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MicroRNA-1(miR-1) inhibits chordoma cell migration and invasion by targeting Slug

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Abstract

Recent studies have revealed that expression of miRNA-1(miR-1) is frequently downregulated in several cancer types including chordoma. Identifying and validating novel targets of miR-1 is useful for understanding the roles of miR-1 in chordoma. We aimed to further investigate the functions of miR-1 in chordoma. Specifically, we assessed whether restoration of miR-1 affects cell migration and invasion in chordoma, and focused on the miR-1 potential target Slug gene. Migratory and invasive activities were assessed by wound healing and Matrigel invasion assays, respectively. Cell proliferation was determined by MTT assay. Slug expression was evaluated by Western blot, immunofluorescence, and immunohistochemistry. Restoration of miR-1 expression suppressed the migratory and invasive activities of chordoma cells. Transfection of miR-1 inhibited cell proliferation both time- and dose-dependently in chordoma. miR-1 transfected cells showed inhibited Slug expression. Slug was overexpressed in chordoma cell lines and advanced chordoma tissues. In conclusion, we have shown that miR-1 directly targets the Slug gene in chordoma. Restoration of miR-1 suppressed not only proliferation, but also migratory and invasive activities, and reduced the Slug expression in chordoma cells. These results collectively indicate that miR-1/Slug pathway is a potential therapeutic target because of its crucial roles in chordoma cell growth and migration.

Keywords

miRNA-1(miR-1); Chordoma; Invasion; Migration; Slug

INTRODUCTION

Chordoma is an uncommon bone cancer, usually arising from embryonic remnants of the axial skeleton with the notochord. This tumor occurs most often in the sacral region, followed by the skull base and the mobile spine region.¹ The peak incidence is around 60 years of age. Chordoma has a long clinical course because these tumors are typically slow-

growing. Therefore, they are often clinically asymptomatic until the advanced stages of disease, tending to destroy the surrounding bone and invade adjacent soft tissue. Moreover, metastases tend to occur several years after the initial diagnosis and have been reported in the follow-up of 40–60% of patients with chordoma.² Surgical resection is the most effective treatment for achieving disease-free survival without the presence of recurrences or metastases.^{3, 4} However, adequate resection is frequently impossible because of the anatomical location of the tumors,⁵ and more than 40% of cases present with local recurrence. Furthermore, chordomas are resistant to chemotherapy and relatively resistant to radiation.³ Therefore, identification and validation of a potential therapeutic target is critical in advancing treatments for patients with chordoma.

Recently, several studies have demonstrated the involvement of microRNAs (miRs) in various diseases including neoplasms such as chordoma.^{6, 7} MiRs are small non-coding RNAs, usually 18–25 nucleotides in length, that bind to the 3'-untranslated region (UTR) of target mRNA of the gene. The target mRNA induces degradation if complementarity produces perfect binding of miR, whereas the target mRNA represses translation if complementarity indicates imperfect binding of miR, and thereby regulates various biological processes including inflammation, the cell cycle, apoptosis, proliferation, differentiation, migration, metabolism, immunity, and development.⁶ MiRs can be divided into either oncogenic or tumor suppressive depending on the target mRNA. Overexpressed miRs can act as oncogenes by inhibiting tumor suppressor genes, while underexpressed miRs can act as tumor suppressors by inhibiting oncogenes.^{6, 8}

A significant number of studies have shown that microRNA-1 (miR-1) can act as a tumor suppressor and is involved in various biological functions, including cell growth, cell migration and invasion, apoptosis, and cell cycle distribution.^{9, 10} However, the mechanisms by which miR-1 functions via target genes in chordoma remain largely unknown. We previously demonstrated that expression of miR-1 and miR-206 were significantly reduced, or even absent, in chordoma tissues and cell lines as compared with normal cells. The restoration of miR-1 inhibited the growth of chordoma cells, resulting in repression of MET expression.⁷ As human cancer is in general a multigenic, multipathway disease, each miR is believed to interact with up to 200 potential target genes. Therefore, further identifying and validating novel targets of miR-1 would be useful for understanding the tumorigenesis process in chordoma.

