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CONTENT OF STEM TUMOR CD133$^+$ CELLS IN BRAIN NEOPLASMS OF DIFFERENT HISTOLOGICAL TYPE

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Today, there are conflicting data on the content of cancer stem cells responsible for recurrence and resistance to chemotherapy in tumors of human brain. The *aim* of the study was to analyze the content of CD133$^+$ cells in different brain tumors by immunofluorescence assay and immunohistochemical method. *Materials and Methods*: The samples of different brain tumors removed during neurosurgical operations were studied for CD133 expression. *Results*: Immunofluorescence assay of tumor imprints revealed CD133$^+$ cells in 40–85% of tumors regardless of histological type. In malignant tumors, the count of CD133$^+$ cells was higher than in benign tumors. Immunohistochemical method used for detection of CD133$^+$ cells was less sensitive than immunofluorescence technique. The number of CD133$^+$ cells may vary even in tumors of the same histological type. In 20–30% of malignant tumors (glioblastomas, medulloblastomas), the content of CD133$^+$ cells was very low or not detected at all. *Conclusions*: In tumors of the brain of different genesis and degree of anaplasia CD133$^+$ cells are found out. In malignant tumors (glioblastomas and medulloblastomas), CD133$^+$ cells are much more frequently detected than in benign brain tumors. The content of CD133$^+$ cells in brain tumors is highly variable being small and some malignant tumors, indicating low predictive and diagnostic value of cancer stem cell content in clinical practice.
***Key Words*: brain tumors, stem tumor cells, CD133$^+$ cells, immunofluorescence method.**

Presently a special attention of many researchers is focused on the study of tumor cells that have the properties of stem cells. It is believed that this area of research will provide a new understanding of the nature of cancer, the causes of inefficiency of various methods of its treatment, and will contribute to the development of the effective therapeutic approaches.

One of the first evidences on the existence of stem cell tumor cells (STCs) was found by Bonnet and Dick in 1997. They investigated acute myeloid leukemia, in which a subpopulation of 0.01–1% of the total population of cells phenotypically characterized as CD34$^+$ could cause leukemia after transplantation to immunodeficient mice [1].

The data obtained in recent years have shown the presence of STCs in tumors of the colon, breast, pancreas, liver, lung and brain (gliomas, medulloblastomas, ependymomas, etc.) [2–5]. Until recently it was assumed that the STCs counts in a number of malignancies yield up to 0.01–1% of the total population of tumor cells, but presently it is supposed that the number of STCs in various tumor types ranges from 1 to 20%, and in some cases is even higher. STCs of the brain tumors are characterized by unlimited capacity for self-renewal, the formation of tumors in experimental immunodeficient mouse, genetic damage and the generation of new tumor cells [6]. STCs have high ability to invade, stimulate the formation of blood vessels and promote cell migration [2, 3]. They are also involved in the carcinogenesis, and there is growing evidence that these cells contribute to tumor progression [7] and metastasis [8]. This type of cells is responsible for resistance of tumors to chemo- and radiotherapy, recurrence after surgical intervention and radiation therapy. There remains a disputable issue regarding the origin of STCs. Neuronal stem cells (NSCs), neuronal cells, precursors and differentiated glioma cells are considered as the sources of STCs. Shiras et al. confirmed the possibility of the spontaneous transformation of CD133$^+$ normal stem cells to STCs with a potential to produce invasive brain tumors in immunodeficient mice [9]. Junier and Sharif [10] demonstrated that after exposure to transforming growth factor (TGF-α), which is expressed at the early stages of tumor progression, astrocytes can acquire the phenotype of neuronal precursor cells becoming malignant after irradiation. This indicates the possibility of malignant transformation in mature glial cells and the formation of gliomas [11].

Several methods of obtaining and identifying the cerebral STCs are now described including the expression of the CD133 (prominin-1) molecule; the identification of the "side population" (SP); the formation of the neurospheres in cell culture *in vitro* and the ability of STCs to reproduce the tumor histologically identical to the primary tumor in immunodeficient animals. The most commonly used method is the determination of CD133 molecule (prominin-1) as a marker for many STCs identified to date in the tumors of brain, lung, liver, prostate, etc. [4, 12]. Using a flow cytofluorometry, it is possible to isolate STCs by positive expression of CD133. With the use of flow cytofluorometry it was demonstrated [13] that the content of CD133$^+$ cells in glioblastomas ranged from 0.1 to 46.8%, in medulloblastomas from 6.1 to 45.4%, and pilocytic astrocytomas from 3.5 to 37.1%. In another study [14] using immunohistochemical approach the high (more than 60%) content of CD133$^+$ cells was shown in tumor tissue. Data on the prognostic value and correlation between the level of CD133$^+$ cells and the survival time of patients with gliomas are contradictory. Pallini et al. demonstrated that the increased count

