Tumor suppressive microRNA-424 inhibits osteosarcoma cell migration and invasion via targeting fatty acid synthase

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Abstract. Numerous studies have recently suggested that miRNAs contribute to the development of various types of human cancer as well as to their invasive and metastatic capacities. The aim of this study was to investigate the functional significance of miR-424 and to identify its possible target genes in osteosarcoma (OS) cells. Previously, inhibition of fatty acid synthase (FASN) has been shown to suppress OS cell proliferation, invasion and migration. The prediction was made using the microRNA.org and TargetScan.human6.0.database. The results showed that FASN is a promising target gene of miR-424. FASN may be a direct target of miR-424 as shown by the luciferase reporter assays. Furthermore, miR-424 expression was increased in osteosarcoma cells by transfection with has-miR-424. FASN mRNA and protein expression levels were measured by RT-PCR and western blot analysis. Cell migration and invasion was measured using Transwell migration and Transwell invasion assays. Expression levels of FASN mRNA and protein were greatly decreased in U2OS cells transfected with has-miR-424. The migration and invasion of cells was significantly decreased by the upregulation of miR-424. These findings suggested that miR-424 plays a key role in inhibiting OS cell migration and invasion through targeting FASN.

Introduction

Osteosarcoma (OS) is one of the most common primary malignant bone tumors in childhood and adolescence. It was not until the early 1970s that the introduction of doxorubicin and methotrexate with leucovorin rescue revealed the potential to improve survival. With the advent of effective chemotherapy, the 5-year survival rate of patients treated with intensive multidrug chemotherapy and aggressive local control have been reported as 55-80% (1-3). Despite the encouraging trend towards longer survival many patients still face a dismal outcome. Numerous articles have reported that the 5-year survival rate of patients with metastatic diseases is <20% (4-6). Clearly, the impact of identifying factors that govern metastasis is significant in the management of osteosarcoma.

Fatty acid metabolic pathways play an important role in carcinogenesis (7). Fatty acid synthase (FASN) is an enzyme crucial for endogenous lipogenesis in mammals responsible for catalyzing the synthesis of long-chain fatty acids. FASN is critical to sustain the biological features of cancer cells (8). FASN is expressed at high levels in a variety of human tumors (9-13) with low levels in normal tissues. Various reports have shown that inhibiting expression of FASN suppresses cancer cell proliferation in vitro and in vivo (14-19). Recent studies revealed that FASN may contribute to cancer cell metastasis (20-22). FASN is, thus, considered a novel promising target for anticancer therapy.

miRNAs are small endogenous RNAs averaging 20 to 24 nucleotides, transcribed from non-protein-coding genes or introns, which mediate translational suppression or cleavage of their target mRNAs by binding to complementary sites in their 3’UTR (23-25). A large number of miRNAs are located inside or close to fragile chromosomal sites that are frequently lost or amplified in cancer (26). miRNAs have been characterized as oncogenes, tumor suppressors or as components of regulatory pathways critical for tumorigenesis. miRNAs play an important role in tumorigenesis and metastasis.

The aim of this study was to investigate the functional significance of miR-424 and to identify its possible target genes in osteosarcoma (OS) cells.

Materials and methods

Cell culture and transfection. Human OS cell line U2OS (Shanghai Cell Bank, Chinese Academy of Sciences, China) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO2. U2OS cells were seeded in six-well plates at 30% confluence on the day before transfection. Transfection with has-miR-424 or negative miRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfection complexes were prepared according to the
Upregulation of miR-424 inhibits cell migration in vitro. To corroborate the effect of the upregulation of miR-424 on U2OS cell migration, migration was measured by Transwell migration assay. U2OS cells were transfected with mir-424 or negative miRNA. The results showed that the migration of cells transfected with has-miR-424 was significantly inhibited when compared with cells transfected with negative miRNA (Fig. 1A and B, P<0.05). These results suggested that upregulation of miR-424 inhibited the migration of U2OS cells.

Upregulation of miR-424 inhibits cell invasion in vitro. To examine the effect of upregulation of miR-424 on U2OS
cell migration, the Transwell invasion assay was performed. U2OS was transfected with has-miR-424 or negative miRNA. The results showed that the invasion of cells transfected with has-miR-424 was significantly inhibited when compared with cells transfected with negative miRNA. (Fig 1C and D; \(P<0.05\)). These results suggested that upregulation of miR-424 inhibits the invasion of U2OS cells.

