MicroRNA-224 promotes the sensitivity of osteosarcoma cells to cisplatin by targeting Rac1

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Abstract

Osteosarcoma is the most common primary bone tumour in children and adolescents. Accumulating evidence has shown that microRNAs (miRNAs) participate in the development of almost all types of cancer. Here, we investigated the role of miR-224 in the development and progression of osteosarcoma. We demonstrated that miR-224 was down-regulated in osteosarcoma cell lines and tissues. Lower miR-224 levels were correlated with shorter survival in osteosarcoma patients. Furthermore, overexpression of miR-224 suppressed osteosarcoma cell proliferation, migration and invasion and contributed to the increased sensitivity of MG-63 cells to cisplatin. We identified Rac1 as a direct target gene of miR-224 in osteosarcoma. Rac1 expression was up-regulated in the osteosarcoma cell lines and tissues, and there was an inverse correlation between Rac1 and miR-224 expression in osteosarcoma tissues. Furthermore, rescuing Rac1 expression decreased the sensitivity of miR-224-overexpressing MG-63 cells to cisplatin. We also demonstrated that ectopic expression of Rac1 promoted the proliferation, migration and invasion of miR-224-overexpressing MG-63 cells. These data suggest that miR-224 plays a tumour suppressor role in the development of osteosarcoma and is related to the sensitivity of osteosarcoma to cisplatin.

Keywords: osteosarcoma • cisplatin • microRNA • miR-224 • Rac1

Introduction

Osteosarcoma is the most frequent primary bone tumour that arises from osteoid tissues in young adults and children [1–4]. Most patients with osteosarcoma are diagnosed at a late stage, and many patients need radiotherapy or/and chemotherapy [5–8]. However, the response to chemotherapy is usually poor in these patients [9–12]. The molecular mechanisms of chemotherapeutic drugs used for osteosarcoma treatment are unknown.

MicroRNAs (miRNAs) are small noncoding RNAs that can suppress the expression of protein coding genes by targeting mRNAs for cleavage and/or translational repression [13–16]. Recent evidence has demonstrated that miRNAs play critical roles in many biological processes, such as lineage determination and cell proliferation, migration, apoptosis and differentiation [2, 17–19]. Deregulation of miRNAs is involved in the initiation, progression and metastasis of various types of cancer, in which miRNAs can act as oncogenes or tumour suppressors [20–23]. Moreover, accumulating evidence has demonstrated that miRNA expression is significantly associated with chemosensitivity in cancer [24–27].

miR-224 is a well-known miRNA that plays important roles in the development of multiple tumours [28–32]. For example, Liao et al. found that miR-224 was up-regulated in colorectal cancer and that high miR-224 expression was associated with poor prognosis and an aggressive phenotype. Moreover, miR-224 overexpression increased colorectal cancer cell proliferation by targeting PHLPP1 and PHLPP2 expression [33]. However, the exact role of miR-224 remains unknown. In this study, we determined that miR-224 expression was down-regulated in osteosarcoma cell lines and tissues and that lower miR-224 levels were correlated with shorter patient survival. We also observed that overexpressing miR-224 suppressed osteosarcoma cell proliferation, migration and invasion. Moreover, miR-224 overexpression contributed to the increased sensitivity of MG-63 cells to cisplatin. We identified Rac1 as a direct target gene of miR-224 in osteosarcoma.

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Materials and methods

Clinical tissues and cell lines

The osteosarcoma tissues were obtained at our department from 2012 to 2015. The clinical characteristics of the patients are listed in Table 1. Tissues were obtained with informed consent, and our study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. The human osteosarcoma cell lines (MG-63, U2OS, SOSP-9607 and SAOS-2) used in this study were cultured as described in our previous study [34].

Cell transfection

The miR-224 and scramble mimics were synthesized by GenePharma (Shanghai, China) and transfected into the cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions.

