Short Academic Review

from

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in his capacity as a member of the scientific jury based
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on the dissertation thesis entitled

PROGNOSTIC AND PREDICTIVE FACTORS IN GLIOBLASTOMA MULTIFORME

for awarding the educational degree "Doctor"
to. Georgi Stoyanov Stoyanov, MD

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Materials on paper and electronic media have been submitted for the competition - dissertation, abstract, biographical and professional data, administrative documents, copies of publications by Dr. Georgi Stoyanov Stoyanov.

Dr. Georgi Stoyanov Stoyanov was born in 1992. In 2011 he graduated from high school, and in 2017 he completed his studies at the Faculty of Medicine, MU-Varna, obtaining a master's degree in medicine. After that, he worked as a part-time assistant in the Department of Anatomy and Cell Biology, in the Department of Physiology and Pathophysiology, and voluntarily in the Department *of* general and clinical pathology, forensic medicine and deontology, and subsequently as a regular assistant in the same from 2018 until today.
The dissertation of Dr. Georgi Stoyanov is dedicated to a current problem in the field of human pathology - glioblastoma multiforme. This is one of the most malignant tumors, in which, despite significant scientific and clinical studies over the past 50 years, patient survival has increased by an average of only a few months, as the author rightly wrote in his publication.

Therefore, studying aspects related to this malignant neoplasm of the CNS is an essential task of modern medicine. For this reason, the study of prognostic and predictive factors, as set by Dr. Stoyanov, makes his dissertation relevant.

The dissertation is written on 113 standard typewritten pages; it contains 65 figures and 13 tables and is distributed in the following standard way: 2 pages of abbreviations used, 22 pages of literature review, goal and tasks - 2 pages, materials and methods - 7 pages, results - 33 pages, discussion - 17 pages, conclusions and contributions - one page each. The cited works are from 250 literature sources. Eleven of them are in Cyrillic, and it makes a good impression that the works of Bulgarian researchers have been taken into account.

The literature review is well structured and comprehensive. After an interesting historical review, the author presents the current opinion of the WHO that the diagnosis of tumors of the nervous system and, in particular, GBM is complex - localization, macroscopic and histological type have to be taken into account. According to some authors, "glioblastoma multiforme should be viewed more as a nosological group of diseases with varying biological behavior, with no definitive markers of biological potential yet identified, except for IDH and MGMT status". Some of these characteristics are due not only to the tumor's biological potential but also to the body's response to it. Molecular knowledge in these tumors is also crucial for understanding this potential. It is an important starting point for classifying CNS tumors, which is constantly improving, with increasing knowledge in this area. The last one is from 2021, which is duly presented in the review. The microscopic features of GBM and their IHC are described in detail. It is emphasized that molecular testing is crucial for the determination of the tumor, and conventional morphological examination does not lose its importance. Despite the significant progress in the molecular-genetic understanding of the processes of gliomagenesis and driver mutations, at this stage, the prognostic and predictive markers concerning GBM are extraordinarily scarce and partly contradictory. It is pointed out that multiple factors have not been studied in glioblastoma multiforme with not well-established prognostic and predictive significance. Such are, e.g., the body's immune response and its correlation with tumor growth and the role of Diaph3 as a diagnostic marker for tumor proliferation. The MGMT profile of the tumor, which is a predictive factor for the response to treatment of patients with glial tumors, is of constant clinical significance, although outside the classifications. MGMT is a protein with a stabilizing and reparative role for DNA. Tumors without mutation in MGMT have an inadequate response to therapy, while those with MGMT mutation show an excellent response.

Based on these conclusions made at the end of the review, the dissertation indicates the purpose of the work, which I find relevant - to make a comprehensive analysis of age and gender characteristics of glioblastoma multiforme, tumor size and location, the importance of intense immune response and survival in primary diagnosed tumors as they are compared with the MGMT profile of the tumors and the importance for the diagnosis and prognosis of the forms and levels of expression of Diaph3 in it.

To realize this goal, the following tasks are logically set: 1. Selection of materials from glial tumors. 2. Reclassification of tumors according to the newly introduced classification. 3. Determining the demographic characteristic. 4. Analysis of preoperative blood tests. 5. Establishing the MGMT profile. 6. Establishing the neuroradiological characteristics of tumors. 7. Preparation of three-dimensional reconstructions of the selected tumors. 8. Diaph3 expression study. 9. Study the intensity of expression in the rhythmic (Scherer) growth structures. 10. Statistical analysis and correlation of the obtained data.

