miR-34c-3p acts as a tumor suppressor gene in osteosarcoma by targeting MARCKS

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Abstract. Previous studies have demonstrated that microRNA (miR)-34c-3p is important in human cancer progression. However, the function of miR-34c-3p in osteosarcoma (OS) remains to be elucidated. In the present study, miR-34c-3p level was measured by reverse transcription-quantitative polymerase chain reaction in OS tissues and the associated prognostic value for overall survival was determined. The function of miR-34c-3p was examined in vitro and in vivo. A luciferase reporter assay was used to identify the targets of miR-34c-3p. The results of the present study revealed that miR-34c-3p was downregulated in OS tissues and cell lines, and decreased levels of miR-34c-3p were associated with a high mortality rate in patients with OS. Furthermore, restoration of miR-34c-3p expression reduced cell growth in vitro and suppressed tumorigenesis in vivo. Conversely, inhibition of miR-34c-3p stimulated OS cell growth in vitro and in vivo. Myristoylated alanine-rich protein kinase C substrate (MARCKS) was identified as a direct target of miR-34c-3p and its overexpression partly reversed the suppressive effects of miR-34c-3p. Furthermore, MARCKS was revealed to be upregulated and inversely correlated with miR-34c-3p levels in OS tissues. These data suggested that miR-34c-3p acts as a tumor suppressor via regulation of MARCKS expression in OS progression and miR-34c-3p may be a promising therapeutic target for this type of cancer.

Introduction

Osteosarcoma (OS) is one of the most common malignancies in children and young adults worldwide (1). The surgical techniques and chemotherapeutic treatments for OS have markedly improved; however, 30% of children diagnosed with OS will not survive for >5 years (1-3). Therefore, a comprehensive understanding of OS biology is required to optimize the diagnosis, therapy and prognostic predictions of this disease.

MicroRNAs (miRNAs) are a class of non-coding small RNAs, 18-22 nucleotides in length, which regulate target gene expression at the post-transcriptional level, via imperfect base-pairing with the 3' untranslated region (3'UTR) of specific target mRNAs (4). It has previously been demonstrated that miRNAs may function as oncogenes or tumor suppressors in cancer development (5). A large number of miRNAs have previously been identified; however, their roles in OS development and the underlying molecular mechanisms remain to be fully elucidated.

miRNA (miR)-34c-3p is one of the mature miRNAs of the miR-34c family, which are critical modulators of the p53 pathway and potential tumor suppressors in human cancer (6). Downregulation of miR-34c-3p expression was previously revealed in several types of cancer, including non-small cell lung cancer (NSCLC) (7,8), cervical cancer (9) and glioma (10). It has previously been demonstrated that miR-34c-3p functions as a tumor suppressor via the targeting of numerous signaling pathways (7,8). However, compared with other types of cancer, the role of miR-34c-3p in the pathogenesis of OS remains to be elucidated.

The aim of present study was to investigate the expression pattern and the biological effects of miR-34c-3p in OS. The findings suggested that miR-34c-3p was downregulated and associated with poor prognosis in OS patients. Forced miR-34c-3p expression suppressed cell growth in vitro and tumorigenesis in vivo. In addition, myristoylated alanine-rich protein kinase C substrate (MARCKS) was identified as a direct target of miR-34c-3p and its overexpression partly reversed the suppressive effects of miR-34c-3p. The results support a tumor suppressor role of miR-34c-3p in OS via inhibition of MARCKS, and may thus be a promising therapeutic target for the treatment of OS.

Materials and methods

Human tissue samples. A total of 50 paired fresh surgically resected OS tumor and adjacent non-tumor tissues were collected from the Xi'an Honghui Hospital (Xi'an, China)
between September 2010 and November 2012. Of the 50 OS patients, 32 were male, 28 were female, with a median age of 32 years old (range 19-51). None of the patients received pre-operative chemotherapy or radiotherapy prior to surgery. Specimens were gently washed with normal saline and flash-frozen in liquid nitrogen immediately following collection and stored at -80°C until use. Tumor and non-tumor tissue samples were confirmed by pathological examination. The present study was approved by the Research Ethics Committee of Xi’an Honghui Hospital.