Protein and mRNA quantification

Quantitative RT-PCR was performed to detect mRNA and miRNA expression, and the primer sequences are shown in Table 2. Total RNA was isolated from cells or tissues with TRizol reagent (Invitrogen, California, USA). Western blotting was performed to detect protein expression as previously described [34]. The following primary antibodies were used: Rac1 and GAPDH (Abcam, Cambridge, UK).

Cell proliferation, migration and invasion assays

Cell Counting Kit-8 (CCK-8) assays (DOJINDO, Kyushu, Japan) were used to detect cell proliferation according to the manufacturer’s instructions. Scratch assays were used to measure cell migratory potential. The scratches were generated using a pipette tip, and the cells were then cultured in fresh medium. Invasion assays were used to assess cell invasion ability based on passage through a Matrigel-coated matrix membrane (BD Biosciences, New York, USA), as previously described [34].

Dual luciferase assays

The cells were cotransfected with the reporter construct, control vector and scramble or miR-224 mimics. Cells were collected after 24 hrs and analyzed with the Dual Luciferase Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Firefly luciferase data were normalized to Renilla luciferase data, and Firefly/Renilla ratios were calculated.

Statistical analysis

One-way ANOVA was performed for data involving 3 groups, and sets of two groups were analyzed using Student’s t-test (two-tailed). All the statistical analyses were performed with SPSS 17.0 (SPSS, Inc., IBM, Chicago, IL, USA). P < 0.05 was considered statistically significant.

Result

miR-224 increased the sensitivity of MG-63 cells to cisplatin

Osteosarcoma cell lines (MG-63, U2OS, SOSP-9607 and SAOS-2) had lower miR-224 mRNA expression than hFOB cells (Fig. 1A). miR-224 expression was up-regulated in MG-63 cells after transfection with a miR-224 mimic (Fig. 1B). The response of MG-63 cells to
cisplatin increased after transfection with the miR-224 mimic compared with scramble-transfected cells (Fig. 1C).

**miR-224 was down-regulated in osteosarcoma tissues**

Compared with non-tumour tissues, miR-224 was down-regulated in osteosarcoma tissues (Fig. 2A) from most osteosarcoma patients (26/35) (Fig. 2B). Higher miR-224 levels were correlated with longer patient survival (Fig. 2C).

**miR-224 overexpression suppressed osteosarcoma cell migration, invasion and proliferation**

Ectopic expression of miR-224 suppressed MG-63 cell migration (Fig. 3A). In addition, we performed cell invasion assays to ascertain the invasion ability of MG-63 cells after transfection with the miR-224 mimic. Overexpression of miR-224 decreased MG-63 cell invasion (Fig. 3B). CCK-8 assays showed that the growth rate of miR-224 mimic-transfected MG-63 cells was inhibited compared with that of scramble-transfected cells (Fig. 3C).

**Rac1 is a direct target of miR-224**

As predicted by TargetScan, complementarity existed between has miR-224 and the Rac1 3'UTR (Fig. 4A). Ectopic expression of miR-224 suppressed Rac1 expression in MG-63 cells (Fig. 4B and C). The effect of miR-224 on Rac1 mRNA translation into protein was confirmed by a luciferase reporter assay (Fig. 4D). Ectopic expression of miR-224 reduced the luciferase activity of a wild-type reporter but not of a mutant Rac1 3'UTR reporter, suggesting that miR-224 can directly target the Rac1 3'UTR (Fig. 4D). Furthermore, we showed that osteosarcoma cell lines (MG-63, U2OS, SOSP-9607 and SAOS-2) expressed higher Rac1 levels than hFOB cells (Fig. 4E).

**Rac1 was up-regulated in osteosarcoma tissues with low levels of miR-224**

Compared with non-tumour tissues, Rac1 was up-regulated in osteosarcoma tissues (Fig. 5A) from 28 of 35 osteosarcoma patients (Fig. 5B). Kaplan–Meier survival analysis of osteosarcoma patients with high or low miR-224 expression (N = 35). Patients with low miR-224 expression exhibited significantly longer overall survival (OS).
Interestingly, among pairs of osteosarcoma tissues and non-tumour tissues, Rac1 expression was inversely correlated with miR-224 expression (Fig. 5C).

