SYMPOSIUM: 2013 MEETINGS OF THE MUSCULOSKELETAL TUMOR SOCIETY AND THE
INTERNATIONAL SOCIETY OF LIMB SALVAGE

MicroRNA Regulates Vascular Endothelial Growth Factor Expression in Chondrosarcoma Cells

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Published online: 9 August 2014
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Abstract

Background Systemic treatments to prevent or treat chondrosarcoma metastasis are lacking and targeted therapy has yet to be developed. Hypoxia develops in tumors as they grow and hypoxia-related alterations in gene expression underlie some of the traits of cancer. One critical trait is the ability to induce sustained angiogenesis, which is usually related to expression of vascular endothelial growth factor (VEGF). A potential hypoxia-related mechanism resulting in altered gene expression involves microRNA.

One or more of the authors (LW, QC, RMT) has received funding from the National Institutes of Health and from the National Center for Research Resources, a component of the National Institutes of Health: Grant R01AR059142 (LW), Grants 1P20RR024484-01 and 9P20GM104937-06 (QC), and Grants 1P20RR024484-01 and 1R01CA166089-01A1 (RMT).

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Little is known about microRNA expression in chondrosarcoma and its potential role in regulation of VEGF expression.

Questions/purposes Our purposes were (1) to determine if there is hypoxia-regulated microRNA overexpressed in chondrosarcoma; (2) if that contributes to increased VEGF expression; and (3) can VEGF expression be inhibited with a specific antagomir?

Methods MicroRNA expression was analyzed in two primary human chondrosarcomas and articular cartilage using array analysis and a cutoff of a fourfold difference in expression between tumor and normal tissue. The effects of hypoxia and hypoxia-inducible factor-1 α (HIF-1 α) transfection and silencing with siRNA on expression of candidate microRNAs were analyzed in chondrosarcoma cell line JJ. VEGF expression was measured with quantitative polymerase chain reaction and enzyme-linked immunosorbent assay after specific microRNA transfection and knockdown.

Results miR-181a was identified by array analysis and confirmed with quantitative reverse transcription-polymerase chain reaction, which showed that miR-181a was overexpressed in both human chondrosarcomas (33- and 55-fold) and the JJ cell line (sixfold) compared with cartilage and chondrocytes, respectively. In vitro, hypoxia and HIF-1 α transfection each further increased miR-181a expression twofold in JJ cells. miR-181a transfection of JJ cells doubled

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expression of VEGF mRNA and increased secreted VEGF protein by 46% in normoxia, an effect that could be either direct or indirect. Similar enhancement of VEGF expression by miR-181a was found during hypoxia. Transfection with the antagomir anti-miR-181a decreased VEGF protein by 27% in normoxia and 23% in hypoxia.

Conclusions miR-181a is a hypoxia-regulated microRNA that is overexpressed in chondrosarcoma and enhances VEGF expression, an effect that could be inhibited by anti-miR-181a.

Clinical Relevance Systemic treatment options for chondrosarcoma are limited. Antiangiogenic strategies could potentially be effective in limiting tumor progression. One method of inhibiting VEGF expression and associated angiogenesis could be an antagomir-based therapy targeted at miR-181a or other oncogenic microRNAs, although methods of systemic delivery are still under development. The effectiveness of antagomirs also needs to be compared with other antiangiogenic modalities in pre-clinical models.

Introduction

Chondrosarcoma is a disease in need of systemic treatment. Like in other sarcomas, morbidity is primarily related to the development of metastatic disease, which is mostly to the lungs and is usually fatal. Because conventional cytotoxic chemotherapy is considered ineffective, the search is on for targeted therapy [29]. Sustained angiogenesis is one of the characterizing traits of neoplasia and blocking angiogenesis may inhibit tumor progression, including growth of the primary tumor and metastatic behavior [11, 12]. Angiogenesis is largely mediated by vascular endothelial growth factor (VEGF). Strategies to block the effects of VEGF include antibodies, decoy receptors, receptor blockade, and molecular techniques aimed at inhibiting the expression of VEGF. In prior work, we have shown that despite the fact that cartilaginous tissue is avascular, Grades II and III chondrosarcoma have more microvasculature and VEGF expression than Grade I or benign tumors. Also, regulation of VEGF expression by the major hypoxia-driven transcription factor hypoxia-inducible factor-1 (HIF-1) is intact in chondrosarcoma [15, 19, 20]. HIF-1 is a heterodimer of HIF-1 α and HIF-1 β . HIF-1 α is rapidly degraded during normoxia but accumulates during hypoxia, allowing for binding with HIF-1 β , which is present in excess, to form the active transcription factor HIF-1. Although hypoxia is a driving force behind the aggressive phenotype in cancer, agents to block the effects of hypoxia have yet to be developed and therefore blocking downstream effects may be necessary. For example, we