Cell culture. Human OS cell lines (MG-63, U2OS, HOS and Saos-2), and normal human osteoblast (NHOst) cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, in a humidified incubator in an atmosphere containing 5% CO₂.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from tissues and cells using miRNeasy Mini kit (Qiagen China Co., Ltd., Shanghai, China), according to the manufacturer’s protocol. Total RNA concentration was assessed by measuring absorbance at a wavelength of 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 2 µg total RNA was reverse transcribed using the PrimeScript RT reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan) and miRNA-specific stem-loop RT primer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The stem-loop RT primer sequence was: 5'-GTC GTA TCCAGTGAGGTCGCCAGTAGTTGCACTGATACCCACCTGGC-3', for miR-34c-3p. Gene-specific amplification was performed using Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR-Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Inc.) with a first step at 95°C for 10 min followed by 40 cycles with 95°C for 15 sec and 60°C for 1 min with a fluorescent reading at the end of this step. The gene-specific primers used were as follows: MiR-34c-3p, forward 5'-GGTGGAGATCTACAACCCACACG-3' reverse 5'-GTGCCAGGTCCGAGGTT-3'; MARCKS, forward 5'-AGCCCGGTAGAGAAGGAGG-3' reverse 5'-TTGGGGCAAGAAAGCTGCAGGA-3'; U6 small nuclear RNA, forward 5'-AGAAGCC TGTGGTGTCCG-3' reverse 5'-CTCATTCTCAAGACCTTCCCT-3'; and GAPDH, forward 5'-CATGTTGCTGTAGGTTGTA GAACCA-3' and reverse 5'-AGTGATGGTCAAGACTGTTGTTGTC-3'. The relative expression levels of miR-34c-3p and MARCKS were normalized to that of internal control U6 and GAPDH using the comparative 2-ΔΔCt method (11). Each sample was analyzed in triplicate and the mean expression level was calculated.

Cell transfection. The miR-34c-3p mimics, miR-34c-3p inhibitor and negative control were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). For transfection, 2x10⁵ MG-63 and Saos-2 cells were seeded into a 6-well plate in growth medium without serum and antibiotics at a density of 30-40% and incubated overnight. Subsequently, the cells were transfected with miR-34c-3p mimics, miR-34c-3p inhibitor or negative control using HiPerFect Transfection Reagent (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The mixture was added to cells at a final concentration of 100 nM. Following incubation at 37°C in an atmosphere containing 5% CO₂ for 4-6 h, the serum-free medium was removed, and cells were maintained in DMEM supplemented with 10% FBS.

Cell proliferation assay. A total of 24 h post-transfection, cells were harvested and seeded into 96-well plates at a density of 5x10³ cells/well and cultured at 37°C in an atmosphere containing 5% CO₂ for 1, 2, 3 and 4 days. A total of 10 µl Cell Counting Kit-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added into the culture medium in each well. After 1 h incubation, optical density values were measured using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 nm. Each time point was repeated in three wells and the experiment was independently performed three times.

Colony formation assay. A total of 24 h post-transfection, cells were harvested, seeded into 24-well plates at a density of 5x10³ cells/well and cultured at 37°C in an atmosphere containing 5% CO₂. During colony growth, the culture medium was replaced every 3 days. After 12 days, the plates were stained for the formation of cell colonies with crystal violet in 70% ethanol and counted under a microscope (Nikon AZ100; Nikon Corporation, Tokyo, Japan). The colony was counted only if it contained >50 cells. The experiment was independently performed three times.

Animal experiments. A total of 12 female BALB/c nu/nu mice (6-8 weeks old and weight 18-20 g; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were housed together in specific pathogen-free conditions with a temperature of 25°C, 55-65% relative humidity, and a 12-h light-dark cycle with standard chow and water ad libitum. A total of 2x10⁶ MG-63 cells transfected with miR-34c-3p agomir or agomir-negative control (Shanghai GenePharma Co., Ltd.) were injected subcutaneously into the flank region of 6-8 week-old female nude mice (n=6/group). Tumor growth was monitored every 3 days, measured with fine digital calipers. Tumor volume was calculated using the following formula: Tumor volume=0.5 x width² x length. After 4 weeks, the mice were sacrificed by cervical dislocation and tumor weights were measured. All animal procedures were performed in accordance with protocols approved by the Institute Research Ethics Committee of Xi’an Honghui Hospital.