**FASN is a direct target of miR-424.** To validate whether miR-424 regulates FASN directly through a putative binding site in U2OS cells, we cloned FASN 3′-UTR in the predicted miRNA binding site into the luciferase gene (pSiCHECK2; Promega). Following cotransfection with the pSiCHECK2 vectors and miR-424 or negative control miR-001, the upregulation of miR-424 in U2OS cells transfected with has-miR-424 resulted in a significant decrease in the luciferase activity of the wild-type FASN 3′-UTR (Fig. 2; \(P<0.05\)). The results indicate that FASN is a direct target of miR-424.

**miR-424 negatively regulates FASN mRNA expression in U2OS cells.** To investigate the effect of upregulation of miR-424 on the expression of FASN mRNA, miR-424 was upregulated in U2OS cells by treatment with has-miR-424 for 48 h. The expression level of FASN mRNA was measured by qRT-PCR. The data (\(2^{-\Delta\Delta C_{t}}=0.254\pm0.01157\)) showed that the FASN mRNA expression in cells transfected with negative control vector was four-fold that in cells transfected with has-miR-424. It indicated that miR-424 may negatively regulate the expression of FASN mRNA.

**miR-424 inhibits FASN protein expression in U2OS cells.** To investigate the effect of upregulation miR-424 on the expression of FASN protein, miR-424 was upregulated in U2OS cells by treatment with has-miR-424 for 48 h. The expression level of FASN protein was measured using western blot analysis. The results revealed that the upregulation of miR-424 in OS cells resulted in decreasing the expression of FASN protein.
(Fig 3A and B). It suggested that miR-424 may negatively regulate the expression of FASN protein.

Discussion

miR-424, one of the miR-16/15/195/424/497 family members, induces muscle differentiation and promotes cell cycle quiescence and differentiation (27-29) and regulates cell-autonomous angiogenesis (30-32). Recent evidence demonstrated that miR-424 plays an important role in tumorigenesis (33-34). miR-424 has been reported to be downregulated in cervical cancer (33), senile hemangioma (30), tongue cancer (35), chronic myelogenous leukemia (36) and acute myeloid leukemia (34). However, miR-424 is upregulated in human colorectal cancer (37-38) and atypical chronic myeloid leukemia (37). Deregulation of miR-424 may be different in different types of cancer and the roles of miR-424 in carcinogenesis and progression should not be assumed as a tumor suppressor or oncogene. The roles of miR-424 deregulation in cancer development remains to be further investigated. In the present study, miR-424 was downregulated in the human osteosarcoma cell line U2OS. The inhibiting effect on OS cell migration and invasion by upregulation of miR-424 was observed. It suggested that miR-424 plays a role as a tumor suppressor in inhibiting OS cell migration and invasion.

A previous study reported that inhibition of FASN causes a decrease in OS cell invasion and migration. The prediction was made using software (microRNA.org and TargetScan, human6.0). It revealed that miR-424 may be targeting FASN. In this study, to investigate the molecular mechanism that inhibits the migration and invasion by restoration of miR-424 in OS cells, RT-PCR and western blot analysis were performed to detect the expression level of FASN mRNA and protein in the OS cell line U2OS. The results showed that FASN expression was significantly inhibited in cells transfected with has-miR-424 compared with the control group (Fig. 4). This suggested that restoration of miR-424 expression could inhibit FASN expression in OS cells. Furthermore, to identify whether miR-424 regulates the expression of FASN, the FASN 3'-UTR was cloned into the pSiCHECK2, placing the 3'-UTR with miR-424 or negative miR-001. Overexpression of miR-424 significantly reduced luciferase activity from the reporter construct containing the FASN 3'-UTR. It indicated that FASN is a direct miR-424 target. There are hundreds of predicted targets of miR-424 in the TargetScan prediction and a single miRNA is demonstrated to target multiple mRNAs to regulate gene expression (39), therefore it is probable that other targets of miR-424 may also participate in OS migration and invasion. miR-424 may also target different molecules in different types of cancer. Additionally, the tumor microenvironment may influence tumor progression, invasion and migration. This study indicates that expression of FASN is negatively regulated by miR-424 through a special binding site of the FASN 3'-UTR. Moreover, miR-424 inhibits cell invasion and migration in OS cells in vitro. These results suggest that miR-424 plays a key role in inhibiting OS cell migration and invasion by targeting FASN. Further research is necessary to identify the entire roles of miR-424 in osteosarcoma metastasis.

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