RESEARCH ARTICLE

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

Shao-Wen Zhu[1,2&], Jian-Peng Li[3&], Xin-Long Ma[2*], Jian-Xiong Ma[2], Yang Yang[2], Yang Chen[2], Wei Liu[1]

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

Asian Pac J Cancer Prev, **16 (11)**, 4509-4513

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

*[1]Tianjin Medical University, [2]Department of Orthopedics, Tianjin Hospital, [3]Department of Orthopedics, the Fifth Center Hospital of Tianjin, Tianjin, China [&]Equal contributors *For correspondence: maxinlongtjh@126.com*

oligos were purchased from Genepharma. For GCIP overexpression, CDS region of GCIP were cloned to pcDNA3.1 plasmid. For GCIP knockdown, siRNA targeting 5'-GATTTGGCTCTGAGCATATAT-3' were obtained from Genepharma. For luciferase activity assay, a wild-type 3'-UTR segment of GCIP were cloned in to pmirGLO (Promega) plasmid. The mutated sequences in the complementary site for miR-9 were generated by site-specific mutagenesis. All transfection was performed using lipofectamine 2000 (Invitrogen) as manufacture instructions.

Colony formation assay

Cells were transfected with indicated plasmid or antisense oligos. Twenty-four hours after transfection, 400 cells per well were placed in a six-well plate and were cultured for 2 weeks, and the culture medium was replaced every 3 days. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 20 min.

Luciferase reporter assay

HOS or U-2 OS cells were plated in 48-well plate and cotransfected with either miR-9 antisense oligos or negative control antisense oligos and either pmirGLO-GCIP-3'-UTR-WT or pmirGLO-GCIP-3'-UTR-MUT and pRL-TK. 48 hours after transfection, luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega) using pRL-TK as an internal control. Each experiment were repeated at least three times independently.

Proliferation assay

Osteosarcoma cells transfected with indicated plasmid or antisense oligos were seeded, 3×10^4 cells/well in 6-well plates, and cell numbers determined every 2 days for 8 days by Countess (Invitrogen). Each experiment was carried out in triplicate, at least 3 times.

qPCR and western blot assay

miR-9 and mRNA expression for indicate gene were analyzed by qPCR assay. Total RNA was extracted by using Trizol (Invitrogen). For miR-9 expression analysis, a Taqman miRNA assay kit and Taqman miRNA assay supplies were used to quantify the expression of mature miRNAs according to the manufacturer's instructions (Applied Biosystems). For mRNA, cDNA was synthesized using First Strand cDNA Synthesis Kit (Thermo). Quantitative real-time PCR analysis was performed using the 7500 Real-Time PCR System (Applied Biosystems). U6 and GAPDH were used as endogenous control. The primers used in this study are shown below: GCIP, GACCACCGAGGAGTTTAATCG and GGGTGATCCCCTGATCCTTTG. GAPDH, GGAGCGAGATCCCTCCAAAAT and GGCTGTTGTCATACTTCTCATGG. The following antibodies were used for western blot assay from the indicated suppliers, anti- GCIP (R and D Systems), cyclin E1 (CST), anti-Cdc6 (Abcam), anti- Rb, mouse anti-underphosphorylated Rb (BD Biosciences), and rabbit anti-GAPDH (Sigma).

Statistical analysis

Statistical analysis was performed using Student's t-test to determine significance between groups. In this context, significant differences were defined as $p<0.05$.

Results

miR-9 is upregulated in osteosarcoma cells and regulates 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

