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Targeting Aurora kinase-A downregulates cell proliferation and angiogenesis in neuroblastoma

Carmelle Romain [a], Pritha Paul [a,b], Kwang Woon Kim [a], Sora Lee [a,b], Jingbo Qiao [a], Dai H. Chung [a,b,*]

[a] Department of Pediatric Surgery, Vanderbilt University Medical Center, Nashville TN
[b] Department of Cancer Biology, Vanderbilt University Medical Center, Nashville TN

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ABSTRACT

Purpose: Aurora kinase A (AURKA) overexpression is associated with poor prognosis in neuroblastoma and has been described to upregulate VEGF in gastric cancer cells. However, the exact role of AURKA in the regulation of neuroblastoma tumorigenesis remains unknown. We hypothesize that AURKA-mediated stabilization of N-Myc may affect VEGF expression and angiogenesis in neuroblastoma. Therefore, we sought to determine whether inhibition of AURKA modulates neuroblastoma angiogenesis.

Methods: Cell viability and anchorage-independent growth were determined after silencing AURKA or after treatment with MLN8237, AURKA inhibitor. Immunofluorescence was used to determine N-Myc localization. Human umbilical vein endothelial cells (HUVECs) were used to assess angiogenesis *in vitro*. Real time-PCR and ELISA were performed to determine VEGF transcription and secretion, respectively.

Results: Knockdown of AURKA significantly reduced cell proliferation and inhibited anchorage-independent growth. It also decreased N-Myc protein levels and nuclear localization. AURKA inhibition also decreased HUVECs tubule formation along with VEGF transcription and secretion. Similarly, MLN8237 treatment decreased neuroblastoma tumorigenicity *in vitro*.

Conclusions: Our findings demonstrate that AURKA plays a critical role in neuroblastoma angiogenesis. AURKA regulates nuclear translocation of N-Myc in neuroblastoma cells, thus potentially affecting cell proliferation, anchorage-independent cell growth, and angiogenesis. Targeting AURKA might provide a novel therapeutic strategy in treating aggressive neuroblastomas.

Clinical heterogeneity is a hallmark of neuroblastoma with certain tumors undergoing spontaneous regression and others progressing to advanced disease. To help guide treatment, patients are stratified into high- and low-risk groups based on *MYCN* status, age at diagnosis, and DNA ploidy [1]. Despite advances in treatment modalities, high-risk group of patients remains difficult to cure with dismal long-term survival of 40% [1,2]. In light of this, we and others continue to discern intracellular signaling in neuroblastoma that is associated with aggressive tumor phenotypes with the goal of developing highly specialized treatment against specific biologic targets.

Aurora kinase A (AURKA), also known as *STK15*/*BTAK*, is part of a family of serine/threonine kinases that are integral to the regulation of mitosis and cytokinesis. Amplification of AURKA has been reported in breast [3], and colon [4] cancers, as well as in neuroblastoma cell lines [5]. Dysregulation of this oncogene is associated with chromosomal instability, aneuploidy, cell cycle delay and centrosomal abnormalities [6]. In neuroblastoma, AURKA overexpression is associated with high-risk group of tumors, *MYCN* amplification, disease-relapse and decreased progression free survival [7]. Furthermore, AURKA has been shown to stabilize *MYCN* protein levels in neuroblastoma [8]. Previously, we have demonstrated that N-Myc regulates PI3K-mediated vascular endothelial growth factor (VEGF) and angiogenesis in neuroblastoma [9]. Besides the established role that AURKA plays in promoting carcinogenesis, AURKA overexpression is associated with increased VEGF transcription [10]. AURKA contributes to poor prognosis in neuroblastoma via its own overexpression and by directly interacting with N-Myc to stabilize its protein levels.

Several AURKA inhibitors are currently being used in clinical settings. Specifically, MLN8237 is a second generation, orally bioavailable, selective AURKA inhibitor that has been shown to induce cytotoxicity and cell cycle arrest in multiple myeloma [11], enhance chemosensitivity in esophageal cancer, medulloblastoma, and neuroblastoma [12,13]. Preclinical studies using MLN8237 showed significant *in vitro* growth inhibition and a positive impact on *in vivo* event-free survival in several pediatric cancers, including neuroblastoma [14], thus prompting phase I clinical trials [15]. Based on these, MLN8237 shows a promise for clinical use; however, it remains critical to elucidate the signaling pathways involved in AURKA-mediated tumorigenesis in neuroblastoma.

In this study, silencing AURKA, with shRNA or MLN8237, inhibited cell proliferation and anchorage-independence. For the first time in

* Corresponding author. Departments of Pediatric Surgery and Cancer Biology Vanderbilt University Medical Center 2200 Children's Way Suite 7100 Nashville TN 37232. Tel.: +1 615 936 1050; fax: +1 615 936 1046.
E-mail address: dai.chung@vanderbilt.edu (D.H. Chung).

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neuroblastoma, to our knowledge, we also show that targeting AURKA decreases *in vitro* angiogenesis. Here, we demonstrate that knockdown of AURKA results in decreased nuclear translocation and expression of N-Myc and decreased VEGF secretion, suggesting that AURKA may be upstream of this critical oncogene and indirectly regulating angiogenesis in neuroblastoma. Our results further solidify the role that AURKA has in promoting malignant neuroblastoma and the rationale behind creating and using biologic inhibitors, such as MLN8237, as part of the treatment for children with this disease.

