Diaph3 Underlines Tumor Cell Heterogeneity In Glioblastoma Multiforme With Implications For Treatment Modalities Resistance

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Abstract

Introduction: Glioblastoma multiforme (GBM) is the most malignant astrocytic tumor of the central nervous system (CNS) with a growth pattern mimicking that of the developing brain. Diaphanous homolog (Diaph3) has been established to play a role in CNS maturation and cancer progression as it is required both for cell migration and division. Furthermore, Diaph3 has been shown to play a role in malignant disease progression through hyperactivation of the EGFR/MEK/ERK in loss of expression and its overexpression correlating to hyperactivity of the mTOR pathway, both of which are with a well-established role for GBM. Herein we set out to establish the role of Diaph3 immunohistochemistry expression pattern in GBM.

Materials and methods: The study utilized a retrospective nonclinical approach. Results of Diaph3 expression were compared morphologically as well as for statistical significance with neuroradiological measured tumor size and patient survival.

Results: GBM samples showed a heterogeneous positive reaction to Diaph3, mean number of positive cells 62.66%, median 61.5, range 12-96%. Areas of migrating cells showed a strong reaction, while centrally located tumor areas were negative, as well as areas of submeningeal aggregation. Statistical analysis revealed no correlation with tumor size or patient survival.

Conclusion: Despite Diaph3 expression not correlating with survival and tumor size, there is an accumulating body of evidence that Diaph3 correlates with mTOR activity. Thus, Diaph3 can predict response to rapamycin and taxanes, clinical studies of which have shown promising, if mixed results in GBM.

Introduction:

Glioblastoma multiforme (GBM) is a high-grade malignant central nervous system (CNS) tumor with predominantly astrocytic differentiation, no isocitrate dehydrogenase (IDH) mutation, nor alteration in histone 3 (H3) as per the 2021 World Health Organization (WHO) classification of tumors of the CNS [1,2]. GBM is a common tumor and one of the most aggressive entities with a dismal patient prognosis [3,4].

At the time of presentation, the tumors have a very high tumor volume, occupying large parts of the cerebral hemispheres, often more than one lobe. Part of the growth phenomena and patterns of GBM are explained by the so-called secondary Scherer structures - patterns of growth and invasion of the brain parenchyma by the tumor cells, which mimic the embryonic and fetal process of formation and maturation of the CNS where the cells migrate through blood vessels and the cytoplasmic projections of mature cells [5–8]. It has recently been suggested that these are due to the reactivation of neural stem cell genes in the GBM stem cells (GBMsc) [3,5,9,10]. As a result, these allow the fast growth of the tumor through the CNS by means of axon and blood vessel migration and limit neuronal death, as they preserve the microenvironment [11,12]. Hence GBM presents clinically when the tumor has already reached an immense size and infiltrates a significant part of the CNS in contrast to metastatic disease, which results

in rapid neuronal death and presents clinically with local neurological deficit, when the tumors are with significantly smaller in size [13–16].

One gene which has been established to have a significant role in the formation and maturation of the CNS is diaphanous homolog (Diaph3), also referred to as aka mDia2 [17–19]. Diaph3 codes a protein that is key in the cytoskeleton stabilization required both for cell migration and division in the formation of the CNS. As GBMsc and mature GBM cells have been established to be mobile migrating cells and originate from progenitor cells of the CNS, the role of Diaph3 can be vital in understanding the behavior of this highly heterogeneous tumor entity.

Furthermore, Diaph3 has been established to have a role in the progression of hepatocellular carcinoma, breast malignancies and prostate carcinoma, with its loss correlating with hyperactivation of the EGFR/MEK/ERK signaling pathways and its overexpression correlating to hyperactivity of the mTOR pathway, both of which are with a well-established role for GBM [20–22].

The EGFR/MEK/ERK pathway is one of the most well-understood mechanisms of GBM tumorigenesis and has been a critical focus of pharmacological inhibition of GBM progression. However, GBMsc have been reported to be resistant to EGFR/MEK/ERK inhibition and responsible for poor treatment outcomes, despite initial tumor response to treatment.

This makes Diaph3 a novel candidate for identifying active migrating tumor cells in GBM and pharmacologically refractive ones.

Furthermore, several studies have suggested that the inactivation of Diaph3 plays a role in the ameboid migration of tumor cells in malignancies, including GBM [21,23,24].

