Knockdown of neuropilin-1 suppresses invasion, angiogenesis, and increases the chemosensitivity to doxorubicin in osteosarcoma cells – an in vitro study

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Abstract. – OBJECTIVES: Neuropilin-1 (NRP-1) is a novel co-receptor for vascular endothelial growth factor (VEGF). NRP-1 expression in osteosarcoma tissues was significantly higher, and high NRP-1 expression was more frequently occurred in osteosarcoma tissues with advanced clinical stage, positive distant metastasis and poor response to chemotherapy. We tested a hypothesis that the NRP-1 gene plays a role in the invasiveness, angiogenesis and chemoresistance of human OS.

MATERIALS AND METHODS: To determine the role of NRP-1 in OS, NRP-1 was stably transfected into the human OS cell line MG-63 to increase the NPR-1 level, and NRP-1 siRNA was stably transfected into the human OS cell line SaOS-2 to knockdown of NRP-1. The effect of NRP-1 on invasion and angiogenesis was assessed by Matrigel invasion assay and in vitro angiogenesis assay. Chemosensitivity to doxorubicin was assessed by MTT assay in the MG-63 and SaOS-2 cells following NRP-1 overexpression or siRNA-induced downregulation of NRP-1.

RESULTS: The NRP-1 transfected MG-63 cells showed a markedly higher level of invasion in Matrigel invasion assay. The capillary-like structure formation of endothelial cells was also increased by coculture with the NRP-1 transfected MG-63 cells. On the contrary, the NRP-1 siRNA transfected SaOS-2 cells showed a markedly lower level of invasion in Matrigel invasion assay. The capillary-like structure formation of endothelial cells was also repressed by coculture with the NRP-1 siRNA transfected SaOS-2 cells. NRP-1 overexpression in MG-63 cells increased survival of cells after exposure to doxorubicin. In contrast, downregulation of NRP-1 expression in SaOS-2 cells markedly increased chemosensitivity after exposure to doxorubicin.

CONCLUSIONS: We suggest that NRP-1 could be used as a biomarker for OS progression and a novel therapeutic or chemopreventive target for human OS treatment.

Key Words: Neuropilin-1, Invasion, Angiogenesis, Chemotherapy.

Introduction

Osteosarcoma is the most common form of childhood and adolescent cancer, comprising 2.4% of all malignancies in pediatric patients and approximately 20% of all primary bone cancers. The gold standard for therapy consists of a combination of multi-agent neoadjuvant chemotherapy, followed by radical surgery and adjuvant chemotherapy. With this aggressive regimen, 5-year survival rates of approximately 65% are obtained in patients with localized disease. However, in the case of metastatic or recurrent disease, 5-year survival rates are reduced to only 20%. The lack of responsiveness to chemotherapy due to intrinsic or acquired chemoresistance is the major reason for poor survival and disease relapse of osteosarcoma patients. However, the mechanisms underlying osteosarcoma chemoresistance remain largely unknown. Therefore, the identification of prognostic factors that allow risk stratification at the time of diagnosis and elucidation of the mechanisms underlying chemoresistance will be pivotal in the development of new therapeutic strategies.

Neuropilin-1 (NRP-1) was initially characterized as an axonal membrane glycoprotein involved in neuronal guidance and development. Neuropilin serves as a coreceptor for semaphorin 3a (Sema3a) and vascular endothelial growth factor (VEGF). In neuronal cells, NRP-1 regulates axon guidance, in part by acting as a coreceptor with Plexin-A1 and the ligand Sema3a but may also guide the accurate migration of somata by binding VEGF164. NRP-1 is also expressed in
endothelial cells, where it is involved in the regulation of angiogenesis and endothelial cell migration, possibly via its function as a coreceptor for VEGF isoforms.\textsuperscript{9,11}

NRP-1 is expressed in various human tumors, including human nasopharyngeal carcinoma, squamous cell carcinoma of the esophagus, prostate cancer, breast cancer, melanoma, and pancreatic adenocarcinoma, but not in corresponding normal epithelial tissues.\textsuperscript{12-17} In some model systems, NRP-1 expression has been shown to increase tumorigenicity, possibly by promoting VEGF-mediated angiogenesis.\textsuperscript{18,19}

Zhu et al.\textsuperscript{20} has found that NRP-1 expression in osteosarcoma tissues was significantly higher than that in corresponding noncancerous bone tissues at both mRNA and protein levels. In addition, high NRP-1 expression more frequently occurred in osteosarcoma tissues with advanced clinical stage, positive distant metastasis and poor response to chemotherapy. However, the functional significance of NRP-1 on OS tumour cells has not been elucidated.