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Abbreviations used: NSCs – neuronal stem cells; SP – side population; STCs – stem tumor cells.

of CD133$^+$ cells and CD133/Ki-67 co-expression were associated with poor survival in patients with glioblastoma [15]. Zeppernick et al. [16] found that the presence of CD133 clusters and large amounts of CD133$^+$ cells correlated with a short survival period. However, Kim et al. did not establish the dependence between the level of CD133$^+$ cells and the survival of patients with glioblastoma multiforme [17, 18].

Another technique for STC analysis consists in delineation of SP based on the ability of STC for the efflux of fluorescent dyes *in vitro* due to the activity of ABC transporters.

It should be noted that until now, the panel of specific markers of the STC has not been clarified. It is shown that stem cells isolated from gliomas express such markers as CD133, CD105, CD90, CD15, CD24, CD20, CD44, Nanog, Oct3/4, CXCR4 (CD184), NF (neurofilament protein), GAPDH (glyceraldehyde 3-phosphate dehydrogenase). In addition, STCs express the markers characteristic for the NSC, such as GFAP (glial fibrillary acid protein), Nestin, Sox-2, Musashi-1, Bmi-1 and do not express the early (Tuj1) and late (NeyN) neuronal markers and oligodendroglial marker Olig-1 [19]. Antigen CD15, which is identified in normal tissues and in different types of cancers, including gliomas [20] is regarded as a marker of STC. Implantation of CD15$^+$ cells from the glioma into the brain of a mouse causes the formation of new tumors [21]. According to the literature, there are significant differences in the frequency and number of STCs in tumors depending on different methods of their determination.

The aim of our study was to determine STC content in different brain tumors using immunohistochemical and immunofluorescence techniques.

MATERIALS AND METHODS

Patients. The biopsies of intracerebral tumors of various histological types obtained during neurosurgery operations performed at Romodanov Neurosurgery Institute (Kyiv, Ukraine) were used. The experiments were done in accordance with the bioethics regulations and under permission of Bioethics Commission of RNI. In all cases, a written informed consent was obtained from the patients. Histological diagnosis of brain tumors was done according to the latest WHO classification of tumors of the CNS (2007) [22].

Immunocytofluorescence detection of CD133 in the imprints-smears of brain tumor samples was performed by a standard method using specific primary anti-CD133 antibodies (clone 17A6.1) from Millipore (USA) in a working dilution of 1:100. Anti mouse-FITC labeled antibodies (Labdiagnostika, Russia) were used as secondary antibodies. The samples were examined using fluorescence microscope Zeiss (Germany) with wide-band and narrow-band filters.

Immunohistochemical study. For an immunohistochemical study, histological sections of tumor samples as well as imprints were used. The specimens were routinely processed according to the instructions of the manufacturer (Thermo scientific, UK). The primary anti-CD133 antibodies (clone 17A6.1) of Millipore, (USA) were used in a working dilution of 1:100. For immunostaining, HRP Polymer Qundit, DAB Substract Quonto and DAB Chromogen Quonto (Sigma, Germany) were used. After development of the reaction, the slides were counterstained with methyl green.

Statistical analysis. The software package Statistica 6.0 was used. Statistical analysis was performed with Pearson χ^2 test. A difference between means was considered significant if p value was less than 0.05.

RESULTS AND DISCUSSION

Immunofluorescent detection of CD133 expression on the surface of cells of CNS tumors of different histological type and degree of malignancy has shown the substantial differences, in particular in the intensity and nature of specific fluorescence. The results of immunofluorescent analysis of tumor imprints are shown in Fig. 1. Differences were also found in the morphological features of the cells Namely, CD133$^+$ cells were smaller compared to other cells of the test tumor sample. We found that the percentage of CD133$^+$ samples was higher in malignant tumors (glioblastoma, medulloblastoma, anaplastic astrocytoma) than in benign ones (Table 1).