This study aimed to establish the expression pattern of Diaph3 on immunohistochemistry in cases diagnosed with GBM, IDH-wildtype, WHO CNS grade 4 and establish a histogenic pattern related to tumor growth, patient survival and possible therapeutic implications.

Materials And Methods:

Ethical approval

All procedures included in the study adhered to the ethical standards of the Declaration of Helsinki 1964 and its seventh revision from 2013. The study was approved by the Committee on Ethics for Scientific Research, Medical University—Varna "Prof. Dr. Paraskev Stoyanov," — Protocol no. 93/21.05.2020.

Funding

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Study design and patient selection

A retrospective nonclinical approach for patient selection was chosen for the study. Inclusion criteria were the diagnosis of GBM, IDH-wildtype, WHO CNS grade 4 diagnosed in the period February 2018 to February 2021. Cases with repeat surgery in the time period for previously diagnosed GBM were excluded as well as those with a second malignancy. Neuroradiology parameters (location and size) were pulled from the hospital database to compare statistically with Diaph3 expression levels.

A control group of five cases with astrogliosis and 15 autopsy cases of non-pathologically affected CNS were included as controls for antibody expression and comparison.

Slide preparations

Tumor samples from the formalin-fixed paraffin-embedded (FFPE) tissue blocks used for the patients' initial diagnosis were used for the means of the study. Histological slides with a 6µm thickness were prepared and stained immunohistochemically (IHC) with a polyclonal rabbit IgG anti-Diaph3 antibody (catalog number HPA032151, Sigma-Aldrich, St. Louis, Missouri, United States). Antibody was diluted with antibody diluent to a ratio of 1:700.

Slide digitalization

The IHC slides were digitally scanned using a Leica Aperio AT2 (Leica Microsystems, Wetzlar, Germany) whole slide scanning microscope. Glass slides were scanned at 400x magnification using the precalibrated scanner settings.

Cell interpretation

Counting of positive cells was performed using the open-source software QuPath (University of Edinburgh, Edinburgh, Scotland, United Kingdom), using the precalibrated setting for automatic positive cell count for IHC slides. Cells were counted on the whole scanned slide and interpreted as a percent of positive tumor cells to total tumor cells present.

Statistical analysis

Data for patient survival was obtained from the central hospital information system and compared with the percent of Diaph3 positive tumor cells by the Kaplan-Meier method using the MedCalc version 19.7.1 (MedCalc Software Ltd, Ostend, Belgium) statistical software for biomedical analysis.

Results:

Diaph3 expression in healthy brain parenchyma

Diaph3 showed a positive cytoplasmic reaction in only a small fraction of cells in healthy brain parenchyma (Figure 1). The percentage of positive cells in the brain parenchyma, counted automatically, was less than 1% (Figure 1). Positive cells presented with astrocytic and individual ones with neuronal morphology and were located in the cerebral cortex, while white matter did not show cell positivity (Figure

1 A-B). Low-intensity reaction to Diaph3 was observed throughout the endothelial cells of the blood vessels of the brain as well as the meninges (Figure 1 C-D).

Diaph3 expression in reactive gliosis

In the reactive gliosis controls, a more intense background staining was observed and high-intensity fibrillar expression mainly in the cytoplasmic projections of cells with astrocytic morphology (Figure 2). There was also a low-intensity nuclear expression in individual cells with lymphocyte morphology located in the perivascular areas (Figure 2).

Diaph3 expression in GBM

In our cohort, all tumors showed a positive reaction to Diaph3 (Figure 3). The expression was moderate to highly intense, granular, and predominantly in the cytoplasm of tumor cells, with individual cells also showing a moderately intense nuclear expression (Figure 3A). In terms of diffusion, the reaction was heterogeneous. Individual areas of the tumor parenchyma located centrally in the tumor formation itself showed low intensity to no complete lack of reaction (Figure 3B).

