miR-9 Modulates Osteosarcoma Cell Growth by Targeting the GCIP Tumor Suppressor

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Abstract

Osteosarcoma is the most common primary bone tumor in humans, especially in childhood. However, the genetic etiology for its pathogenesis remains elusive. It is known that microRNAs (miRNAs) are involved in the development of tumor progression. Here we show that microRNA-9 (miR-9) is a potential oncogene upregulated in osteosarcoma cells. Knockdown of miR-9 in osteosarcoma resulted in suppressed colony formation and cell proliferation. Further study identified GCIP, a Grap2 and cyclin D interacting protein, as a direct target of miR-9. In addition, GCIP overexpression activated retinoblastoma 1 (Rb) and suppressed E2F transcriptional target expression in osteosarcoma cells. Moreover, GCIP depletion reversed miR-9 knockdown induced colony formation and cell proliferation suppression. In sum, these results highlight the importance of miR-9 as an oncogene in regulating the proliferation of osteosarcoma by directly targeting GCIP and may provide new insights into the pathogenesis of osteosarcoma.

Keywords: miR-9 - osteosarcoma - GCIP - proliferation - GCIP - tumor suppressor

Introduction

Osteosarcoma is a rare malignancy but the most common histological form of primary bone sarcoma in children and adolescents (Dorfman and Czerniak, 1995; Moore and Luu, 2014). The five-year survival rate for osteosarcoma patients is around 70%. More than 80% of patients will progress to develop metastasis following resection of the primary tumor and the five-year survival rate drops to only 20-30%. Lung is the most frequent metastasized tissue. Although several predisposing environmental and genetic factors have been identified, the exact etiology of the disease remains unknown (He et al., 2014; Li et al., 2014).

microRNAs (miRNAs) has been defined as short double-stranded RNA hairpins which target the 3’-untranslated region (UTR) of specific mRNA to downregulate the target gene expression (John et al., 2004; Calin and Croce, 2006; Ha and Kim, 2014; Kavitha et al., 2014). miRNAs are found expressed in both normal and malignant tissues and can function as oncogenes or tumor suppressors (Calin and Croce, 2006; Jansson and Lund, 2012). miR-9 was first found involved in TLR4 activation by targeting NFKB1 transcript (Bazzoni et al., 2009). Recent studies indicate miR-9 oncogenic role in kidney, breast, stomach and lung cancer, which is suggested to modulate cell proliferation migration and angiogenesis (Delaloy et al., 2010; Ma et al., 2010; Zhuang et al., 2012; Gao et al., 2013; Zhao et al., 2014). However, its functional role in osteosarcoma still remains unknown.

GCIP is a HLH leucine zipper protein, which interacts with Grap2 and Cyclin D and regulates E2F transcriptional activity. In the current study, we found that miR-9 was upregulated in osteosarcoma cells. Knockdown of miR-9 in osteosarcoma cells resulted in suppressed colony formation and cell proliferation. Furthermore, GCIP was characterized as a direct target of miR-9. In addition, further study indicated miR-9’s oncogenic function is GCIP dependent. Taken together, these results suggest miR-9 as a potential oncogene in osteosarcoma.

Materials and Methods

Cell culture

Established osteosarcoma lines HOS and U-2 OS used in this study were obtained from the American Type Culture Collection (ATCC) and were maintained as manufacture instruction. Human Mesenchymal Stem Cells (MSCs) and Normal Human Osteoblasts (NHOsts) were purchased from Lonza and were maintained as manufacture instruction.

Plasmid and RNA oligo transfection

miR-9 antisense oligos and negative control
miR-9 is Upregulated in Osteosarcoma Cells and Modulates Cell Proliferation.

Mesenchymal stem cells (MSCs) and osteoblast precursors are suggested to be the cells of origin of osteosarcoma (Mutsaers and Walkley, 2014). To investigate the potential role of miR-9 in osteosarcoma, we first characterized its expression in osteosarcoma cell lines, HOS and U-2 OS, MSCs and osteoblast precursors. Interestingly, miR-9 is overexpressed in osteosarcoma cell lines, compared with MSCs and osteoblast precursors (Figure 1A). These results indicate that miR-9 may play an oncogenic role in osteosarcoma. To confirm this hypothesis we inhibited its expression in HOS and U-2 OS cells with antisense oligos (anti-miR-9). And the inhibition efficiency was confirmed by qPCR assay (Figure 1B). Then colony formation assay was performed. As we expected, miR-9 inhibited cells displayed much fewer and smaller colonies compared with negative control cells (Figure 1C). To further confirm the above results, miR-9 was reintroduced into these cells. Proliferation assay showed that miR-9 knockdown inhibited colony formation and cell proliferation, compared with negative control (Figure 1D). These results indicated that miR-9 overexpression is associated with cell proliferation and colony formation in osteosarcoma. To further investigate this hypothesis, we performed luciferase reporter assay to study the targets of miR-9. As shown in Figure 1E, miR-9 targets GCIP, a cell cycle regulatory gene, and negatively regulates its expression. These results indicated that miR-9 may play an oncogenic role in osteosarcoma by targeting GCIP.
miR-9 targets the 3′-UTR of GCIP transcripts and downregulates GCIP expression

It is widely accepted that miRNAs exert their functions through imperfect pairing with target messenger RNAs (mRNAs) of protein-coding genes and the transcriptional or post-transcriptional regulation of their expression (Calin and Croce, 2006). To explore the molecular mechanism through which miR-9 exerts its function we predicted and identified the candidate target genes of miR-9. miRNA target prediction program, TargetScan, revealed that GCIP, a 40-kDa HLH leucine zipper protein, was a potential target of miR-9. The GCIP segments contained a complementary site for miR-9 binding sequence (Figure 2A). In addition, GCIP is a putative tumor suppressor in breast, colon and liver cancers. We predicted that miR-9 might carry out its function by suppressing GCIP expression.