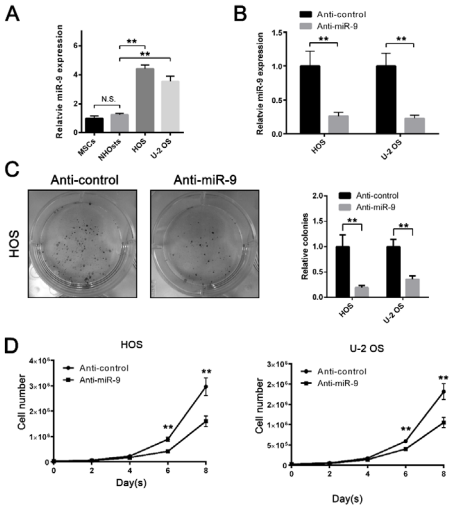

Figure 1. miR-9 is Upregulated in Osteosarcoma Cells and Modulates Cell Proliferation. A) qPCR assay was applied to measure the expression of miR-9 in MSCs, NHOsts and osteosarcoma cell lines, HOS and U-2 OS. **B)** HOS and U-2 OS cells were transfected with either miR-9 antisense oligos (anti-miR) or negative control antisense oligos (anti-control). 48 hours after transfection, cells were harvested and miR-9 knockdown efficiency was analyzed by qPCR assay. **C)** Effect of miR-9 on colony formation of osteosarcoma cell lines. HOS and U-2 OS cells were transfected with either miR-9 antisense oligos (anti-miR) or negative control antisense oligos (anti-control) and then were subjected to colony formation assay. **D)** Effect of miR-9 on cell proliferation of osteosarcoma cell lines. HOS and U-2 OS cells were transfected with either miR-9 antisense oligos (anti-miR) or negative control antisense oligos (anti-control) and then record cell number every two days. n=3. *$p<0.05$, *$p<0.01$, and n.s., not significant

findings, we determined cell proliferation curve of both miR-9 depletion cells and negative control cells. As is shown in Figure 1D, both HOS and U-2 OS cells exhibited suppressed cell proliferation after miR-9 depletion. Taken together, these data indicate that miR-9 may promote osteosarcoma progression.

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 contained 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.

GCIP active retinoblastoma 1 (Rb) and suppresses its downstream protein expression in osteosarcoma cells

GCIP was reported to suppress cell proliferation via regulating Rb activation and E2F mediated transcriptional activity (Xia et al., 2000). Yet whether these function is also the case in osteosarcoma is unknown. So we examined GCIP expression and its effects on Rb activation in osteosarcoma cells. qPCR and western blot analysis demonstrated that mRNA and protein levels of GCIP were lower in osteosarcoma cells than in MSCs and osteoblast precursors (Figure 3A and 3B). Then we examined whether GCIP could activate Rb in osteosarcoma cells. There are two forms of RB, an underphosphorylated form that is mainly found in resting or fully differentiated cells and a hyperphosphorylated form that is present in proliferating cells. We overexpressed GCIP in HOS and U-2 OS cells to check out whether GCIP expression could alter Rb phosphorylation state. As is shown in Figure 3C, GCIP overexpression significantly upregulated underphosphorylated form of Rb in both cell lines. Rb interacts with transcriptional activator E2F and suppresses

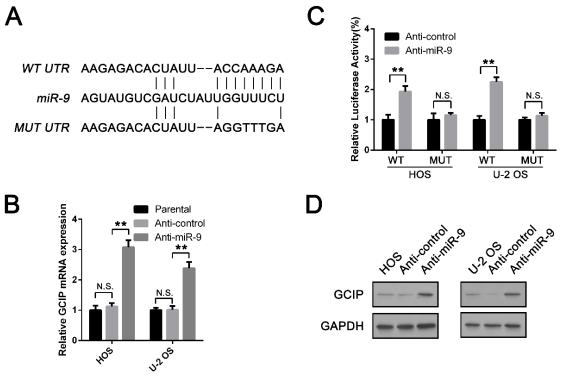

Figure 2. GCIP is a Direct Target of miR-9 in Osteosarcoma Cells. A) Putative miR-9 binding site in 3'-UTR of GCIP mRNA. Mutated 3'-UTR of GCIP mRNA for miR-9 binding site was generated as indicated. **B)** HOS and U-2 OS cells were cotransfected with Renilla luciferase expression construct pRL-TK, luciferase reporter plasmid with either wild-type (WT) or mutant GCIP 3'-UTR (MUT), and either miR-9 antisense oligos (anti-miR) or negative control antisense oligos (anti-control). 48 hours later, luciferase activity was measured. **C and D)**, miR-9 expression was suppressed in HOS and U-2 OS cells and then GCIP expression was determined by qPCR and western blot assay. n=3. *p<0.05, *p<0.01

