REVIEW OF THE DISSERTATION WORK OF GEORGI STOYANOV STOYANOV, M.D. ON THE TOPIC: PROGNOSIS AND PREDICTIVE FACTORS IN GLIOBLASTOMA MULTIFORME

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Glioblastoma /GBM/ is one of the most malignant tumors in human pathology, characterized by exceptional histological and genetic heterogeneity. The desire to understand the mechanism of this variability and the inability to control its aggressive evolution maintains a great interest in its study and provokes different opinions about its nature.

The discovery of the IDH1/2 mutation in the last decade has proved to be a key factor in the development of glial tumors. Gradually genetic alterations in gliomas and many other CNS tumors have become significant and even gained parity in their diagnosis along with histological features even according to the revised classification from 2016. The latest classification of glial tumors, 2021 led to the dominance of genetic characteristics in the competition between the two methodologies cited. GBM, described histologically more than 100 years ago, maintained an almost uniform definition with a large volume of diverse subspecies, variants and patterns, but it always contained the triad - cellular and nuclear atypism, violent neovascularization, which gave rise to a vaso-occlusive model of origin and necrosis of the palisade and ischemic phenotype. Until recently, GBM was divided into two groups - IDH mutant and IDH non-mutant, and some differences in their clinical characteristics were found. In the latest WHO classification, 2021 GBM is transformed into two separate tumor species, despite their similar histological picture. A small number of the so-called GBMs dropped out of it and were renamed astrocytoma, grade 4, based on a genetic defect - an IDH mutation. The large group retained its name glioblastoma multiforme also due to its IDH status, which is non-mutant. Histological criteria have receded into the background for the identification of malignant astroglial tumors.

Whether this taxonomic change will have a positive effect on the study of GBM behavior and its effect on future treatment remains to be seen. The study of Dr. Stoyanov with reclassification of GBM, in line with recent changes, is a step in this direction. The candidate`s rapid reflex to reorient to the new classification schemes provides updated information on the demographic characteristics, location and some prognostic factors of the newly named GBM. The fact that GBM has been examined as different entity in the new classification with updated clinical and immunohistochemical methods are a prerequisite for fruitful results and original conclusions.

The topic of the dissertation "Prognostic and predictive factors in GBM" is very broad and is related to the entire gliomalogy history. The prognostic indicators of GBM are based on the classification of Bailey and Cushing, which confirms the clinical and morphological direction, coded by its authors pathologist and neurosurgeon.

All subsequent works by Kernohan, who introduced grading, Scherer, Russell and Rubinstein, are essentially histobiological, as they combined the histology with a prognosis. This trend crystallized in the first WHO classification edited by Zulch, 1979 /where the tumor

grading is determined by patients' survival/ and is maintained to this day. Hundreds of histological, histochemical, immunohistochemical and fluorometric data have been accumulated, as well as genetic alterations, studied to determine their prognostic and later predictive significance. In the review, Dr. Stoyanov narrows the information and predictably directs it to several parameters with possible prognostic and predictive significance, on which he will focus his research.

The survey is based on 250 sources, mostly from the last few years. After essential historical notes and data on the latest classification, 2021, Dr. Stoyanov reviewed the epidemiological publications on the distribution of GBM by sex and age and their topography, determined according to neuroradiological studies. New trends, related to the three-dimensional examination of gliomas, presenting their exact volumetric characteristics, have also been announced. The review includes the role of IDH, which has established itself as a leading genetic factor in the revised 4-th edition, WHO 2016 and MGMT, whose importance as a prognostic and predictive biomarker for gliomas has also been widely discussed since the last decade. The role of Diaph3 in non-CNS malignancies has been extensively discussed, which provoked Dr. Stoyanov to study its importance in GBM. Based on the key position of Diaph3 on the formation and function of the cytoskeleton, he suggests that in GBM as a highly malignant tumor with impaired genetic stability, an alteration of this gene is possible. Recent data on the systemic inflammatory response in malignant tumors including GBM indicate that impaired ratios of each of neutrophils, platelets, and macrophages with lymphocytes, have a prognostic effect and may direct treatment toward the immune response.

