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Descriptive analysis of tumor cells with stem like phenotypes in metastatic and benign adrenal tumors

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ABSTRACT

Background: Neuroblastoma (NB) comprises 7% of all childhood cancers. Here we report a descriptive analysis of key cellular markers that have "stem-like" properties which theoretically represents the self-renewing population of cells responsible for generating new tumor cells. Samples are obtained from freshly isolated tissue from nonmetastatic NB, metastatic NB, benign adrenal adenoma and a ganglioneuroma. In addition, in metastatic NB, descriptive analysis of the tumor cells after 3D culture as well as reanalysis of fresh tumor obtained after surgical excision posttreatment was performed.

Methods: Cells were isolated from primary tissue and characterized via immunohistochemistry and flow cytometry for markers associated with stem-like properties. In two patients, reanalysis was performed in freshly isolated tissue after chemotherapy. In three patients, freshly isolated tumors were cultured in 3 dimensions for 7–10 days and changes in stem-like marker expression were characterized.

Results: Flow analysis of metastatic NB revealed elevated levels of markers CD133, CD24, CD44, Oct4, CXCR4 and Nestin. In addition, some markers such as CD133 and CXCR4 maintained increased expression after chemotherapy.

Conclusions: The expression profile of cells with "stem-like" properties has individual variability and differs depending on the tumor type. In metastatic NB, expression of "stem-like" markers Nestin, Oct4, and CXCR4 are maintained in a higher percentage of cells and this persists even after chemotherapy. In addition, culture of freshly isolated tissue maintained the individual expression profile of stem-like markers for at least 7 days.

There are more than 12,000 children diagnosed with cancer each year, making cancer the leading cause of nontraumatic deaths among children [1]. Despite significant advances in the treatment of pediatric malignancies, overall survival rate for certain tumors, such as high grade neuroblastoma (NB), remains poor [1]. In general, cancer arises from an aberrant proliferation of cells within normal tissues. Replacement of the mature cells in these tissues is accomplished by a highly orchestrated process in which a relatively small population of self-renewing adult stem cells gives rise to progenitor cells. These progenitor cells undergo limited rounds of mitotic division prior to terminal differentiation [2]. Recent literature has documented isolation and characterization of cancer cells with stem-like phenotypes in tumors such as multiple myeloma, neuroblastoma, medulloblastoma, and glioblastoma [3–5]. In addition, inhibitors and other pharmacologic agents have been described for use in reducing these cell populations [6], lending credence to new treatment options based on reducing these cell populations [7,8]. Determining the expression profile of cells with "stem-like" phenotypes in individual tumors may lend itself to novel targets and personalized medicine.

In NB, the aggressiveness of the population of cells with stem-like properties is associated with increased self-renewal [9]. A microarray time course analysis from metastatic NB cells shows an up-regulation of CD133, ABC transporter, WNT and NOTCH genes [10]. CD133 is believed to play a role in membrane protrusions, like microvilli, and is expressed by hematopoietic stem cells, neural stem cells and other progenitor cells [11,12]. In addition it has been described that CD133 + cells upregulate antiapoptotic factors, which could explain their resistance to current treatments [12]. Other studies utilize a combination of markers such as CD133 and Nestin; or CD24, CD44 and CD166 in combination with CD133 to identify cells with "stem-like" populations [5,9,13–18]. Based on these studies, it is evident that detailed characterization of the expression profile of stem-like markers in freshly isolated patient specific NB may enhance the understanding of the tumor biology. Herein we compare the expression profiles of markers associated with stem-like properties in freshly isolated tumors of 2 metastatic NB patients, 2 nonmetastatic NB patients, a benign adrenal adenoma and a ganglioneuroma. In addition, we characterized the profile of these markers over time in a nonadherent 3 dimensional culture system in an effort to maintain cell phenotype *in vitro* for downstream drug testing applications. Finally, in the two metastatic NB patients, we were able to compare the expression of key markers associated with stem-like properties before and after treatment.

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1. Materials and methods

1.1. Isolation of NB primary cells

Tumor tissue was obtained from patients diagnosed at the Connecticut's Children Medical Center after informed consent and in agreement with institutional ethical regulations (CCMC IRB# 13-035). A summary of patient demographics is presented in Table 1. Tumor tissue was processed within 2 hours of resection or biopsy into a single-cell suspension by enzymatic and mechanical dissociation utilizing the Miltenyi Neural Tissue Dissociation Kit and MACS Dissociator System according to manufacturer's instructions (Miltenyi Biotech, Cambridge, MA).

1.2. Histology and immunofluorescence analysis

Fresh frozen tissue was embedded in optimal cutting temperature compound (OCT) (Fisher Scientific, Pittsburgh PA), sectioned at 5 μm thickness, and fixed in 50% methanol/50% acetone for 10 minutes. Sections were then stained with hematoxylin and eosin. For immunofluorescence, tissue sections were permeabilized and blocked in 1× PBS with 0.1% Tween, 0.5% Triton X-100 and 2% FBS for 1 hour at room temperature. Primary antibody staining for Nestin (eBioscience, San Diego, CA), CD133 (Biorbyt San Francisco, CA), Ki-67 (BD Bioscience San Jose, CA) and cleaved caspase 3 (Cell Signaling Beverly, MA) was performed at 4 °C overnight. Secondary antibodies used were of specific isotypes conjugated to Alexa 546 (Life Technologies, Grand Island, NY). Tissue was counterstained with DAPI to visualize cell nuclei. Tissue sections were imaged on a Zeiss Observer Z1 Inverted Microscope utilizing Carl Zeiss ZEN Blue Software (Carl Zeiss Microimaging, LLC Thornwood, NY). All images were processed using Carl Zeiss ZEN Blue Software (Carl Zeiss Microimaging, LLC Thornwood, NY).

