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MiR-485-5p inhibits metastasis and proliferation of osteosarcoma by targeting CX3CL1

F.-R. WANG, S.-H. XU, B.-M. WANG, F. WANG

Department of Trauma Emergency Surgery, Shandong provincial hospital affiliated to Shandong University, Ji'nan, China

Abstract. – OBJECTIVE: To investigate the potential effect of miR-485-5p on the development of osteosarcoma (OA) and its relevant mechanism.

PATIENTS AND METHODS: The expression level of miR-485-5p was detected in OA tissues and cells (MG-63) comparing with corresponding adjacent normal tissues and normal human osteoblastic cell lines (Hfob1.19), respectively. Luciferase assay was performed to evaluate the interaction between miR-485-5p and CX-3CL1, the effects of miR-485-5p on MG-63 cells were determined by subsequent experiments including cell proliferation, expression level of CX3CL1, detection of invasion and migration capacities.

RESULTS: In our present research, miR-485-5p was down-regulated in OA tissues and we got the same result in OA cells. In order to obtain potential target of miR-485-5p, we checked it in three publicly available algorithms, TargetScan, miRDB and microRNA. We found that CX-3CL1 is a direct target of miR-485-5p, and Luciferase assays confirmed our hypothesis. The results showed that decreased expression of CX-3CL1 resulting from the up-regulation of miR-485-5p could decelerate cell proliferation, invasion and migration in OA cells.

CONCLUSIONS: We showed the suppressor function of miR-485-5p in OA by targeting CX-3CL1, indicating that miR-485-5p/CX3CL1 axis might be a potential therapeutic target for the treatment of OA.

Key Words:
miR-485-5p, Osteosarcoma (OA), CX3CL1.

Introduction

Osteosarcoma (OA) is common in adolescents and children and is a common primary malignancy, whose incidence is high, with a rapid progress. It has a high degree of malignancy and is prone to distant metastases with a low long-term survival rate[1-3]. OA occurs predominantly in the metaphyseal of long bones of the extremities. In addition, it often occurs in other parts such as the ilium and spine[4-7]. The treatment of early OA is dominated by surgical amputation. However, this type of surgery has large trauma and high disability rate, and the patient's long-term survival rate is low. With the advancement of surgery and the introduction of preoperative and postoperative chemotherapy concepts, the clinical remission rate and long-term survival rate of OA have been greatly improved. Although surgery and neoadjuvant chemotherapy have achieved great progress, most patients still have poor clinical outcomes. The main reason for this result is lung metastasis and drug resistance of OA cells. Because the exact mechanism of the occurrence and metastasis of OA is not yet clear, how to elucidate the molecular mechanism of OA has important significance for the diagnosis and treatment of OA. MicroRNAs are a category of micromolecule non-coding ribonucleic acid (RNA) with about 18-22 nucleotides in length, acting as the expressions of regulatory factors and controlling genes after transcription[8]. MiRNAs play an extremely important role in the occurrence and development of tumors. Its molecular mechanism regulates the expression of key functional proteins by combining with the 3' untranslated regions (UTR) of gene to exert its effects on the biological functions of tumor cells, including the functions of tumor cell's proliferation, migration, invasion, cell cycle and apoptosis[9-12]. Researches have shown that the abnormal expression of miRNAs is closely related to the biological behaviors of OA such as proliferation, metastasis, drug resistance and prognosis. For example, miRNA-150 can inhibit the proliferation and metastasis of OA cells by targeting Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1)[13]. Mir-34a-5p can increase the resistance of OA to chemotherapy by mediating angiotensin II receptor type 1 (AG-

Corresponding Author: Shihong Xu, MM; e-mail: 15168887120@163.com

TR1) gene[14]. MiR-490-3p was an independent prognostic factor in OA significantly associated with distant metastasis, advanced clinical stage and long-term survival rate[15]. As a member of the miRNA family, miR-485-5p has shown its unique advantages in the diagnosis and treatment of a variety of cancers, such as liver cancer[16], gastric cancer[17, 18] and breast cancer[19, 20]. However, there are few reports on the role of miR-485-5p in the occurrence and development of OA and its related molecular mechanism. In this study, the role of miR-485-5p in the occurrence and development of OA and its related molecular mechanism were clarified through analyzing the expression of miR-485-5p and the effects of miR-485-5p on biological behaviors of OA.