The survey unites the historical data and contemporary studies on GBM and some aspects of other malignancies and gives grounds for Dr. Stoyanov to conduct his research on discussion problems focused on the prognosis, as well as in the search for new prognostic and predictive factors. I agree with inferences of the review of insufficient data for predictive markers in GBM and for contradictive results of the multiple biomarkers with predictive significance.

The aim of the author is "Against the background of unsolved problems to study the cohort of patients with GBM by making a comprehensive analysis of age and sex characteristics, tumor size and location, importance of the intense immune response and survival in primary diagnosed tumors and compare with MGMT profile of tumors and the importance for the diagnosis and prognosis of the forms and levels of expression of Diaph3". The aim corresponds to the tasks, the topic and the content of the dissertation, but for its formulation a more synthesized form can be desired. In fact, the aim is to analyze a number of clinicobiological characteristics /demographic, topographic, laboratory and neuroradiological/ and IHC biomarkers MGMT and Diaph3 in relation to their role as prognostic and/or predictive markers.

The tasks are related to the goal and described in points: selection of tissue biocks, reclassification of GBM, demographic characteristics, analysis of the ratio of neutrophil leukocytes, platelets and monocytes to lymphocytes, determination of imaging anatomical parameters - size and topography, examination and topography and statistical data analysis.

Material. 62 blocks of glioblastomas and 62 control groups were studied, 57 with normal brain parenchyma and 5 with reactive gliosis, exclusion criteria were specified, data from the medical record of the patient, KT and MRI, necessary for the task of the study, were used.

The methods are actual, wide-ranging, directed to comprehensive histological, clinical and neuroradiological examination and IHC studies in full accordance with the tasks. The methods include the following: reclassification of all IDH-1 R132H-verified tumors, reference to lab result for neutrophil, platelet, and monocyte-to-lymphocyte ratios, determination of tumor size, volume and location by three-dimensional KT/MRT reconstruction, IHC study of MGMT and Diaph3, comparison of Diaph3 expression in tumors, reactive gliosis and normal brain, statistical analysis. Three-dimensional reconstruction is described detailly in two stages and authors' model, which strikes better tumor size and relation with adjacent brain structures because of color marking. The methodology of the IHC follows the protocol of the producer and tests are validated with appropriate positive and negative controls and specification of the working concentration.

These methodological clarifications are a guarantee of high quality and relevance of the results. The preparations are digitized with an automatic scanner, allowing to view many slices at the desired magnification - a modern method that can provide computer transmission of images. Robotic expression reading was performed using an automated algorithm, which also certifies the accuracy of the results and statistical analysis. The introduction of digitalization of scientific facts by Dr. Stoyanov is an innovative approach to research that deserves to be encouraged and disseminated in the diagnostic practice of pathologists.Statistical analysis is applied, ensured by wide set of methods.

Results. After refining the tissue blocks, the number of cases was adjusted to 55. The reclassification of GBM with the requirement for no mutations in IDH1 R132H resulted in the elimination of 5 patients in the age group under 50, which coincides with the results published so far for the ratio of non-mutant / mutant GBM 10: 1. The chosen illustration of the changes is very typical - in both cases there is almost the same histology /slight difference in cellularity/, but the figure 5.1 shows a tumor that until recently belonged to GBM - with patterns of *astrocytoma /GBM* (WHO, 2016). Important demographic indicators were studied, namely distribution of GBM by sex and age.

The results describe the exact location and size in 45 patients according to preoperative neuroradiological indicators. A slight predominance of left-sided /55.55%/tumors, affecting more than one lobe /40%/and one multicentric tumor in both hemispheres have been found. The most common are GBM in the temporal lobe /37.78%/, followed by the parietal /28.89%/, decrease in the frontal /24.44%/ and the least in the occipital lobe /8.89%/.

A complete topographic map of the distribution according to their size and ratio with the lobe localization was made with insignificant statistical differences. Three-dimensional reconstructions were carried out in two stages. At the first stage, they reveal the volume of the tumor nucleus and the spatial relationships with the adjacent brain structure and optimize the operative approach. In the second stage, by applying an author's model, tumor sizes and relationships with neighboring brain structures are better emphasized due to color marking and may be preferred in medical education, preoperative planning and postoperative follow-up of the patient. There is no statistically significant difference

between primary and secondary three-dimensional reconstructions with neuroradiologically reported tumor parameters.