To validate whether GCIP is a bona fide target of miR-9, a human GCIP 3′-UTR fragment containing wild-type or mutant miR-9 binding sequence (Figure 2A) was cloned downstream of the firefly luciferase reporter gene. miR-9 antisense oligos (anti-miR-9) or negative control antisense oligos (anti-control) were then cotransfected with firefly luciferase reporter in osteosarcoma cells. Results showed that the relative luciferase activity of the reporter containing wild-type 3′-UTR was significantly enhanced by miR-9 antisense oligos, compared with negative control antisense oligos (Figure 2B). In contrast, the reporter vector carrying the mutated GCIP 3′-UTR could restore luciferase activity when this construct was cotransfected with miR-9 antisense oligos, indicating that miR-9 may suppress gene expression through miR-9-binding sequence at the 3′-UTR of GCIP (Figure 2B).

The effect of miR-9 on the endogenous expression of GCIP was further examined by qPCR and western blot analysis. We found that transfection with miR-9 antisense oligos in osteosarcoma cells resulted upregulated endogenous mRNA and protein levels of GCIP (Figure 2C and 2D). Taken together, these results indicate that miR-9 may suppress the expression of GCIP by directly targeting the 3′-UTR of GCIP transcript.

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miR-9, we wonder whether miR-9’s oncogenic effects are GCIP dependent. We knockeddown expression of GCIP in osteosarcoma cells transfected with miR-9 antisense oligos to normal level. Knockdown efficiency was confirmed by qPCR and western blot analysis (Figure 4A and 4B). Then colony formation assay was performed. Results showed that GCIP knockdown reversed colony numbers and size in miR-9 antisense oligos transfected cells (Figure 4C). Furthermore, we examined whether GCIP could affect cell proliferation in miR-9 depletion cells. In agreement with colony formation assay, GCIP knockdown reversed miR-9 depletion induced cell proliferation suppression. Together, these results suggest that miR-9 may exert oncogenic effects by directly targeting GCIP 3′-UTR fragment.

**Discussion**

miRNA alterations are involved in the initiation and progression of human cancer. In osteosarcoma, miRNA alterations are also reported (Song et al., 2010; Zhao et al., 2013; Gao et al., 2014; Lv et al., 2014). miR-9 was first reported as a proinflammatory signals regulator in monocytes and neutrophils (Bazzoni et al., 2009). Further study revealed that miR-9 also participated in neural progenitor cells proliferation and migration regulation (Delaloy et al., 2010). Recent studies focused on its potential role in malignant diseases. However, functions of miR-9 in tumor progression exhibit controversial in different tissue context (Hildebrandt et al., 2010; Ma et al., 2010; Zhuang et al., 2012; Visani et al., 2014). In this work, we identified miR-9 as an oncogenic miRNA in osteosarcoma. MSCs and NHOsts are supposed to be the cells of origin of osteosarcoma (Mutsaers and Walkley, 2014). We showed that miR-9 was upregulated in osteosarcoma cells compared with MSCs and NHOsts. Knockdown of miR-9 in osteosarcoma cells could suppress colony formation and cell proliferation. These suggest miR-9’s oncogenic effects in osteosarcoma.

We further characterized GCIP as a direct target of miR-9. Bioinformatics analysis revealed that the 3′-UTR of GCIP mRNA had a complementary site for miR-9 binding. Luciferase reporter assay showed that miR-9 knockdown upregulated luciferase activity of reporter vector carrying wild type 3′-UTR of GCIP. In contrast, there was no effect of miR-9 knockdown on luciferase activity of reporter vector carrying mutant 3′-UTR of GCIP. qPCR and western blot assay also confirmed that miR-9 knockdown upregulated GCIP expression in osteosarcoma cells. Furthermore, GCIP depletion reversed colony formation and cell proliferation suppression in in miR-9 knockdown cells. These data suggest miR-9 may exert its oncogenic effects by directly targeting GCIP in osteosarcoma.

GCIP is a Grap2 and Cyclin D Interacting Protein, which is reported as a tumor suppressor in breast, prostate, liver and colon tumor tissues (Ma et al., 2006; Chang et al., 2008; Chen et al., 2008; Lee et al., 2010; Chen et al., 2014). However, its role in osteosarcoma has not been characterized. Our results showed that GCIP was downregulated in osteosarcoma cells and could upregulate underphosphorylated form of Rb in osteosarcoma, which in turn suppressed E2F targeting gene, Cdc6 and Cyclin E1 expression.

In summary, our current data identify a new oncogenic miRNA in osteosarcoma and help to understand the biology role of miR-9 in different tissue context. Furthermore, we found a new direct target of miR-9, GCIP. These results may provide new insights into the pathogenesis of osteosarcoma.

**References**


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