,e). For further confirmation, miR-375 inhibitor oligos were transfected into MG-63 cancer cells and significant miR-375 down-regulation ($p < 0.05$, 7.2 fold) was observed with respect to respective inhibitor-NC transfected cells (Fig. 1f). Silencing of miR-375 was shown to significantly enhance ($p < 0.05$) growth and viability, *in vitro* (Figs. 1g,h).

There is growing evidence about the molecular dysregulation and therapeutic utility of micro-RNAs (miRs) in human cancer (11, 21). The group of RNAs has been shown to regulate a number of crucial target genes which are involved in the growth, proliferation and metastasis of human cancers (22). Moreover, recent research data indicate that several miRs exhibit abnormal expression patterns in osteosarcoma and profoundly affect osteosarcoma tumorigenesis (23). As per a few research reports, microRNA-375 (miR-375) has been shown to display frequent down-regulation in osteosarcoma as per few research reports. The sponging action of long non-coding RNAs has been established to be one of the molecular factors that mediate the miR-375 repression in osteosarcoma (16–18).

Recently, miR-375 was reported to be associated with cancer development; this miRNA was down-regulated in some malignancies, including liver cancer, gastric cancer (24, 25), head and neck squamous cell carcinoma (26) and pancreatic adenocarcinomas, and was up-regulated in an ERα^+ breast cancer cell line (27).

