Cancer Letters 355 (2014) 210–216

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

# Silencing of CDC42 inhibits neuroblastoma cell proliferation and transformation

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ARTICLE INFO

*Article history:*
Received 1 April 2014
Received in revised form 18 August 2014
Accepted 21 August 2014

*Keywords:*
CDC42
AKT2
Twist
N-myc
Transformation
Neuroblastoma

ABSTRACT

Cell division cycle 42 (CDC42), a small GTPase of the Rho-subfamily, regulates diverse cellular functions including proliferation, cytoskeletal rearrangement and even promotes malignant transformation. Here, we found that increased expression of CDC42 correlated with undifferentiated neuroblastoma as compared to a more benign phenotype. CDC42 inhibition decreased cell growth and soft agar colony formation, and increased cell death in BE(2)-C and BE(2)-M17 cell lines, but not in SK-N-AS. In addition, silencing of CDC42 decreased expression of N-myc in BE(2)-C and BE(2)-M17 cells. Our findings suggest that CDC42 may play a role in the regulation of aggressive neuroblastoma behavior.



## Introduction

Neuroblastoma is the most common solid tumor malignancy in infancy and children with two-thirds of patients presenting with distant organ metastasis at the time of diagnosis [1]. More importantly, metastatic disease is associated with 'high-risk' group category and an overall dismal survival of 40% [2,3]. Our ongoing research continues to focus on identifying pathways that promote malignant tumor transformation and progression in neuroblastoma, but much remains to be elucidated. The Rho family of GTPases represents a subgroup of the Ras superfamily of GTPases whose aberrant regulation has been associated with key features of aggressive tumor behavior including increased cell migration, invasion and metastasis [4]. Specifically, Rho GTPases are integral in the regulation of cellular functions such as cell proliferation, survival and migration and cytoskeleton development, which all have important implications in aggressive tumor development [5,6].

CDC42 (cell division cycle 42), a member of the Rho family, is known to contribute to tumorigenesis and cancer progression. Although there are no known activating mutations of CDC42, which result in its proto-oncogenic behavior, it is overexpressed in several different cancers [7]. Specifically, CDC42 has been shown to induce cellular transformation, invasion and metastasis in several tissue types including melanoma, breast and colon cancers [6,8,9]. Furthermore, genetic knockdown of CDC42 results in cell cycle arrest and apoptosis in colorectal cancer [10]. However, the exact role of CDC42 in neuroblastoma tumorigenicity has not been fully elucidated.

Downstream signaling for CDC42 is mediated via many different pathways. One of the effectors of CDC42-induced cell transformation is the PI3K/AKT pathway [11]. It has been well established that AKT2, an isoform of the AKT family, promotes cell survival, invasiveness and metastasis in cancer cells [12]. Similarly, work done in our lab has shown AKT2 is critically important in regulating the metastatic potential of neuroblastoma cells [13]. Another upstream regulator of AKT2 cited in the literature is the transcription factor Twist [14]. Twist is a key regulator of neural crest cell migration during development [15], and its activation has been linked to aggressive adult cancer by promoting AKT2, SNAIL and SLUG to promote cancer initiation and progression [16,17]. Twist is constantly overexpressed in neuroblastoma and inhibits cell apoptosis by cooperating with the oncoprotein N-myc [18]. To date, there is no known established link providing evidence that CDC42 acts in concert with AKT2 or Twist to regulate neuroblastoma tumor progression or transformation. Based on the cited interactions between CDC42/AKT2 and Twist/AKT2, we hypothesized that silencing CDC42 would inhibit neuroblastoma tumorigenesis via the regulation of Twist and AKT2.

### Materials and methods

#### *Materials*

Antibody against CDC42 was purchased from Cytoskeleton, Inc. (Denver, CO) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against AKT2, AKT, survivin,

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http://dx.doi.org/10.1016/j.canlet.2014.08.033
0304-3835/

N-myc, cleaved caspase-3, cleaved PARP and cell lysis buffer were obtained from Cell Signaling Technology (Beverly, MA). Twist antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti β-actin monoclonal antibody and fetal bovine serum (FBS) were from Sigma-Aldrich (St. Louis, MO). NuPAGE Novex 4–12% Bis–Tris Gel and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Santa Clara, CA). Agarose (SeaPlaque®) was from Lonza, Inc. (Allendale, NJ). Cell Death Detection ELISA$^{Plus}$ was purchased from Roche Applied Science (Indianapolis, IN).

*Cell culture and transfection*

Human neuroblastoma cell lines BE(2)-C, BE(2)-M17 and SK-N-AS 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. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% $CO_2$. For transfection, cells were plated in 6-well plates and transfected with plasmids (total of 4 μg) using Lipofectamine 2000 according to the manufacturer's protocol. Plasmid pCDNA3.1 was obtained from Invitrogen (Carlsbad, CA), and pcDNA3-Myr-HA-AKT2 (Addgene plasmid 9016, Cambridge, MA), pCMV6-XL5-Twist, and pCMV6-XL4-N-myc were purchased from OriGene (Rockville, MD). Plasmid pLKO.1-shRNA against CDC42 and its non-targeting control vector SHC002 were purchased from Sigma-Aldrich (St. Louis, MO). For stable transfection, cells were selected with puromycin (2.5 μg/mL). Cells were seeded on 6-well plates for protein preparation and 96-well plates for DNA fragmentation or cell growth assays. The experiments were repeated on at least three separate occasions.