Patients and Methods

Non-Small Cell Lung Cancer Cases and Cells

This study included 40 OA patients undergoing a surgical procedure at Shandong Provincial Hospital Affiliated to Shandong University, which were underwent pathological diagnoses to be confirmed OA. Preoperative chemotherapy or radiotherapy treatment was forbidden. The liquid nitrogen was used to freeze OA tissues and corresponding adjacent normal tissues were kept at -80ºC in a refrigerator. The adjacent normal tissues have to be concerned by biological biopsy to be sure that they do not include OA cells. This study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University and followed the Declaration of Helsinki. Signed written informed consents were obtained from all participants before the study. The human osteosarcoma cell lines (MG-63) together with normal human osteoblastic cell lines (Hfob1.19) were purchased from the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) complemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 cell culture incubator.

Luciferase Reporter Assays

In TargetScan, miRDB and microRNA websites, it was found that CX3CL1 is the target gene of miR-485-5p. The binding sequence of miR-485-5p at the 3'-end of CX3CL1 was mutated using a point mutation kit (Agilent Technologies, Foster City, CA, USA), and the mutated CX3CL1 (Mut-type) and non-mutant CX3CL1 (WT-type) were connected with the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vector with mutant CX3CL1 was transfected into MG-63 cells after lentivirus intervention on the 24-well plate. The same treatment was performed on the pGL3-Basic vector connected with the non-mutant CX3CL1 according to steps in the Luciferase Reporter Gene Assay Kit (Yeasen, Shanghai, China). Next, the luciferase activity was detected in a multi-function microplate reader.

Transfection

MiR-485-5p mimics and si-CX3CL1 were synthesized and transfected to OA cell line to analyze biological function of miR-485-5p. Three groups were established to investigate the potential relevance between miR-485-5p and MG-63 cell: NC group (negative control), miR-485-5p mimics (MG-63 cell transfected by miR-485-5p mimics) and mimics + CX3CL1 (MG-63 cell transfected by miR-485-5p mimics and si-CX3CL1). All the materials were purchased from RiboBio (Guangzhou, China), and were transfected by using Lipofectamine RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

MG-63 cells were detected *via* Real-time fluorescence quantitative polymerase chain reaction (qPCR). Total RNA was procured by TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. SYBR green qPCR assay was used to measure the level of CX3CL1, expression and endogenous controlled by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were used to measure the level of miR-488 expression normalized to miRNA U6. Primer sequences used in this study were as follows: CX3CL1, F: 5'- ACGATGCCCTCCTACGATCA -3', R: 5'-TAATTCAACACGCAGGACAG-3'; miR-488, F: 5'-GCATAAAGACATACTCCAATCCC-3', R: 5'- TCCTCCACGATCATGCTGTGT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-GGAAGGTGAAGGTCGGAGTCA-3', R: 5'-GTCATTGATGGCAACAATATCCACT -3'.

Western Blot Analysis

MG-63 cells were collected and lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The total protein was extracted, and its concentration was determined according to instructions of the bicinchoninic acid (BCA) protein concentration kit (Pierce, Rockford, IL, USA). The same amount of total protein was separated *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane, sealed with 5% skim milk and incubated with rabbit anti-rat CX3CL1 primary antibodies (1:1000) at 4°C overnight. After the membrane was fully washed with Tris-buffered saline and Tween-20 (TBST), anti-rabbit secondary antibody (coupled by horseradish peroxidase) was added for incubation at room temperature for 2 h, followed by development *via* enhanced chemiluminescence (ECL) (Thermo Fisher Scientific, Waltham, MA, USA), exposure in gel imaging system, fixation and observation of results. With GAPDH as an internal reference, the relative changes in protein expression were detected.