To solve the set tasks, materials from tissue sections of histologically proven GBM and an equal volume control group of non-tumor processes in the University Hospital "Sveta. Marina"- Varna, Varna, Bulgaria were used from February 2018 - February 2021 as n = 62 cases, main group and n = 62 cases, control group. Data from the medical documentation of the diagnosed patients and their imaging studies of the CNS were used.
The methods used briefly are analysis of histological sections and staining, immunohistochemical labeling with IDH1 R132H antibody and MGMT antibody, Diaph3 antibody, all according to standard protocol, as well as analysis of computer tomographic and magnetic resonance imaging, calculation of the ratios of neutrophils to lymphocytes, platelets to lymphocytes and monocytes to lymphocytes, in order to determine the equilibrium of the immune response to tumor growth, digitization and automatic processing of the obtained images, digitization of the obtained IHC slides using an automatic scanner, software ImageScope V12.1.0.5029 for statistical analysis implemented using MaxStat Pro v3.6 software package and including the methods - (1) descriptive analysis to determine statistical values: mean, standard error of the mean, minimum and maximum values, median, (2) non-parametric methods for testing the null hypothesis, (3) parametric methods - Student t-test, statistical study by correlation analysis and Cox regression model for univariate analysis. The methods used in this way guarantee the objectivity of the obtained results.

The results of the study show that the demographic characteristics of patients with GBM are: 56% (n = 28) males and 44% (n = 22) females; the m:f ratio is 1.27: 1; mean age is 65.3 years, median - 65 years, range 43-86 years. Out of 50 cases after immunohistochemical examination of MGMT mutation status positive cases are 35% (n = 17), the average age of patients with MGMT positive GBM is comparable to that of the general cohort, with no statistically significant difference, females show a small predominance. Regarding the tumor localization, it was found that it is slightly more common in the left cerebral hemisphere; at most, 37.78% (n = 17) GMB are found in the temporal lobe. The average size of the tumors is 50.51 mm (range 20-76 mm), and in terms of tumor size and laterality of the process, there is no statistically significant difference. Tumors of the largest size are located in the temporal lobe. The methodology of three-dimensional reconstructions better outlines the characteristics of tumor growth, especially its rough outlines. The median survival in the overall cohort (n = 50) from the day of surgery to the day of death was 255.96 days (8.41 months). The survival range is from 18 to 1061 days, with the longest overall survival seen in a living patient, 1150 days (37.78 months) after surgery. Data from preoperative differential blood counts (in only 22 patients) showed that the mean neutrophil count in the cohort was 15.81, the median was 6.77, eight patients had NLR> 4, five patients had PLR> 200, five patients had MLR> 0.45, which is

evidence that a small number of patients have intense immunity. This is the result that a combined synergistically increased NLR, PLR and MLR index are found in three patients. In the studied cohort (n = 50 - 100%), all tumors showed a positive reaction to the Diaph3 antibody. The reaction is moderate to highly intense, granular, and predominantly localized in the cytoplasm of tumor cells, but single cells also show a mild to moderate fibrillar nuclear response. The average life expectancy of patients in the cohort was 255.96 days (8.41 months), higher in men. The largest difference in survival was found based on MGMT status, where the average survival of patients in the cohort of MGMT-positive tumors was 477.77 days (15.7 months). Compared to tumor localization, parietally located tumors have the longest survival - 310.23. Furthermore, compared to the age groups, the highest average survival in the group is 41-50 years - 584.75 days. A statistically significant difference in survival, with p = 0.0044, was found only in patients with MLR> 0.45, and in these patients, the survival was significantly lower - 103.83 days against 313.13 in patients without an increase in the index. No correlations were found between Diaph3 expression levels for age, sex, tumor location, and neuroradiologically determined tumor volume by correlation analysis.

Based on these data and information from the literature, the author discusses that glioblastoma multiforme is a nosological unit that represents a significant diagnostic difficulty from a clinical point of view, with significant variability in histological findings, with a variety of molecular and genetic alterations that are intensively studied, but where, despite the great concentration of efforts on the topic, there are still no positive results in therapy. Based on the significant molecular genetic research and the reports presented on this basis by cIMPACT-NOW, in the fifth of them, the most significant change in the classifications of tumors of the nervous system since its introduction is presented. With the idea of unifying the WHO classifications and the way of answering, the grade was proposed to be marked with Arabic (1-4) and not with Roman numerals (I-IV). Also, IDH mutant tumors are separated by their diagnostic category, and the morphological criterion for grade 3 tumors is specified - pronounced mitotic activity. The sixth report presents the principles for modifying future classifications by introducing types and subtypes to some of them, and the last seventh report presents minor clarifications for some of the new molecular profiles of tumors. IDH R132H mutant phenotype was found in 9.1% (n = 5) of n = 55 patients in the primary cohort, allowing reclassification of tumors according to the new classification. These data are fully comparable to data from large cohorts, where the incidence of IDH mutants, currently diffuse astrocytoma, WHO CNS grade 4, varies around 10%.