The aim of the present study was to investigate whether NRP-1 is directly involved in the invasion, angiogenesis, and the development of chemoresistance in osteosarcomas and whether inhibition of NRP-1 gene expression might usefully improve the outcome of therapy.

\textbf{Materials and Methods}

\textbf{Cell Culture}

The human osteosarcoma cell line MG-63 and SaOS-2 (sarcoma osteogenic-2) was obtained from the American Type Culture Collection (ATCC, Shanghai, China) and was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 U/mL) at 37°C in an atmosphere of 5% CO\textsubscript{2}. Transfected cells were maintained in medium supplemented with 200 g ml\textsuperscript{-1} G418 (neomycin; Life Technologies Corp., Carlsbad, CA, USA). \textit{In vitro} experiments were performed at 60-80% cell confluence, and cells were used at passages 0-7 days after transfection.

\textbf{Stable Transfection of NRP-1 siRNA}

RNA against NRP-1 RNA (NRP-1-siRNA) and control plasmid were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). When 80%-90% confluence, SaOS-2 cells were transfected with the plasmids using the Lipofectamine 2000 according to the manufacturer’s protocol. The stable clones (SaOS-2/NRP-1-siRNA and control SaOS-2/siRNA) were selected by culturing transfected cells in the presence of 400\textsuperscript{g} m\textsuperscript{-1} G418 (InvivoGen, San Diego, CA, USA) for 10 days. Stable pooled populations of SaOS-2/NRP-1-siRNA and control SaOS-2/siRNA cells were maintained in culture using 200 g ml\textsuperscript{-1} of G418. The knockdown effect was verified by Western Blotting analysis. Clones expressed the lowest levels of NRP-1 and were selected for further study.

\textbf{Stable Transfection of NRP-1 cDNA}

The full-length human NRP-1 cDNA (Sino Biological Inc., Beijing China) was subcloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) by standard techniques. Orientation was confirmed by restriction enzyme analysis and DNA sequencing. The resulting vector was stably transfected into human MG-63 cells using the Lipofectamine 2000 transfection reagent according to the manufacturer’s protocol. Control cells were transfected with the native pcDNA3.1 vector (mock transfectants). After 48h, the medium was replaced by selective medium containing 400 g ml\textsuperscript{-1} G418. Clones were expanded, and successful transfection was confirmed by Western blot analysis using anti-NRP-1 antibodies. Clones expressed the highest levels of NRP-1 and were selected for further study.

\textbf{Matrigel Invasion Assay}

The invasiveness of OS cells was tested after stable transfection. The cells (1 x 10\textsuperscript{5}/mL) were added to the upper wells coated with Matrigel (1 mg/mL; Collaborative Research, Inc., Boston, MA, USA) with serum-free medium containing 25 \textmu g/mL fibronectin as a chemoattractive agent in the lower wells. After a 24-h incubation period, cells that migrated through the filters into the lower chamber were counted by the number of cells on the lower side of the membrane in five random fields after staining with Hema-3 kit.

\textbf{In vitro Angiogenesis Assay}

OS cells (2x10\textsuperscript{5}/mL) were transfected with various plasmids for 48 h and the conditioned medium was filtered off for future research. Human microvascular endothelial cells-1 (HMEC-1, 4x10\textsuperscript{4}) were seeded onto eight-well chamber slides and the aforementioned conditioned medium was added. Cells were cultured for 72 h until
capillary network formation was observed. The number of branch points and total number of branches per point were counted after H&E staining to quantify the degree of angiogenesis.

**Analysis of Effect of NRP-1 Transfection on Chemosensitivity**

Transfected MG-63 or SaOS-2 cells (4×10⁶) were plated in 96-well plates. Following cell attachment after 24h, medium was changed to 10% fetal calf serum-minimal essential medium (FBS-MEM) with various concentrations of doxorubicin, and cells were incubated for 24h. In order to quantitate surviving/proliferating cells, 0.5 mg ml⁻¹ of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added and incubated for an additional 90 min. Medium and MTT were removed, dimethyl sulfoxide (DMSO) was added for 1min to solubilize cells, and absorption was read at 570 nm in a spectrophotometer. In each experiment, cells were plated in quadruplicate and the average of the relative absorption (OD₅₇₀) was used as an estimate of the number of metabolically active cells. Percentage of treated cells surviving compared to control cells not exposed to chemotherapy was calculated from the average OD₅₇₀ values obtained in each experiment. Each experiment was performed at least in triplicate for statistical analysis.