In an effort to maximize the significance of each parameter of GBM, the author seeks evidence to support tumor growth and cites two cases of repeated MRI scans of the brain in which the tumor grows by 8 months to 51 mm in one case and in the other for 28 days up to 26 mm.

It is important to note that growth according to radiological image of the tumor may be due to pseudoprogression and it is recommended to determine it in combination with histological examination. Rapid growth also depends on the size of the residual tumor as well as the high proliferative capacity. My personal experience proves an extremely rapid increase until the doubling of GBM in the postoperative period /up to 2 months/, depending on the histological correlates of this growth.

The analysis of important features of GBM based on the neuroradiological picture with emphasis on the size, topic and spread of the tumor provides useful information and largely justifies the treatment behavior. The comparison of the histological and imaging results made by Dr. Stoyanov, is a necessary element of the integrated diagnosis and deserves a positive assessment.

The undisputed success of the dissertation is the study of patients whose survival is known to us - this is rare in our conditions, but it is essential to draw conclusions about the prognostic and predictive effect of certain biomarkers. The author found a median survival of 8.41 months ranging from 18 days to 1061 days, with one patient surviving 37.78 months after surgery. Survival is 26% in the first year, decreases to 8% in the second and is only 4% in the third year, which coincides with the results of large clinical trials. Statistically significant differences in the survival of both sexes, the laterality of the process, tumor localization on the lobes and different tumors'size ranging have not been established. There is a statistically significant difference in survival only in the 41-50 age group, which lives longer compared to the 71-80 and 81-90 age groups. The average survival is 47 days in the age group of 40 to 90 years.

The MGMT tumor study showed positivity in 17 patients /35%/, 8 men and 9 women. The age of both sexes is higher in the negative cases - average age 66.79 years in the negative against 62.42 years in the positive patients. The results lead convincingly to the conclusion that survival correlates with MGMT status and is increased in positive patients. The median survival in the patients with MGMT positive status was 15.7 months versus 4.65 months in MGMT negative patients with a statistical significance of $p < 0.0001$. In MGMT-positive tumors, first-year survival is approx. 59%, the second and third decreased to 24% and 12%. Its average value is higher for men - 9.02 months against 7.64 months for women.

The results of the **study of the immune response** were analyzed in great detail according to the ratio of leukocytes, platelets and monocytes to lymphocytes individually and in combination with established standards for intense immunity. They show statistically confirmed lower survival in patients with increased circulating monocytes – at value of MLR>0.45 the survival is 103 days versus 313 days in patients without change of this index. Statistically important differences are not found in the survival and the relation neutrophils/lymphocytes and platelets/lymphocytes. The combined study of all indexes is showed that patients with increasing of circulated monocytes and at least one of the other markers have significantly lower survival. With others words, disturbance relation between

monocytes and lymphocytes independently weather they are alone or are combined with other indicators reveal statistically significant shorter survival.

The expression of the Diaph3 antibody. The analysis of the reaction is accurate regarding the spread, intensity, localization in the individual components of the tumor - tumor cells, zones of growth, palisade structures, endothelium. The reaction is described by similar indicators in normal brain matter, meninges, vessels and reactive glia. Diaph3 expression was found in all 50 GBM tested. Changes in the immunoreactivity are described in detail in different areas of the tumor. The heterogeneity of the reaction with different intensity in the central /weak to absent/ and peripheral areas, incl. Scherer's growth phenomena. The high intensity in the palisades is correctly associated with the active migration of cells outside the necrotic zone. Strong immunoreactivity has been observed in angiocentric macrorosettes around large vessels, which the author explains by suggesting that "tumor cells first engage large vessels and then spread to their small branches". This phenomenon can be explained by the rearrangement of tumor cells around the vessels, which provide them with better conditions for survival, and this requires stimulation of the cytoskeletal structures in which Diaph3 participates; the penetration of tumor cells takes place at the level of the microcirculation. An expression gradient was reported in the transition zone with a non-involved parenchyma correlating with perifocal edema; in the same area, expression is moderate to high in single cells from vascular satellite, comparable to lower physiological expression in the small vessel endothelium. A high-intensity reaction is also found in the tract area (Fig. 5.23), which microscopically is apparently not involved. A sharp decrease in expression was found by approaching the cerebral cortex to a negative reaction in the subpial area. With the algorithm of robotic reading the author finds extremely high variability of positive cells in the tumor with a range of 12-96% and with an average percentage of expressing cells of 62.66%.