**miR-224 increased the sensitivity of MG-63 cells to cisplatin and suppressed osteosarcoma cell proliferation, migration and invasion by down-regulating Rac1**

The Rac1 vector promoted the expression of Rac1 in MG-63 cells (Fig. 6A and B). The responses of MG-63 cells and miR-224-overexpressing MG-63 cells to cisplatin were decreased after transfection with the Rac1 vector compared with the control vector (Fig. 6C and 6D). Overexpression of Rac1 promoted MG-63 cell proliferation (Fig. 6E). Moreover, ectopic expression of Rac1 increased the proliferation (Fig. 6F), migration (Fig. 6G) and invasion (Fig. 6H) of miR-224-overexpressing MG-63 cells.

**Discussion**

In this study, we demonstrated that miR-224 was down-regulated in osteosarcoma cell lines and tissues and that lower miR-224 levels
were correlated with shorter patient survival. We also observed that overexpression of miR-224 suppressed osteosarcoma cell proliferation, migration and invasion and contributed to the increased sensitivity of MG-63 cells. We next identified Rac1 as a direct target gene of miR-224 in osteosarcoma. Rac1 was up-regulated in osteosarcoma cell lines and tissues, and there was an inverse correlation between Rac1 and miR-224 expression in the osteosarcoma tissues. Furthermore, overexpression of Rac1 decreased the sensitivity of miR-224-overexpressing MG-63 cells to cisplatin. We also demonstrated that ectopic expression of Rac1 promoted the proliferation, migration and invasion of miR-224-overexpressing MG-63 cells. These data suggest that miR-224 plays a tumour suppressor role in osteosarcoma development and is related to the sensitivity of osteosarcoma to cisplatin.

miR-224 is a well-known miRNA that plays important roles in the development of multiple tumours [28–32]. For example, Liao et al. found that miR-224 was up-regulated in colorectal cancer and that high miR-224 expression was associated with poor prognosis and an aggressive phenotype. Moreover, miR-224 overexpression increased colorectal cancer cell proliferation by targeting PHLPP1 and PHLPP2 expression [33]. However, Ke et al. showed that miR-224 expression levels were lower in colorectal cancer tissues and that overexpression

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**Fig. 4** Rac1 is a direct target of miR-224. (A) The 3' UTR of Rac1 contains one predicted miR-224 binding site. The mutagenesis nucleotides are indicated in red. (B) Overexpression of miR-224 suppressed Rac1 mRNA expression in MG-63 cells. (C) Overexpression of miR-224 suppressed Rac1 protein expression in MG-63 cells. (D) MG-63 cells were cotransfected with 3'UTR-reporter constructs and a miR-224 mimic or a scramble sequence, and dual luciferase reporter assays were performed. (E) Rac1 expression was measured in osteosarcoma cell lines (MG-63, U2OS, SOSP-9607 and SAOS-2) and hFOB cells by qRT-PCR. **P < 0.01 and ***P < 0.001.