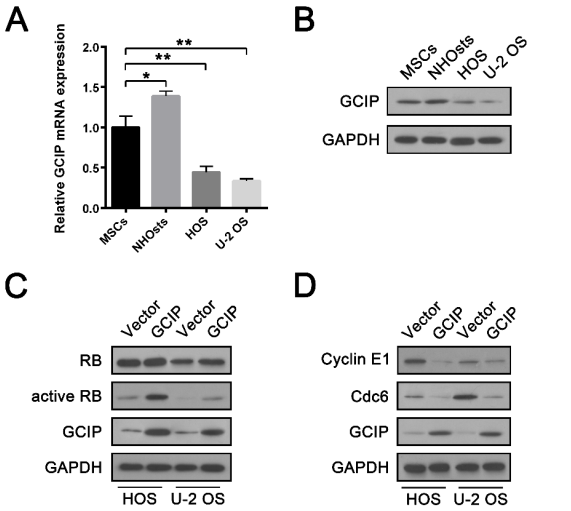

Figure 3. GCIP Active Rb and Suppresses its Downstream Protein Expression in Osteosarcoma Cells. A and B) qPCR and western blot assay were applied to measure the expression of GCIP in MSCs, NHOsts and osteosarcoma cell lines, HOS and U-2 OS. **C and D)** HOS and U-2 OS cells were transfected with either GCIP overexpressing plasmid or vector plasmid. 48 hours later, cells were harvested and subjected with western blot assay using indicated antibodies. n=3. *p<0.05, *p<0.01

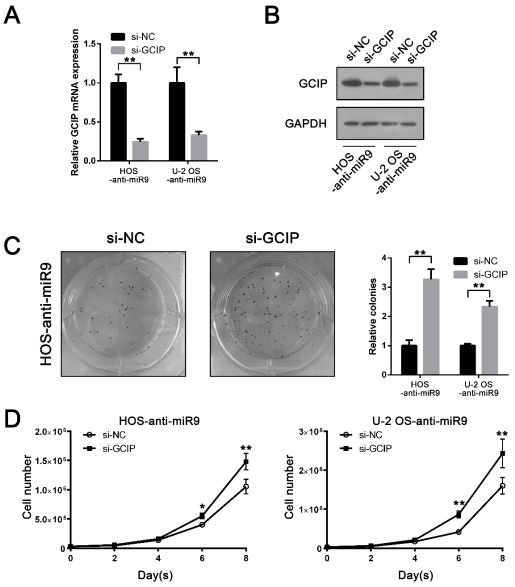

Figure 4. GCIP is Involved in miR-9's Oncogenic Effects in Osteosarcoma Cells. A and B) miR-9 depleted osteosarcoma cells were transfected with siRNA targeting GCIP (si-GCIP) or scramble siRNA (si-NC). GCIP knockdown efficiency was analyzed by qPCR and western blot assay. Then cells were subjected to colony formation assay **C)** and cell proliferation assay **D)** to examine whether GCIP can reverse the effect of miR-9 depletion. n=3. *p<0.05, *p<0.01

its transcriptional activity. We examined expression of E2F downstream target genes, Cdc6 and cyclin E1, in GCIP overexpressed cells and negative control cells. Results showed that GCIP overexpression suppressed Cdc6 and cyclin E1 in both cell lines. Together, these results indicate that GCIP suppresses oncogenes such as Cdc6 and cyclin E1 through Rb-E2F pathway.

GCIP is involved in miR-9's oncogenic effects in osteosarcoma cells

Previous results indicated GCIP is a bone fide target of miR-9, we wonder whether miR-9's oncogenic effects are GCIP dependent. We knockdowned 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 knockdown 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 tumor promotion functions by 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

Bazzoni F, Rossato M, Fabbri M, et al (2009). Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci USA*, **106**, 5282-7.

Calin GA, Croce CM (2006). MicroRNA signatures in human cancers. *Nature Rev Cancer*, **6**, 857-66.

Chang TW, Chen CC, Chen KY, et al (2008). Ribosomal phosphoprotein P0 interacts with GCIP and overexpression of P0 is associated with cellular proliferation in breast and liver carcinoma cells. *Oncogene*, **27**, 332-8.

Chen KY, Chen CC, Tseng YL, et al (2014). GCIP functions

as a tumor suppressor in non-small cell lung cancer by suppressing Id1-mediated tumor promotion. *Oncotarget*, **5**, 5017-28.

Chen WC, Su PF, Jin YT, et al (2008). Immunohistochemical expression of GCIP in breast carcinoma: relationship with tumour grade, disease-free survival, mucinous differentiation and response to chemotherapy. *Histopathol*, **53**, 554-60.

