Introduction

In the current classification of the World Health Organization, gliomas are classified into four grades (G1-4). The most malignant in this group of CNS tumors is Glioblastoma Multiforme (GBM). Despite advances in neurosurgery, radiation and chemotherapy, the average survival rate is only several months [1]. GBM is a cancer with a very diverse morphological image with features of pronounced anaplasia.

The previous decade has been a period of interest in cancer stem cells, identified in a variety of primary tumors. Stem cells in GBM may be isolated from other cells in the primary tumor of the brain with the use of markers of neural stem cells, i.e. CD133, Nestin, βIII-tubulin, as well as markers of glial cells, for example GFAP. Currently, it is being suggested that transcription factors such as Nanog, Oct-4, Rex and Olig2 can be used as biomarkers of cancer stem cells and help identify these cells in a tumor mass [3-5]. CD133 is a transmembrane cell-surface glycoprotein. Cells expressing this marker comprise from 3.5% to 46% of the total cells in a tumor and are more frequent in tumors with a higher grade of malignancy [6]. CD133+ cells repair DNA damage significantly faster than CD133- cells [7]. Nestin is an intermediate filament protein playing an important role in organogenesis, cell metabolism and cytoskeletal organization. It may be re-expressed in neoplastic transformation [8]. βIII-tubulin is one of the two globular proteins

Material and Methods: 21 GBM samples were tested. In the assessment of GBM histological features, the following were considered: nuclear pleomorphism, Ki-67 labeling index and endothelial proliferation. The expression of CD133, GFAP, Nestin, βIII-tubulin, Oct-4, Nanog, Olig2 and Rex was analyzed by reverse transcription polymerase chain reaction.

Results: A significantly higher expression of CD133 (p = 0.0007) and Nestin (p = 0.0216) were found in GBM with evident nuclear pleomorphism. We found also significantly higher expression of CD133, Nestin and Olig2 in patients with a Ki-67 labeling index over 15% (p = 0.0033, p = 0.0101, and p = 0.0066).

Conclusion: The expression of biomarkers of stem cells in GBM is associated with a greater severity of anaplasia and tumor proliferative activity. So, it can be assumed that the aggressiveness of GBM is at least partly due to the presence of cancer stem cells in the tumor mass. Due to the resistance of these cells to radio- and chemotherapy, the described phenomena may be associated with lower therapeutic efficacy in patients with GBM.
which form microtubules in combination with α-tubulin. This protein is predominantly expressed in neurons and is involved in neurogenesis. Overexpression of βIII-tubulin has been reported in various cancers, including GBM [9].

GFAP is an intermediate filament protein. Its expression depends on the maturity of tumor cells. Therefore, primitive glioma cells may fail to exhibit reactivity with antibodies detecting the antigen, while mature cells are characterized by a high expression of GFAP [10]. Oct-4 and Nanog transcription factors are necessary for keeping stem cells in the state of pluripotency. Clinical studies have demonstrated the expression of Oct-4 and Nanog in a variety of cancers, including GBM [11-14]. Olig2, in turn, a transcription factor, whose expression is largely limited to the CNS, where it acts as a both anti-and pro-neurogenic agent, depending on the degree of cell development. It is understood that Olig2 is the most specific marker of GBM stem cells [15]. Rex is a marker of pluripotency of embryonic cells. Its expression decreases with the degree of cell differentiation [4]. A number of stem cell markers, including cancer and specifically GBM, have been identified and well described in literature. Previous research has revealed a relationship between some GBM stem cell markers and clinical features of cancer, such as a response to treatment and survival. However, no attempts have been made to determine a relation between the expression of stem cell markers in GBM and morphological features of this tumor.

**Material and Methods**

**The Clinical Material**

The study involved material containing cancer tissue collected during a routine neurosurgical treatment of 21 patients of both genders (10 women, 11 men) aged 30 to 77 years (median 56 years). The study protocol did not include another procedure during surgery or collection of more material than necessary for tumor removal. Nervous tissue comprising cancer tissue was frozen directly after removal in liquid nitrogen and frozen at -80°C. Once the diagnosis was confirmed by histopathological examination, detection of stem/progenitor cells was performed with the use of molecular assessment.