Slug, a member of the snail family of transcription factors, is reportedly one of the direct targets of miR-1, and is associated with various biological functions such as proliferation, cell migration, cell invasion, angiogenesis, adhesion, and drug resistance in lung cancer.^{9, 11} Furthermore, overexpression of Slug correlates with poor outcomes, recurrence, distant metastasis, and the histological grade of various cancers.^{11–15} More recently, overexpression of Slug has been found in the tissue of an aggressive chordoma patient. However, the association of miR-1 and biological functions of Slug has not yet been investigated in chordoma.

This study aimed to investigate the functions of miR-1 including cell migratory and invasive activities in chordoma. Specifically, we focused on the miR-1 target Slug gene to determine

the expression of Slug in chordoma cell lines and tissues, and assessed whether restoration of miR-1 affects Slug expression, and cell migration and invasion in chordoma.

MATERIALS AND METHODS

Cell lines and cell culture

UCH1 and UCH2 are established human chordoma cell lines and were kindly provided by Dr. Silke Bruderlein (University Hospitals of Ulm, Germany).^{16, 17} Another human chordoma cell line, CH22, was established in our laboratory as previously reported.¹⁸ These cell lines were cultured in the medium, RPMI1640 (Invitrogen, CA) for UCH1 and UCH2, DMEM (Invitrogen, CA) for CH22, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, CA). Cells were cultured in a humidified incubator with a 5% CO₂-95% air atmosphere at 37°C and passaged every 3–4 days using trypsin-EDTA when cell confluence reached 90–100%.

Pre-miRNA-1 precursor, miR-1 mimics, and transfection

The chordoma UCH1 and CH22 cells were transiently transfected with LipofectamineTM RNAiMAX (Invitrogen, CA) and pre-miR-1 precursor (pre-miR-1) or miR-1 mimics or non-specific control miRNA (NS-miRNA) according to the manufacturer's instructions. Pre-miR-1 (Ambion[®] hsa-miR-1 Pre-miRTM Precursor, TX), miR-1 mimics (Ambion[®] mirVanaTM miRNA mimic miR-1 Positive Control, TX) and NS-miRNA (Ambion[®] mirVanaTM miRNA mimic Negative Control #1, TX), as a negative control, were transfected at different concentrations. The sequences of the mature miRNA for pre-miR-1 and mimic are 5'-UGGAAUGUAAAGAAGUAUGUA-3'.

Wound healing assay

Cell migration activity was evaluated by wound healing assay. In brief, 2×10⁵ cells were seeded onto 12-well plates and transfected with pre-miR-1 or NS-miRNA. After cells reaching 100% confluence, the cells were wounded by scraping of three parallel lines with a 200 µl tip, and then washed 3 times in serum-free medium and incubated in regular medium. The wounds were observed at 0, 8 and 24h after wounding, and photographed using a microscope (Nikon Instruments Inc, NY). Three images were taken per well at each time point using a 10× objective, and the distance between the two edges of the scratch (wound width) was measured at 10 sites in each image. The cell migration distance was calculated by subtracting the wound width at each time point from the wound width at the 0 h time point and then dividing by two.

Matrigel invasion assay

Cell invasion activity was evaluated by Matrigel invasion assay and evaluated using a BD BioCoatTM MatrigelTM Invasion Chamber (Becton-Dickinson, MA) according to the manufacturer's instructions. In brief, 5×10⁴ cells were seeded into the upper chamber of each well in serum-free medium and transfected with pre-miR-1 or NS-miRNA at an increasing concentration, and the bottom chambers were filled with medium containing 10% FBS without antibiotics. After a 48h incubation period, the non-invading cells were removed by scrubbing from the upper surface of the membrane with a cotton swab. Invading cells

that had been fixed using 100% methanol and stained with hematoxylin were evaluated. The number of invading cells was counted in three images per membrane by microscopy using a 20× objective.

MTT cell proliferation assay

Cell proliferation was determined using the MTT assay. In brief, 2×10^3 cells were seeded onto 96-well plates in medium containing with 10% FBS without antibiotics. For reverse transfection, the cells were incubated with pre-miR-1 or miR-1 mimics or NS-miRNA added at different concentrations for 24–96h. After incubation, 20μl of MTT (5mg/ml, Sigma, MO) were added, followed by incubation for another 4h at 37°C. The MTT formazan products were dissolved in acid-isopropanol. In turn, the absorbance at 490nm was read on a SPECTRAMax Microplate Spectrophotometer (Sunnyvale, CA). All procedures were repeated for four days. The MTT assay was performed in triplicate.