Delaloy C, Liu L, Lee JA, et al (2010). MicroRNA-9 coordinates proliferation and migration of human embryonic stem cell-derived neural progenitors. *Cell Stem Cell*, **6**, 323-35.

Dorfman HD, Czerniak B (1995). Bone cancers. *Cancer*, **75**, 203-10.

Gao F, Zhao ZL, Zhao WT, et al (2013). miR-9 modulates the expression of interferon-regulated genes and MHC class I molecules in human nasopharyngeal carcinoma cells. *Biochem Biophys Res Commun*, **431**, 610-6.

Gao Y, Luo LH, Li S, et al (2014). miR-17 inhibitor suppressed osteosarcoma tumor growth and metastasis via increasing PTEN expression. *Biochem Biophys Res Commun*, **444**, 230-4.

Ha M, Kim VN (2014). Regulation of microRNA biogenesis. *Nature Rev Molecular Cell Biol*, **15**, 509-24.

He JP, Hao Y, Wang XL, et al (2014). Review of the molecular pathogenesis of osteosarcoma. *Asian Pac J Cancer Prev*, **15**, 5967-76.

Hildebrandt MA, Gu J, Lin J, et al (2010). Hsa-miR-9 methylation status is associated with cancer development and metastatic recurrence in patients with clear cell renal cell carcinoma. *Oncogene*, **29**, 5724-8.

Jansson MD, Lund AH (2012). MicroRNA and cancer. *Mol Oncol*, **6**, 590-610.

John B, Enright AJ, Aravin A, et al (2004). Human microRNA targets. *Plos Biology*, **2**, 1862-79.

Kavitha N, Vijayarathna S, Jothy SL, et al (2014). MicroRNAs: biogenesis, roles for carcinogenesis and as potential biomarkers for cancer diagnosis and prognosis. *Asian Pac J Cancer Prev*, **15**, 7489-97.

Lee I, Yeom SY, Lee SJ, et al (2010). A novel senescence-evasion mechanism involving Grap2 and cyclin D interacting protein inactivation by ras associated with diabetes in cancer cells under doxorubicin treatment. *Cancer Res*, **70**, 4357-65.

Li CJ, Cong Y, Liu XZ, et al (2014). Research progress on the livin gene and osteosarcomas. *Asian Pac J Cancer Prev*, **15**, 8577-9.

Lv H, Guo J, Li S, et al (2014). inhibitor reduces the proliferation and migration in osteosarcoma MG-63 cells. *Exp Ther Med*, **8**, 1575-80.

Ma L, Young J, Prabhala H, et al (2010). miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol*, **12**, 247-56.

Ma W, Xia X, Stafford LJ, et al (2006). Expression of GCIP in transgenic mice decreases susceptibility to chemical hepatocarcinogenesis. *Oncogene*, **25**, 4207-16.

Moore DD, Luu HH (2014). Osteosarcoma. *Cancer Treat Res*, **162**, 65-92.

Mutsaers AJ, Walkley CR (2014). Cells of origin in osteosarcoma: mesenchymal stem cells or osteoblast committed cells? *Bone*, **62**, 56-63.

Song B, Wang Y, Titmus MA, et al (2010). Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. *Mol Cancer*, **9**, 96.

Visani M, de Biase D, Marucci G, et al (2014). Expression of 19 microRNAs in glioblastoma and comparison with other brain neoplasia of grades I-III. *Mol Oncol*, **8**, 417-30.

Xia C, Bao Z, Tabassam F, et al (2000). GCIP, a novel human grap2 and cyclin D interacting protein, regulates E2F-mediated transcriptional activity. *J Biol Chem*, **275**, 20942-8.

Zhao H, Li M, Li L, et al (2013). MiR-133b Is down-regulated in human osteosarcoma and inhibits osteosarcoma cells proliferation, migration and invasion, and promotes apoptosis. *PLoS ONE*, **8**, 83571.

Zhao SF, Zhang X, Zhang XJ, et al (2014). Induction of microRNA-9 mediates cytotoxicity of curcumin against SKOV3 ovarian cancer cells. *Asian Pac J Cancer Prev*, **15**, 3363-8.

Zhuang G, Wu X, Jiang Z, et al (2012). Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *EMBO J*, **31**, 3513-23.