*Western blot analysis*

Whole-cell lysates were prepared using cell lysis buffer with 1 mM PMSF. Total protein (30 μg/lane) was resolved on NuPAGE Novex 4–12% Bis–Tris gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding sites were blocked with 5% milk in TBS-T (120 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 2 h at room temperature or overnight at 4 °C. Target proteins were detected using rabbit or mouse anti-human antibodies (1:500–1000 dilution) for 2 h at room temperature 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 ECL system. Equal loading and transfer were confirmed with β-actin. Data are representative of three independent experiments.

*Reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA was isolated using Trizol and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit according to manufacturer's protocol (Applied Biosystems, Foster City, CA). qRT-PCR was performed using the Bio-Rad Thermocycler CFX96. SsoFAST EvaGreen Supermix, cDNA and specific 3′ and 5′ primers were incubated together using the manufacturer's protocol (Bio-Rad). GAPDH and β-actin were used as internal controls.

*DNA fragmentation assay*

Apoptosis was measured using a DNA fragmentation assay as previously described [19]. Briefly, $5 \times 10^3$ cells per well were plated in triplicate. Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were detected using a Cell Death Detection ELISA$^{plus}$ kit according to manufacturer's recommended protocol. The experiments were repeated on at least three separate occasions.

*Cell proliferation and cell death assay*

Cells were seeded in 96-well plates at a density of $3 \times 10^3$ cells per well in RPMI 1640 culture medium with 10% FBS and grown for up to 4 days. Cell proliferation was assessed using CCK-8 daily. Each assay point was performed in triplicate, and the experiment was repeated three times. The values, corresponding to the number of viable cells, were read at OD450 with the FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA). Cell death induced by CDC42 shRNA transfection was evaluated by trypan blue exclusion assay. Cells were stained with 0.25% trypan blue solution and the percentages of dead cells were determined by TC10$^{TM}$ Automated Cell Counter (Bio-Rad).

*Soft agar colony formation assay*

Using a 12-well plate, cells were trypsinized and resuspended in RPMI 1640 containing 0.4% agarose and 7.5% FBS and then overlaid onto a bottom layer of solidified 0.8% agarose in RPMI 1640 containing 5% FBS at a concentration of $3 \times 10^3$ cells per well and incubated for 3 weeks. Colonies were stained with 0.05% crystal violet dissolved in 70% methanol before being photographed and quantified.

*Cell migration assay*

For Transwell migration assay, polycarbonate Transwell filters (8 μm; Corning, Inc., Corning, NY) were coated on the lower side with 5 μg/mL collagen type I (BD Biosciences) overnight and then blocked with 2.5% BSA in PBS for 1 h. 50,000 cells in 100 μL of serum-free media were added to the Transwell and allowed to migrate for 24 h at 37 °C under tissue culture conditions. Media with 10% FBS was added to the lower chamber. Cells that failed to migrate through the filter after incubation were scraped out using a sterile cotton swab. Cells that migrated to the bottom surface of the filter were fixed with 4% paraformaldehyde, stained with DAPI, and counted. Each substrate was repeated in duplicate wells, and within each well counting was done in five randomly selected microscopic fields (200× magnification).

*Immunohistochemistry*

Vanderbilt University Institutional Review Board reviewed IRB# 091331 (Surgical Studies on the Role on Gastrin-releasing Peptide in Neuroblastoma) and determined the study does not qualify as "human subject" research per §46.102(f)(2). Samples of discarded tissue without identifiable private information were obtained from Surgical Pathology at Vanderbilt University.

Tissues were fixed in formalin for 3 days and embedded in paraffin wax. Paraffin-embedded sections (5 μm) were deparaffinized in three xylene washes followed by a graded alcohol series, antigen retrieval performed with 10 mM sodium citrate buffer, and then blocked with solution for 1 h at RT. They were incubated with primary antibody against CDC42 overnight at 4 °C, washed with PBS, incubated with secondary antibodies for 30 min at RT, and developed with DAB reagent. All sections were counterstained with hematoxylin, and then dehydrated with ethanol and xylene. Coverslips were mounted and slides observed by light microscopy.

*Statistical analysis*

Statistical analyses were performed using Student's paired *t*-test. A $P$ value of $<0.05$ was considered significant. Image J was used for densitometric analysis of immunoblot band intensities.

## Results

### *Constitutive CDC42 expression in human neuroblastoma tissues and cell lines*

We first wanted to determine whether CDC42 expression correlated with malignant phenotype of neuroblastoma. Using paraffin-embedded tumor sections from five undifferentiated neuroblastomas and two ganglioneuromas, a benign phenotype of neuroblastoma, we performed immunohistochemical analysis to assess for CDC42 expression. We observed an increase in CDC42 expression in undifferentiated neuroblastomas when compared to benign ganglioneuromas (Fig. 1A). Next, we also examined the constitutive protein levels of CDC42 in several human neuroblastoma cell lines. Several of the neuroblastoma cell lines demonstrated high CDC42 protein levels (Fig. 1B), with the highest expression found in BE(2)-C. We chose BE(2)-C for further experiments since it overexpresses N-myc and is known to have increased malignant potential and tumorigenic properties [20]. Additionally, we compared BE(2)-M17, another N-myc overexpressing cell line, and SK-N-AS, which does not overexpress N-myc, to better characterize the role of CDC42 in neuroblastoma.

### *Inhibition of CDC42 decreased neuroblastoma cell proliferation and increased cell death*

To determine a functional role for CDC42 in neuroblastoma, BE(2)-C cells were transfected with plasmids containing shRNA specific for CDC42 (shCDC42) as well as a separate group of non-targeted shRNA for control, and stable subclonal populations were cultured. After transfection, there was reduced expression of CDC42 utilizing two separate RNA sequences for silencing (shCDC42 #1, shCDC42 #2) as compared to cells transfected with control vector (shCON) (Fig. 2C). We then focused on identifying the phenotypic