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Research paper

The potential function of microRNA in chordomas

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ABSTRACT

Little is known about the molecular biology of chordomas, which are rare, chemoresistant tumors with no well-established treatment. miRNAs regulate gene networks and pathways. We aimed to evaluate the effects of dysregulated miRNA in chordomas would help reveal the underlying mechanisms of chordoma initiation and progression. In this study, miR-31, anti-miR-140-3p, anti-miR148a, and miR-222 were transiently transfected to chordoma cell lines and an MTS assay, apoptosis assay, and cell-cycle analysis were conducted to evaluate the effects. The mRNA level of predicted and confirmed targets of each miRNA, as well as the EMT and MET markers of U-CH1 and MUG-Chor1, were assessed with real-time polymerase chain reaction. Transient transfection of miRNA mimics was achieved, as each mimic increased or decreased the level of its corresponding miRNA. miR-31 decreased cell viability in MUG-Chor1 and U-CH2 after 72 h, which is consistent with previous findings for U-CH1. Both miR-31 and anti-miR-148a induced apoptosis in all three cell lines. Although each miRNA had a similar pattern, miR-31 had the most effective S-phase arrest in all three cell lines. *RDX*, *MET*, *DNMT1*, *DNMT3B*, *TRPS1*, *BIRC5*, and *KIT* were found to be targeted by the selected miRNAs. The level of miR-222 in chordoma cell lines U-CH1 and MUG-Chor1 correlated positively with EMT markers and negatively with MET markers. This study uncovered the potential of miR-31, miR-140-3p, miR-148a, and miR-222-3p to be key molecules in the cell viability, cell cycle, and apoptosis in chordomas, as well as initiation, differentiation, and progression.

1. Introduction

A chordoma is a primary bone tumor observed on the axial skeleton and arising from notochordal remnants. It is a rare tumor with an incidence of 0.08 in 100,000 and most frequently occurring in patients at the fourth decade of life (McMaster et al., 2011; Almefty et al., 2007). The most common sites for chordomas are sacral (29.1%), spinal (32.8%), and cranial (32%), with a male–female ratio of about 2:1 (McMaster et al., 2011; Papagelopoulos et al., 2004). Chordomas are highly resistant to chemotherapy and radiotherapy, which makes surgery the first choice for treatment. These tumors have a high rate of relapse but a low tendency to metastasize. Median survival of patients is 6.29 years (Chugh et al., 2007; Walcott et al., 2012). Knowledge about the molecular biology of initiation and progression of the disease is poor.

miRNAs are short RNA sequences of 20–30 nucleotides known to regulate the expression of eukaryotic genes. Their dysregulation may drastically alter mRNA expression and, consequently, the pathways that contribute to cancer initiation and progression. The role of miRNAs in differential gene regulation has been noted in several types of cancer, including non-small-cell lung, prostate, and breast cancer (Yu et al., 2008).

Studies of miRNA profiling of chordoma samples has been carried out on different types of control tissues: muscle, embryonic notochord, and nucleus pulposus (Duan et al., 2010; Long et al., 2013; Bayrak et al., 2013). Previously, our group has compared the miRNA expression profile of human skull-base chordoma tissues with nucleus pulposus cells. We discovered that the miR-31, miR-140-3p, miR-148a, and miR-221/222 gene clusters were differentially regulated and that miR-31 targeted MET in the U-CH1 cell line. This follow-up study extends the experiments with newly developed cell lines and functional studies. The levels of miRNAs of interest were observed in the new cell lines, and we examined the effects of ectopic expression or downregulation of these miRNAs on cell

Abbreviations: miRNA, microRNA; MET, mesenchymal to epithelial transition; mRNA, messenger RNA.

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viability and apoptosis. The mRNA targeting of the selected miRNAs was also evaluated.

2. Materials and methods

2.1. Ethics statement

Our study design received approval from the institutional review board of Yeditepe University, Faculty of Medicine (Istanbul, Turkey).

2.2. Cell culture

Chordoma cell lines U-CH1 (Scheil et al., 2001), U-CH2 (Bruderlein et al., 2010), and MUG-Chor1 (Rinner et al., 2012) available at ATCC bioresource center, were kindly provided by the Chordoma Foundation (Durham, NC, USA). The cell lines were cultured in culture flasks or well plates coated with 0.1% gelatin (SIGMA-ALDRICH cat. no. G1890-100G) with IMDM/RPMI (4:1) 10% FBS containing 10% fetal bovine serum and 1% antibiotics (100 μg/mL streptomycin and 10,000 units/mL penicillin). Eight nucleus pulposus tissue samples obtained from patients with an acute disk hernia were used as the healthy controls. These primary cultures were developed as described by Bayrak et al. (2013).

2.3. Total RNA isolation

Cell lysates were acquired from 6-well plates and T75 flasks with the TRIzol method, according to the manufacturer's protocol, by directly adding the TRIzol reagent (Invitrogen) on monolayer cells or by pelleting after trypsinization with 0.05 trypsin EDTA solution (Life Technologies). RNA was quantified using a NanoPhotometer (IMPLEN).