have previously shown that VEGF expression is also mediated by chemokine (C-X-C motif) receptor 4 (CXCR4), a cell surface receptor frequently overexpressed in cancer, whose activity can be blocked by the drug AMD3100. However, CXCR4 blockade with AMD3100 is only partially effective in decreasing VEGF and matrix metalloproteinase 1 expression [25, 27], the latter an important factor in metastasis and a negative prognostic marker in chondrosarcoma [3]. A relatively new layer of gene regulation involves microRNA (miRNA) [9]. Mis-expression of miRNA plays a role in cancer biology and interestingly, hypoxia is responsible for some altered miRNA expression [14]. MicroRNA are short, noncoding RNA molecules, 18 to 22 nucleotides in length, that bind to the three prime untranslated region (3'-UTR) of mRNA through base pair complementarity, resulting in mRNA degradation or inhibition of translation. The net result can be a gain of function or loss of function depending on whether the miRNA is over- or underexpressed and the function of the target gene(s). The net result is similar to the effects of an oncogene or tumor suppressor mutation, respectively. An antagomir has the complementary sequence of a miRNA and can be used to counteract the effect of an overexpressed miRNA. Loss of expression of a miRNA can be restored through miRNA transfection. Both strategies are being developed for various diseases including cancer. One advantage of targeting miRNA misexpression is that multiple genes and related pathways can be regulated by one miRNA so that the effect of using one antagomir or one miRNA is amplified. However, a liability of targeting miRNA may be a greater chance of off-target, unintended effects.

Our objective is to understand metastatic pathways in this tumor and our long-term goal is to develop targeted therapy. Working on the assumptions that hypoxia is a major factor in driving the malignant phenotype, that hypoxia alters miRNA expression, and that regulation of VEGF is multifactorial, we undertook a study of miRNA expression in chondrosarcoma. The purposes of this project were (1) to determine if there is hypoxia-regulated miRNA overexpressed in chondrosarcoma; (2) if that contributes to increased VEGF expression; and (3) can VEGF expression be inhibited with a specific antagomir? Addressing these objectives would support further investigation into the contribution of miRNA to angiogenesis and metastasis in chondrosarcoma and the plausibility of an antagomir-based approach for targeted therapy.

Materials and Methods

This study is based on analysis of miRNA expression in primary chondrosarcoma tissue and in vitro analysis in a chondrosarcoma cell line, evaluation of the role of hypoxia

and specifically HIF-1 in the regulation of miRNA expression, and analysis of the effect of select miRNA on VEGF expression using miRNA and antagomir transfection.

Cell Lines and Cell Culture

Human primary chondrocytes and chondrosarcoma cell line JJ were cultured as previously described [14]. JJ was derived from a human Grade II chondrosarcoma [4]. The cell line was authenticated using short tandem repeat profiling and was performed (ATCC, Manassas, VA, USA) on the source cell line in 1999, 2007, and repeated in 2012. There is 94% similarity between the different time points, the cells are human, and there are no matches with any cell lines in the ATCC database. Chondrocyte cell line C-28/I2 was cultured in F12/DMEM medium with 10% fetal bovine serum [7]. All cells were cultured in a humidified incubator (NuAire Inc, Plymouth, MN, USA) under 5% CO₂ and either normoxia or hypoxia (2% O₂) [15].