Cell Proliferation

When cells grew to the logarithmic growth phase, they were collected, diluted into 1×10^6 cell suspension, and added into a 96-well cell culture plate (5×10^3/100 μL per well). The wells only added with medium were used as blank controls. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA). 15 μL MTT reagents (500 μg/mL) were added into each well for culture for another 2 h, after which the absorbance (A) was measured using an enzyme-labeled spectrophotometer, followed by zero setting using blank wells.

Cell Invasion and Migration Assays

Cell invasion assay was performed by Transwell plates (Corning, Corning, NY, USA) with 8-μm-pore size membranes with Matrigel. Briefly, 2 x 10^4 cells were planted into the upper chambers with serum free medium, On the other hand, the lower chamber was offered with medium containing 10% fetal bovine serum as chemoattractant. After 2 days incubating, cells on the top of membrane were wiped by a brush. Subsequently, the membrane was stained by 0.2% crystal violet followed drenched by 95% ethanol. The invading cells were noted by an inverted microscope.

Cell migration assay was performed by scratch-wound assay. MG-63 cells were seeded in six-well plates and allowed to reach confluence. After transfection, each well was scraped with a 10 μL pipette tip to create a linear region devoid of cells, Subsequently, the cells in each well were cultured with RPMI-1640 medium with 2% FBS (both from Gibco, Rockville, MD, USA), We monitored wound healing at 48 h after scraping. Five random fields of each well were selected for analysis

Statistical Analysis

Statistical analysis was performed with a Student's *t*-test or *F*-test. All *p*-values were two-sided and $p < 0.05$ were considered significant and analyzed by Prism 6.02 software (La Jolla, CA, USA).

Results

MiR-485-5p Expression Found Reduced Both Tissues and Cells of OA

To examine the role of miR-485-5p in OA development, we detected the level of miR-485-5p expression in OA tissues and the adjacent normal tissues by qRT-PCR, We found that the expression of miR-485-5p was pretty lower in OA tissues by comparing with the adjacent normal tissues (Figure 1A). The same results were obtained in cellular level (Figure 1B). However, the level of CX3CL1 expression was significantly up-regulated in OA cells (Figure 1C). Taken together, we thought miR-485-5p and CX3CL1 might have some correlation on the effect during the progression of OA.

CX3CL1 Was a Direct Target of miR-485-5p in OA Cell

In order to investigate the potential target of miR-485-5p, we checked it in three publicly available algorithms, TargetScan, miRDB and microRNA to elucidate the putative and possible targets of miR-485-5p. We found the CX3CL1 was checked as supposed target of miR-485-5p (Figure 2A). Thus, CX3CL1 had caught our attention and was implemented to further investigations. We established luciferase reporter vectors containing the wild or mutant-type miR-485-5p seed sequences of the