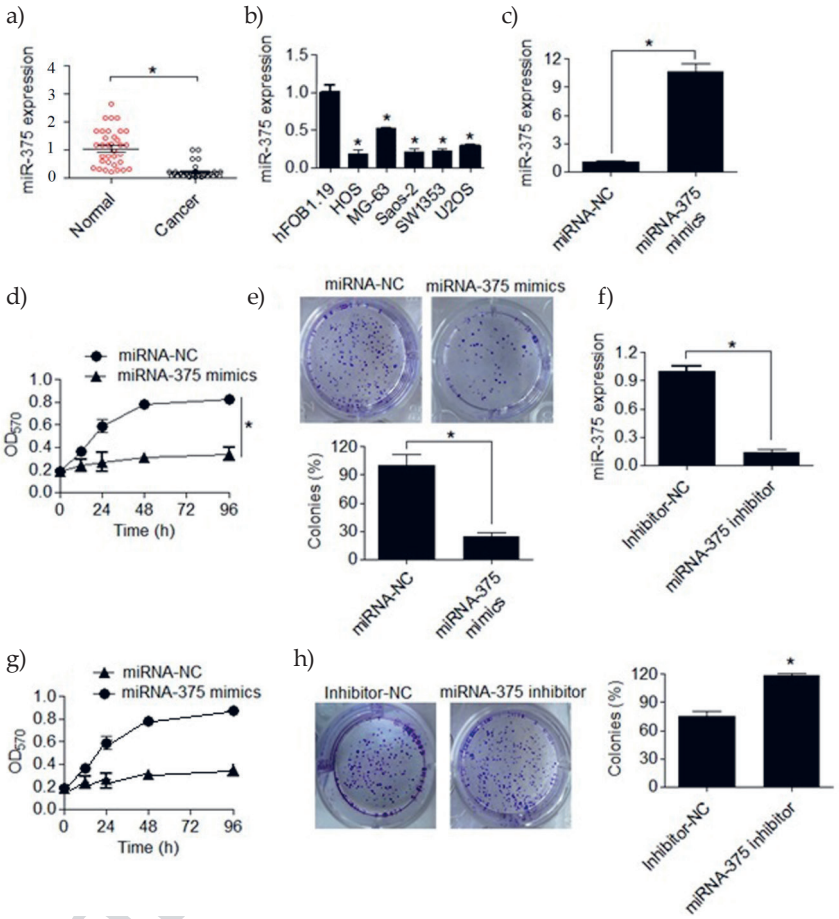

Fig. 1. MiR-375 inhibits osteosarcoma cell growth both *in vitro* and *in vivo*. a) Expression of miR-375 in normal and osteosarcoma tissues; b) qRT-PCR expression analysis revealing significant down-regulation of miR-375 in osteosarcoma cell lines (HOS, MG-63, Saos-2, SW1353 and U2OS) in comparison to normal Hfob1-19 cells; c) MG-63 cells transfected with miR-375 mimics showing significant up-regulation of miR-375 in comparison to respective miR-NC transfected cells; d) MG-63 cells over-expressing miR-375 exhibit limited growth *in vitro* than the respective miR-NC transfected cells; e) MG-63 cells overexpressing miR-375 exhibit significantly lower colony formation than the respective negative control cells; f) expression analysis of miR-375 in cells transfected with either miR-375 inhibitor or inhibitor-NC; g) inhibition of miR-375 in MG-63 cells exhibit enhanced growth *in vitro* than the respective miR-NC transfected cells; h) inhibition of miR-375 in MG-63 cells exhibit significantly higher colony formation than the respective negative control cells. Experiments carried three replicates and results were considered significant at $p < 0.05$.

SP1 acts as the functional target of miR-375 in osteosarcoma

Bioinformatics analysis identified SP1 as the potential target of miR-375 and predicted that miR-375 binds to a specific binding site in 3′-UTR of SP1 (Fig. 2a). The significant decline ($p < 0.05$) in the luciferase activity of MG-63 cancer cells co-transfected with miR-375

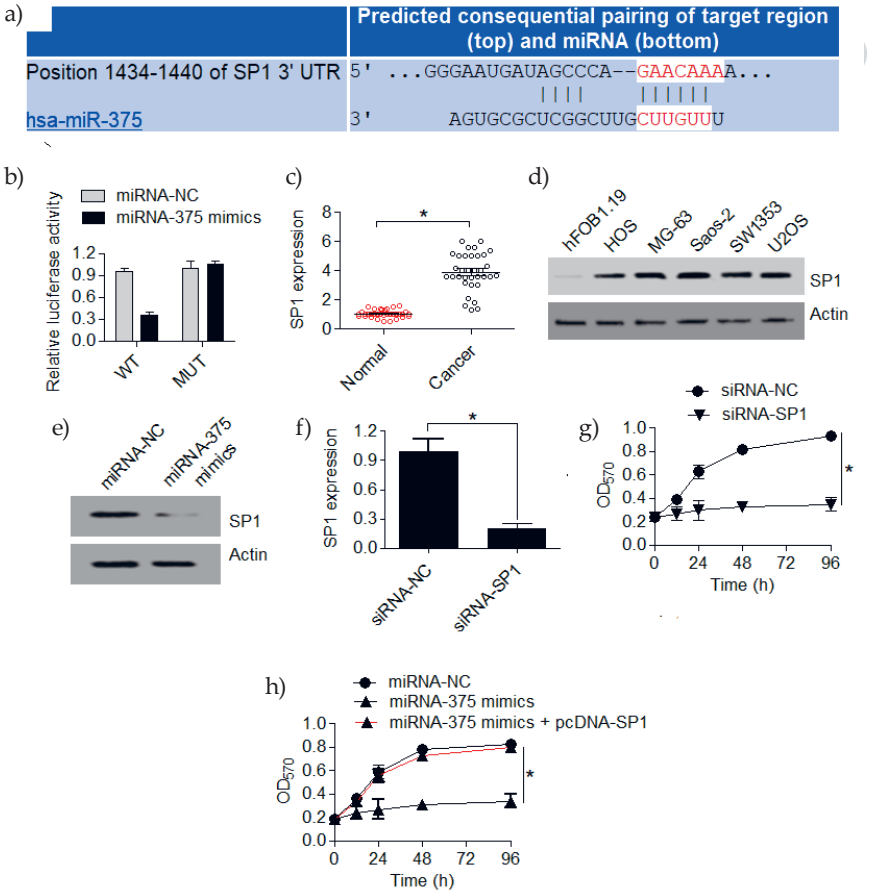

Fig. 2. MiR-375 targets SP1 in osteosarcoma. a) *In silico* analysis predicted specific miR-375 binding site in SP1 3′-UTR; b) a significant decline in luciferase activity of MG-63 cells was witnessed under miR-375 mimics and SP1-WT transfection signifying miR-375 interaction with SP1; c) expression of SP1 in normal and osteosarcoma tissues; d) expression analysis of SP1 in different osteosarcoma cell lines (HOS, MG-63, Saos-2, SW1353 and U2OS) in comparison to normal Hfob1-19 cells; e) expression of SP1 in MG-63 cells transfected with miRNA-NC and miRNA-375 mimics; f) expression of SP1 in MG-63 cells transfected with siRNA-NC and siRNA-SP1; g) inhibition of SP1 cells exhibit declined growth *in vitro* than the respective siRNA-NC transfected cells; h) inhibition of miR-375 cells exhibit declined growth *in vitro* than the respective siRNA-NC transfected cells, however, forced expression of SP1 significantly increased cell growth than the control. Experiments carried three replicates and results were considered significant at * $p < 0.05$.