RNA Isolation and MicroRNA Microarray

Total RNA including miRNA was isolated from two human chondrosarcoma (Grades II and III), the same patients' normal articular cartilage, which were then pooled, primary chondrocytes, chondrocyte cell line C-28/I2, and chondrosarcoma cell line JJ using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The concentration and purity of total RNA were determined by a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Human microarray assay containing 894 human miRNA sequences was performed (LC Sciences, Houston, TX, USA) using miRNA from the individual tumors and the pooled normal cartilage samples. The specific miRNA that were over- and underexpressed by a factor of four or more in both chondrosarcoma samples compared with the pooled normal articular cartilage (with $p < 0.01$, Student's *t*-test) were identified for further analysis. A more stringent cutoff of fourfold was used in contrast to the commonly used twofold difference in expression to minimize false-positives. Institutional review board approval was obtained for the study.

cDNA Synthesis and Quantitative Polymerase Chain Reaction

Total RNA was reverse-transcribed using the miScript Reverse Transcription kit (Qiagen) and miRNA expression levels were analyzed using the miScript SYBR Green PCR

kit (Qiagen). U17a, a ubiquitously expressed miRNA, was used as an internal control and is expressed consistently in normoxia and hypoxia. VEGF mRNA was measured using the Reverse Transcription System (Bio-Rad, Hercules, CA, USA) followed by real-time polymerase chain reaction (qRT-PCR) with SYBR Green Master Mix (Qiagen). 18S was used as an internal control [1, 2]. The primers for VEGF and 18s have been previously published and the data analysis was performed as previously described [18, 26].

Transfection

JJ cells were cultured in six-well plates for 1 day, then transfected with control miRNA, hsa-miR-181a mimic, anti-hsa-miR-181a (Applied Biosystems Inc, Foster City, CA, USA), or HIF-1 α construct, EV, control siRNA, or HIF-1 α siRNA (Qiagen), all using GenMute transfection reagent (SignaGen Laboratories, Gaithersburg, MD, USA). After 2 days of culture, conditioned medium was collected for enzyme-linked immunosorbent assay or the cells were lysed in RIPA buffer (Cell Signaling, Danvers, MA, USA) with Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN, USA) for Western blot.

Western Blot

Equal amounts of protein in cell lysates were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis and probed with anti-HIF-1 α (Cell Signaling Technology, Danvers, MA, USA) or antiactin (Santa Cruz, Dallas, TX, USA). The signals were detected using a fluorescently labeled secondary goat antirabbit IgG (Alexa Fluor 680; Molecular Probes, Eugene, OR, USA) or donkey antigoat IgG (IRDye 800; Rockland, Gilbertsville, PA, USA) and analyzed on a Licor Odyssey Scanner (LI-COR Biosciences, Lincoln, NE, USA).

Enzyme-linked Immunosorbent Assay

Conditioned media from cultured cells were used to measure VEGF content as previously described (R&D Systems, Minneapolis, MN, USA) [25, 27].

Statistics

Statistical analysis was performed with GraphPad Prism, Version 5.0 (GraphPad Software, San Diego, CA, USA). qRT-PCR with two groups and enzyme-linked immunosorbent assay were analyzed with the Student's *t*-test.

Fig. 1 miR-181a is overexpressed in chondrosarcoma. Expression of miR-181a was confirmed with qRT-PCR in chondrosarcoma and cartilage used in the array analysis. CSIII = Grade III chondrosarcoma; CSII = Grade II chondrosarcoma; NCL = pooled normal articular cartilage (N = 2); mean $\pm$ SD, * p < 0.001, both compared with NCL.

qRT-PCR with three or more groups were compared with one-way analysis of variance followed by the Student's *t*-test with Bonferroni correction for individual comparisons. The null hypothesis of no difference was rejected at a significance level of 5%. All experiments were repeated at least three times. Expression of miRNA in array analysis was compared with Student's *t*-test. The null hypothesis of no difference was rejected at a significance level of 1%.

Results

As a first step in identifying miRNAs that are overexpressed in human chondrosarcoma, miRNA array analysis was performed on two human chondrosarcomas (Grade II and III) with their normal articular cartilage used as a control. Using the criteria of a statistically significant fourfold difference in expression between tumor and normal in both tumors, we identified seven overexpressed miRNA. Expression of candidate miRNAs was confirmed in primary tumors and screened for their response to hypoxia and their effect on VEGF expression. Expression of miR-181a was analyzed with qRT-PCR in the primary chondrosarcoma and cartilage used in the array analysis, chondrosarcoma cell line JJ compared with chondrocytes, and in JJ and chondrocytes cultured in normoxia and hypoxia. The analysis confirmed the overexpression of miR-181a in the primary tumors (33- and 55-fold) (Fig. 1), demonstrated overexpression in the JJ chondrosarcoma cell line compared with chondrocytes (sixfold) (Fig. 2A), and showed that hypoxia doubled miR-181a expression in both normal and tumor cells (Fig. 2B). Having shown that miR-181a is overexpressed in primary tumors as well as in the JJ chondrosarcoma cell line, further mechanistic analysis of miR-181a regulation was undertaken in vitro. Because the

