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Characterization of stem-like cells in a new astroblastoma cell line

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ABSTRACT

Cell lines established from tumors are the most commonly used models in cancer research, and their use in recent years has enabled a greater understanding of the biology of cancer and the means to develop effective treatment strategies. Astroblastomas are uncommon neuroepithelial tumors of glial origin, predominantly affecting young people, mainly teenagers and children, predominantly females. To date, only a single study has reported that astroblastomas contain a large number of neural stem-like cells, which had only a partial proliferation capacity and differentiation. Our objective was to establish an astroblastoma cell line to investigate the presence of astroblastic cells and cancer stem-like cells. The migratory and invasion abilities of the cells were quantified with invasion and migration assays and compared to a glioblastoma cell line. The presence of stem cells was detected with surface-marker analysis by using flow cytometry, and measuring the differentiation ability with a differentiation assay and the self-renewal capacity with a sphere-forming assay. These characteristics may determine whether this novel cell line is a model for astroblastomas that may have stem-cell characteristics. With this novel cell line, scientists can investigate the molecular pathways underlying astroblastomas and develop new therapeutic strategies for patients with these tumors.

1. Introduction

Astroblastomas are uncommon neuroepithelial tumors of glial origin. They are generally observed as large and peripheral supratentorial tumors with a “bubbly” appearance. Brat and associates showed that all of 20 astroblastomas they studied were categorized histologically by astroblastic pseudorosettes, and most displayed noticeable perivascular hyalinization (glassy appearance), regional hyaline changes, and aggressive borders in regard to neighboring brain tissue [1]. Tumor cells were found to be highly immunoreactive: positive for S-100, fibrillary acidic protein (GFAP), and vimentin. Half of the cases were considered to be "well differentiated" and others "malignant," because of increased mitotic indices, vascular growth, and necrosis.

Astroblastomas predominantly affect young people, mainly teenagers and children, with a high female affinity [2]. In contrast to Fu and colleagues [3], a recent study done by Ahmed and associates [4] shows that patients of different ages might have dissimilar prognoses because of genetic variations between young and elderly people. Since the definition of this tumor type by Bailey and Cushing in 1924, rare cases have been reported that cause difficulty in terms of diagnosis and typing among other brain tumors [5].

On the basis of their recurrence rate, anaplastic astroblastomas can be divided into three subtypes: low-grade, well-differentiated, and high-grade [6]. Patients with a low-grade astroblastoma do not usually have symptoms of the tumor after surgery, but may have a recurrence in 1–2 years. At this point, the size of the tumor determines the follow-up treatment, [7,8] which most of the time does not require another operation, and other treatment methods remain sufficient. These patients usually do not go through strict treatment regimens [9,10]. One report describes a low-grade group with infiltration into brain parenchyma and high mitotic activity and yet no necrosis [11].

The survival rate for patients with high-grade astroblastomas is quite high, although a number of patients with these tumors died due to the recurrence [12]. The inefficacy of treatment options has led researchers to seek alternative methods such as targeting specific cells that may be responsible for the malignancy of astroblastomas.

Cancer comprises heterogeneous cell populations, and specifically includes a small group involved in self-renewal and drug resistance that recapitulate the original tumor [13–15]. Based on their similarity to the cancer cells, these subgroups of cells are called cancer stem cells. The

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cancer stem-cell phenomenon has two distinct but related components. The first component involves the origin of tumors at a cellular level, and researchers question whether tumors result from tissue stem cells. The second component is that tumors originate from cellular factors that show "stem cell properties." To date, only a single study has reported that astroblastomas contain a high number of neural stem-like cells which have only a partial proliferation capacity and differentiation [16]. The authors concluded that the functional role of this stem-like characteristic is currently unknown. This conclusion inspired us to investigate the possible roles of these stem-like cells in the development, progression and chemotherapy resistance of astroblastomas. In this study, we aimed to define the characteristics of this novel cell line and investigate the possible existence of stem-like cells among these very heterogeneous cell populations.

2. Materials and methods

2.1. Patient history

A 24-year old male presented with headache and nausea. Magnetic resonance imaging showed a contrast-enhancing lesion in the left frontal lobe, and the patient underwent gross total resection. The pathological report defined a glial tumor with some aspects of astroblastoma, positive for GFAP and S-100, negative for epithelial membrane antigen (EMA) and P53, and with a Ki-67 index of 2–3%. Microscopic analysis showed few mitotic figures although the labeling index was high, as in an aggressive tumor. Once discharged, the patient was not re-admitted to the hospital for any reason, and died 5 years after surgery of an unknown cause.