**Laboratory Methods**

**Preparations for Histopathological Evaluation**: The material collected during surgery was fixed in a buffered formalin solution for up to 24 hours, then dehydrated in acetone, put in xylene and embedded in paraffin. Analysis was conducted on paraffin sections of 4μm, stained with hematoxylin-eosin. In the assessment of GBM histological features, the following were considered:

a) Nuclear pleomorphism (size, shape and hyperchromasia of nuclei): relatively small vs. evident – Figures 1a & 1b; in four consecutive fields of view in the hot spot area, magnification 40x

b) Ki-67 labeling index: up to 15% vs over 15% - Figures 1c & 1d; in four consecutive fields of view in the hot spot area, magnification 40x

c) Endothelial proliferation: thin-walled vessels vs vessels with marked proliferating endothelium – Figures 1e & 1f; in four consecutive fields of view in the hot spot area, magnification 40x

**Isolation of total RNA and Quantitative Reverse Transcription Reaction (qRT-PCR)**: Total RNA obtained from tumor tissue fragment was isolated using TRizol® kit plus RNA Purification Kit (Life Technologies, USA). 0.5 g of RNA was subjected to reverse transcription process using a set of First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Analysis of expression of target genes and a housekeeping gene β-actin was performed using the apparatus CFX96 Real Time System (Bio-Rad, USA). In the reaction, we used primers specific for the tested genes, the cDNA template and a ‘mix’ containing SYBR Green - iQ™ SYBR® Green Supermix (Bio-Rad, USA). Relative quantification of mRNA expression of genes tested was calculated using the comparative Ct method. The primers designed for qRT-PCR.

**Statistical Methods**

There were two main types of variables assessed: measurement variables and qualitative variables. The results were presented as mean and standard deviation. Data were compared between groups using the nonparametric Mann-Whitney U-test for continuous variables. All statistical tests were two-tailed, and p < 0.05 was considered to indicate statistical significance. All statistical analysis was performed with STATISTICA 12 software.
Results

a) Nuclear pleomorphism and the expression of cancer stem cell markers: We found significantly higher expression of the markers of CD133 (p = 0.0007) and Nestin (p = 0.0216) in patients with evident nuclear pleomorphism in hotspot regions.

b) Ki-67 labeling index and expression of cancer stem cell markers: A significantly higher expression of CD133, Nestin and Olig2 were found in patients with a Ki-67 labeling index over 15% (respectively p = 0.0033, p = 0.0101 and p = 0.0066).

c) Endothelial proliferation and expression of cancer stem cell markers: In the hotspot regions, there were no significant differences between endothelial proliferation and the expression of cancer stem cell markers.

Discussion

In conclusion it should be emphasized that despite a relatively small number of patients in this study we were able to identify cells in the GBM tissue which, using appropriate markers of cancer stem cells, it can be assumed with high probability that these are GBM stem cells. They coexisted significant with the pleomorphism and proliferative index of this tumor. The cancer stem cells are most likely responsible, at least in part, for the recurrence of tumor and shorter survival. This hypothesis is supported by experimental results, which show that cancer stem cells are characterized by high activity of membrane proteins (ABC transporters) and increased ability to repair DNA, also after radiotherapy [16-18,4,19]. In view of the above, the present study compared the expression of cancer stem cell markers with selected histopathological features of GBM, such as nuclear pleomorphism, pathological proliferation of endothelium including the formation of glomerular-shaped vessels and tumor proliferative index. Significantly higher expression of CD133 and Nestin were found in GBM with evident nuclear pleomorphism. Such a role of CD133 protein may be promoted by its ability to quickly repair damaged DNA [7].

Here we would like to pinpoint one of the mechanisms that most likely contributes to the resistance of stem cells in the tumor to the currently available treatment. GBM has features that play an important role in tumor progression, such as invasiveness and the ability to create specific vascularity. This allows cancer cells to adapt to the anoxic environment.

Persano et al. [26] showed that poorly differentiated cancer stem cells of glioblastomas are located mainly in the layers of weakly oxygenated cells, while more differentiated cancer cells are found in better vascularized regions, and therefore, at least theoretically, better supplied with oxygen and better nourished. Importantly, cytostatics, including temozolomide, have a much worse reach into areas with less blood.

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