Depending on the percentage of Diaph3-expressing tumor cells, patients are divided into two groups: over 60% have high levels of expression, and less than 60% have low levels. The number of patients in both groups is close /26 versus 24/, and the difference in survival is statistically negligible /246 days in the group with high levels versus 267 days - in the group with low levels/. No significant differences were found between Diaph3 expression and demographics, tumor size, and location.

The discussion is purposeful and made in-depth by analyzing the extensive and up-to-date available literature on the subject. The author made comparison with his own data and in accordance with the obtained results, made logical conclusions and suggested a topic for prospective studies. I will review some important characteristics of GBM with a focus of them.

Based on a **mutation in IDH**, it was found that 9.1% were reclassified as mutant GBM, which is comparable to the ratio of non-mutant GBM in large statistical studies. This suggests that much of the clinicobiological parameters for GBM in the previous classification will not show significant deviations in the newly formed oncological unit defined as non-mutant GBM in the previous classification. It is known that the two forms of GBM mutant and non-mutant have been characterized in great detail with regard to age and sex differences.

The frequency of GBM is discussed on the base of previous author's examination. The author correctly notes that the incidence of GBM will not change dramatically if only 10% of

tne group drop out. According to his own data, the frequency is 2.03% per 100,000 people, significantly lower than the CBRITUS data /3.23 per 100,000, which puts us in the group of countries with a high rate of hidden morbidity. It is logical to expect that there will be differences in the two indicators due to the removal of the mutant GBM, which starts at an earlier age and has a longer survival. Dr. Stoyanov's observations confirm that the average age of onset of GBM is 65.3 years compared to the previous study - 59.18 years, and the average survival is significantly lower /8.41 months/ compared to CBITRUS data /16.9 months/. There were differences in the survival of men /15 months/ and women /25.5 months/ in the data of Ostrom and the findings in the group of Stoyanov, who did not find a significant difference in sexual survival.

Dr. Stoyanov finds a bad trend in our country compared to developed countries - lower incidence of GBM and lower survival. Although the analysis of GBM incidence is beyond the direct objectives of the study, from an epidemiological point of view, it is important to clarify the reason for these facts and this can be the aim of the future study of the national measure. In my opinion, the causes should be sought in organizational weaknesses, early diagnosis, and gaps in effective treatment and follow-up of patients. The registration of CNS tumors is not done properly - just not all gliomas are registered in the National Cancer Registry and the diagnosis is not always correct. These facts should provoke our healthcare system for more in-depth epidemiological studies and improved therapeutic behavior. However, it should be noted that the slightly higher survival of patients in the USA analyzed in CBRITUS does not significantly affect the quality of life and mortality in GBM, which is also high in developed countries.

A detailed study of the localization of GBM on lobes showed the same frequency for frontal and temporal lobes, a slight difference in parietal lobes and did not reveal, and is not expected, a relationship with survival. The lack of statistical regularity between the exact location and survival of patients is among the few publications devoted to this problem. Determining the exact location of the tumor in the lobes is of practical importance, as GBM rarely develops in the occipital lobes according to literature sources and Stoyanov's data. This fact may help the pathologist not to include GBM in the working diagnosis of occipital tumors. The results may provide an appropriate framework for further studies.

The prognostic significance of the size of the tumor resection is a debatable problem, which is also addressed by Dr. Stoyanov, but specific conclusions from his own material have not been made.