Western Blot Assay

Expression of Slug protein was evaluated by Western Blot. Protein lysates from chordoma cell lines were extracted using 1× Cell Lysis Buffer (Cell Signaling Technology, MA). The protein concentrations were determined using Protein Assay Reagents (Bio-Rad, CA) and a SPECTRAMax Microplate Spectrophotometer from Molecular Devices (Sunnyvale). The primary antibodies for Slug (1:800 dilution) and actin (1:2000 dilution) were purchased from Cell Signaling and Santa Cruz, respectively. Secondary antibodies were bound to IRDye® 800CW or IRDye® 680LT (LI-COR Biosciences, NE). Western blots were carried out as previously described.¹⁹ Normalization was performed using actin as an endogenous control. Membrane signals were scanned using an Odyssey infrared imaging system and analyzed using Odyssey 3.0 software (LI-COR Biosciences, NE). The protein levels were quantified with NIH Image J software.

Immunofluorescence

Expression of Slug protein was also evaluated by immunofluorescence. In brief, chordoma cell line UCH1 was transfected with pre-miR-1 and NS-miRNA for 48h. The cells were then incubated in 4% paraformaldehyde, and fixed in ice-cold methanol and blocked with 1 % bovine serum albumin (BSA), and bound with Slug (1:50) and actin (1:400) antibodies. The cells were incubated with anti-rabbit IgG (1:1000), anti-mouse IgG (1:1000) and Hoechst 33342 (Life Technologies Corp, NY). The cells were captured on a Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments Inc, NY) equipped with a SPOT RT™ digital camera.

Chordoma Tissues and Immunohistochemistry

Pathologically confirmed chordomas were obtained from 7 patients who had undergone surgical resection in our hospital, and were conducted according to the policies of the institutional review board of the hospital. All patients were adequately achieved negative surgical margins. In brief, slides were warmed at 65°C, then immersed in xylene for deparaffinization, and finally immersed in a graded ethanol series for rehydration.

Immunohistochemistry using the HRP-DAB System Cell and Tissue Staining Kit (R&D Systems, MN) was performed according to the manufacturer's instructions.

RESULTS

MiR-1 suppressed the migratory activity of chordoma cells

Recent studies have shown that the miR-1 pathway is important for cell migration in several types of tumors.^{9, 10, 20–28} We assessed whether transfection of miR-1 into chordoma cells could affect their migratory activity using a wound healing assay. Because the wound was covered in control cells after 48h, we observed the wound distance after 0, 8 and 24h (Fig. 1A). During 24h incubation, the UCH1 and CH22 cells transfected with pre-miR-1 migrated only 52.8µm and 10.8µm, respectively. In contrast, UCH1 and CH22 cells transfected with NS-miRNA migrated 150µm and 75.8µm, respectively, from the scratch defect (Fig. 1B). These data demonstrated that chordoma cell migratory activities were significantly decreased in pre-miR-1 transfected cells as compared with non-specifically transfected and control cells.

MiR-1 suppressed the invasive activity of chordoma cells

We next assessed whether miR-1 transfection of chordoma cells affected their invasive activity using a Matrigel invasion assay. After transfection, the Matrigel invasion assay revealed significant dose-dependent inhibition of the invasive activities of pre-miR-1 transfected chordoma cells (Fig. 1 C). The number of invasive chordoma cells transfected with 10nM, 20nM, 40nM, 60nM and 80nM of miR-1 was decreased by 58%, 39.1%, 27.7%, 15.7% and less than 6.6%, respectively, as compared to invasion by non-transfected chordoma cells (Fig. 1D).