mimics and WT-UTR of SP1 confirmed the interaction of miR-375 with its binding site in 3′-UTR of SP1 (Fig. 2b). Also, as expected; the SP1 expression was significantly higher ($p < 0.05$) in osteosarcoma tissues and cell lines in comparison to the normal tissues and cells (Figs. 2c,d). Additionally, the miR-375 over-expressing MG-63 cancer cells exhibited significantly lower ($p < 0.05$) SP1 protein levels in comparison to the negative control cancer cells (Fig. 2e). MG-63 cancer cells were transfected with si-SP1 silencing oligos and 6.8 fold SP1 down-regulation ($p < 0.05$) was observed with respect to the silencing control (si-NC) transfected MG-63 cells (Fig. 2f). CCK-8 proliferation assays were performed to examine if miR-375 performs its growth inhibitory role in osteosarcoma *via* SP1 repression. The results showed that silencing of SP1 significantly inhibited ($p < 0.05$) the proliferation of MG-63 cells *in vitro* (Fig. 2g). Subsequent over-expression of SP1 in miR-375 over-expressing MG-63 cancer cells enabled the host cells to proliferate at levels comparable to miR-NC transfected control cells (Fig. 2h). Additionally, silencing of SP1 in miR-375 inhibitor transfected MG-63 significantly declined their proliferation in vitro when compared with MG-63 transfected with miR-375 inhibitor oligos alone (Fig. 2i). The results confirm that miR-375 experiences its regulatory role in osteosarcoma *via* the post-transcriptional silencing of SP1.

At molecular level, miR-375 was shown to exert its regulatory role *via* the functional targeting of Sp1 transcription factor. The Specificity protein 1 (SP1) transcription factor, belonging to C2H2- zinc finger protein family, is involved in several metabolic and cellular processes (28). SP1 has been shown to be up-regulated in a number of human cancers including osteosarcoma and exhibits a tumor-promoting role (29, 7). The present study thus indicates that alleviation of SP1 suppression by miR-375 resulting from down-regulation of the latter might be one of the contributing factors for osteosarcoma development and progression. MiR-375/SP1 molecular might emerge as an effective molecular therapeutic target against osteosarcoma.

MiR-375 over-expression induced osteosarcoma cell cycle arrest at G2-phase via *SP1*

The cell cycle analysis was performed using flow cytometry to investigate the mechanism of growth inhibitory action of miR-375 against the osteosarcoma cells. The results revealed that miR-375 over-expressing MG-63 cells showed significantly higher ($p < 0.05$) abundance at the G2-phase of the cell cycle in comparison to the negative control cells indicative of induction abortion of cell cycle at G2/M transition (Fig. 3a). Conversely, such effect was not observed under miR-375 silencing (Fig. 3b). Whether the cell cycle arrest was introduced *via* SP1, the cell cycle of MG-63 cancer cells repressing SP1 was studied with reference to si-NC transfected control cells. The MG-63 cancer cells down-regulating SP1 again showed the induction of G2/M phase cell cycle arrest (Fig. 3c). The co-transfection studies confirmed that the induction of cell cycle arrest was exclusively exerted *via* the SP1 silencing (Fig. 3d).