Prediction of miR-34c-3p target genes and luciferase reporter assay. Putative miR-34c-3p targets were predicted using several different algorithms, including TargetScan (www.targetscan.org), Pictar (http://www.pictar.org/) and miRanda (http://www.microrna.org). The 3'-UTR of MARCKS containing the putative miR-34c-3p binding sequence was amplified by PCR and cloned downstream of the firefly luciferase gene in the pMIR-Report vector (Promega Corporation, Madison, WI, USA) (12). Mutations in the miR-34c-3p seed
regions of the MARCKS 3’UTR were generated using the QuikChang Multisite-directed mutagenesis kit (Promega Corporation). The mutated MARCKS 3’-UTR fragment was cloned into the pMIR-Report vector to develop the pMIR-MARCKS-3’-UTR-mut vector. For the luciferase assay in the OS cells, MG-63 or Saos-2 cells were co-transfected with 50 ng/well MARCKS-3’-UTR or MARCKS 3’-UTR-mut, and 50 nM/well miR-34c-3p mimic or scrambled microRNA negative control using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were collected after 48 h for analysis using the Dual Luciferase reporter assay system (Promega Corporation).

Western blotting. MG-63 and Saos-2 cells transfected with miR-34c-3p mimics, miR-34c-3p inhibitor and negative control were lysed in radioimmunoprecipitation assay buffer with protease inhibitor at 72 h post-transfection. Lysate protein concentrations were obtained by BCA assay (Thermo Fisher Scientific, Inc.). A total of 50 µg protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes and blocked for 1 h in 5% bovine milk diluted in Tris-buffered saline with Tween-20 at room temperature. The mouse monoclonal antibody against MARCKS (catalog no. sc-100777; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse monoclonal antibody against β-actin (catalog no. sc-69879; 1:2,000; Santa Cruz Biotechnology, Inc.) were incubated with the blot overnight at 4˚C. Following extensive washing with phosphate-buffered saline containing 0.1% Triton X-100, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (catalog no. sc-2005; 1:5,000, Santa Cruz Biotechnology, Inc.) for 30 min at room temperature. The bands were visualized using an enhanced chemiluminescence system (EMD Millipore, Billerica, MA, USA).

Statistical analysis. Statistical Package of the Social Sciences version 19.0 for Windows (IBM SPSS, Armonk, NY, USA) was used for statistical analyses. The survival rate of patients with OS was calculated using Kaplan-Meier survival analysis. Differences between experimental groups were assessed using the two-tailed unpaired Student’s t-test. The relationship between miR-34c-3p and MARCKS expression was assessed using Spearman’s correlation analysis. Data are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-34c-3p is downregulated in human OS and correlates with poor prognosis. To understand the role of miR-34c-3p in OS, miR-34c-3p expression was examined by RT-qPCR in 50 OS and corresponding healthy bone tissues. The results demonstrated that miR-34c-3p was downregulated in the majority of OS tissues (Fig. 1A). Furthermore, miR-34c-3p was downregulated in MG-63, Saos-2, HOS and U2OS human OS cell lines, compared with the NHOst cell line (Fig. 1B). Furthermore, patients with lower expression of miR-34c-3p revealed a poor prognosis compared with those exhibiting a high expression of miR-34c-3p (P=0.015; Fig. 1C). These results suggested that miR-34c-3p is essential in OS development.

miR-34c-3p suppresses OS growth in vitro and in vivo. The present study hypothesized that miR-34c-3p was implicated in OS growth, based on the aforementioned findings. The effects of miR-34c-3p on OS cell growth were investigated following overexpression or inhibition of miR-34c-3p in MG-63 cells (Fig. 2A). As presented in Fig. 2B, overexpression of miR-34c-3p significantly inhibited MG-63 cell growth (P=0.009 at 72 h and P=0.003 at 96 h). Conversely, inhibition of miR-34c-3p stimulated MG-63 cell growth (P=0.007 at 72 h and P=0.001 at 96 h). In addition, colony formation assays revealed that the overexpression of miR-34c-3p suppressed MG-63 colony formation (P=0.031), whereas inhibition of miR-34c-3p promoted MG-63 colony formation compared with the control (P=0.036; Fig. 2C). These results were further confirmed using another OS cell line Saos-2 (data not shown).
To determine if this effect was also apparent in vivo, these in vitro results were confirmed using a xenograft model. MG-63 cells transfected with agomiR-34c-3p or negative control (agomiR-NC) were subcutaneously injected into the flank region of nude mice. As presented in Fig. 2D, overexpression of miR-34c-3p inhibited tumor growth compared with the control group (P=0.024), and the tumor weights in the agomiR-34c-3p group were significantly decreased compared with the agomiR-NC group (P=0.008; Fig. 2E). These data revealed that miR-34c-3p suppresses OS cell growth in vitro and in vivo.