**Western Blot Assay**

Transfected MG-63 or SaOS-2 cells (1×10⁶) were lysed on ice with lysis buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulphate (SDS). Lysates were cleared of insoluble material by centrifugation at 14000 rpm, 4°C for 10 minutes. Protein content was measured with a standard Bradford assay and 40 µg of total protein were resolved by SDS-PAGE. Resolved proteins were then transferred to Immobilon-P membranes for immunoblotting. NRP-1 was detected using an anti-NRP-1 (Cell Signaling, Danvers, MA, USA). β-actin was detected with an β-actin antibody. Horseradish peroxidase conjugated (HRP) secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Results**

**NRP-1 Overexpression by Transfection in MG-63 Cells**

Western blot assay showed NRP-1 is expressed at higher levels in SaOS-2 cells than that of in MG-63 cells (Figure 1). We first chose to study the effect of overexpression of NRP-1 in MG-63 cell with relatively low endogenous expression. Western blot analysis demonstrated high levels of NRP-1 expression in NRP-1-transfected MG-63 cells compared to parental or mock-transfected MG-63 cells (Figure 1 A). Similar results were confirmed after repeating experiments several times.

![Figure 1](image-url)  
**Figure 1.** Effect of NRP-1 /siRNA transfection on NRP-1 levels in OS cells. **A,** Western blot analysis of lysates from mock-transfected and NRP-1-transfected MG-63 clones confirmed overexpression of NRP-1 in transfectants. **B,** Western blot analysis of lysates from mock-transfected and NRP-1-siRNA1 and NRP-1-siRNA2-transfected SaOS-2 clones confirmed NRP-1 silencing in transfectants. Data shown are representative of results from multiple experiments.
Knockdown of NRP-1 by siRNA Transfection in SaOS-2 Cells

We next chose to study the effect of NRP-1 silencing by siRNA transfection in SaOS-2 cell with relatively high endogenous expression. Western blot analysis demonstrated low levels of NRP-1 expression in NRP-1 siRNA-transfected SaOS-2 cells compared to parental or mock-transfected SaOS-2 cells (Figure 1B). Similar results were confirmed after repeating experiments several times.

NRP-1 Overexpression Promotes Invasion and Angiogenesis in MG-63 Cells

The result (Figure 2A) from Matrigel invasion assay indicates that NRP-1 overexpression significantly promotes the invasion of MG-63 cells by 78.4% as compared with mock-transfected and control vector-transfected cells. Furthermore, the results (Figure 2B) showed that HMECs treated with conditioned media from NRP-1 transfected MG-63 cells were able to form more capillary-like structures than mock- and pcDNA3.1-transfected MG-63 cells.

Knockdown of NRP-1 Inhibits Invasion and Angiogenesis in SaOS Cells

Knockdown of NRP-1 by siRNA significantly inhibits the invasion of SaOS-2 cells by 89.7% as compared with mock-transfected and control vector-transfected cells (Figure 2C). Furthermore, the results (Figure 2D) showed that HMECs treated with conditioned media from NRP-1 siRNA transfected SaOS-2 cells were able to form less capillary-like structures than mock- and pcDNA3.1-transfected SaOS-2 cells. HMECs cultured with mock- and pcDNA3.1-transfected cells exhibited capillary networks, whereas the formation of capillary-like structures in NRP-1 -transfected siRNA SaOS-2 cells was almost completely suppressed (82.3%; Figure 2D).

Effect of NRP-1 Overexpression on Chemosensitivity of MG-63 Cells

After 24h of doxorubicin exposure, MTT assay demonstrated improved survival in both NRP-1-overexpressing clones relative to the mock transfectants at doses around 50 mol/L, near the 50% inhibitory concentration (IC50) of doxorubicin in MG-63 cells (p < 0.01) (Figure 3A). An IC50 dose was not achieved in the NRP-1 transfected cells at this time point, even at supratherapeutic doses. The survival advantage was even more marked (p < 0.01) at 5000 mol/L. At this dose, at least 70% more of the NRP-1-transfected cells survived compared to control cells (30%).