1. Materials and methods

1.1. Materials

Antibodies against AURKA, N-Myc and cell lysis buffer were obtained from Cell Signaling Technology (Beverly, MA). Antibody against β-actin and fetal bovine serum (FBS) were from Sigma (St. Louis, MO). NuPAGE Novex 4%–12% Bis–Tris Gel and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Horseradish Peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Chemiluminescence (ECL) HRP substrate was purchased from Millipore (Immobilon Western) and Perkin Elmer (Western Lightning). MLN8237 was from Selleckchem (Houston, TX). Human VEGF antibody and VEGF neutralizing antibody were from R&D Systems, Inc. (Minneapolis, MN).

1.2. Cell culture, plasmids and transfection

Human neuroblastoma cell lines were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium with L-glutamine (CellGro Mediatech, Inc. Herndon, VA) supplemented with 10% FBS. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Human umbilical vein endothelial cells (HUVECs, obtained from Dr. M. Freeman, Vanderbilt University Medical Center) were cultured in EMM-2 supplemented with growth factors (EGM-2 SingleQuot kit, Lonza, Walkersville, MD) at 37 °C and humidified 5% CO_2. shRNAs against AURKA (shAURKA) and non-targeting control (shCON) were purchased from Sigma-Aldrich. For transfection, cells were plated in 6-well plates and transfected with shRNA using Lipofectamine 2000 as per manufacturer's protocol.

1.3. Cell viability and soft agar colony formation assays

Cells were seeded onto 96-well plates at a density of 5,000 cells per well in RPMI culture media with 10% FBS, and cell number was assessed using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) for cell viability. For soft agar assay, cells were trypsinized and resuspended in RPMI 1640 media containing 0.4% agarose and 10% FBS. Cells were overlaid onto a bottom layer of solidified 0.8% agarose in RPMI 1640 media containing 5% FBS, at cell concentrations of 3×10^3 cells per well of a 12-well plate, and incubated for 3 weeks. Colonies were stained with 0.05% crystal violet, photographed, and quantified.

1.4. Immunoblotting and immunofluorescence

Whole-cell lysates were prepared using cell lysis buffer with 1 mM PMSF and incubated on ice for 30 to 60 min. Total protein (50 μg/lane) was resolved on NuPAGE Novex 4%–12% Bis–Tris gels and electrophoretically transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked with 5% milk in TBST (120 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature (RT) or overnight at 4 °C. Target proteins were detected by using rabbit or mouse anti-human antibodies (1:500 to 1000 dilution) for 3 h at RT or overnight at 4 °C. The membranes were washed three times and incubated with secondary antibodies (1:5000 dilution) conjugated with HRP. Immune complexes were visualized using the enhanced ECL system. Equal loading and transfer were confirmed by blotting the same membrane with β-actin antibody. Data are representative of three independent experiments.

For immunofluorescence, transfected BE(2)-C cells were plated on glass coverslips and incubated for 48 h. The treated cells were washed once with phosphate buffered saline (PBS) and fixed in formalin for 20 min at RT. Fixed cells were rehydrated for 30 min at RT. Samples were then blocked in 1% BSA/PBS buffer for 30 min, and incubated for 60 min in 1:100 dilution of anti-N-Myc antibody. Cells were washed three times with PBS and incubated 30 min in PBS containing 1% BSA and 1:500 secondary antibody Alexa Flour 488 goat anti-rabbit, then stained with DAPI for 5 min. Coverslips were mounted onto the slide glasses. The mounted cells were viewed with a fluorescence microscope using a 40× objective lens.

1.5. Endothelial cell tubule formation assay

HUVECs grown to ~70% confluence were trypsinized, counted, and seeded with 48,000 cells per well in 24-well plates coated with 300 μl of Matrigel (BD Biosciences, Bedford, MA). These cells were periodically observed by microscope as they differentiated into capillary-like tubule structures. After 6 h, cells were stained with Hematoxylin & Eosin and photographs were taken via microscope. The average number of tubules was calculated from examination of three separate microscopic fields (200×) and representative photographs obtained.

1.6. VEGF ELISA

The supernatant of cultured cells was collected at various time points, and subsequently frozen at −70 °C. For assay, samples were thawed and centrifuged, and then VEGF levels were measured using a human VEGF ELISA kit according to manufacturer's instructions (R&D Systems, Inc.).

1.7. Reverse transcription and Real Time-PCR

Total RNA was isolated using RNAqueous™ kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Isolated RNA was used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Austin, TX, USA). Primers designed to amplify human VEGF fragment (NM_003376) were: forward primer 5′-AGGAGGAGGGCAGAATCATCAC-3′; reverse primer 5′-ATGTCCACCAGGGTCTCGATTG-3′. Glyceraldehyde 3-phosphate dehydrogenase-specific oligonucleotide primers used were previously published [16]. *VEGF* mRNA levels were measured by quantitative real-time PCR using SsoFast™ EvaGreen Supermix with CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA).

1.8. Statistical analysis

Scoring index were expressed as means ± SEM; statistical analyses were performed using one-way analysis of variance for comparisons between the treatment groups. A P value of <0.05 was considered significant. Image J was used for densitometric analysis of immunoblot band intensities.