As for the incidence of glioblastoma multiforme, according to the author, it will not change drastically, but there will be a difference in the average age of onset of the disease, given that mutant forms are more common in young people under 50 years of age, as well, it is believed that there will be a statistical increase in cases in men. Another direct consequence, according to Dr. Stoyanov of the change in the classification, is a decrease in the average survival of patients, given the inclusion of longer surviving patients in another nosological unit. With these novelties in the classification, the changes, according to the dissertation, can be summarized as glioblastoma is a tumor characteristic of old age, more common in men, with a poor prognosis. For this reason, glioblastoma multiforme is defined from a clinical and epidemiological point of view and as a neurological disease characteristic of older men. Despite higher survival in observations other than those presented in the dissertation, glioblastoma multiforme is a tumor

with an abysmal prognosis. According to WHO criteria for CNS tumors, a significant factor essential for the survival of patients with glioblastoma multiforme from 2021 remains the MGMT mutation status, indicating the possibility of tumor response from temozolomide therapy. In the study group of Dr. Stoyanov, the incidence of MGMT-positive tumors was 35% (n = 17), which is comparable to the literature data, where the incidence varies between 30% and 60%. Given the rapid growth of the tumor and its diffuse nature, which cannot be well established neuroradiologically, neurosurgical interventions in glioblastoma multiforme are not only tricky methodologically but often with consequent neurological deficits. Differences are found in the localization, which in the cases presented in the dissertation is most often in the temporal area, in contrast to those in the literature, which find that it is more often frontal. The most significant statistical significance in terms of survival in terms of immune response was reported in the MLR index, alone and in combination with other inflammatory response markers, and similar results were obtained in other studies. From the available data on the role of Diaph3 in malignant processes, it was found that the loss of expression leads to the so-called amoeboid phenotype - cells acquiring plastic properties, which allows them to move in the extracellular matrix without destroying it. As a vital molecule whose loss allows cytoskeletal remodeling in amoeboid cell migration, Diaph3 plays a significant role in invading surrounding tissues and developing lymphonodular metastases and glioblastoma multiforme, a tumor with a heterogeneous expression of Diaph3. It has been found that its expression will play an increasing role in treating GBM.

At the end of the thesis, he openly admits that " this dissertation by no means exhausted all the possibilities for stratification of the problems" the author concludes that glioblastoma multiforme, according to WHO criteria for CNS tumors from 2021, is a malignant tumor with predominantly astrocytic differentiation, cell polymorphism, microvascular proliferation and/or pseudopalisadic necrosis, which is non-mutated in IDH, that the main factor determining the prognosis of patients is the mutational status of MGMT, that the survival of patients with acute and tense immunity varies and that glioblastoma multiforme is a Diaph3 positive tumor, the positivity is heterogeneous, but there are expectations for future therapy related to its expression.

As presented by Dr. Stoyanov, the thesis contains the following contributions:

1. Clinical-diagnostic contributions: three-dimensional reconstructions confirmed the correlation between neuroradiological parameters in glioblastoma multiforme and performed volumetric analyzes, identified predilection sites for tumor development, established its average neuroradiological size - just over 50 mm, the importance of expression of the anti-Diaph3 antibody, no correlation was found between Diaph3 expression levels and survival and primary tumor size.
2. Therapeutic: the role of the systemic immune response has been established, the critical importance of MGMT mutation status has been confirmed, the importance of Diaph3 expression as a possible predictive factor for treating GBM with taxanes and rapamycin has been argued.

The dissertation results are presented in thirteen scientific publications, eight in peer-reviewed journals, two of them with IF, three in international nonindexed journals, two in national nonindexed journals, and one in a scientific forum report.

Based on the above, I believe that the dissertation of Dr. Stoyanov is a fully completed work, containing significant results and conclusions of clinical-diagnostic and therapeutic significance. The work is detailed and comprehensive; it has a broad scope, including a review of foreign and Bulgarian sources; it is an important study on the problem of GBM and glial tumors in the domestic scientific literature and fully meets the requirements of the Law on Academic Development in Bulgaria and the conditions and the procedure for obtaining scientific degrees in MU-Varna.

Therefore, I give a positive assessment of the dissertation, I will vote for, and I would like to recommend to the members of the esteemed Scientific Jury to support the award of the educational and scientific degree "Doctor" of Dr. Georgi Stoyanov Stoyanov.

22.03. 2022

Prof. Dr. Belovezhdov