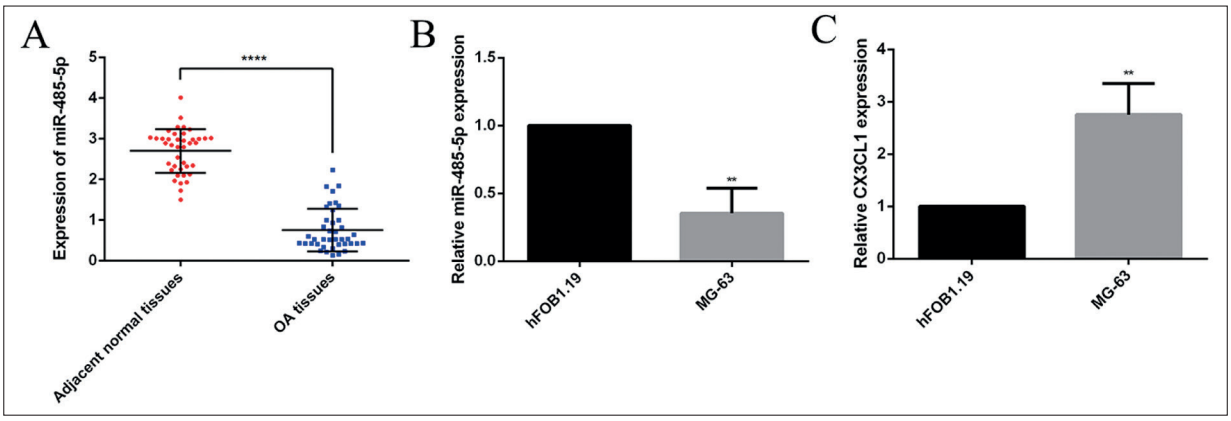

Figure 1. The expressions of miR-485-5p and CX3CL1 in osteosarcoma (OA) tissue samples and cells compared with corresponding adjacent normal tissues and normal human osteoblastic cells. ***A,*** Difference in the expression of miR-485-5p between OA tissues and corresponding adjacent normal tissues (****$p < 0.0001$ compared with adjacent normal tissue). ***B-C,*** The expression of miR-485-5p and CX3CL1 in OA cells (MG-63) and normal human osteoblastic cells (hFOB1.19) (**$p < 0.01$ compared with hFOB1.19).

CX3CL1 30UTR. The expression of miR-485-5p increased with mimics result in the decrease of the luciferase activity of the wide-type CX-3CL1 30UTR reporter gene, but it had no effect on mutant-type (Figure 2B), suggesting the expression of CX3CL1 could be regulated by miRNA 485-5p.

MiR-485-5p Decreased the Expression Level of CX3CL1

We set up three groups to take the similar experiments (miR-NC group, miR-485-5p mimics group and the mimics + CX3CL1 group) in MG-63 cell. We found that the expression level of CX3CL1 was decreased by up-regulation of