Fig. 2A–B MiR-181a is overexpressed in chondrosarcoma cells and increased by hypoxia. Expression of miR-181a was measured with qRT-PCR in (A) chondrosarcoma cells and chondrocytes under normoxic conditions and (B) chondrosarcoma cells and chondrocytes in normoxic and hypoxic conditions. JJ = chondrosarcoma cell line; Ch = chondrocytes; C-28 = C-28/I2 chondrocyte cell line; n = normoxia; h = hypoxia (2% O₂); Mean $\pm$ SD, # p < 0.002, ** p < 0.05, * p < 0.001, * p < 0.01.

transcription factor HIF-1 mediates many of the hypoxia-related changes in gene expression, JJ cells were transfected with an expression construct for HIF-1 α and cultured in normoxia or transfected with siRNA directed at HIF-1 α during hypoxic culture conditions. The results show that HIF-1 upregulates miR-181a expression 2.4-fold during normoxic conditions and HIF-1 α knockdown inhibits miR-181a expression 42% during hypoxia (Fig. 3A). The effect of the transfections on HIF-1 α protein expression was confirmed with Western blot (Fig. 3B).

JJ cells were transiently transfected with individual miR, anti-miR, or control sequences and the effect of VEGF mRNA determined. miR-181a increased VEGF mRNA twofold (Fig. 4A).

JJ cells were transfected with control miRNA, miR-181a, or anti-miR-181a and cultured during normoxia and hypoxia and VEGF protein in conditioned media was measured. Transfection with miR-181a increased VEGF protein in normoxia by 46% and enhanced the increase in VEGF mediated by hypoxia an additional 36%. Anti-miR-181a inhibited secreted VEGF during normoxia and hypoxia by 27% and 23% compared with the control,

Fig. 3A–B HIF-1 regulates miR-181a expression. (A) HIF-1 α was overexpressed in JJ cells in normoxia or knocked down in hypoxia and miR-181a expression was quantitated with qRT-PCR (mean $\pm$ SD; * p < 0.001, # p < 0.01); (B) Western blot demonstrating effect of HIF-1 α overexpression or knockdown on HIF-1 α protein. EV = empty vector; control-si = control siRNA; HIF1 α -si = siRNA directed at HIF-1 α .

respectively. During hypoxia, VEGF concentration after control transfection was elevated to a similar level as resulted from miR-181a transfection during normoxia (Fig. 4B).

Discussion

Systemic treatment options for chondrosarcoma are limited. Because miRNAs may be master regulators of the malignant phenotype [8], identification of overexpressed miRNAs in chondrosarcoma, particularly those that might be involved with angiogenesis, is important and may provide a target for therapy using antagomirs. miR-181a was identified as a potential oncomir (a miRNA associated with cancer) by performing array analysis of primary human chondrosarcoma. Microarray analysis is an efficient method to broadly screen tumors for proteins, genes, or miRNAs that could be biomarkers or targets for therapy. Overexpression of miR-181a was also present in chondrosarcoma cell line JJ and was further increased by hypoxia and specifically HIF-1. Inhibition of miR-181a decreased expression of VEGF. Our results show that miR-181a is a hypoxia-regulated miRNA that upregulates expression of VEGF in chondrosarcoma.

There are several limitations to our study. The initial miRNA array screen was based on two tumors. Although

Fig. 4A–B miR-181a regulates VEGF expression. JJ cells were transiently transfected with miR-181a, anti-miR-181a, or control sequences and cultured in normoxia or hypoxia. (A) VEGF mRNA was quantified with quantitative PCR in normoxia (mean $\pm$ SD; * p < 0.001). (B) VEGF protein secretion into culture media was evaluated with enzyme-linked immunosorbent assay (mean $\pm$ SD; * p < 0.001, ** p < 0.05, # p < 0.01).