2.4. Expression analysis for quantifying selected miRNAs

A previous study found the expression levels of four miRNAs – hsa-miR-222, hsa-miR-140-3p, hsa-miR-148a, and hsa-miR-31 – to be significantly dysregulated in chordomas (Bayrak et al., 2013). With a two-step real-time polymerase chain reaction (PCR) using miRNA PCR primers (Exiqon), the cell lines U-CH1, U-CH2, and MUG-Chor1 were checked for the relative level of the selected miRNAs compared with nucleus pulposus samples by using a LightCycler 480 Instrument II device (Roche). 5S RNA was used as the housekeeping control. The data was analyzed with the $2^{-\Delta\Delta Ct}$ method.

2.5. Transfection of miRNA and anti-miRNA mimics

Ambion FAM3 Dye-Labeled Pre-miR Negative Control #1 (Life Technologies) has been used in a previous study to evaluate the ability of X-tremeGENE siRNA Transfection Reagent (Roche) to transfect U-CH1 cells (Bayrak et al., 2013). We used the same method to evaluate the efficiency of transfection of U-CH2 and MUG-Chor1 cell lines. After transfection, cells were imaged with fluorescence microscopy. The pre-miRNA mimics hsa-miR-31 and hsa-miR-222-3p (Ambion Pre-miR miRNA Precursors) and anti-miRNA mimics anti-hsa-miR 140-3p and anti-hsa-miR-148a (Ambion Anti-miR miRNA Inhibitor) were transfected into U-CH1, U-CH2, and MUG-Chor1 cells with X-tremeGENE siRNA Transfection Reagent in 6-well plates, according to the manufacturer's protocol. To control the efficiency of transfection and to determine the intracellular availability of the ectopically expressed or downregulated miRNAs, total RNA isolation and miRNA reverse transcription was performed, followed by real-time PCR after transfection. The miRNA levels were measured after 8 h with miRNA real-time PCR.

2.6. Cell viability assay

To evaluate the effect of pre-miRNA mimics hsa-miR-31 and hsa-miR-222-3p, and anti-miRNA mimics anti-hsa-miR 140-3p and anti-hsa-miR-148a, on cell viability, chordoma cell lines U-CH1, U-CH2, and MUG-Chor1 were transfected. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used according to the manufacturer's protocol. MTS analysis was done 24, 48, 72, and 96 h after transfection. As the controls, the X-tremeGENE transfection reagent, the scrambled miRNA group (Ambion Pre-miR miRNA Precursor Molecules or Anti-miR Negative Control #1), and the negative control group, which contained only opti-MEM, were used. Results were obtained by detecting absorbance at 490 nm with an ELx800 Elisa microplate reader (BioTek).

2.7. Apoptosis detection with flow cytometry

To evaluate apoptosis and necrosis with flow cytometry, pre-miRNA mimics hsa-miR-31 and hsa-miR-222-3p, and anti-miRNA mimics anti-hsa-miR 140-3p and anti-hsa-miR-148a, were transfected into U-CH1, U-CH2, and MUG-Chor1 in six-well plates using X-tremeGENE siRNA Transfection Reagent according to the manufacturer's protocol. Only the miRNAs that decreased viability were chosen for each cell line. Cells were harvested 48 or 72 h after transfection, followed by staining with annexin V and 7-AAD, according to the manufacturer's protocol, with the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen) Flow cytometry was done using FACSAria III (BD Biosciences).

2.8. Cell cycle analysis

Cell cycle analysis was done 72 h after transfection of each cell line. Cells were trypsinized, collected, and fixed in ethanol at −20 °C for 1 h. Cells were permeabilized by using Triton X-100 (Sigma) and treated with RNAse A (Qiagen). Propidium iodide stain was used to visualize the amount of DNA content in the cells by using FACSAria III.

2.9. Expression analysis to quantify target and downstream genes

For hsa-miR-31, the *RDX* (validated), *MET* (predicted), *PIK2C2A* (predicted), and *SEPHS1* (predicted) genes were chosen. In a previous study, *RDX* and *MET* were shown to be targeted by *MET* in U-CH1 (Bayrak et al., 2013). Several website databases (mirdb.org/miRDB/, mirtarbase.mbc.nctu.edu.tw, http://targetscan.org/, pictar.mdc-berlin.de, http://www.microrna.org/, diana.cslab.ece.ntua.gr) were used to choose validated and predicted targets of hsa-miR-31, hsa-miR-140-3p, hsa-miR-148a, and hsa-miR-222-3p (Table 1). The predicted targets were chosen to balance prediction match scores and the relevancy of the given genes in potential chordoma initiation and progression. The expression level of the selected target genes was measured 72 h after transfection. Additionally, the *CDH1*, *CK19*, *EMA*, *SNAI1*, *SNAI2*, *VIM* and *CDH2* levels were evaluated. All mRNA levels were measured through two-step real-time PCR with TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The control group was the transfection reagent (X-Treme) group

Table 1
The validated and predicted targets of selected miRNAs.

miRNA	Validated target genes	Predicted target genes
hsa-miR-31	RDX	MET
hsa-miR-140-3p	NRIP1	MAPK1, GOLT1B, CBL, SCAMP1
hsa-miR-148a	DNMT1, DNMT3B	BCL2L11, USP33
hsa-miR-222-3p	TRPS1, BIRC5, STAT5A, MMP1	KIT, CDKN1B