Of interest is the study of **the role of the immune response** according to the ratio of neutrophil leukocytes, platelets and macrophages to lymphocytes. The author finds statistical significance for the survival of the MLR index alone and in combination with other markers. The immune status of GBM has been the subject of many years of research and is manifested in many aspects, from the study of lymphocytic tumor stroma to the study of checkpoint molecules, incl. conducting immune therapy, as there is experience in our country.Rarely do pathologists focus on interpreting this type of clinical and laboratory research. Dr. Stoyanov's approach is innovative and in line with the desire to study tumors in many ways. The results are indicative of the role of these indices as prognostic factors. As these studies are relatively easy to perform, their introduction into practice can be stimulated as indicators of a systemic immune response.

Diaph3 research plays a central role to the dissertation for many reasons. The presumption that a tumor as heterogeneous as GBM may have damage to a gene associated

with basic cell function is original, as is the search for analogies with other highly malignant tumors. Studies on this gene in CNC diseases are negligible. The choice is not accidental, because there is a predictive element and the possibility of influencing therapy. In the discussion, the dissertation demonstrates its in-depth awareness of the predictive role of Diaph3 in the treatment of extracerebral malignancies. Clarifies the functional significance of Diaph3 as part of the group of proteins involved in the polymerization of actins and stabilization of microtubules, contact with cell growths and the cytoskeleton as a whole.

Diaph3 studies have shown a correlation with the prognosis, but in opposite directions - to improve or worsen the prognosis. The results of the dissertation reveal that Diaph3 is expressed only in some GBM /interspecific heterogeneity/, in part of the individual GBM /intratumoral heterogeneity/ ie. it is necessary to conclude that GBM is heterogeneous with respect to the studied marker. Another confirmation of its extreme heterogeneity appears. Second, the type of expression in GBM and in reactive gliosis is different, and therefore Diaph3 may serve as a DD mark between the two processes.

Of practical diagnostic value is the detection of strong expression of the Diaph3 antibody in peripheral areas or in areas of infiltration. According to the author, this fact is due to the predominant presence of stem cells in the growth zones. The extent to which there is a strict distribution of stem and mature cells and the extent to which there are preserved mature cells in the center of the tumor requires further evidence given the non-strict growth of malignant tumors.

One of the well-highlighted issues in the dissertation is the role of Diaph3 in the mTOR signal transduction pathway, which is related to the possible role of Diaph3 as a predictive marker for rapamycin and taxane therapy. In cell cultures, decreased expression of Diaph3 has been shown to be associated with decreased expression of mTOR-associated proteins. However, clinical trials for the treatment of GBM with rapamycin have led to conflicting results - about half of patients do not respond to treatment. Based on good information and his own results, Dr. Stoyanov makes a logical assumption that only patients with a high percentage of antibody-positive tumor cells would benefit from rapamycin.

This is a valuable guideline for the selection of suitable patients in prospective therapeutic studies. The role of Diaph3 in taxane therapy based on extraclinical studies that low levels of hypersensitivity to taxanes has also been discussed. Clinical studies for the treatment of mammary carcinoma with taxanes show that the loss of Diaph3 is associated with a longer survival in taxane therapy, i.e. it is a positive predictive factor in taxane treatment. There has been a careful suggestion that similar mechanisms work in GBM, and there is even more evidence that patients with GBM treated with taxanes have an increased survival rate.

Most of the conclusions are a logical synthesis of Dr. Stoyanov`s own results.

They present the most important facts, established in the study, a part of which are original. Only the first conclusion is postulated by definition by the WHO, 2021 and is not related to Dr. Stoyanov's research.

It was found that the primary tumor size and the exact location on the lobes and laterality have no prognostic role. The age factor is important as a statistical significance between the age groups 41-50 years and over 70.

MGMT status has a certain prognostic and predictive significance - the difference in survival between the groups with and without expression of MGMT is significant and applies

not only to the criterion of average median survival, but also to survival of the each of the first three years.

The statistical significance in the survival of patients with acute phase immune response is great, especially in the ratio of monocytes to lymphocytes in the blood picture, and the increase in this coefficient is a poor prognostic sign.

GBM has been shown to be a Diaph3 positive tumor, with marked heterogeneous positivity within the individual tumor. No correlation was found between expression levels versus prognosis and tumor size.