we confirmed overexpression with PCR, the results will need to be confirmed in a larger series of tumors. Ideally, an annotated database of tumor specimens and clinical followup could potentially validate miR-181a as a biomarker. Although miR-181a regulates VEGF expression, we did not perform phenotypic assays or in vivo testing, although in prior work, VEGF expression correlated with in vitro and in vivo angiogenesis assays [25, 26]. In addition to VEGF, there may be additional miR-181a targets related to tumor progression yet to be identified. We have not worked out the mechanism by which miR-181a regulates VEGF expression. Implementation of antagomir-based therapy will require a systemic delivery vehicle and potential off-target effects of miR-181a will need to be evaluated. Most importantly, the effects of anti-miR-181a need to be tested in vivo. We used a conservative threshold of a fourfold increase in miRNA expression to decrease the likelihood of false-positives. Nevertheless, there may be additional miRNAs with lesser changes in expression that

have biologic effects. We did not study the effect of restoring underexpressed miRNAs.

Our results are consistent with other studies in which expression of certain miRNAs was elevated in specific cancers and contribute to the malignant phenotype. miR-181a has been reported to be underexpressed in lung cancer [10] but overexpressed in breast cancer [28]. miR-181a has not been previously reported as overexpressed in any sarcomas, including chondrosarcoma [23, 31].

In these studies of chondrosarcoma, different techniques, cell lines, and expression criteria were used. miR-181a has been noted to be upregulated by hypoxia; however, the mechanism of its regulation during hypoxia has not been investigated [13]. Our results show that the HIF-1 transcription factor regulates miR-181a. Another reported mechanism of miR-181a upregulation in breast cancer involves transforming growth factor- β [28].

Functional effects of miR-181a overexpression are varied and include modulation of epithelial to mesenchymal transition (EMT) in ovarian carcinoma [22], enhanced chemoresistance to cisplatin by targeting protein kinase C, delta, a promoter of apoptosis [6], and promotion of proliferation and inhibition of apoptosis in gastric cancer by targeting the tumor suppressor ataxia telangiectasia mutated [32]. Enhancement of VEGF expression by miR-181a and potentially angiogenesis has not been previously reported. MicroRNA works by targeting the 3'UTR of a gene resulting in translational repression or mRNA degradation. Rarely does miRNA cause increased translation. We have not been able to identify putative binding sites in the 3'UTR for the VEGF gene, suggesting that the mechanism of miR-181a activity is indirect and could involve the inhibition of an inhibitor of VEGF expression. For example, in lung carcinoma, overexpression of miR-483-5p inhibits Rho GDP dissociation inhibitor alpha (RHOGDI1), an inhibitor of the transcription factor SNAIL, resulting in increased expression of SNAIL, which promotes EMT, which in turn promotes metastasis [24]. Other mechanisms of VEGF expression in chondrosarcoma include the direct regulation by HIF-1 [15], CXCR4 signaling [25], RUNX2 [21, 26], and endothelin-1 [30].

The fact that miR-181a transfection in normoxia has a similar effect as hypoxia on VEGF expression indicates the robustness of the effect of miR-181a overexpression. MicroRNAs involved with VEGF expression in other systems include miR-150, which also increases VEGF expression [17]; miR-126, which directly targets VEGF with loss of miR-126 expression resulting in increased VEGF expression in lung cancer [16]; and loss of miR-26a, which also increases VEGF with phosphatidylinositol-4,5-bisphosphate 3-kinase as the downstream target in hepatocellular carcinoma [5]. Thus, altered miRNA expression

is another mechanism of increased VEGF expression and antagomirs may be a way to inhibit VEGF expression.

In conclusion, miR-181a is expressed at high levels in chondrosarcoma and is involved with hypoxic regulation of enhanced VEGF expression. These results suggest that inhibition of miR-181a may have therapeutic applications; however, additional investigation is needed. The mechanism of activity and other potential targets need to be evaluated. Anti-miR-181a needs to be tested in vivo for its effect on angiogenesis, tumor progression, and potentially metastasis. Then the effectiveness of anti-miR-181a needs to be compared with other anti-VEGF strategies alone and potentially in combination with these agents. Lastly, a method of targeted delivery of antagomirs to the tumor needs to be optimized.

Acknowledgments We thank Drs Joel Block for the JJ chondrosarcoma cell line, Mary Goldring for the C-28/I2 chondrocyte cell line, and Hiroyuki Kato for the HIF-1 α construct.

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