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RESEARCH ARTICLE

MiR-143 inhibits EGFR-signaling-dependent osteosarcoma invasion

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Abstract The molecular regulation of the invasion of osteosarcoma (OS) remains elusive. Here, we reported significant lower level of miR-143 and significant levels of phosphorylated EGFR and MMP9 in the resected OS from the patients, compared to the adjacent normal tissue. Moreover, strong correlation was detected among these three factors. We thus hypothesized existence of a causal link, which prompted us to use two human OS cell lines to study the interaction of miR-143, MMP9, and activation of EGFR signaling. We found that EGF-induced EGFR phosphorylation in both lines activated MMP9, and consequently cancer invasiveness. Both an inhibitor for EGFR phosphorylation and an inhibitor for ERK1/2 phosphorylation significantly inhibited the EGF-induced activation of MMP9. Moreover, miR-143 levels did not alter by EGF-induced EGFR phosphorylation, while overexpression of miR-143 antagonized EGF-induced MMP9 activation without affecting EGFR phosphorylation. Taken together, our data suggest that miR-143 inhibits EGFR signaling through its downstream ERK/MAPK signaling cascades to control MMP9 expression in OS. Thus, miR-143, EGFR, and MMP9 are therapeutic targets for inhibiting OS invasion.

Keywords Osteosarcoma · Epidermal growth factor receptor · miR-143 · MMP9

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Introduction

Osteosarcoma (OS) is the most common primary bone malignancy and accounts for 60 % of all malignant childhood bone tumors. The distal femoral and proximal tibial metaphyses are the most common sites for development of osteosarcoma. Although combined chemotherapy and surgery have been applied, the 5-year survival of patients with no metastatic disease at diagnosis reaches about 70 %. Nevertheless, the 5-year survival of patients with metastatic cancer becomes as low as about 20 % [1, 2]. Therefore, for controlling and treating metastasis of osteosarcoma, new therapeutic targets are extremely needed. However, the mechanisms underlying the metastatic process in OS are not fully clarified. To that point, there is an urgent need for studying the tumor biology of OS in order to increase our understanding so as to treat it more efficiently [2–4]. A number of human OS cell lines have been established, among which U2OS [5] and HOS [6] cell line have been extensively characterized and widely used in research.

Activation of the epidermal growth factor receptor (EGFR) signaling is critical for many biological events [7–10], and has been reported in OS in that ErbB2 expression has even been proved to predict for poor outcome in OS patients [11]. However, correlation of EGFR expression and molecules that control OS metastasis has not been drawn. Some therapies have been developed aiming to interfere with intracellular pathways regulated by EGFR in glioblastoma [12–14], in lung cancer [15], in larynx carcinoma [16], and in gastric cancer [17]. Moreover, EGFR as a potential therapy target is further justified by the fact that protein expression in these tumors is associated with significantly higher metastasis.

The growth and metastasis of OS have been shown to depend on the expression of matrix metalloproteinase-9 (MMP9) [18–21]. Generally, cancer cells either secrete proteinases or acquire proteinase activity from host stromal cells

or inflammatory cells to allow them to break through collagenous protein barriers. MMP9 is an important matrix proteinase that degrades collagen type IV, the major component of the basement membrane. Overexpression of MMP9 often facilitates metastatic spread of various tumors and appears to be one of the most important molecules to directly promote cancer metastasis. Nevertheless, the precise molecular pathways controlling the activation of MMP9 in OS remain unclarified.

MicroRNA (miRNA) belongs to a class of endogenously expressed, non-coding small RNA of about 22 nucleotides. MiRNA can regulate the expression of protein-coding genes at the posttranscriptional level through imperfect base pairing with the 3′-untranslated region (3′-UTR) of target mRNA [22, 23]. It is noteworthy that miRNA is predicted to regulate the expression of at least 30 % of all genes, and deregulation of miRNA may contribute to development of many diseases, including cancer in that miRNA has been implicated in the regulation of various cellular processes that are often deregulated during tumor development and progression [14, 17, 24].

