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The value of cell cycle analysis by propidium-iodine staining of CD56+ cells in pediatric brain tumors

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ABSTRACT

Purpose: Cell cycle analysis by flow cytometry has not been adequately studied in pediatric brain tumors. We investigated the value of cell cycle analysis by propidium-iodine (PI) staining of CD56+ (gated) cells for the characterization of pediatric brain tumor's malignancy.
Methods: Patients that underwent surgery for an intracranial lesion and tissue sample was available were included in the study. All tumor samples were histopathologically verified before flow cytometric analysis.
Results: There were 46 pediatric brain tumor cases. As control we used samples from three cases of brain tissue obtained during surgery for epilepsy. All tumors had significant lower G_0/G_1 and significant higher S-phase, G_2/M fraction, $S+G_2/M$ and $S+G_2/M/G_0/G_1$-phase fraction than normal brain tissue. Low-grade tumors had significant lower S-phase than high grade tumors. Grade IV tumors had significant lower G_0/G_1 fraction and higher $S+G_2/M/G_0/G_1$ index than grade III tumors. DNA diploidy was detected in 24 tumors and DNA aneuploidy was detected in 23 tumors. There was a significant correlation between Ki-67 index and both $S+G_2/M$ and $S+G_2/M/G_0/G_1$ phase fraction.
Conclusions: Cell cycle analysis by PI staining of CD56+ cells is a promising method for the assessment of pediatric brain tumors malignancy and could be a valuable adjunct to histopathological evaluation.

1. Introduction

Brain tumors constitute the most common solid tumors in childhood and the second most common cancer after leukemia. Astrocytomas are the most frequent, followed by medulloblastomas, the most aggressive tumor, and ependymomas [1]. Flow cytometry is the method of choice for the evaluation of hematologic malignancies [2]. Until now, the study of cells derived from solid tissue has been rather limited, although protocols exist for disaggregating solid tumors to single cell suspensions. The analysis of cell cycle distribution of neoplastic cells has been consistently used and in adult brain tumors, cell cycle analysis provided important information for the differentiation of low from high-grade tumors and for the assessment of glioma prognosis [3]. Furthermore, using a rapid cell-cycle analysis protocol, intraoperative identification of tumor margins was made possible [4,5]. In pediatric brain tumor few studies on cell cycle analysis have been conducted to date [6–9].

An important issue when solid tumors are subjected to flow cytometric analysis pertains the quality of sample itself. Flow cytometry requires monodispersed cell suspensions. Although histopathologically verified tumor tissue can be used, the specimen might contain tissue elements that are not target for analysis, such as cell debris. Thus, a method to select the targeted cells would be ideal. The neural cell adhesion molecule (NCAM), also known as CD56, is involved in the intercellular junctions of neurons and glial cells and is also expressed on the surface of a subset of lymphocytes, the natural killer cells [10]. Furthermore, several brain tumors express CD56 such as gliomas, medulloblastomas and ependymomas [10,11]. Herewith, we investigated the value of cell

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cycle analysis by propidium-iodine (PI) staining of CD56+ (gated) cells in a panel of pediatric brain tumors, with emphasis on the characterization of tumor's malignancy.

2. Materials and methods

Forty-six brain tumor specimens from patients treated at our institute were obtained and graded using the World Health Organization's 2007 criteria. All samples were preserved in 2 ml of RNA later solution until flow cytometric analysis. Seventeen specimens classified as medulloblastoma (MB) (11 classical, 3 large cell and 3 desmoplastic/nodular), 12 classified as anaplastic ependymomas, 9 classified as atypical teratoid/rhabdoid tumors (AT/RT), 2 primitive neuroectodermal tumors (PNET), 1 glioblastoma, 2 low-grade astrocytomas, 1 atypical meningioma and 2 classified as atypical papillomas were used for this study (Table 1). As controls we used samples from three cases of brain tissue obtained during surgery for epilepsy. The histopathological analysis was performed in Children's Hospital "Agia Sofia". Regarding flow cytometry analysis, the tumors were classified as low-grade (WHO grade I and II) and high-grade (WHO grade III and IV). There were 27 males and 19 females. Patient ages ranged from 6 months to 14 years (mean age 6.2 ± 4.1 years). The study had the approval of our Institutional Review Board and was in accordance with the principles of the Declaration of Helsinki.

2.1. Immunohistochemical analysis

Immunohistochemical staining of Ki-67 was performed using the Bond Max Autostainer (Leica Microsystems, Illinois, USA). The immunohistochemical expression of Ki-67 was evaluated by two independent experienced pathologists. Results were expressed as the percentage of positive tumor cells out of the total number of counted cells (approximately 3000 counted cells) in the highest density of stained areas. All cells with staining of any intensity were considered positive, irrespectively of staining intensity. Any discrepancy between the two pathologists was solved by consensus.

