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Mesenchymal change and drug resistance in neuroblastoma

Jessica A. Naiditch, MD,[a,b] *Chunfa Jie, PhD,*[c] *Timothy B. Lautz, MD,*[a,b] *Songtao Yu, PhD,*[b] *Sandra Clark, BS,*[b] *Dimitry Voronov, BS,*[b] *Fei Chu, MD, PhD,*[a,b,*] *and Mary Beth Madonna, MD*[a,b]

[a] *Department of Surgery, Ann & Robert H. Lurie Children's Hospital of Chicago, Northwestern University, Chicago, Illinois*

[b] *Cancer Biology and Epigenomics, Stanley Manne Children's Research Institute, Chicago, Illinois*

[c] *Whole Genome Core Facility, Northwestern University, Chicago, Illinois*

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ABSTRACT

Background: Metastatic initiation has many phenotypic similarities to epithelial-to-mesenchymal transition, including loss of cell–cell adhesion, increased invasiveness, and increased cell mobility. We have previously demonstrated that drug resistance is associated with a metastatic phenotype in neuroblastoma (NB). The purpose of this project was to determine if the development of doxorubicin resistance is associated with characteristics of mesenchymal change in human NB cells.

Materials and methods: Total RNA was isolated from wild type (WT) and doxorubicin-resistant (DoxR) human NB cell lines (SK-N-SH and SK-N-BE(2)C) and analyzed using the Illumina Human HT-12 version 4 Expression BeadChip. Differentially expressed genes (DEGs) were identified. Volcano plots and heat maps were generated. Genes of interest with a fold change in expression >1.5 and an adjusted $P < 0.1$ were analyzed. Immunofluorescence (IF) and Western blot analysis confirmed microarray results of interest. Matrigel invasion assay and migration wounding assays were performed.

Results: Volcano plots and heat maps visually demonstrated a similar pattern of DEGs in the SK-N-SH and SK-N-BE(2)C DoxR cell lines relative to their parental WT lines. Venn diagramming revealed 1594 DEGs common to both DoxR cell lines relative to their parental cell lines. Network analysis pointed to several significantly upregulated epithelial-to-mesenchymal transition pathways, through TGF-beta pathways via RhoA, PI3K, and ILK and via SMADs, as well as via notch signaling pathways. DoxR cell lines displayed a more invasive phenotype than respective WT cell lines.

Conclusions: Human SK-N-SH and SK-N-BE(2)C NB cells display characteristics of mesenchymal change via multiple pathways in the transition to a drug-resistant state.

* *Corresponding author*. Cancer Biology and Epigenomics, Stanley Manne Children's Research Institute, Ann & Robert H. Lurie Children's Hospital of Chicago, Northwestern University, 225 East Chicago Ave, Box 224, Chicago, IL 60611. Tel.: +1 773 755 6519; fax: +1 773 755 6518.

E-mail address: fchu@luriechildrens.org (F. Chu).

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1. Introduction

Neuroblastoma (NB) is the most common extracranial solid malignancy in infancy and childhood, accounting for 6%–10% of childhood cancers and 15% of cancer deaths in children [1]. The disproportionate rate of cancer death from NB is attributable to the many patients who present with late stage widely disseminated NB, carrying a poor prognosis of only 20%–35% survival despite aggressive therapy [1,2]. The majority of these late stage patients succumb to their metastatic disease due to inherent or acquired drug resistance, enabling tumor aggressiveness with consequent NB progression.

There are a number of mechanisms through which drug resistance arises including upregulation of drug efflux pumps [3], expression of cytoprotective molecules [4], and decreased susceptibility to apoptosis [5–7]. Although 50% of NB patients present with metastatic disease, little is known about invasion and metastases in NB [1]. There is a paucity of data accounting for the development of metastases and its relationship to the acquisition of drug resistance. Previous work has suggested NB's metastatic potential arises from those innate to the neuroectodermal origin of NB, apparent during the formation and delamination of the embryonic neural crest [8]. The role of mesenchymal transition and its relation to the acquisition of drug resistance has not previously been examined.