Here, we reported significant lower level of a member of miRNA family, miR-143, and significant levels of phosphorylated EGFR and MMP9 in the resected OS from the patients, compared to the adjacent normal tissue. Moreover, strong correlation was detected among these three factors. We thus hypothesized existence of a causal link, which prompted us to use two human OS cell lines to study the interaction of miR-143, MMP9, and activation of EGFR signaling. We found that EGF-induced EGFR phosphorylation in both lines activated MMP9, and consequently cancer invasiveness. Both an inhibitor for EGFR phosphorylation and an inhibitor for ERK1/2 phosphorylation significantly inhibited the EGF-induced activation of MMP9. Moreover, miR-143 levels did not alter by EGF-induced EGFR phosphorylation, while overexpression of miR-143 antagonized EGF-induced MMP9 activation without affecting EGFR phosphorylation.

Material and methods

Cell line culture and transfection and reagents

U2OS [5] and HOS [6] are two human OS cell lines purchased from ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Both lines were transfected with a miR-143 overexpressing plasmid using Lipofectamine 2000 (Invitrogen, Canada) transfection reagents, according to the manufacturer's instructions. Cells were analyzed 48 h after transfection. Recombinant EGF, AG1478, and PD98059 were all purchased from Sigma (USA).

Patient tissue specimens

A total of 18 resected specimens from OS patients were collected for this study. All specimens had been histologically and clinically diagnosed at Department of Orthopedics of Jinling Hospital from 2008 to 2013. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

ELISA

The concentration of MMP9 in the conditioned media from cultured cells was determined by a MMP9 ELISA Kit (Calbiochem/Oncogene, Cambridge, MA, USA). ELISAs were performed according to the instructions of the manufacturer. Briefly, the collected condition media was added to a well coated with MMP9 polyclonal antibody, and then immunosorbented by biotinylated monoclonal anti-human MMP9 antibody at room temperature for 2 h. The color development catalyzed by horseradish peroxidase was terminated with 2.5 mol/l sulfuric acid, and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

Transwell matrix penetration assay

Cells (3×10^5) were plated into the top side of polycarbonate transwell filter coated with Matrigel in the upper chamber of the BioCoatTM Invasion Chambers (BD, Bedford, MA, USA) and incubated at 37 °C for 22 h. The cells inside the upper chamber with cotton swabs were then removed. Migratory and invasive cells on the lower membrane surface were fixed, stained with hematoxylin, and counted for ten random 100× fields per well. Cell counts are expressed as the mean number of cells per field of view. Five independent experiments were performed and the data are presented as mean± standard deviation (SD).

Western blot

The protein was extracted from the resected gastric cancer or adjacent normal tissue (NT), or cultured cells. Primary antibodies were anti-EGFR, anti-phosphorylated EGFR (pEGFR), anti-phosphorylated ERK1/2 (pERK1/2), anti-MMP9 (the phosphorylated form), and anti-β-actin (all purchased from Cell Signaling, USA). β-actin was used as a protein loading control. Secondary antibodies were HRP-conjugated anti-rabbit and were all purchased from Jackson Lab (USA).

RT-qPCR

MiRNA and total RNA were extracted from resected patient tissue or from cultured cells with miRNeasy mini kit (Qiagen, Hilden, Germany), or RNeasy kit (Qiagen) and used for cDNA synthesis. Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Values of genes were normalized against β-actin.

Statistical analysis

All statistical analyses were carried out using the SPSS 17.0 statistical software package. All values are depicted as mean± standard deviation from five individuals and are considered significant if $p<0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction. Bivariate correlations were calculated by Spearman's rank correlation coefficients.

