

# REVIEW

by prof. NIKOLAI ELENKOV LAZAROV, MD, PhD, DSc  
on the dissertation of Dr. George Stoyanov Stoyanov, MD  
“Prognostic and predictive factors in *glioblastoma multiforme*”  
for awarding the Doctor of Philosophy (PhD) degree

George Stoyanov Stoyanov, MD is an assistant professor in the Department of General and Clinical Pathology at the Medical University (MU) "Prof. Dr. Paraskev Stoyanov" - Varna. In 2019 he was enrolled as a regular PhD student on a full-time basis (Order No. P-109-51/01.02.2019) under the doctoral program "Pathology and Cytopathology" at the Department of General and Clinical Pathology, Forensic Medicine and Deontology of MU-Varna, where he conducted the main part of the experiments in this dissertation. After completing the training program and successfully passing exams in scientific specialty and English, he was expelled by decision of the Faculty Council of the Faculty of Medicine and Order No. R-109-71/04.02.2022 of the Rector of MU-Varna with the right of its public defense before a scientific jury.

The dissertation entitled “*Prognostic and predictive factors in glioblastoma multiforme*” is written on 113 standard pages, divided into sections as follows: *Title page*, *Contents* – 3 pages, *List of abbreviations* – 2 pages, *Introduction and Literature review* – 23 pages, *Purpose of the research* – 1 page, *Material and methods* – 7 pages, *Results* – 34 pages, *Discussion* – 18 pages, *Conclusions* – 1 page, *Contributions* – 1 page, *Publications on the topic* – 2 pages, *Acknowledgements* – 1 page, *References* – 18 pages including 250 titles, 11 in Cyrillic and 239 in the Latin alphabet. The work is illustrated with 13 tables and 65 figures.

The **topic** of the dissertation is adequately chosen. It is dedicated to a socially significant and extremely topical issue of oncopathology, i.e. the prognosis and prevention of *glioblastoma multiforme*, which is the most common and malignant of all glial tumors of the central nervous system. Although the average survival of patients with glioblastoma has increased over the last 50 years, albeit to a lesser degree, the prognosis of cancer patients is usually gloomy. Therefore, early diagnosis of this very aggressive brain tumor is crucial for a more favorable prognosis of its treatment. In this context, the elucidation of prognostic and predictive factors would be useful in order to find an effective manner to stop the growth of glioblastomas. In search of such an approach, the PhD student has set himself the ambitious task to examine histopathologically tissues from

patients with *glioblastoma multiforme*, to make a comprehensive analysis of their age and sex characteristics, the tumor size and location, to take into account the role of systemic immune response in patients' prognosis and finally to evaluate immunohistochemically the expression of a formin family protein, Diaph3 that is associated with the tumor progression and therefore considered a candidate biomarker of taxane sensitivity and a reliable predictive factor for the treatment and thus for the overall survival of glioblastoma patients.

The **introduction** is integrated in the *Literature Review* section. It is short, concisely written and in a synthesized form introduces the reader to the thesis content.

The **literature review** is detailed and provides in thematic subsections an extensive information about the historical methods for the study of gliomas and the contributions of foreign and Bulgarian scientists to the knowledge of glioblastomas. The current integrated classification of gliomas, incl. genotypic classification of *glioblastoma multiforme* is presented. Exhaustive data on imaging and histopathological criteria for tumor diagnosis, as well as on the most important molecular genetics factors of diagnostic importance are provided. Described are some new and unexplored factors in *glioblastoma multiforme* whose prognostic and predictive roles in other tumors have been identified. The literature data are appropriately illustrated with own photomicrographs, computed tomography and magnetic resonance imaging scans of the tumor. Of all the literature sources cited in the references, 152 (>60% of all) articles are published after 2015, including 60 from 2020-2021, which is another proof of the relevance of the research problem, the completeness of the literature survey and, last but not least, the good literary awareness of the author. The fact that at the end of the literature review in a short concluding paragraph the current state of the considered problem is presented in summary and the open and still unresolved issues are outlined, makes an excellent impression. This allowed Stoyanov to ensure a smooth transition to the next section of the dissertation and thus to set the exact goal of his research.