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**Fig. 5** Rac1 was up-regulated in osteosarcoma tissues with low levels of miR-224. (A) Rac1 was detected by qRT-PCR in tissues from 35 osteosarcoma patients. (B) Of the 35 osteosarcoma tissues, Rac1 was up-regulated in 28 tumour tissues compared with non-tumour tissues. (C) Among pairs of osteosarcoma tissues and non-tumour tissues, there was an inverse correlation between Rac1 and miR-224 expression.
Fig. 6 miR-224 increased the sensitivity of MG-63 cells to cisplatin and suppressed osteosarcoma cell proliferation, migration and invasion by down-regulating Rac1 expression. (A) Rac1 protein expression was detected by western blot analysis. (B) Rac1 mRNA expression was detected by qRT-PCR. (C and D) Cell survival was examined using MTS assays. (E and F) Cell proliferation was evaluated using CCK-8 assays. (G) The wound healing analysis demonstrated that ectopic expression of Rac1 promoted the migration of miR-224-overexpressing MG-63 cells. (H) The invasion analysis demonstrated that ectopic expression of Rac1 promoted the invasion of miR-224-overexpressing MG-63 cells. *P < 0.05, **P < 0.01 and ***P < 0.001.
of miR-224 suppressed colorectal cancer cell migration [32]. He et al. demonstrated that miR-224 was up-regulated in esophageal intra-epithelial neoplasia and esophageal squamous cell carcinoma and that miR-224 overexpression increased esophageal squamous cell carcinoma cell migration, proliferation and invasion by inhibiting PHLPP1 and PHLPP2 [35]. Wang et al. showed that miR-224 expression was higher in meningioma tissues than in normal brain tissue, and high miR-224 expression was associated with an advanced pathological grade [36]. Inhibition of miR-224 decreased meningioma cell proliferation by targeting ERG2 expression. However, the role of miR-224 in osteosarcoma was unknown. In our study, we measured miR-224 expression in 4 osteosarcoma cell lines and 35 osteosarcoma tissues. We found that miR-224 expression was down-regulated in the osteosarcoma cell lines and tissues and that lower miR-224 levels were correlated with shorter patient survival. Furthermore, overexpression of miR-224 suppressed osteosarcoma cell proliferation, migration and invasion and increased the sensitivity of MG-63 cells to cisplatin.

Rac1 is a member of the Ras superfamily of Rho proteins, and it plays an essential role in many cellular processes, including mitogenesis, kinase cascade activation, transcriptional activation, DNA synthesis and cytoskeleton reorganization [37–42]. Rac1 overexpression has been found in various types of cancer, such as lung cancer, gastric cancer, pancreatic cancer, bladder cancer and breast cancer [43–47]. Moreover, activation of Rac1 can increase cancer cell migration, adhesion, invasion, proliferation and metastasis [40, 44, 48–50]. In our previous study, we found that miR-124 repressed osteosarcoma cell proliferation, migration and invasion by targeting Rac1 expression [34]. However, the underlying mechanisms of Rac1 overexpression in osteosarcoma were unclear. In this study, we demonstrated that Rac1 was a direct target gene of miR-224 in osteosarcoma. Ectopic expression of miR-224 reduced the luciferase activity of the wild-type reporter but not of the mutant Rac1 3’UTR reporter, suggesting that miR-224 can directly target the Rac1 3’UTR. Moreover, overexpression of miR-224 repressed Rac1 expression in MG-63 cells. We also demonstrated that Rac1 was up-regulated in osteosarcoma cell lines and tissues, and there was an inverse correlation between Rac1 and miR-224 expression in osteosarcoma tissues. These results suggested that the ability of miR-224 to target Rac1 may represent a mechanism for the post-transcriptional regulation of Rac1 expression.

Accumulating evidence has shown that deregulated miRNA expression plays important roles in drug resistance [51–54]. miRNAs can regulate the expression of various genes and play significant roles in cell proliferation, apoptosis and cell cycle, resulting in different cellular sensitivity to chemotherapeutic agents [55–58]. In our study, we found that miR-224 overexpression contributed to the increased sensitivity of MG-63 cells to cisplatin. Moreover, Rac1 overexpression decreased the sensitivity of miR-224-overexpressing MG-63 cells to cisplatin. These findings indicated that miR-224 acts as a critical predictor of the response of osteosarcoma to chemotherapy.

In conclusion, we are the first to demonstrate that miR-224 plays a tumour suppressor role in osteosarcoma cells. Ectopic miR-224 expression suppressed Rac1 expression, which in turn inhibited osteosarcoma cell proliferation, migration and invasion and may be involved in drug resistance. These results indicate that miR-224-Rac1 is a potential therapeutic target for the prevention of osteosarcoma.

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Conflict of interest
The authors declare no conflict of interest.

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