2.2. Specimen collection and maintenance of a tumor cell line

The tissue sample was obtained in accordance with the approved ethical standards of the institutional review board of Yeditepe University Hospital. The tissue sample was lysed through mincing with a scalpel into 1-mm pieces. The cells were allowed to adhere to the bottom of tissue flasks and grow. They were then placed in culturing media, Dulbecco's Modified Eagle's Medium, (DMEM, #41966-029, Gibco, Invitrogen, ThermoFisher Scientific), supplemented with 10% fetal bovine serum (FBS, #10500-064, Invitrogen) and 1% penicillin/streptomycin/amphotericin (PSA, Invitrogen) in a humidified chamber at 37 °C and 5% CO_2. The name of this cell line – HERK – is a combination of the first, middle, and surnames of the patient.

2.3. Proliferation rate assay

Cells were seeded onto three 96-well plates (each plate defining 1 day) as 1000 cells per well in six (replicate) wells and incubated for 3 days. The proliferation rate was determined via a colorimetric 3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay (#G3582, CellTiter 96 Aqueous One Solution, Promega, Southampton, UK) for three days. Based on each absorbance value and driven by calculations, a diagram graph was created daily. The number of cells in each well was calculated based on the formula driven by the graph. The mean value and standard deviations were determined, and the doubling time for this specific cell type was calculated accordingly.

2.4. Short tandem repeat assay

A short tandem repeat assay, which determines the genetic identification for a person based on a quantifying number of repeats found in the human genome, was done according to the protocol described in AmpFlSTR® Identifiler® PCR Amplification (#4322288, Applied Biosystems, ThermoFisher Scientific).

2.5. Array comparative genomic hybridization (Array CGH)

For array CGH, the CytoScan® Optima Kit (#902533, Affymetrix, Santa Clara, CA) was used.

2.6. Invasion assay

All cells were seeded as 200,000 cells/well onto 8-μm porous chambers coated with growth-factor reduced Matrigel (#356230, Corning, NY). Control inserts and the protocol were performed as described. Cells were stained with Giemsa (CytoSelect™ 24-Well Cell Migration and Invasion Assay, CBA-100-C, Cell Biolabs, San Diego, CA), counted with ImageJ, and compared with the control group.

2.7. Migration assay (wound healing)

A wound healing assay was done to measure the closure of a scratch and the migration rate of the cells according to the protocol described by Liang and associates [17]. Cells were incubated for 2 days and images were taken on each day under inverted microscopy. Closure was measured, then quantified with ImageJ, and comparisons were made.

2.8. Immunocytochemistry

Astroblastoma cells are known to have certain diagnostic markers including GFAP, vimentin, nestin, neuroD, and beta-III tubulin. With immunocytochemistry, these markers were detected on HERK cells.

Cells were seeded onto poly-L-Lysine (PLL, P8920, Sigma-Aldrich, USA) coated microscope slides and incubated in a humidified chamber at 37 °C, with 5% CO_2, and 18% O_2. Cells were washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde (P6148, Sigma-Aldrich) for half an hour at 4 °C. Cells were then washed with PBS and permeabilized with 0.1% Triton-X 100 (X100, Sigma-Aldrich) for half an hour at room temperature. This procedure was followed by blocking with 10% Goat Serum (GS, 31872, ThermoFisher Scientific) for 1 h, primary antibodies (in 3% Goat Serum) at 4 °C overnight, and a secondary antibody solution in the same buffer as used for the primary antibody with phalloidin (A22287, ThermoFisher Scientific) (staining actin filaments), at a concentration of 1/1000. DAPI (5 μg/mL) was used for nuclear staining and slides were viewed under a confocal microscope. Images were taken at 20 X magnification.

2.9. Characterization assay with flow cytometry

The choice of stem cell markers used to distinguish stem cells based on surface marker labeling and detection by flow cytometry was based on the previous studies [18,19]. With flow cytometric analysis, stem-cell markers were detected on antibody-labeled HERK cells. Cells were trypsinized, washed with PBS, and fixed with 2% paraformaldehyde for half an hour at 4 °C. This procedure was followed by permeabilization with 0.1% Triton-X 100 for half an hour at room temperature and incubation with selected antibodies [CD90 (#ab95700, Abcam, Cambridge, MA), CD73 (#ab157335, Abcam), CD34 (#ab18227, Abcam), CD14 (#ab82434, Abcam), CD105 (#ab53321, Abcam), CD31 (#ab27333, Abcam), CD44 (#ab58754, Abcam), CD29 (#ab27314, Abcam), CD45 (#ab134202, Abcam)], and integrin beta at a concentration of 10 μL/10^6 cells at 4 °C overnight. Cells were then re-suspended in 500 μL PBS for analysis with flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA).

2.10. Differentiation assays and gene-expression analyses

HERK cells were exposed to osteogenic (ODM) and chondrogenic (CDM) differentiation [20] and neurogenic differentiation media (NDM) for 21 days, as described in previous studies. Cells were stained with alizarin red to visualize calcium deposits and alcian blue for