MicroRNA-181a Regulates Chondrosarcoma Metastasis By Enhancing Chemokine Receptor Signaling

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Introduction: Chondrosarcoma is a primary bone tumor that remains without an effective systemic treatment. Identifying regulators of metastatic pathways could help develop targeted therapies. Regulation of metastatic pathways may involve microRNAs (miRs), short noncoding RNAs that negatively regulate gene expression. In cancer, miRs can function analogous to tumor suppressor genes or oncogenes, and may be master regulators of the malignant phenotype through complementarity with multiple target genes. We identified candidate miRs using overlapping screens in which miR microarrays were used to compare miR expression in primary human tumors, xenograft tumors, normal cartilage, and chondrosarcoma cell lines cultured under normoxic and hypoxic conditions. We identified miR-181a as a hypoxia responsive miR that is overexpressed in chondrosarcoma. Overexpression of miR-181a increased VEGF and MMP1 expression, factors known to enhance chondrosarcoma metastasis. We hypothesized that anti-miR-181a would inhibit chondrosarcoma angiogenesis, invasion, tumor growth, and metastasis and tested our hypothesis in vitro and in vivo. We demonstrate that the mechanism of miR-181a activity is by inhibiting Regulator of G-protein Signaling (RGS16), which is a negative regulator of C-X-C chemokine receptor type 4 (CXCR4). Thus, miR-181a enhances CXCR4 signaling and functions as an oncomir.

Methods: Primary human tumors (N = 18), normal human cartilage samples (N = 12) and human chondrosarcoma cell line JJ were used in the analysis. JJ cells were transfected with a lentivirus construct expressing either anti-miR-181a, miR-181a, or control sequence, and used for in vitro studies and for xenograft tumors in nude mice (N = 12/group). Results are presented as means ± SEM. Real-time RT-PCR was used to quantify expression of miR-181a and RGS16 mRNA with normalization to U17 and 18s. ELISA was used to measure secreted VEGF and active MMP1 in conditioned media and Western blotting for RGS16 in cell lysates. Luciferase assay was used in conjunction with wild type and mutated pEZX-RGS16 promoter to assess miR-181a regulation of RGS16. In vitro invasion assay, xenograft tumor volume and weight, in vivo bioimaging of angiogenesis and MMP activity, and quantification of lung metastases in a mouse xenograft tumor model were used to measure the effects of anti-miR-181a on invasion, angiogenesis, tumor growth, and metastasis. Statistical analysis: qRT-PCR and Western blot were analyzed with the Student’s t-test. ELISA, luciferase activity, and invasion assays were compared with one-way ANOVA, followed by the Student’s t-test with Bonferroni correction. Lung metastases were quantitated by determining the number of nodules in each lung and compared with Student’s t-test. The number of mice in each group with metastases was compared with Chi Square (Fisher’s Exact test). The null hypothesis of no difference was rejected at a significance level of 5%. The study was approved by the institution’s IACUC and was conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (eighth edition).

Results: miR-181a expression was increased in human chondrosarcoma 43-fold compared to articular cartilage (p < 0.01, Fig. 1), in JJ chondrosarcoma cell line 14-fold compared to chondrocytes (p < 0.002), increased in JJ by hypoxia, and further increased in JJ xenograft tumors. miR-181a knockdown decreased active MMP1 62% (384± 42 vs. 148 ± 26 pg/ml, p < 0.05) and VEGF 47% (400 ± 33 vs. 212 ± 21 pg/ml, p < 0.01) in conditioned media from JJ, whereas miR-181a transfection had the opposite effects. RGS16 was identified as a potential target of miR-181a and cotransfection of RGS16 with miR-181a counteracted the effects of miR-181a overexpression on MMP1 and VEGF. RGS16 had the opposite expression profile as miR-181a: RGS16 mRNA expression was decreased 18-fold in chondrosarcoma compared to normal cartilage (p < 0.003), decreased 50-fold in JJ compared to chondrocytes (p < 0.02), and decreased 20-fold in xenograft tumors compared to JJ in vitro (p < 0.01). Regulation of RGS16 by miR-181a was evaluated with a luciferase reporter construct and Western blot. miR-181a transfection decreased luciferase activity 62% ( p < 0.001 ) and protein 64% ( p < 0.05), whereas anti-miR-181a increased luciferase activity 46% ( p < 0.05) and protein level 102% ( p < 0.05). There was no effect when the RGS16 3’ UTR was mutated in the luciferase reporter.
Bioimaging of mice bearing xenograft tumors was performed at 3 weeks with Fluorescence-based Quantitative Tomography using MMPSense and AngioSense probes, indicators of MMP activity and angiogenesis. MMP activity was decreased 56% in tumors transfected with anti-miR-181a (6.4 ± 0.9 vs. 2.8± 0.7, p < 0.004, Fig. 2), and in vitro, JJ cell invasion index decreased by 70% (p < 0.001). Similarly, AngioSense probe content in xenograft tumors was decreased 65% (17 ± 3.1vs. 6 ±1.4 pmol, p < 0.004). At 5 weeks, xenograft tumor size was decreased by anti-miR-181a transfection: volume (905 ± 112 vs. 349 ± 59 mm$^3$, p < 0.001, Fig. 3a) and weight (850 ± 119 vs. 317 ± 49 mg, p < 0.0004). Most importantly, the number of lung metastatic nodules was less in the treatment group (4.8 ± 1.6 vs. 0.8 ± 0.7, p < 0.04, Fig. 3b) and the number of mice with lung metastases was 7/12 in the control group vs. 2/12 in the treatment group, p < 0.04.

Figure 1. miR-181a expression is increased in human chondrosarcoma tissue (CS, N=18) compared to normal cartilage (CL, N=12), * p<0.012.

Figure 2. Anti-miR-181a decreased MMP activity in xenograft tumors. A. Representative images with Fluorescence-based Quantitative Tomography using MMPSense are shown. B. MMPSense content in xenograft tumors was measured after three weeks, N = 12/group . * p<0.004.
Discussion: In conclusion, our results show that miR-181a is an onco-miR that promotes chondrosarcoma growth and metastasis. Overexpression of miR-181a increases the invasive phenotype by upregulating expression of VEGF and MMP1. Antagomir therapy aimed at miR-181a reduced expression of these pro-angiogenic and metastatic factors, and reduced tumor growth and metastasis. The mechanism of mir-181a is through regulation of RGS16, an endogenous inhibitor of chemokine receptor signaling. Knockdown of miR-181a restores expression of RGS16, thereby inhibiting CXCR4 signaling. We have previously reported increased CXCR4 expression and signaling in chondrosarcoma (1,2). Overexpression of miR-181a is part of the mechanism underlying enhanced CXCR4 signaling and anti-miR-181a may augment receptor blockade. One limitation of our study is that anti-miR-181a was delivered with a lenti virus vector to chondrosarcoma cells before implantation into mice. Targeted delivery methods using nanoparticles and linked antagomirs are currently under development for in vivo delivery.

Significance: In clinical practice, there are no effective treatments to prevent or inhibit chondrosarcoma growth and metastasis. The results of our study suggest that antagomir therapy with anti-miR-181a may be a promising, novel approach for chondrosarcoma treatment.

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