Figure 2. Effect of NRP-1 /siRNA transfection on invasion and angiogenesis in SaOS cells. A, Invasiveness of MG-63 cells transfected with NRP-1 in Matrigel invasion assay. B, HMEC endothelial cells treated with conditional medium from MG-63 cells transfected with NRP-1 by in vitro angiogenesis assay. C, Invasiveness of SaOS-2 cells transfected with NRP-1 siRNA in Matrigel invasion assay. D, HMEC endothelial cells treated with conditional medium from SaOS-2 cells transfected with NRP-1 siRNA by in vitro angiogenesis assay. Mean from three separate experiments; bars, SD (vs control, p < 0.01).
Knockdown of NRP-1 Expression on Chemosensitivity of SaOS-2 Cells

Having demonstrated that NRP-1 overexpression provides a survival advantage for MG-63 cells exposed to doxorubicin, we sought to determine whether decreased NRP-1 expression would render SaOS-2 cells more susceptible to doxorubicin. We utilised the SaOS-2 cell line with endogenously high levels of NRP-1 to develop clones with down-regulated NRP-1 expression, utilising siRNA technology. The results showed siRNA-transfected SaOS-2 cells proved to be much more susceptible to doxorubicin at both 24 compared to the mock or control-transfected SaOS-2 cells (Figure 3 B).

Discussion

Invasive infiltration, distant metastasis and growth are key features in OS, which are accompanied by remodeling of the vasculature and by the destruction of the surrounding tissues. Several reports in human OS showed overexpression of VEGF, which is involved in the adhesion, invasion, metastasis, and angiogenesis of human OS. Numerous studies on interference with the VEGF pathways has shown the successful inhibition of neovascularization and tumor growth in animal trials.

Neuropilin-1 (NRP-1) is a co-receptor for class-3 semaphorins in neuronal guidance, and for the angiogenic cytokine vascular endothelial growth factor (VEGF or VEGF-A) in vascular development. Neuropilin-1 requires plexin-A1 to transduce semaphorin-3A signaling in neuronal cells, which is implicated in chemorepulsion and neuronal cell migration. In endothelial cells, NRP1 enhances VEGFR-2-mediated VEGF functions, including cell migration and angiogenesis.

Since migration of tumour cells plays a key role in neoplastic spread, invasion of surround-
ing tissue, and formation of metastasis, these findings indicate a key role for NRP1 in the motility of carcinoma cells, which may contribute to tumour progression and metastatic potential. We, therefore, hypothesized that direct blocking NRP1 transcriptional activity could contribute to inhibition of tumor invasion, metastasis, and angiogenesis of human OS. Previous study\textsuperscript{20} found NRP-1 was overexpressed in osteosarcoma tissues, and high NRP-1 expression more frequently occurred in osteosarcoma tissues with advanced clinical stage and positive distant metastasis.

In the present study, we investigated the effects of the NRP1 on cell migration and angiogenesis in OS cells \textit{in vitro}. The results showed blocking the NRP1 using siRNA-targeting NRP1 inhibited SaOS-2 cells angiogenesis and invasion. On the contrary, overexpression of NRP1 promotes MG-63 cells angiogenesis and invasion. Therefore, blocking NRP1 might be a potential approach for OS therapy.

The existence or development of intrinsic or acquired chemoresistance represents the principal reason for poor survival and disease recurrence in osteosarcoma patients. Unfortunately, the mechanisms underlying osteosarcoma chemoresistance remain largely unknown. However, knowledge of the mediators that contribute to chemoresistance is pivotal to the identification of high-risk patients and the development of new therapeutic strategies. Wey et al\textsuperscript{29} has reported Neuropilin-1 overexpression in FG cells enhanced anoikis resistance and increased survival of cells after exposure to gemcitabine and 5-FU. In contrast, downregulation of NRP-1 expression in Panc-1 cells markedly increased chemosensitivity to gemcitabine.

In our study, we studied the effects of the NRP1 on chemosensitivity to doxorubicin in OS cells. The results showed inhibiting the NRP1 suppressed proliferation and increased chemosensitivity to doxorubicin in SaOS-2 cells. On the contrary, overexpression of NRP1 promotes MG-63 cells proliferation and increased strong chemoresistance to doxorubicin. These data indicated that NRP1 would be directly involved in the development of chemoresistance. In fact, the findings of the present study demonstrate that overexpression of NRP1 in osteosarcoma cells induces strong chemoresistance to doxorubicin. Inhibition of NRP1 response represents an attractive method for chemosensitization of this lethal malignancy, and the results of the current study confirm the NRP1 as an appropriate therapeutic target. The mechanisms by which NRP-1 increases chemoresistance may be multifactorial.

**Conclusions**

NRP-1 overexpression promotes angiogenesis, invasion, and increased strong chemoresistance to doxorubicin in MG-63 cells. Blocking the NRP-1 using siRNA-targeting NRP-1 inhibited angiogenesis, invasion, and increased chemosensitivity to doxorubicin in SaOS-2 cells. As such, NRP-1 silencing may be useful for the treatment of OS as well as other tumors.

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**Conflict of Interest**

The Authors declare that there are no conflicts of interest.

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