Immunotherapy of Chondrosarcoma with Nanopiece Delivery of Antisense Oligonucleotide against miR-181a Targeting Tumor Macrophage

Yan Xiang Michael Shao[1], Veer Rege[1], Amol Rathore[1], Jing Ding[1,2], Jesse Hart[1], Yajun Liu[1], Richard Terek[1], Qian Chen[1]
[1]Department of Orthopedics, Alpert Medical School of Brown University/Rhode Island Hospital, Providence, RI, [2]NanoDe Therapeutics Inc.
yan_xiang_shao@brown.edu

Disclosures: Yan Xiang Michael Shao (N), Veer Rege (N), Amol Rathore (N), Jing Ding (Y, NanoDe Therapeutics employee), Jesse Hart (N), Yajun Liu (N), Richard Terek (N), Qian Chen (Y, NanoDe Therapeutics co-founder)

INTRODUCTION: Chondrosarcoma is a malignant bone tumor with poor response to traditional therapies, including chemotherapy and radiation therapies. Late-stage chondrosarcoma is lethal, resulting from frequent pulmonary metastasis. A new and effective therapy is urgently needed. miR-181a has been identified as an oncogenic microRNA that contributes to tumor progression by downregulating RGS16, upregulating VEGF and MMP1, and promoting angiogenesis and metastasis [1]. In a human tumor xenograft nude mouse model, systemic delivery of an antisense oligonucleotide (ASO) targeting oncogenic miR-181a via Nanopieces (NPs), a non-viral, non-lipid nucleic acid delivery vehicle, inhibited tumor growth, metastasis, and prolonged survival [2]. In this study, we determined the mechanisms by which NPs-ASO target tumor and its microenvironment for cancer therapy. Specifically, we hypothesized that NPs-ASO not only suppresses tumor growth but also modulates the tumor microenvironment (TME) through regulating M1 macrophage polarization and enhancing its tumor infiltration.

METHODS: Human chondrosarcoma cells were injected subcutaneously to allow tumor growth and metastasis to the lung in 6-8-week-old female nude mice. Males were excluded because they can become aggressive and fight when tumors or wounds are present, and there are no known differences in prognosis between males and females. Treatment with NPs-miR-181a ASO was carried out by multiple IV injections in three weeks. RNA was extracted from xenograft tumor samples (n = 7, ASO-treated; n = 5, scrambled ASO-treated controls) using miRNeasy Mini Kit (Qiagen). Species-specific RNA sequencing was performed by Nanostring. Cell, gene, and pathway profiling was performed using the nCounter® Analysis System with the Human PanCancer IO 360™ and Mouse PanCancer Immune Profiling Panels. Immunohistochemistry (IHC) of tumor samples was performed and quantified using ImageJ. RAW 264.7 cells were transfected with miR-181a ASO (n = 3 per group) using Lipofectamine 3000. miR-181a knockdown was assessed using miRCURY LNA miRNA PCR Assay (Qiagen). M1/M2 polarization of macrophages was quantified using real-time RT-PCR. The comparative threshold cycle (Ct) method, i.e., 2-ΔΔCt method, was used for the calculation of fold change. Statistical comparisons were performed using Student's t-test, with $p < 0.05$ considered significant. All animal studies were approved by the IACUC at Rhode Island Hospital and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (eighth edition).

RESULTS: As shown previously, systemic IV delivery of NPs-ASO in the nude mice with human chondrosarcoma xenografts reduced tumor weight, volume, and metastasis to the lung [2]. To determine the mechanism of NPs-ASO treatment, we isolated RNA from human tumor xenografts after NPs-ASO treatment and performed species-specific RNA sequencing for gene, cell, and pathway profiling. First, human-specific RNA sequencing revealed alteration of gene expression in human chondrosarcoma cells in response to NPs-ASO treatment. The MMP1 mRNA level was significantly inhibited ($p < 0.05$), validating the previous finding by real-time RCR analysis [2]. Furthermore, IL-7R was a top upregulated gene by NPs-ASO in tumor cells ($p < 0.01$), suggesting an activation of the IL-7 pro-inflammatory pathway in tumor cells in response to NPs-ASO treatment. Thus, NPs-ASO inhibition of tumor growth and metastasis is associated with not only inhibition of MMP1 and angiogenesis but also upregulation of IL-7 inflammation signaling in human tumor cells. Second, mouse-specific RNA sequencing revealed the presence of infiltrated immune cells in human tumor xenografts from the nude mouse host. The mouse cell adhesion pathway score was significantly increased (Fig. 1A). This suggests an increase in infiltration of host immune cells within the tumor. Differential expression analysis identified an upregulation of multiple adhesion-related genes in mouse cells within the tumor (data not shown), indicating modulation of the tumor microenvironment following treatment. Immunohistochemistry analysis demonstrated a significant increase in CD-68 positive macrophages in tumors in response to NPs-ASO treatment (Fig. 1B & C). M1 marker IL-1β and IL-7R positive staining were significantly increased (data not shown), supporting an increase of M1 macrophages in the tumor after NPs-ASO treatment. In vitro, RAW 264.7 macrophages transfected with ASO showed 75% knockdown of miR-181a and a significant increase in iNOS mRNA expression (2.28-fold, $p < 0.05$), indicating that ASO induced polarization toward a pro-inflammatory M1 phenotype (Fig. 2A & B).