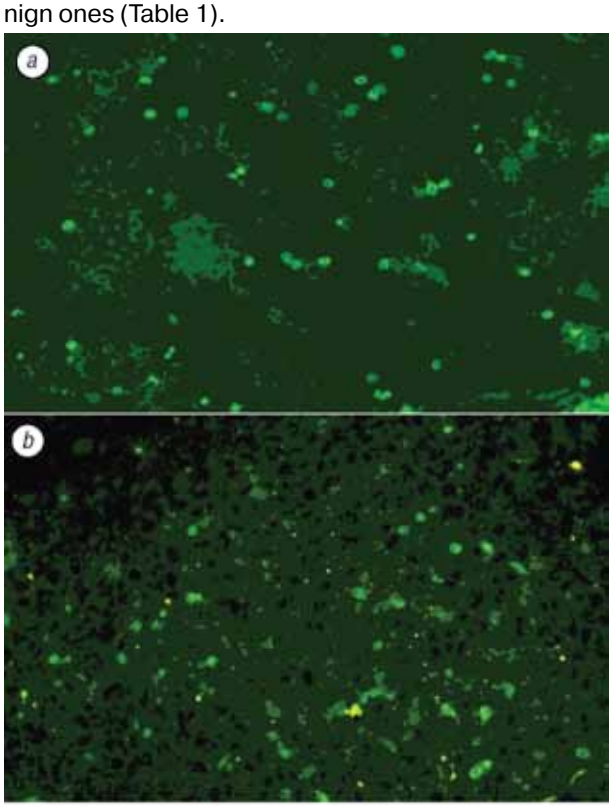

Fig. 1. Immunofluorescent analysis of CD133 expression using a narrow-band filter and FITC labeled antibodies. CD133$^+$ cells glow brightly in light color; CD133$^-$ cells are dull green, × 400. *a* — medulloblastoma; *b* — glioblastoma

Table 1. Frequency of detection of CD133$^+$ cells in human brain tumors

Histological type	CD133$^+$ cases	
	Number of cases	%
Glioblastoma (n = 20)	15	75.0
Anaplastic astrocytoma (n = 8)	8	100.0
Fibrillary-protoplasmic astrocytoma (n = 18)	11	61.1
Medulloblastoma (n = 7)	6	85.7
Primitive neuroectodermal tumors (n = 3)	0	0.0
Other types of benign tumors (n = 10)	4	40.0

Although some researchers argue that the rate of detection of CD133$^+$ cells does not correlate with tumor malignancy, it is definitely well known that CD133$^+$ STCs possess higher (4–5-fold) invasive capacity compared to non-stem tumor cells both *in vitro* and *in vivo* [23]. Accordingly, an increase in the number of cells in this subpopulation contributes not only to tumor angiogenesis but also to metastasis. Our data show that in malignant tumors STCs are detected more frequently, and the tumors of the same histological type had a different quantitative composition of the CD133$^+$ cell subpopulations. Also, in samples from malignant tumors, especially in malignant tumors with a high level of anaplasia such as glioblastoma, meningioma and anaplastic astrocytoma, CD133$^+$ cells are more abundant than in benign tumors (Table 2). The increase in the number of STCs may reflect their increased proliferation and genetic reprogramming of more differentiated glioma cells toward the "stem" phenotype with accompanying increase in the expression of the "stem" markers OCT-4, NANOG, c-MYC, responsible for maintaining the pluripotent properties of the STC [24–26]. It is known that CD133$^+$ cells isolated from neuronal human tumors are tumor cells that undergo various stages of both neuronal and astrocyte differentiation [5, 6]. Consequently, the presence of a large number of CD133$^+$ cells may indicate an active tumor progression, and therefore may be considered as an indicator of unfavorable prognosis.

Table 2. Distribution of CD133$^+$ scores in samples of human brain tumors of various histological type

Histological type	Number and percentage (in brackets) of samples with different scores of CD133-immunopositivity			
	±	+	++	+++
Glioblastoma (n = 15)	5 (33.3)	4 (26.7)	5 (33.3)	1 (6.7)
Anaplastic astrocytoma (n = 8)	2 (25.0)	5 (62.5)	1 (12.5)	–
Fibrillary-protoplasmic astrocytoma (n = 11)	6 (54.6)	5 (45.4)	–	–
Medulloblastoma (n = 6)	1 (16.7)	3 (50.0)	1 (16.7)	1 (16.7)
Other types of benign tumors (n = 4)	3 (75.0)	1 (25.0)	–	–

Note: ± – 1–2 cells per field; + – 3–5 cells per field; ++ – 10–15 cells per field; +++ – 20–30 cells per field.