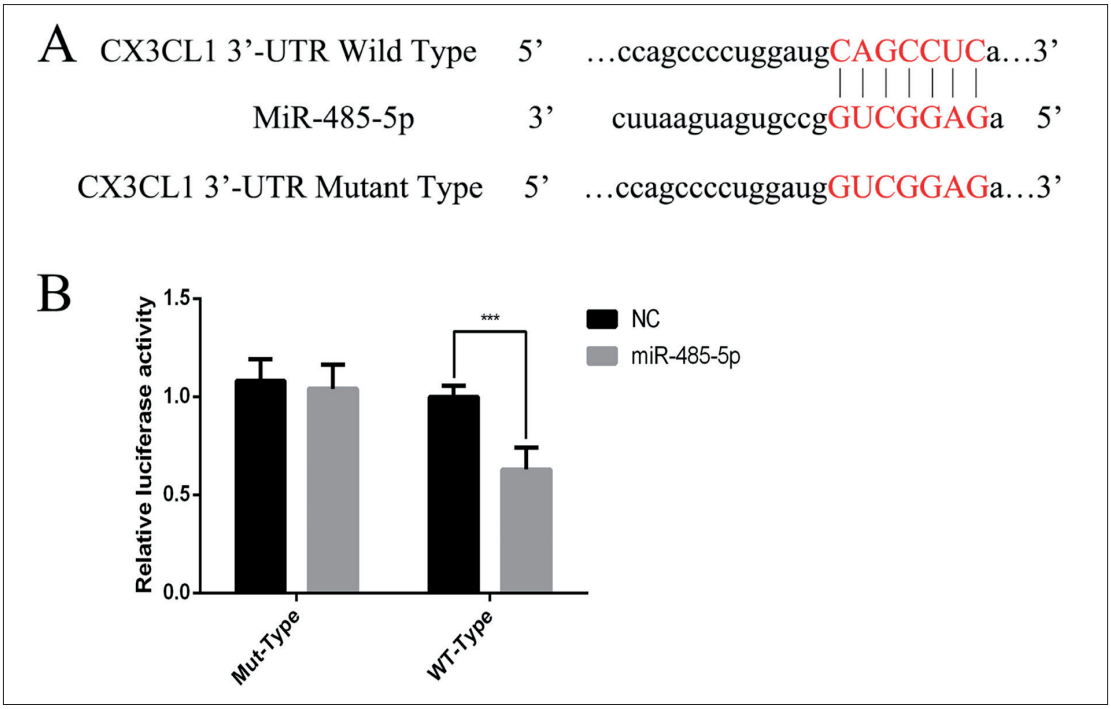

Figure 2. CX3CL1 is a direct and functional target of miR-485-5p. MG-63 cell were transfected with miR-485-5p mimics and inhibitor. ***A,*** Diagram of putative miR-485-5p binding sites of CX3CL1. ***B,*** Relative activities of luciferase reporters (***$p < 0.001$).