Restoration of miR-1 inhibited cell proliferation both time- and dose-dependently in chordoma cell lines

Previous study has shown that transfection of miR-1 into chordoma cells UCH1 inhibits cell growth. In this study, we further examined the effects of miR-1 expression in chordoma cell line CH22 and validated the effects in UCH1. The cells were transfected with pre-miR-1 or miR-1 mimics at increasing concentrations (10nM, 20nM, 40nM, 60nM, and 80nM) for 24–96h. The MTT assay revealed the pre-miR-1 transfected in both UCH1 and CH22 chordoma cell lines demonstrated significant induction of a time-dependent inhibition of cellular growth at various concentrations, whereas NS-miRNA transfected cells and control cells showed no effects on growth during the observation period (Fig. 2A, B). At 72h post-transfection, the pre-miR-1 transfected chordoma cells showed significant dose-dependent inhibition of cellular growth (Fig. 2C). Furthermore, we also validated the effects in miR-1 mimics transfected cells and similar results were observed (data not shown). These results further demonstrated up-regulation of miR-1 to inhibit cell proliferation both dose- and time-dependently in chordoma cell lines.

MiR-1 target gene Slug is overexpressed in chordoma cell lines and advanced chordoma tissues

Slug, one of the potential direct target genes for miR-1, was reported to be strongly expressed in different type tumors including aggressive chordoma.^{9, 23, 29} Therefore, we focused on the association between expression of miR-1 and Slug protein. There is a predicted direct binding site in the 3'-UTR of Slug for miR-1 (Fig. 3A), so miR-1 has the potential to suppress Slug expression. Western blot analysis showed Slug is high expressed in both UCH1 and UCH2 chordoma cell lines (Fig. 3B). However, Slug is low expressed in CH22 (data not shown). After transfection of the pre-miR-1, chordoma cells showed marked and dose-dependent inhibition of Slug expression, whereas NS-miRNA transfected cells showed no effects on Slug expression (Fig. 3C). Expression of Slug was also verified by immunofluorescence staining. The pre-miR-1 transfected chordoma cells showed inhibition of Slug expression (Fig. 4A). We further analyzed Slug expression immunohistochemically using chordoma tissues. Slug was detectable in two of the seven chordoma patients (Fig. 4B). However, these two cases had presented with rapid recurrence and early metastasis, indicating highly aggressive tumors, and both had died of their disease (Fig. 4C).

DISCUSSION

MiR-1 is one of the first miR to be found down-regulated in chordoma cell lines and chordoma tissues as compared with normal tissues. More recently studies have found several other deregulated miRs in chordoma, including miR-31, miR-222, miR-140-3p, miR-148a.^{7, 30} Among these miRs with deregulated expression in chordoma, miR-1 is also a consistently validated and frequently down-regulated in various types of cancers. In the present study, we found miR-1 inhibits chordoma cell migration and invasion by targeting Slug. Our study is consistent with several recent studies that have suggested that Slug is one of the direct miR-1 target genes. Slug has been shown to be strongly expressed in different types of tumors including aggressive chordoma.

Tumor migratory and invasive abilities are essential for the initial metastatic process in cancers such as chordoma. Inhibiting migratory and invasive abilities would presumably decrease local recurrence, which is common in chordoma, thereby improving survival. Table 1 shows that miR-1 directly targets these genes, some of which are involved in migratory and invasive abilities.^{7, 9, 10, 20–28, 31} Therefore, we focused on the Slug gene in this study because overexpression of Slug has been correlated with various biological functions, most notably proliferation, cell migration, cell invasion, apoptosis, angiogenesis, adhesion, and drug resistance in cancer.^{9, 23} Restoration of miR-1 inhibited these migratory and invasive abilities by preventing Slug from functioning in lung cancer.⁹ In this study, we demonstrate for the first time that restoration of miR-1 inhibits cell migratory and invasive activities in chordoma cells.

In malignant tumors, Slug expression was immunohistochemically detected in breast cancer (97%), as well as in esophageal squamous cell carcinoma (70.3%), colorectal carcinoma (37%), gastric cancer (29.9%), and bladder cancer (15.8%). Overexpression of Slug reportedly correlates with recurrence, metastasis, histological grade, and poor outcomes of various cancers.^{11–15} The present study demonstrates that Slug is overexpressed in

chordoma cell lines and tissues. MiR-1 transfected chordoma cells showed dose-dependent decreases in Slug expression. Moreover, Slug was highly expressed in aggressive chordomas as determined by immunohistochemical analyses. Downregulation of Slug expression may have the potential to improve the survival of chordoma patients by reducing rates of both recurrence and metastasis.