Using immunohistochemical technique we have analyzed CD133 expression in the following groups of brain tumor samples: 1) 44 samples of medulloblastoma from adult patients; 2) 20 samples of medulloblastoma from children aged 1 to 12 years; 3) 21 samples of glioblastoma; and 4) 5 primitive neuroectodermal tumors (Fig. 2). The distribution of CD133$^+$ samples within each group is shown in Table 3.

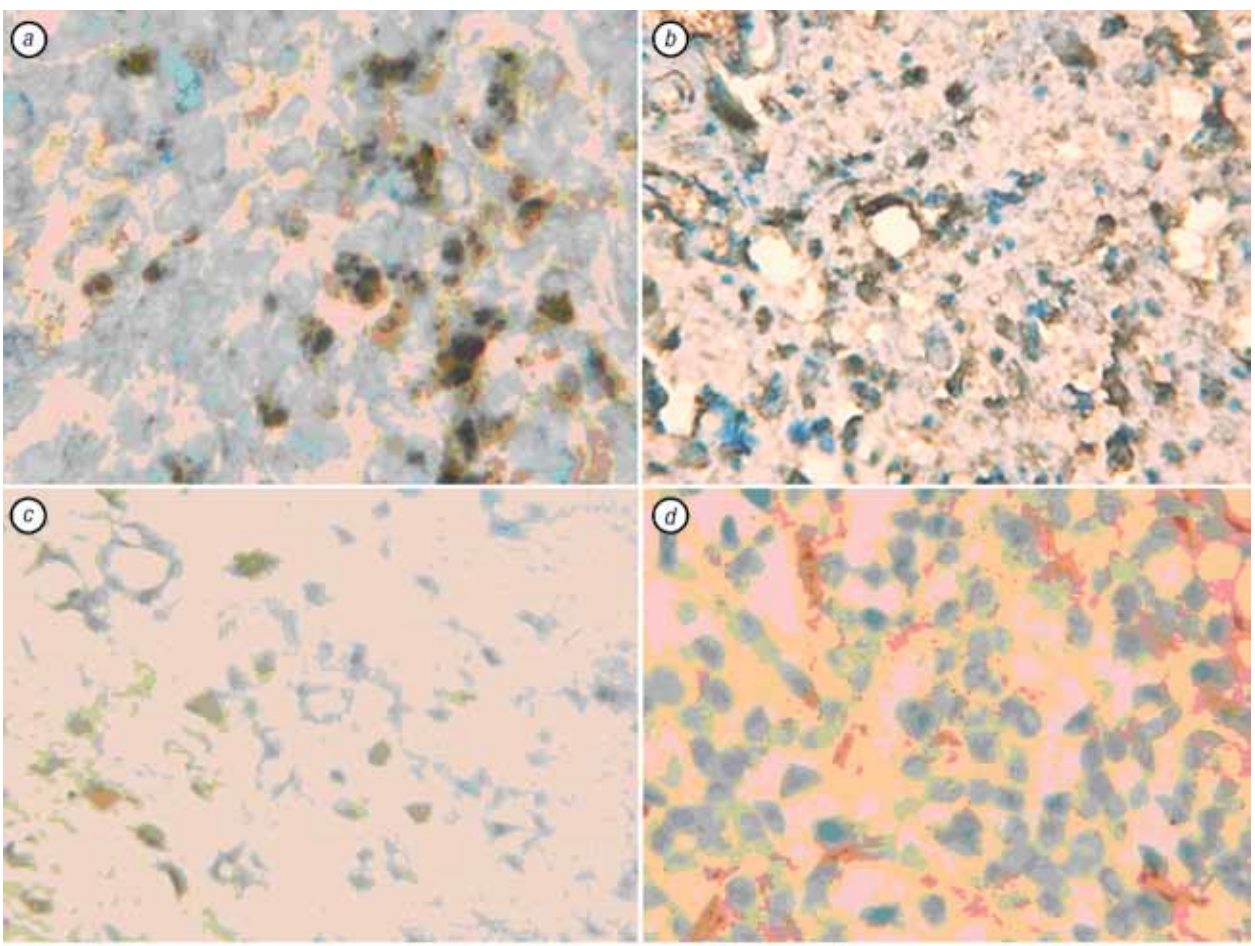

Fig. 2. Immunohistochemical staining of CD133$^+$ cells in samples of various brain tumors. Counterstaining with methylene green, x 400. *a* — non-uniform distribution (focal accumulation) of CD133$^+$ cells in the tissue of medulloblastoma from an adult patient; *b* — distal and superior distribution of CD133$^+$ cells in glioblastoma; *c* — single CD133$^+$ cells in the area of invasive growth of glioblastoma; *d* — single CD133$^+$ cells in the primitive neuroectodermal tumor