The **research goal** is clearly and precisely defined. The **tasks** are set correctly and, as will be understood in the following, they are actually feasible with the methods used. I have no remarks to this section, with the only recommendation to link the set tasks with the specific methods by which they will be achieved.

The **Material & Methods** section is relatively short, but informative enough. The funding sources of the study are correctly indicated. The necessary ethical requirements for working with

human material have been met, there is a permit from the Commission for Scientific Research Ethics at MU-Varna. The total number of samples taken (from 62 cases) and that of the examined (50) tissue samples from them, incl. from the control group is quite sufficient to achieve statistically reliable results. For the immunohistochemical reactions, biopsy material was used, which was promptly fixed, embedded in paraffin blocks, and cut before use. The description of the immunohistochemical protocol with an automated IHC staining system, the analysis of the digital images and the performed statistical analysis of the data are given with accuracy and step-by-step details, thus allowing their reproducibility by other researchers. In my opinion, it would be far clearer for the reader if the necessary information about the primary and secondary antibodies used in the study, the host species, the working dilutions and validated applications, were given in a table. Moreover, no information is provided about the control for the specificity of the antibodies, which are critical for the validation of an immunohistochemical reaction.

The **Results** section is set out on 34 pages, which is more than one-third of the dissertation. It includes separate subsections in a sequence chronologically following the set experimental tasks. At the beginning of the description, the frequency and percentage distribution of the studied cohort, distributed by age and sex, and unified according to the latest WHO classification for CNS tumors, are presented. The frequency of tumor location with left-hemispheric dominance and temporal topographic localization is reported. There are no statistically significant differences in the tumor size related to the laterality of the process, although such is present in the mean tumor size in the temporal lobe in relation to the entire cohort. At the same time, it has been found that the majority (60%) of the tumors located in the left cerebral hemisphere occur in women, while in the right cerebral hemisphere they are predominant in men by a ratio of 3:1. These findings have been statistically confirmed. On the other hand, no significant differences in age have been found between left-sided and right-sided tumor localization. The available data did not allow the PhD student to analyze the dynamics of the tumor growth. By applying an author's model, Stoyanov have managed to create a three-dimensional image reconstruction of the primary tumor, on which he has performed a volumetric analysis of the generated images with an emphasis on the tumor volume and its spatial relationships with neighboring brain structures. The calculated average survival time between the sexes in the cohort has shown a higher value of about 1.5 months in men. In the course of the study, the status of the systemic immune response in patients has been determined by differential counting of leukocytes,

taking into account its main indicator, the number of lymphocytes. When considering the combined index of increased ratios of other leukocytes to lymphocytes, a statistical difference has been found with a marked decrease in the survival time of patients with increased circulating monocytes and/or other markers studied. Applying immunohistochemical staining to the biomarker Diaph3, Stoyanov has found a moderate to high expression in the tumor cells of *glioblastoma multiforme*, with a varying intensity in different areas of the tumor, as well as negative immunostaining in the white matter of intact brain tissue, and also in the control patients with reactive gliosis, with the exception of a weak background staining and nuclear expression in single lymphocytes. An interesting finding is the reported strong immunoreactivity in angiocentric macro-rosettes, which is thought to be closely related to vascular satellites. By statistical analysis with appropriate software, he has demonstrated significant heterogeneity of the expressed biomarker in tumor cells, while the correlation analysis did not establish statistical relationships between the expression levels and the age, sex, tumor location and neuroradiological tumor volume.

I should mention the fact that all the author's findings are supported by sufficient and good quality illustrative material. At the same time, the statistics presented in tables, graphs and charts provide us with rich and clear information about the quantitative data established.

The **discussion**, although not very extensive, is done with skill and understanding, and shows the ability of the author to interpret his own results, comparing them with known facts in the relevant literature, and integrating the available data to draw valuable conclusions and recommendations for the clinical practice. The changes in the tumor taxonomy of the CNS proposed in the latest WHO classification from 2021 and, in particular, the mutant phenotype of glioblastomas identified in this study have been discussed. Relevant to these taxonomic modifications are the related changes in the frequency of *glioblastoma multiforme*, the mean age of the disease onset and the average survival time after its diagnosis. Therefore, the analysis of morphologically verified patients with *glioblastoma multiforme* is important not only for morphological diagnosis, but is also particularly valuable in demographic terms. All this gives the PhD student reasons to conclude that glioblastoma is a malignant brain tumor among the elderly people, that it is more common in men and has an extremely poor prognosis. In this regard, the predictive potential of Diaph3 suggested in this study is also of some practical interest, not only for the selection of patients suitable for pharmacological treatment of the tumor, but also for the identification of tumor proliferations by the neuropathologist. As a final result, the new data obtained in