There is limited knowledge about the exact molecular role and mechanism of action of miR-375 in osteosarcoma. As per the qRT-PCR results, miR-375 bears marked down-regulation in osteosarcoma tissues and cell lines which is in corroboration with the previous research reports. The inhibition of osteosarcoma cell proliferation by miR-375 over-expression *in vitro* signifies the putative tumor-suppressive role of miR-375 in osteosarcoma. Similar inferences have been drawn from several research reports previously though the

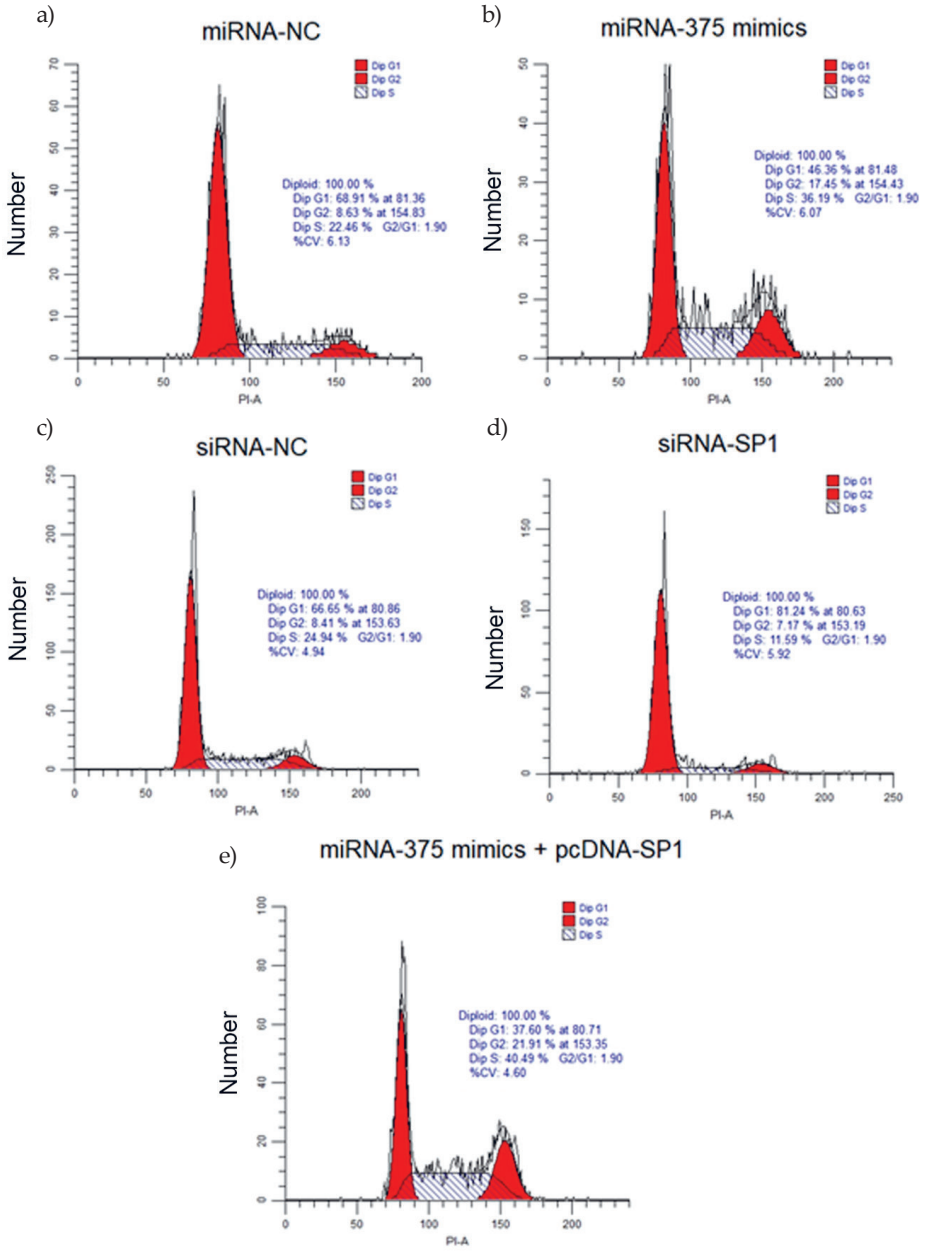

Fig. 3. Cell cycle analysis: a) and b) flow cytometry analysis showed that miR-375 over-expressing significantly enhanced abundance at G2-phase than negative control; c) d) and e) showed that the silencing of SP1 declined abundance at G2-phase than the negative control, however, overexpression of SP1 resumed the enhanced abundance at G2-phase.

oncogenic function of miR-375 has also been concluded (14, 30). To investigate the underlying mechanism mediating the growth inhibitory action of miR-375 against osteosarcoma, cell cycle analysis was made using flow cytometry. The results showed that the re-expression of miR-375 in osteosarcoma inducted the host cells with partial cessation at G2/M transition stage. Targeting the tumor cells for cell cycle arrest is one of the effective anticancer strategies thus highlighting the therapeutic importance. MiR-375 has also been reported to exhibit anti-metastatic regulatory potential (31). Confirming the same, miR-375 inhibited the migration and invasion of osteosarcoma cells *in vitro*.