**MARCKS is a direct target of miR-34c-3p in OS cells.** Bioinformatic research was conducted to find potential...
targets of miR-34c-3p using TargetScan and miRanda. As presented in Fig. 3A, MARCKS was identified as a potential target gene of miR-34c-3p, with the predicted binding site between base positions 412 and 418. To validate whether the 3'-UTR of MARCKS is a functional target of miR-34c-3p, a dual-luciferase reporter system was employed. 3'-UTR sequences were cloned that contained wild type or mutated binding sites of miR-34c-3p into the pMIR-Report vector and co-transfected with miR-34c-3p mimics or miR-NC into OS cells. Data from the luciferase assay demonstrated that overexpression of miR-34c-3p significantly suppressed the luciferase activity of the reporter gene with the wild type construct (P=0.006 and P=0.008, respectively) but not with the mutant MARCKS 3'-UTR construct (Fig. 3B and C). In addition, overexpression of miR-34c-3p resulted in a reduction of MARCKS mRNA and protein expression in OS cells (P=0.042 and P=0.037, respectively, Fig. 3D and E). Therefore, these results suggested that MARCKS is a direct target of miR-34c-3p in OS cells.

**Discussion**

The present study investigated the biological role of miR-34c-3p in the progression of OS. It was revealed that miR-34c-3p was
significantly downregulated in OS compared with matched healthy bone tissue. In addition, functional experiments demonstrated that miR-34c-3p suppressed cell growth in vitro and inhibited tumor growth in vivo by targeting MARCKS. The present study, to the best of our knowledge, has been the first to reveal the role of miR-34c-3p in OS, as a novel tumor suppressor.

It has previously been suggested that miR-34c-3p is downregulated in various tumors, and functions as a tumor suppressor in numerous types of cancer. Zhou et al (7) and Liu et al (8) demonstrated that miR-34c-3p functions as a tumor suppressor in NSCLC partially by inhibiting the pituitary adenylate cyclase 1/mitogen activated protein kinase pathway and eukaryotic translation initiation factor 4E. Wu et al (10) demonstrated that the overexpression of miR-34c-3p suppressed proliferation and invasion of OS cells by targeting the Notch pathway in glioma. In the present study, it was demonstrated that miR-34c-3p suppressed OS cell growth and colony formation in vitro and inhibited OS growth in vivo, suggesting a tumor suppressive role for miR-34c-3p in OS.

To determine how miR-34c-3p acts as a tumor suppressor, the target genes of miR-34c-3p were screened using bioinformatic analysis. MARCKS was selected as a potential target gene of miR-34c-3p based on its particular functions and expression patterns. It had previously remained to be elucidated as to whether miR-34c-3p directly targeted MARCKS in OS. MARCKS is a ubiquitously expressed protein kinase C substrate, which binds actin and calmodulin, and regulates actin dynamics (13). MARCKS is involved in multiple cellular processes, including cell adhesion, migration, metastasis, membrane trafficking and motility via regulation of the actin cytoskeletal structure (14,15). The expression of MARCKS has been demonstrated to be downregulated in hepatocellular carcinoma tissues, and downregulation of MARCKS may increase the migration of human hepatic stellate cells (16,17). It was additionally involved in 12-O-tetradecanoylphorbol-13-acetate-mediated migration of neuroblastoma cells (18). It has previously been suggested that elevated MARCKS phosphorylation contributes to the unresponsiveness of breast cancer to paclitaxel treatment (19). The present study hypothesized that MARCKS was the precise intracellular target of miR-34c-3p by using the miRanda, TargetScan and Pictar databases. Furthermore, MARCKS was verified as a direct target of miR-34c-3p using the luciferase reporter gene assay. Overexpression of miR-34c-3p suppressed mRNA and protein expression levels of MARCKS.
Furthermore, the inhibitory effects of miR-34c-3p on cell proliferation and colony formation were reversed under conditions of MARCKS overexpression. In OS tissues, it was demonstrated that expression of MARCKS was upregulated, and miR-34c-3p levels were inversely correlated with MARCKS expression levels. These data suggested that MARCKS is a direct and functional target of miR-34c-3p in OS cells. However, the role of MARCKS in OS remains to be fully elucidated.

In conclusion, the present study combined clinical and experimental studies to establish a critical role for miR-34c-3p in OS. The results demonstrated that miR-34c-3p directly targeted the 3'UTR of MARCKS, and promoted tumor cell growth, which further contributed to a high mortality rate for patients with OS. Therefore, miR-34c-3p may represent a novel therapeutic target for OS treatment in the future.

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