the study for this aggressive type of brain cancer allow the author to outline the future directions in the application of innovative neurooncological methods of diagnosis and treatment, such as the combination of three-dimensional reconstructions of the tumor with a neuronavigation. Another important practical application would also be to explore the correlation between the systemic immune response and tumor infiltration by circulating immune cells. The clarification of the role of Diaph3 as a potential biomarker for the prediction of taxanes and rapamycin chemotherapy is essential for the practice as well. Despite the limited objectives and shortcomings of the present study, which are correctly indicated at the end of the text, the data obtained are a good basis for the accurate formulation of its **conclusions**. They are written concisely and, although not numbered, accurately reflect the author's findings. In general, I accept without remarks their wording and scientific value. In order to better highlight the scientific achievements of the study, they are presented in a separate section and are divided into contributions of clinical-diagnostic and therapeutic nature. The main **contributions** of the dissertation, most of which are definitely original, could be summarized as follows:

1. The present study provides a comprehensive analysis of the frequency, severity, clinical, morphological and demographic characteristics of *glioblastoma multiforme*, according to the recently revised by the WHO classification of CNS tumors.
2. The most common sites of tumor localization with regard to the sex and age of the patient and lateralization of the process have been identified.
3. The role of the systemic immune response in the prognosis and key importance of mutational status in patients with *glioblastoma multiforme* has been confirmed.
4. For the first time, the immunohistochemical expression of Diaph3 has been shown experimentally in normal brain parenchyma, in brain tissue with reactive gliosis, and in *glioblastoma multiforme* tumor cells.
5. Original data on the potential prognostic role of Diaph3 for the cancer treatment with taxanes and rapamycin are presented.

In the attached documentation there is a declaration of originality of the work, which gives me a reason to believe that this dissertation is a personal work of Dr. George Stoyanov. An additional argument for this assumption are his scientific publications and communications on the topic of the dissertation. The results of the experiments are published in 12 articles, in 11 of which Stoyanov is the first co-author. Two of the papers are published in peer-reviewed specialized journals with an impact factor, six of

them are published in refereed journals without an impact factor, two articles are in international non-refereed journals and two papers are in Bulgarian journals. A list with one communication in a national scientific event is also provided. The reviewer is not aware of citations of these scientific papers in the works of other authors and/or in other sources of bibliographic information.

The dissertation is written in a concise way, the writing style is clear and only single spelling errors are noticed throughout the text. A relatively large number of abbreviations and specific terms are used, some of which are well known, which does not cause difficulty for the reader. The draft of **the thesis abstract** is prepared according to the requirements. It adequately and sufficiently reflects the state of the problem, the purpose of the study, the methods used for its implementation, the results obtained, their analytical description and interpretation of their own data, as well as the author's conclusions and contributions.

In **conclusion**, I found that the dissertation of Dr. George Stoyanov is a complex and in-depth study on an interesting and topical problem of modern oncopathology. It is a well-conceived study, which is precisely methodologically substantiated, has been conducted accurately and is properly illustrated. The obtained data are clearly and adequately discussed, and expand the current knowledge of brain tumors. The research results have an original and significant theoretical contribution to the understanding of the prognostic and predictive factors of *glioblastoma multiforme*, in particular Diaph3 and its relationship to the overall glioblastoma cancer survival. The present work meets the legal requirements for obtaining the scientific and educational degree and the conditions, criteria and indicators for its award in the Regulations to the development of the academic staff at MU-Varna. All of the above-mentioned reasons convince me as a reviewer to confidently express a positive opinion for the dissertation and as a member of the Scientific Jury of the procedure to support with a positive vote the award of the educational and scientific degree "Doctor" in the professional field 7.1. Medicine, and in the scientific specialty "Pathoanatomy and Cytopathology" to George Stoyanov Stoyanov.

Reviewer:



(Prof. Nikolai Lazarov, MD, PhD, DSc)

Sofia, 18.03.2022