In conclusion, our study is the first to show that miR-1 directly targets the Slug gene in chordoma. Restoration of miR-1 suppressed not only the expression of Slug, but also the migratory and invasive activities in chordoma.

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Figure 1.

Transfection of miR-1 suppressed the migratory and invasive activities of chordoma cells. UCH1 cells were transfected with either pre-miR-1 or NS-miRNA at 40nM. (A) Micrographs of chordoma UCH1 cells at 0h, 8h and 24h after wounding. (B) Migration distance of UCH1 and CH22 cells for each time point and condition. (C) Micrographs of chordoma UCH1 cells transfected with 10–80nM pre-miR-1. (D) The average numbers of invasive chordoma cells among those transfected with 10–80nM miR-1. *, $p < 0.05$, **, $p < 0.01$ (Comparison of transfected cells with control cells using Student's t-test).

Figure 2.

Restoration of miR-1 inhibited proliferation of chordoma cell lines in both a dose- and time-dependent manner. UCH1 cells and CH22 were transfected with pre-miR-1 or NS-miRNA at concentrations between 10nM to 80nM and incubated for 24–96h. (A) UCH1, (B) CH22; Time-dependent inhibition of proliferation by pre-miR-1. (C) UCH1, CH22; Dose-dependent inhibition of proliferation by pre-miR-1. *, $p < 0.05$, **, $p < 0.01$.

Figure 3.

(A) Slug is a direct target of miR-1. Sequence of predicted hsa-miR-1 binding site in the 3'-UTR of Slug. (B) Slug is expressed in chordoma cell lines. Quantification of Slug protein by Western blot. (C) Quantification of Slug protein after transfection with pre-miR-1 or NS-miRNA at 10–80nM.

Figure 4.

(A) Immunofluorescence analysis shows transfection of miR-1 into chordoma cells suppress Slug expression. Micrographs of UCH1 cells show control cells (a–d), NS-miRNA transfected (e–h), and miR-1 transfected (i–l). Pictures are merged with Slug green signals (nuclear), Actin red signals (cytoplasm) and nuclear staining by Hoechst 33342 (blue). (B) Expression of Slug protein in chordoma tissues as determined by immunohistochemical staining. Micrographs of patients with chordoma, (C) Clinical information. DOD; dead of disease, N/A; not available.

Table 1

Predicted direct targets of miR-1 in cancer

Gene name	Official name	Chromosome location	Functions	References
MET	met proto-oncogene	7q31	proto-oncogene	7,24
HDAC4	histone deacetylase 4	2q37.3	histone deacetylase activity and represses transcription	7,24
Pim-1	pim-1 oncogene	6p21.2	signal transduction	24
FOXP1	forkhead box P1	3p14.1	the regulation of tissue- and cell type-specific gene transcription during both development and adulthood	24
TAGLN2	transgelin 2	1q21-q25	the earliest markers of differentiated smooth muscle	20,25
PNP	purine nucleoside phosphorylase	14q13.1	the phosphorylase of purine nucleosides	27
PTMA	prothymosin, alpha	2q37.1	enhance cell-mediated immunity	27
CXCR4	chemokine (C-X-C motif) receptor 4	2q21	CXC chemokine receptor specific for stromal cell- derived factor-1	21
CCND2	cyclin D2	12p13	regulators of CDK kinases	21
SRSF9	serine/arginine-rich splicing factor 9	12q24.31	a member of the serine/arginine (SR)-rich family of pre-mRNA splicing factors	28
FN1	fibronectin 1	2q34	involved in cell adhesion and migration processes	26
ETS1	v-ets avian erythroblastosis virus E26 oncogene homolog 1	11q23.3	a member of the ETS family of transcription factors	10
ET-1	endothelin 1	6p24.1	a potent vasoconstrictor	22
CXCL12	chemokine (C-X-C motif) ligand 12	10q11.1	he ligand for the G-protein coupled receptor, chemokine (C-X-C motif) receptor 4	21
PAX3	paired box 3	2q35	a member of the paired box (PAX) family of transcription factors	31
TWFI	twinfilin actin-binding protein 1	12q12	an actin monomer-binding protein	10
Slug	snail family zinc finger 2	8q11	a member of the Snail family of C2H2-type zinc finger transcription factors	9,23