1.3. Flow cytometry characterization of isolated primary NB cells

Following dissociation, viability was determined using Fixable Viability Dye e450 (eBioscience, San Diego, CA) according to the manufacturer's protocol. Surface marker staining was performed by incubating live cells at 4 °C for 30 minutes with directly conjugated primary antibodies diluted according to the manufacturer's instruction. Unstained cells served as a negative control. Cells were rinsed in PBS and fixed in 4% PFA for 10 minutes. Cells were then permeabilized for 30 minutes in a PBS solution containing 2% FBS and 0.1% Saponin. Following permeabilization, directly conjugated antibodies for intracellular and nuclear markers were incubated at 4 °C for 30 minutes. Cells were finally rinsed and analyzed on an LSRII Flow Cytometer (BD Biosciences, San Jose, CA). The data were analyzed using FlowJo software (TreeStar Inc., Ashland, OH). Antibodies used were as follows: stem like markers CD114 eFluor 710 (eBioscience, San Diego, CA), CD24 FITC (eBioscience, San Diego, CA), CD44 APC (eBioscience, San Diego, CA), CD133 PE (Miltenyi Biotec, San Diego, CA), Nestin A488 (eBioscience, San Diego, CA) and pluripotency markers OCT 3/4 eFluor660 (eBioscience, San Diego, CA) and CXCR4 A488 (R&D Systems, Minneapolis, MN). Flow cytometry data were graphed using GraphPad Prism Software (GraphPad Software, La Jolla, WA). A heat map was generated using the LOG function in Microsoft Excel (Microsoft Inc, Redmond, WA) in order to outline positive or negative changes in expression levels. Patients B, C, D, E and F were normalized to the values of patient A. In addition, another heat map was generated in the same manner in which patients A, B, C, D and E were normalized to the values of patient F. Statistical analysis could not be performed on the data collected from these patient samples since only 1–2 patients were analyzed for each category of tumor.

1.4. Culturing patient cells in nonadherent condition

Following dissociation, tumor from patients A, B, and C were isolated and approximately 1×10^5–5×10^5 live cells were cultured in each well of a six well low attachment culture plate (Corning Tewksbury, MA). Medium was composed of DMEM/F12 (Life Technologies, Grand Island, NY), 3 mM L-glutamine (Life Technologies, Grand Island, NY), 1× Primocin (InVivoGen, San Diego, CA), and 2× StemPro Neural Supplement (Life Technologies, Grand Island, NY). Cells were cultured in normal tissue culture incubators at 21% oxygen and 5% CO_2 for 7–10 days. At the time of harvest cells were stained and analyzed by flow cytometry as previously described.

2. Results

Descriptive analysis of markers associated with “stem-like” tumor characteristics was performed in four pediatric cancer patients with neuroblastoma, 2 patients had metastatic NB and 2 were nonmetastatic. In addition, we compared the NB patient profiles with a patient with a benign adrenal adenoma and a patient with a ganglioneuroma (Table 1). Heat maps illustrating relative expression of markers in NB patients B, C, D and E were calculated using either patient A or patient F. Patient A served as a nonneural benign tumor control (adrenal adenoma) and patient F served as a neural-based benign tumor (ganglioneuroma). The two colorimetric heat maps generated are depicted in Table 2. Values highlighted in shades of green demonstrate a positive relative fold change in expression, while values in shades of red demonstrate a negative fold change in expression. Those values highlighted in yellow demonstrate little to no change in expression. When comparing NB expression patterns against patient A (nonneural benign adrenal adenoma) Nestin, CD114, and CXCR4 were minimally upregulated in metastatic NB (patients B and C). In addition CD133, CD114 and CD133/Nestin were upregulated in the nonmetastatic NB patients (patients D and E). In comparing a benign ganglioneuroma (patient F) against a benign adrenal adenoma (patient A) there was decreased expression of CD133, Nestin, CD114 and CD133/Nestin. When comparing NB expression patterns against patient F (neural benign ganglioneuroma) CXCR4, CD24/CD44, CD44/CD133 and CXCR4/OCT4 populations were upregulated in metastatic NB (patients B and C) compared to nonmetastatic NB, which did not contain any markers that were consistently upregulated.

Table 1
Summary of case and patient information.

Patient	Age/gender	Biopsy site	Pathology	N-myc amp	Urine VMA (normal 1.7–6.5 mg/g creat)	Urine HVA (normal 2.1–23 mg/g creat)
A	9 y/F	Right adrenal gland	Adrenal cortical adenoma	N/A	N/A	N/A
B	5 y/M	Cervical Lymph node	Metastatic NB, poorly differentiated, intermediate MKI, unfavorable histology	No	77.5	70.8
C	5 y/F	Cervical Lymph node	Metastatic NB, poorly differentiated, intermediate MKI, unfavorable histology	No	38.9	21.6
D	1 mo/M	Right adrenal gland	Nonmetastatic NB, poorly differentiated, low MKI, favorable histology	No	39.5	105.9
E	3 mo/M	Chest	Nonmetastatic NB, poorly differentiated, low MKI, favorable histology	No	26.8	21.4
F	4 y/F	Chest	Ganglioneuroma; favorable histology	N/A	7.4	16.3