MiR-375 negatively regulates migration, invasion and EMT of osteosarcoma cells

Whether miR-375 regulates the migration and invasion of the osteosarcoma cells, the transwell assays were performed. It was shown that MG-63 cancer cells over-expressing miR-375 exhibit significantly lower ($p < 0.05$) migration *in vitro* as compared to that of the negative control cells (Fig. 4a). MiR-375 over-expressing MG-63 cancer cells showed 32 percent relative migration with reference to the negative control cells. Similarly, the miR-375 over-expression also declined the relative invasion of MG-63 cancer cells by around 70 percent in comparison to the negative control MG-63 cells (Fig. 4b). Moreover, miR-375 over-expression enhanced the expression of E-cadherin and α-catenin (epithelial markers) while it significantly reduced the expression of fibronectin and Vimentin (both mesenchymal marker proteins) (Fig. 4c). miR-375 was shown to exhibit a negative role in regulating the epithelial-to-mesenchymal transition (EMT) of osteosarcoma cells. EMT is one of the key processes driving the invasion of cancer cells (32).

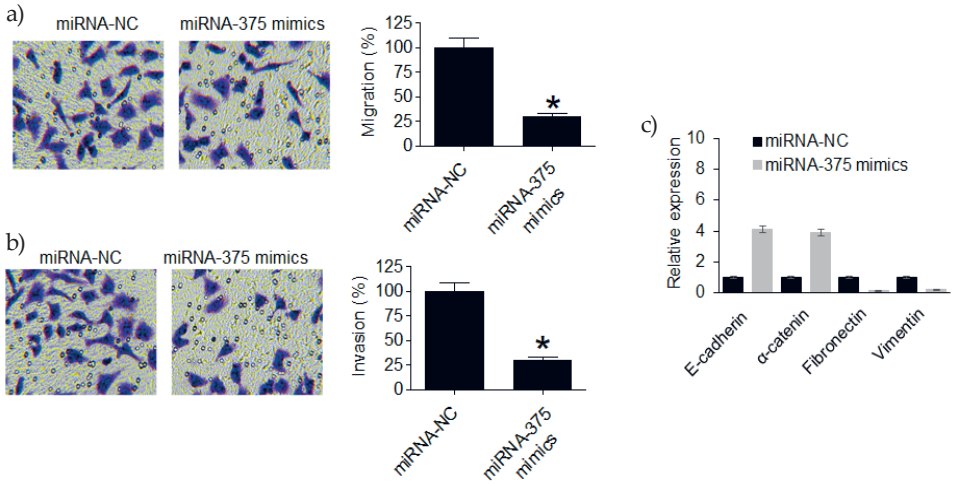

Fig. 4. a) Cellular migration of MG-63 cells transfected with miRNA-375 mimics or miRNA negative control vector; b) transwell chamber invasion assay of MG-63 cells transfected with miRNA-375 mimics or miRNA negative control vector; c) mRNA expression analysis of E-cadherin, α-catenin, fibronectin and vimentin in MG-63 cells transfected with miRNA-375 mimics and miRNA-NC. Three replicates were used for performing the experiment (* $p < 0.05$).

CONCLUSIONS

Osteosarcoma tissues and cell lines exhibit significant down-regulation of miR-375. The re-expression of miR-375 inhibits the growth and viability of osteosarcoma cells *in vitro* by inducing G2/M cell cycle arrest *via* post-transcriptional targeting of SP1. In addition, the results indicated that miR-375 negatively regulates the migration, invasion and EMT of osteosarcoma cells.

Conflicts of interest. – The authors declare no conflict of interest.

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Authors contributions. – Conceptualization, methodology and investigation, C.X and R.W; original draft preparation, R.W and Y.Z, review and editing, Y.Z and R.S. All the authors have read and agreed to the published version of the manuscript.

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