Results

Significant changes in miR-143, pEGFR, and MMP9 in the resected OS from the patients

Since miRNAs, EGFR signaling and MMP9 have been reported in the pathogenesis of OS; first, we examined the expression of different miRNAs by RT-qPCR, and we found a significant decrease in the level of miR-143 in OS from the patients, compared to the miR-143 level in the adjacent normal tissue (NT) (Fig. 1a). We then examined the phosphorylated EGFR (pEGFR) and MMP9 levels by Western blot, showing significant increases in pEGFR (Fig. 1b) and MMP9 (Fig. 1c) levels in the resected OS, compared to those in the adjacent NT

Strong correlations among miR-143, pEGFR, and MMP9 were detected in the resected OS from the patients

We were then prompted to examine the inter-relationship among these three molecules. We thus performed correlation tests for relative miR-143 level, MMP9 level, and pEGFR level in the resected OS from 18 patients. Strong negative correlations were detected between pEGFR and miR-143 (Fig. 1d, $=-0.59$; $p<0.01$), and between MMP9 and miR-143 (Fig. 1e, $=-0.65$; $p<0.01$), while a strong positive correlation was detected between pEGFR and MMP9 (Fig. 1f, $=0.69$; $p<0.01$), suggesting that the MMP9 levels may be positively regulated by activation of EGFR signaling, and negatively controlled by miR-143 in OS.

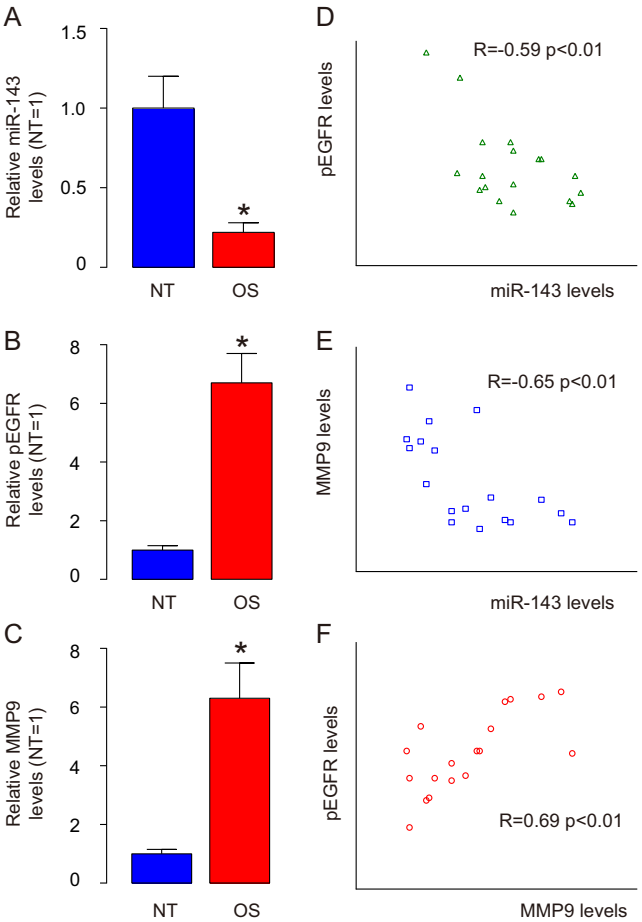

Fig. 1 MiR-143, pEGFR, and MMP9 levels in OS from the patients. **a**–**c** Examination of miR-143 level by RT-qPCR (**a**), and pEGFR (**b**) and MMP9 (**c**) by Western blot from the resected OS tissue, compared with those from the adjacent normal tissue (NT). **d**–**f** Correlation tests between pEGFR and miR-143 (**d**), between MMP9 and miR-143 (**e**), and between pEGFR and MMP9 (**f**). *$p<0.05$