2.2. DNA analysis protocol

Tumor samples (0.5–2 mm^2) are minced (Medimachine System, BD Bioscience) for 1 min in PBS buffer (Ca^{2+} and Mg^{2+} free, with 0.5 mg/ml RNase) and a cell suspension is obtained. The suspension is then filtered (Consult No. 10, Medicons, BD Bioscience) and cells are counted using an automated hematology analyzer to a final concentration of 1.0×10^6 cells/ml. A CD56/propidium iodide double staining technique was performed: 100 μl suspension of cells, labeling of surface antigens CD56 recognizes an extracellular immunoglobulin-like domain common to three molecular weight forms – 20, 140, and 180 kDa – of the neural cell adhesion molecule NCAM, (Becton-Dickinson). Twenty μl of CD56 FITC antibody and 50 μl of PI (125 μg/ml) were incubated for 15 min at room temperature (in the dark) and then flow cytometric analysis was performed. All the stained samples were analyzed within 1 h on a FACSCalibur (Becton-Dickinson) flow cytometer, equipped with 2 lasers (488 nm, 635 nm) and 6 parameters (FSC, SSC, FL1–FL4) and using CellQuest software (Becton-Dickinson), for at least 10,000 cells/sample. Chicken red blood cells and normal brain tissue were used as the standard to define the position of the diploid G_0/G_1 peak in the DNA histograms. Fluorescence compensation between FITC and PI was established using a mixture of PI stained chicken erythrocyte nuclei and FITC-beads (CALIBRITE beads, Becton-Dickinson). The percentage of CD56 positive cells was calculated after excluding cell doublets on a FL, Area/FL, Wight dot plot using the paint "a gate" plus software. The DNA index was calculated as the ratio between the modal channel of the G_0/G_1 peak of the aneuploid cells (tumor cells) and that of the diploid cells. We examined the role of G_0/G_1, S-phase, G_2/M, $S+G_2/M$-phase fraction and of proliferation index ($S+G_2/M/G_0/G_1$) as biological markers of tumor's aggressiveness.

Table 1
Detailed patients data.

Age, years (mean ± SD)	6.2 ± 4.1
Gender	
Male	27 (58.7%)
Female	19 (41.3%)
Histology	
Medulloblastoma	17 (37%)
Anaplastic ependymoma	12 (26.2%)
AT/RT	9 (19.6%)
PNET	2 (4.3%)
Low-grade astrocytoma	2 (4.3%)
Atypical papillomas	2 (4.3%)
Others	2 (4.3%)

Abbreviations: AT/RT, atypical teratoid/rhabdoid tumor; PNET, primitive neuroectodermal tumor.

2.3. Statistical analysis

We used the Mann–Whitney *U* test to compare the G_0/G_1, S-phase, G_2/M, $S+G_2/M$-phase fraction and the proliferation index ($S+G_2/M/G_0/G_1$) of the low-grade vs. high-grade tumors and tissue from neoplastic and non-neoplastic samples. Receiver operating characteristic (ROC) analysis was used to define the threshold value most efficiently discriminating neoplastic from non-neoplastic tissue and low-grade from high-grade tumors. Correlation among G_0/G_1, S-phase, G_2/M, $S+G_2/M$-phase fraction, proliferation index and Ki-67 was analyzed statistically using Pearson's correlation coefficient. Continuous data are expressed as mean ± standard deviation. The level of significance was defined as a probability value less than 0.05.

3. Results

All tumors had significant lower G_0/G_1 (73.1 ± 19.4% vs 96.5 ± 0.7%, $P = 0.017$) and significant higher S-phase (11.1 ± 11.5% vs 1.5 ± 0.7%, $P = 0.02$), G_2/M fraction (15.2 ± 15.5% vs 1.5 ± 0.5%, $P = 0.017$), $S+G_2/M$-phase fraction (25.8 ± 18.8% vs 1.5 ± 0 7%, $P = 0.017$) and proliferation index (0.75 ± 2.1% vs 0.03 ± 0.007%, $P = 0.017$) than normal brain tissue. Using ROC curve analysis no neoplastic lesion had more than 91% G_0/G_1 phase fraction (100% sensitivity, 100% specificity) and G_2/M fraction lower of 2% (100% sensitivity, 100% specificity). For the S-phase fraction a value of

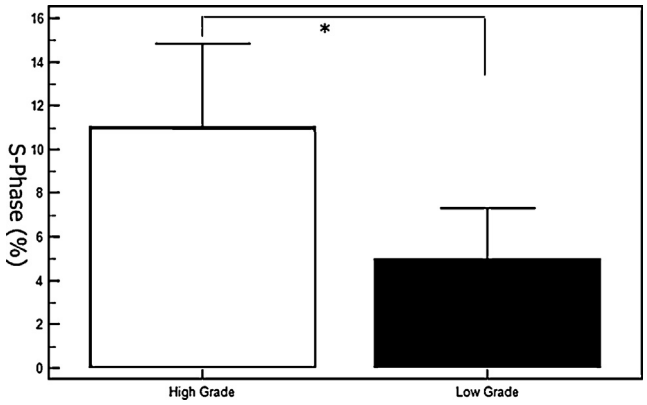

Fig. 1. The S-phase fraction was higher in high-grade than low-grade tumors. The data were represented as the mean ± standard deviation (SD). *$P < 0.05$. Data calculated by Mann–Whitney *U* test.