DISCUSSION: Our data suggested that NPs-ASO not only affects human tumor cells but also modulates the tumor microenvironment by activating immune-related activities and driving macrophage polarization towards the anti-tumor M1 phenotype. Notably, the treatment upregulated the mouse cell adhesion pathway and facilitated immune cell recruitment and infiltration in the tumor. We took advantage of the property of nude mice, which do not have mature T cells but possess normal macrophages. We demonstrated that it was the mouse M1 macrophages that infiltrated the tumor and activated inflammation signaling of tumor cells. Such immune activation was due to the ASO cargo but not the NPs delivery vehicle because the control group also contained NPs delivery vehicle but with scrambled ASO cargo. Indeed, we showed that ASO transfection by Lipofectamine knocked down miR-181a successfully in RAW 264.7 macrophages, which upregulated the expression of iNOS, an M1 marker. It has been shown that iNOS enhances nitric oxide (NO) production and promotes M1 macrophage polarization [3]. While our in vivo data suggest that NPs delivered ASO to not only human tumor cells but also mouse host macrophages, our in vitro data suggest that ASO induced M1 macrophage polarization. These findings provide mechanistic insights into the mechanisms by which NPs-ASO delivery enhances immune engagement, alters tumor microenvironment, and inhibits chondrosarcoma progression and metastasis.

SIGNIFICANCE/CLINICAL RELEVANCE: Our study suggested that NPs-ASO therapy could be used to enhance cancer treatment efficacy for chondrosarcoma patients by affecting both tumor cells and the immune cell microenvironment.

REFERENCES: [1] Sun et al. *Molecular Cancer Research* (2015) 13(8):1347–1357 [2] Sun et al. *Molecular Cancer Therapeutics* (2019) 18:3 [3] Li et al. MedComm (2023) 4:e349

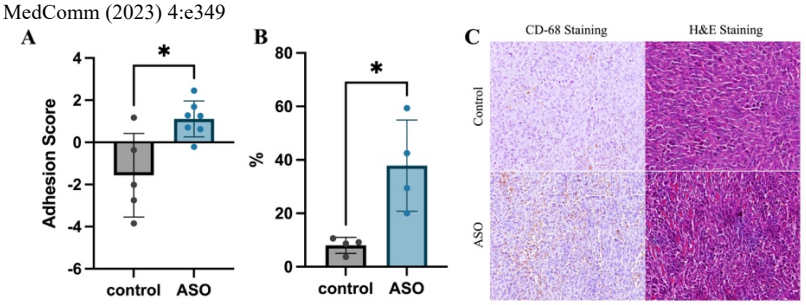

Fig. 1. (A) Adhesion pathway scores from the mouse RNA panel show a significant increase in anti-miR-181a–treated tumors compared to controls. (B) Positive CD-68 area percentage in viable tumor cells following anti-miR-181a treatment showed a significant increase compared to controls. (C) Microscopic comparison images of CD-68 and H&E in ASO-treated samples vs control samples. *p < 0.05.

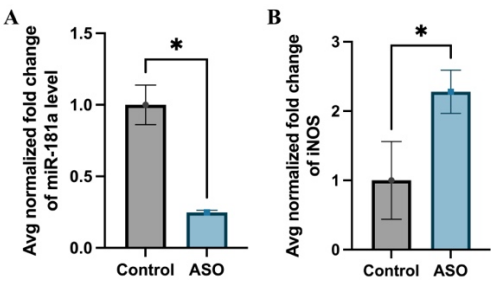

Fig. 2. Real-time RT-PCR analysis of levels of miR-181a (A) and iNOS mRNA (B) in RAW 264.7 macrophages after transfection of miR-181a-ASO with Lipofectamine. *p < 0.05.