Dr. Stoyanov's conclusion that Diaph3 is a marker with strong predictive potential and can be used in the selection of patients for treatment with rapamycin and taxanes is reasonable, based on the author's wide awareness of its functional interactions, predictive role in target selection therapy in non-neurogenic malignancies, as well as his own results for its expression in half of the studied GBM.

It is of practical importance to accurately account for the deposition and intensity of Diaph3 expression (intense background staining and intense fibrillar response in astrocyte growths and lymphocyte nuclei) for the differential diagnosis with reactive gliosis.

Contributions. The first comprehensive study on the demographic and topographic parameters of glioblastoma multiforme in patients operated in a single neurosurgical institute in the country is conducted.

Modern methods for processing neuroradiological parameters and digitization of histological images have been introduced.

The correlation between the neuroradiological parameters in GBM and the volume analyzes of three-dimensional reconstructions was confirmed.

Full topographic map of tumor's distribution is made. The predilection sites of GBM development have been confirmed, regardless of gender, age and laterality of the process.

The average neuroradiological size of glioblastoma at the time of initial diagnosis is established.

The role of the systemic immune response as a prognostic factor has been confirmed.

The prognostic and predictive role of MGMT status for patients with GBM has been confirmed.

Immunohistochemical study of Diaph3 in glioblastoma multiforme has been introduced of full value and detailed characterization of the expression in tumors, growth zones and normal brain structures. Diagnostic role of the expression of Diaph3 is proved.

The role of Diaph3 as a potential predictive factor for the treatment of GBM with rapamycin and taxanes has been substantiated by extensive scientific information.

No correlation has been found between the expression of Diaph3 with survival and primary tumor size.

The importance of Diaph3 expression in the diagnosis of GBM and for differential diagnosis with reactive gliosis has been demonstrated.

Recommendations. The review presents some suggestions that would improve the quality of the dissertation, related to the formulation of the topic and purpose of the study, which can be clarified. It is recommended to study also the expression of MGMT and Diaph3 in diffuse astrocytoma, 4th degree / former IDH mutant GBM / to compare the results. Since the predictive effect of MGMT is analyzed, it is good to apply the treatment regimen to the

patients - was it the same for the positive and negative ones for MGMT. This is especially important given that patients who have a negative MGMT status treated with temozolomide also perform well. Given the interpretation of the results of the immune response as a predictive factor, information on whether any immune treatment has been performed based on these indicators would be useful, at least according to the literature. The extension of the study of the role of Diaf3 with genetic methods is highly recommended and is envisaged by the author.

In conclusion: Dr. Stoyanov's research is thorough, relevant and consistent with modern concepts of integrative diagnosis, based on analysis of clinical, laboratory, neuroradiology diagnostic, histological and molecular pathology studies. The importance of a number of important GBM parameters as prognostic or predictive factors has been clarified. The ratio between the parts of the work is proportional as the largest share is set aside for own results. The review is up-to-date with publications mainly from recent years. The goal and tasks are well formulated and correspond to the results and conclusions. The selection of materials is done strictly. The methodologies are described precisely, with IHC studies being particularly precise. The results are presented clearly, richly illustrated and supported by statistical analysis. The discussion shows the high awareness of the dissertation, its ability for comparative analysis and inferences that can be used for further research. In general, I support the correctness of the author's conclusions and contributions to his dissertation. It is important to note his self-criticism, as well as the outlined guidelines for future research.

The 13 publications made, of which 11 are leading authors, incl. in refereed journals, reveal his scientific activity, rich information, analytical thinking and striving to master modern methods aimed at solving basic problems in the study of glioblastoma multiforme.

I highly appreciate the work of Dr. Stoyanov, his systematic and consistent research, emphasized interest in innovation since his student years, his scientific achievements, which reveal prospects for even more prominent activities of national and international importance.

With great conviction I will give my positive vote and I recommend to the esteemed jury to support the choice of Dr. Georgi Stoyanov to receive the scientific educational grade of "Doctor of Medicine".

Reviewer:
Assoc. prof. Margarita Kamenova, MD