EGF-induced MMP9 expression via ERK/MAPK pathway

We thus used two human OS cell lines, U2OS and HOS, to study the molecular pathways that control MMP9 activation in OS. First, we examined the effect of activation of EGFR signaling on the expression of MMP9 in both cell lines. Cells were cultured in DMEM starvation media for 24 h and then stimulated with 25 ng/ml recombinant EGF. One hour later, the expression of MMP9 was examined by RT-qPCR (Fig. 2a), by Western blot (Fig. 2b), and by MMP9 secretion into the conditioned media by ELISA (Fig. 2c). Our data demonstrate that EGF stimulation significantly increased MMP9 levels in both cell lines (Fig. 2a–c), and as a consequence, cell invasiveness in a transwell matrix penetration assay (Fig. 2d). Moreover, the effect of EGF-stimulated MMP9 activation and increase in cell invasiveness was significantly inhibited by using a specific EGFR inhibitor, AG1478, at a concentration of

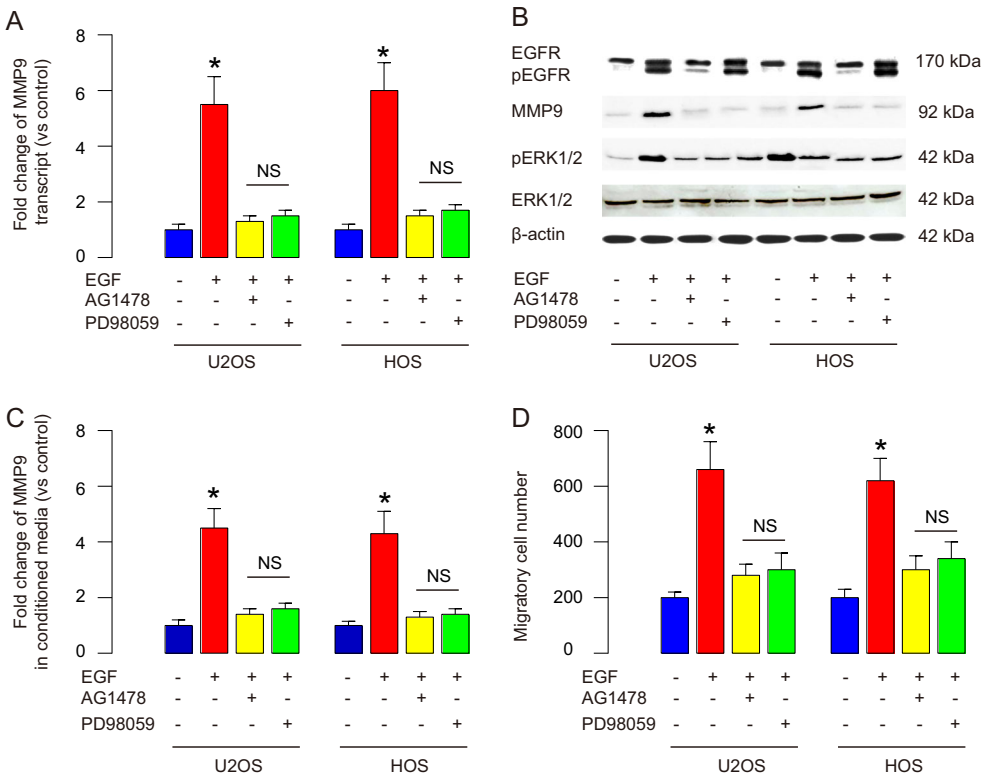

Fig. 2 EGF-stimulated MMP9 expression via ERK/MAPK signaling pathway. **a–d** Expression of MMP9 was examined in two human OS cell line, U2OS and HOS, in response to 25 ng/ml EGF stimulation, by RT-qPCR (**a**), by Western blot (**b**), by ELISA on conditioned media (**c**), and by cell invasiveness in a transwell matrix penetration assay (**d**). AG1478 is a specific inhibitor for EGFR phosphorylation. PD98059 is a specific inhibitor for ERK1/2 phosphorylation. *pEGFR* phosphorylated EGFR, *pERK1/2* phosphorylated ERK1/2, *NS* non-significant. *$p<0.05$.

60 μmol/l (Fig. 2a–d), suggesting that activation of EGFR signaling pathway activate MMP9 to increase cancer cell invasiveness in OS.