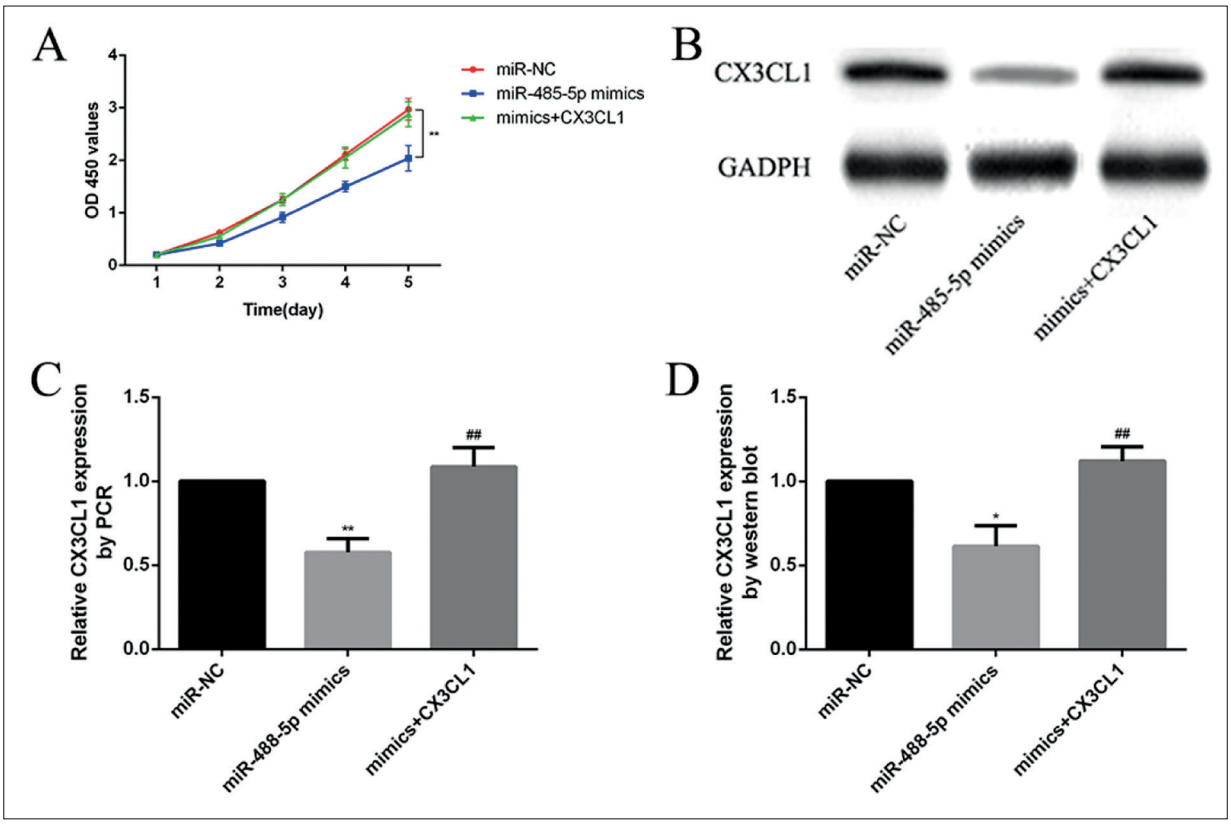

Figure 3. ***A,*** MiR-485-5p inhibits the proliferation of OA cell. (**$p < 0.01$), ***B-D,*** MiR-485-5p decrease the expression level of CX3CL1, Data were presented as means ± standard deviations. (*$p < 0.05$, **$p < 0.01$ *vs.* NC group; $^{\#\#}p < 0.01$ *vs.* Mimics group).

miR-485-5p in MG-63 cell in both Real-time PCR and Western blot analysis (Figure 3B-3D). The data further illustrate the regulating effect of miR-485-5p on the expression of CX3CL1.

MiR-485-5p Suppressed Proliferation of OA Cell

MTT assay was employed to detect the cell proliferation rates after the transfection. The MTT results showed that the cell proliferation rates of MG-63 cell were decrease by up-regulated miR-485-5p using mimics transfection. In contrast, down-regulated miR-485-5p expedited cell growth of OA cells (Figure 3A).

MiR-485-5p Inhibited Invasion and Migration of OA Cell

Migration and invasion are two most key factors in cancer cell proliferation. In the transwell experiments, the capacity of invasion of MG-63 cell was restricted by up-regulation of miR-485-5p with mimics. Meanwhile, the results from scratch-wound assay showed that the cell migration was limited by intervened of miR-485-5p (Figure 4).

Discussion

MicroRNA is a key regulatory factor of tumors and plays an important role in tumor cell's proliferation, migration, and invasion. Research on OA has shown that microRNA has crucial functions in the occurrence, development, differentiation, tumor migration and invasion, and chemotherapy resistance of OA[21]. In addition to the functions of oncogenes and tumor suppressor genes, it can also act as a regulatory factor of tumor's key genes to exert its regulatory effect[22-25]. Plenty of microRNAs are considered to have potential as molecular targets for the diagnosis, treatment, and prognosis of OA[26-28]. C-X3-C motif chemokine receptor 1 (CX3CL1) protein is a member of the CX3C chemokine family that not only has chemotaxis, but also has cell adhesion function. Tsang et al[29] have found that the CX3CL1 is highly expressed in ovarian and breast cancer cells, and its expression level is closely related to tumor cell proliferation and metastasis. In oligodendrogliomas with high-grade malignancy, the expression level of CX-