Table 3. The frequency of detection of CD133+ cells in different tumor types

Groups of patients	Number of CD133$^+$ samples to the total number of samples (percentage in brackets)
Group 1 (adult patients with medulloblastoma, n = 44)	30/44 (68.18) $\chi^2_{1-2} = 1.99$, $p = 0.26$
Group 2 (children with medulloblastoma, n = 20)	17/20 (85.00)
Group 3 (glioblastoma, n = 21)	19/21 (90.47) $\chi^2_{1-3} = 1.64$, $p = 0.19$
Group 4 (primitive neuroectodermal tumors, n = 5)	3/5 (60.00) $\chi^2_{1-4} = 0.2$, $p = 0.83$

According to data of Table 3, 85–90% of the total number of studied histological samples of glioblastomas and medulloblastomas in children are CD133$^+$ which coincides with the known literature data reporting the presence of CD133 cells in > 80% of samples of glioblastomas. In adult medulloblastomas, the number of tumors with CD133$^+$ cells was lower (68.13%), although there was no statistically significant difference between adult and pediatric medulloblastomas ($p > 0.05$). But analysis of the distribution of CD133$^-$positivity scores in these groups demonstrated the significant differences (Table 4). Thus, among adults with medulloblasomas, the samples with a low count of CD133$^+$ cells were found more often than in childhood medulloblastomas. When compared with glioblastomas, it was found that in adult medulloblastomas, the number of samples with low STC counts was 6-fold higher than in glioblastomas. At the same time, the percentage of tumors with high content of STC in medulloblastomas from adults and children was approximately the same and amounted to 23% contrasting to more than 50% in glioblastomas.

Table 4. Distribution of CD133-positivity scores in brain tumors of various histological type

Groups of patients	Total number of CD133$^+$ tumors	CD133-positivity scores, %		
		1	2	3
Adult patients with medulloblastoma (n = 30)	30	30.00 (n = 9) $\chi^2_{1-2} = 0.34$ $p = 0.5$	46.67 (n = 14)	23.33 (n = 7) $\chi^2_{1-2} = 0.23$ $p = 0.73$
Children with medulloblastoma (n = 17)	17	5.89 (n = 1)	70.58 (n = 12)	23.53 (n = 4)
Glioblastoma (n = 19)	19	5.26* (n = 1) $\chi^2_{1-3} = 4.2$ $p = 0.038$	36.84 (n = 7)	57.89%* (n = 11) $\chi^2_{1-3} = 4.5$ $p = 0.032$

Note: *The differences are significant compared to the group with adult medulloblastomas ($p < 0.05$).

Analyzing the data on the frequency and extension of tumor infiltration with STC one could conclude that intracerebral malignant tumors contain different amounts of these cells. In adult medulloblastomas, about one third of samples does not contain STCs, and the other third contains only a small amount of CD133$^+$ cells. The percentage of such tumors with low content of STC among adult medulloblastomas is twice higher than in childhood medulloblastomas and six times higher than in glioblastomas. Such patterns of CD133-positivity may reflect some biological differences between glioblastomas and childhood medulloblastomas, namely, fewer CD133$^+$ cell counts in these tumors may indicate a lower proliferation rate of cancer cells and slow-down of tumor growth that leads to clinical manifestations of these tumors later in life and the easier course of the disease in adults than in children, according to numerous literature data. One should mention that other factors, such as particular genetic changes, viral, and environmental carcinogens along with the STCs play a role in the development of medulloblastomas. This suggests that the larger STC count in glioblastoma as well as medulloblastoma is not entirely typical feature for these tumors.

The frequency of detection and the patterns of score distribution of STCs in the tumor samples largely depend on the method used in the study. We have shown that the immunofluorescence is more accurate and sensitive than immunohistochemical technuique. More important in our study is the fact of STC detection in benign tumors. This calls into question the hypothesis that STCs are characteristic only for malignant tumors but not for benign ones. On the other hand, even in tumors of the same histological type, for example medulloblastomas or glioblastomas, a significant percentage of samples lacks STCs. There is no reasonable explanation in the latter observation, which indicates both the need for further research of this problem and low predictive and diagnostic value of the STC content in clinical practice.

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