To figure out the downstream signaling pathways of EGFR activation that control MMP9 activation, we used several specific inhibitors to PI3k/Akt/mTor pathway, JNK pathway, and ERK/MAPK pathway. We found that only application of 20 μmol/l PD98059, a specific inhibitor to the phosphorylation of ERK1/2, was capable of inhibiting the EGF-induced MMP9 activation and OS invasiveness in both lines (Fig. 2a–d), without affecting the phosphorylation of EGFR by EGF stimulation (Fig. 2b). These data suggest that EGF may induce MMP9 expression through activation of ERK/MAPK signaling cascades, which was confirmed by the analysis of phosphorylated ERK1/2 (pERTK1/2) levels in response to EGF (Fig. 2b). Taken together, our results suggest that activation of EGFR signaling in OS may induce MMP9 expression by activation PI3k/Akt signaling cascades.

EGF stimulation did not affect miR-143 expression

We then examined whether activation of EGFR signaling may affect the levels of miR-143. Interestingly, stimulation with 25 ng/ml EGF did not change the miR-143 levels in both lines, suggesting that expression of miR-143 is not regulated by EGFR signaling (Fig. 3).

MiR-143 inhibited ERK1/2 phosphorylation

To determine whether the levels of miR-143 may affect EGFR signaling and activation of MMP9, we transfected both cell lines with a miR-143-expressing plasmid. Transfected cells expressed significantly higher levels of miR-143 in both lines (Fig. 4a). We found that miR-143-overexpressing cells significantly decreased MMP9 expression, by RT-qPCR (Fig. 4b), by Western blot (Fig. 4c), and significantly decreased secreted MMP9 in the conditioned media by ELISA (Fig. 4d). Moreover, MMP9 did not increase in response to EGF stimulation in miR-143-overexpressing cells (Fig. 4b–d), although the phosphorylation of EGFR was not affected (Fig. 4c). These

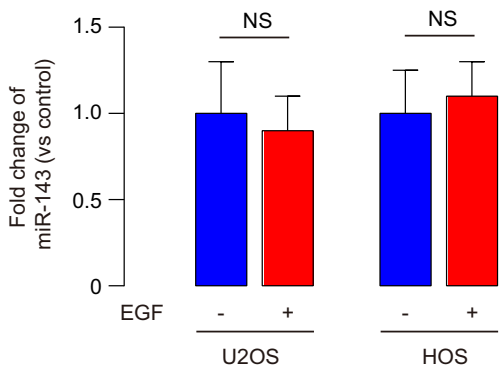

Fig. 3 EGF stimulation did not affect miR-143 expression. RT-qPCR for examining miR-143 levels in response to EGF stimulation. *NS* non-significant