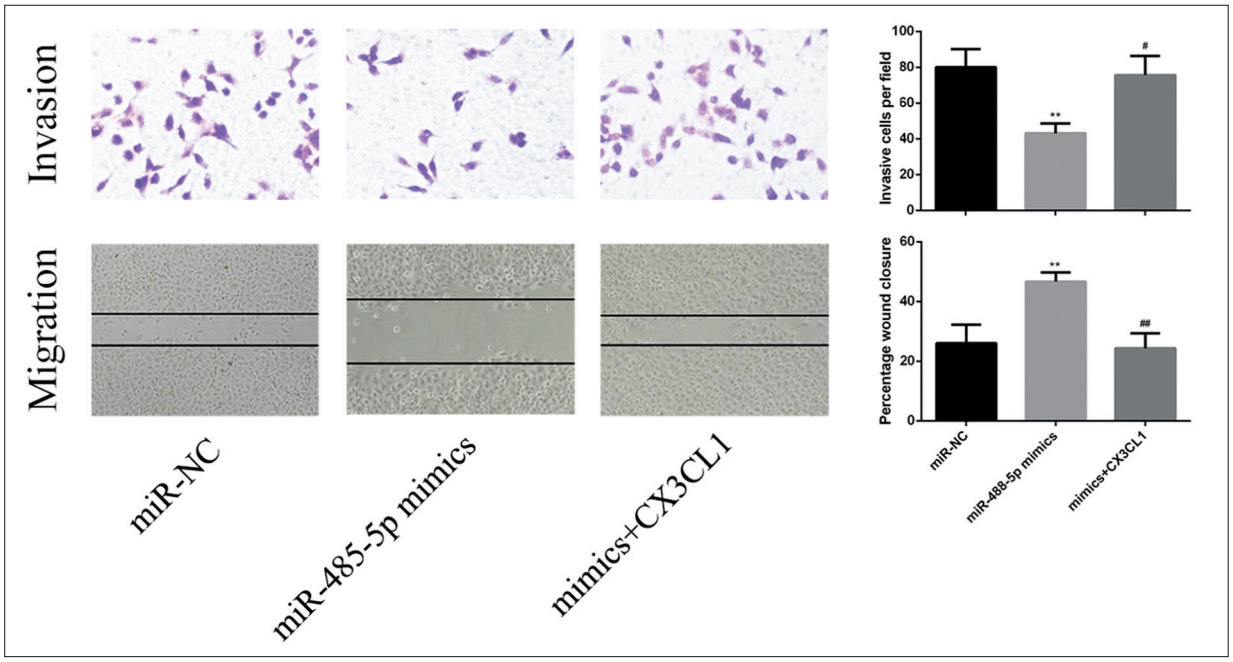

Figure 4. MiR-485-5p/CX3CL1 axis inhibits the invasion and migration of OA cell. CX3CL1 overexpression attenuates the suppressive effect of miR-485-5p on MG-63 cell. ***A***, The invasion test by transwell assay. ***B***, The migration test by scratch-wound assay (**$p < 0.01$ *vs.* NC group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* Mimics group).

3CL1 is negatively correlated with the overall survival rate of patients[30]. The expression level of CX3CL1 is also associated with prostate cancer metastasis and patient's prognosis. When CX3CL1 expression is blocked by neutralizing antibodies, the osseous metastasis of prostate cancer cells is significantly reduced[31]. In this study, we identified the binding sites of the miR-485-5p and 3'-UTR of CX3CL1 genes through target gene prediction. It was speculated that CX3CL1 may be a downstream target gene of miR-485-5p, and the reliability of the prediction was further verified by test of dual luciferase reporter gene. At the same time, the test results of polymerase chain reaction (PCR) also confirmed the results. The essential process of carcinogenesis is that the normal cells get rid of the intrinsic regulatory mechanisms of cells and eventually form an out-of-control, continuous breeding colony. Obtaining abnormal proliferation ability is a key step in cancerization process. The MTT results showed that the cell proliferation rates of MG-63 cell were decrease by miR-485-5p using mimics transfection. In contrast, down-regulated miR-1182 expedite the cell grow.

The 5-year survival rate of patients with laryngeal cancer stays at a high level mainly due to the recurrence and metastasis of patients in advanced stage, and the fundamental features of malignant tumors different from benign tumors are the invasive growth and metastatic potential. When falling off the tumors and invading the blood flows and surrounding tissues, the tumor cells need some movement ability. In general, tumors that are highly invasive are usually also highly motile. In our study, after up-regulating expression of miR-485-5p in OA cells, the migration and invasion capacities of OA cells were significantly decreased, indicating the inhibiting effect of miR-485-5p on cell metastasis.

Conclusions

We observed that miR-485-5p intervention can affect the proliferation, metastasis and invasion of OA cells, and change in such an invasive ability correlates with the regulation of miR-485-5p on CX3CL1 expression. Thus, miR-485-5p/CX3CL1 axis may become a new target for targeted therapy of OA with the further development of the research.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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