Epithelial-to-mesenchymal transition (EMT) is a process of transdifferentiation whereby epithelial cells lose their cell–cell contacts and apical–basal polarity, acquiring mesenchymal characteristics with a fibroblastic migratory phenotype. EMT plays an important role in human development, key in gastrulation and migration of neural crest cells [9]. EMT has also been implicated as a pathologic mechanism whereby carcinomas acquire migratory and invasive capabilities leading to disseminated disease [10,11]. A similar phenotypic transition has been described in glioblastoma whereby tumor cells of neurogenic origin undergo transition to a mesenchymal phenotype described as mesenchymal change (MC) [12–15].

We found that SK-N-Be(2)C and SK-N-SH NB cells with acquired doxorubicin resistance have greater invasive and migratory characteristics relative to their wild type (WT) parental cell line. The simultaneous acquisition of drug resistance and increased migratory and/or invasive phenotype led us to hypothesize that NB cells undergo an MC during prolonged exposure to chemotherapeutics. The present study explores this hypothesis through whole genome microarray analysis of DoxR cell lines and their parental WT cell lines with the goal of identifying differentially expressed genes (DEGs) and enriched biological pathways related to MC/EMT.

2. Materials and methods

2.1. Cell lines and reagents

Human NB SK-N-Be(2)C (male) and SK-N-SH (female) cell lines were purchased from American Type Culture Collection (Rockville, MA). SK-N-SH cells were grown at 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Mediatech), penicillin 100 U/mL, and streptomycin 100 μg/mL. SK-N-Be(2)C cells were cultured in DMEM/F12 (Hyclone Laboratories, Logan, Utah) 1:1 with the same supplements as those listed previously for SK-N-SH cells. DoxR cell lines were generated by incubating the parental cells with stepwise concentrations of doxorubicin (Sigma, St. Louis, MO), beginning 2 Log below the IC_{50}. Cells were deemed resistant after surviving five passages in a concentration of drug approximately 2 Log above the IC_{50}. The time course for this process occurred over 6–12 months and varied based on cell line doubling time. Doxorubicin resistance and doubling time were confirmed by 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide calorimetric (MTT) assay as previously described [16].

2.2. Invasion and migration assay

BD BioCoat growth factor reduced Matrigel invasion chambers (BD Bioscience, Bedford, MA) were used to assess the invasive properties of the cells. Assays were performed according to the manufacturer's instructions. In brief, Matrigel inserts and control inserts (no Matrigel) were rehydrated with serum-free culture medial at 37°C at 5% CO_2 for 2 h. Inserts were then moved to a 24-well plate with 0.75 mL of 10% FBS media in the well. The serum-free media were removed from the inserts and 2.5×10^5 WT or DoxR cells suspended in serum-free media were seeded on the apical side of the insert. Plates were incubated 37°C at 5% CO_2 for 22 h. Matrigel inserts were then wiped with a cotton tipped swab to remove any noninvasive cells and stained using Diff-Quick. Inserts were air dried and placed on coverslips for microscopic imaging and cell counting in 12 high powered fields at 20×. Invasion percentage was calculated as the ratio of the mean number of invasive cells in the Matrigel compared with the mean number of adherent cells on the control insert × 100%. Assays were completed in triplicate for each cell line. Each cell count was independently confirmed by two laboratory members.

2.3. Scratch assay

For the scratch wound assay, six-well collagen-coated plates were preemptively scratched horizontally with a diamond-tipped sterilized probe to create defects in the collagen coating. Cells were seeded onto these plates at 250,000 cells per well and allowed to adhere overnight in the presence of 10% FBS. The "wound" was generated by scratching each well with a sterile glass–tipped pipette vertically. Images were taken from each well immediately after wounding and at 8 h. Wound areas in pixels over a fixed vertical length were analyzed using Adobe Photoshop CS6 (Adobe Systems Inc, San Jose, CA). The change in cleared area was averaged more than three iterations. SK-N-Be(2)C cells are not able to grow to confluence and as such were not amenable to this assay.