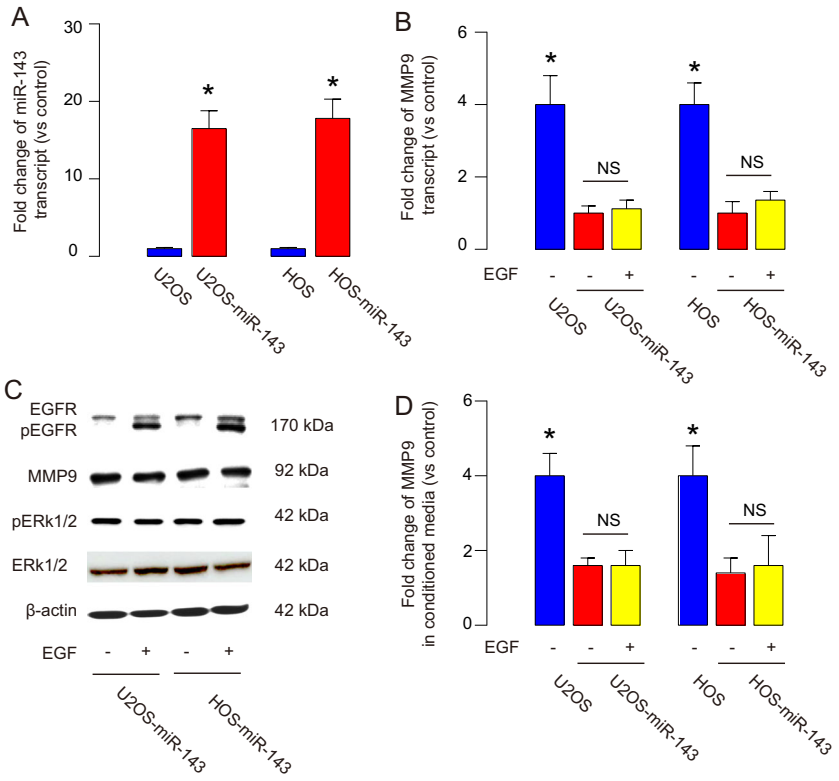

Fig. 4 MiR-143 inhibited MMP9 activation through inhibition of ERK1/2 phosphorylation. **a** RT-qPCR confirming overexpression of miR-143 in U2OS and HOS. **b**–**d** MMP9 levels in response to EGF stimulation was examined in miR-143-overexpressing cells, by RT-qPCR (**a**), by Western blot (**b**), and by ELISA on conditioned media. *$p<0.05$. *NS* non-significant

data suggest that miR-143 may inhibit MMP9 at a level downstream of EGFR phosphorylation. Indeed, we found that the phosphorylation of ERK1/2 was also inhibited in miR-143-overexpressing U2OS and HOS cells (Fig. 4c), suggesting that miR-143 may inhibit phosphorylation of ERK1/2 to abolish MMP9 activation.

Discussion

Understanding the molecular basis of OS metastasis is potentially important for its efficient therapy. EGFR was frequently expressed in OS, and the growth and metastasis of OS have been shown to depend on MMP9. Nevertheless, the molecular pathway controlling the activation of MMP9 in OS is completely understood. MiRNAs are crucial for cell proliferation, apoptosis, differentiation, signaling pathways, and carcinogenesis, especially in antagonizing tumor formation and progression, since they may reduce the aberrant signals produced by overexpressed oncogenes or alternatively to forcefully increase the expression of tumor-suppressor genes. However, the precise role of different miRNAs and their interaction with EGFR signaling pathway, or with MMP9, have not been studied before.

We did a screening of miRNAs in the OS samples and we specifically detected significant decrease in miR-143 levels. Therefore, we were prompted to study MMP9 and miR-143, and their crosstalk with activation of EGFR signaling in OS. Since we found significant decrease in miR-143, and significant increase in phosphorylated EGFR and MMP9 in the resected OS from the patients, compared to the adjacent normal tissue, and since strong correlations were detected among these three factors, we hypothesized that there might be a regulation loop.

We then used two human OS lines to study the molecular basis. We found that EGF-induced EGFR phosphorylation in these cells activated MMP9, and consequently cancer invasiveness. Both an inhibitor for EGFR and an inhibitor for ERK1/2 phosphorylation significantly inhibited the EGF-induced activation of MMP9, suggesting an ERK/MAPK signaling cascades dependent pathway. Of note, we also checked JNK and PI3K signaling pathways, and our data did not support such an involvement.

We also found that miR-143 levels were not affected by EGF-induced EGFR phosphorylation. On the other hand, overexpression of miR-143 antagonized EGF-induced MMP9 activation without affecting EGFR phosphorylation in OS cells. Also, we have examined several other lines, and essentially have same results, which exclude a possibility of cell-line-dependence. Taken together, our data suggest that miR-143 inhibits EGFR signaling through downstream ERK/MAPK signaling cascades to regulate MMP9 expression in OS. Our work thus provides further insights into the molecular basis underlying the EGFR signaling regulated metastasis of OS, suggesting that modulation of miR-143 or

ERK1/2 phosphorylation may affect the MMP9-dependent OS invasiveness.

Conflicts of interest None

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