Heterogeneity in terms of expression was also found in all types of Scherer figures with high to moderately intense expression in single parenchymal cells correlating with some of Scherer's secondary observed in these gradient zones. The most intense expression was observed in the areas of pseudopalisadic necrosis - primary Scherer figures (Figure 3C). Close to the physiological expression levels observed were seen in the glomeruloid vascular proliferation endothelial cells (Figure 3D). In the border zone of transition from tumor parenchyma to the grossly non-involved brain parenchyma, correlating with the neuroradiological phenomenon of perifocal edema, an expression gradient was observed towards the physiological levels of expression levels of the normal parenchyma (Figure 3E). Of Scherer's secondary figures, expression measurement was not possible only in the case of the phenomenon of neuronal satellitosis, given the presence of Diaph3-positive neurons in healthy brain controls. Moderate to high-intensity expression in single cells in the border zone was observed in the areas of vascular satellitosis, where the expression of Diaph3 in spreading tumor cells was higher than the physiological expression of small blood vessel endothelial cells (Figure 3F). Migrating tumors cells in the tractal aggregation pattern of growth had identical characteristics of antibody immunoexpression, with parallel arrangement of longitudinally located small cells with moderate to high-intensity immunoreactivity (Figure 3G). Significant gradients in antibody expression were observed in tumors that grossly and histologically invaded the cerebral cortex to its border with the meninges. Of particular interest here is that the described gradient is towards complete loss of immunoexpression in the areas of submeningeal tumor cell aggregation (Figure 3H). Beyond the diagnostic criteria and growth phenomena, strong immunoreactivity for the antibody was observed in macrorosettes around large blood vessels with no tumor cell invasion towards the vascular wall (Figure 3I). This phenomenon is most likely related to vascular satellitosis, with tumor cells first engaging large vessels and then spreading to their small branches (Figure 3 F and I).

On the background of this strong heterogeneity of Diaph3 expression in different areas of the tumor, using the algorithm for automatic machine-based cell counting of positive cells, extremely high variability in the ratio of positive cells was seen in the cohort, with a mean value of 62.66%, median 61.5, range 12-96%.

Tumor size

Data from in-hospital neuroradiology was present for 45 patients in the cohort, with a mean tumor size of 50.51mm, range 20-76mm. A total of 25 cases had the tumor located in the left hemisphere, with the remaining 20 having a right-sided tumor. Lobar predilection revealed that 13.33% (n=6) of the tumors are located in the left frontal lobe and 11.11% (n=5) in the right one; in temporal lobe tumors, 17.78% (n=8) are located on the left and 20% (n=9) on the right; in parietal lobe tumors, 17.78% (n=8) are located on the left and 11.11% (n=5) on right; in occipital lobe tumors 6.67% (n=3) are located on the left and 2.22% (n=1) on the right.

Patient survival

The mean survival of the cohort is 255.96 days (8.41 months), with five patients still alive. The survival range varies from 18 days to 1150 days. One-year survival rates are 26%, two-year survival rates are 8%, and three-year survival rates are 4%.

Statistical analysis

Regarding the percentage of expressing Diaph3 tumor cells, the cases in the cohort were divided dichotomously, with an expression of 60% as the cut-off value. Thus, the two groups with a low and high expression included the closest number of patients - n=26 for those with high expression levels and n=24 for those with low expression levels. Analysis of the significance of expression levels on the survival of patients Kaplan-Meier survival analysis no correlation ($p > 0.05$), with a mean life expectancy in the high expression group of 246.19 days versus 267.17 days in the low expression group (Figure 4A). No correlations between the levels of Diaph3 expression and age, sex, tumor location, and neuroradiologically determined tumor volume were found by correlation analysis, $p > 0.05$ (Figure 4B).

Discussion:

Based on the expression levels as seen by our results, Diaph3 as a marker with clinical significance can be used to distinguish between reactive gliosis and tumor proliferation in the central nervous system in cases of insufficient biopsy material. Furthermore, as a molecule with multifaceted cellular interaction, it can signal different cellular switches, with implications for pharmacological tumor growth control.

Diaph3 is a protein belonging to the group of formins, which in turn are a cytoplasmic group of proteins involved in the polymerization of actin filaments in the processes of proliferation, migration and cell division, and they are also an essential interface of signal transduction and intracellular regulation [25,26].

In the human body, the formin group is composed of a total of 15 proteins divided into seven subfamilies, many of which are co-expressed in a large number of tissues [27]. All formins have two distinct domains: the formin (FH) 2 homology domain, which catalyzes the polymerization of actin, and the FH1 domain, which binds profilin to provide monomeric actin to the FH2 domain. Other regions and domains may differ between subfamilies by participating in regulatory mechanisms or specific interactions with other proteins. In addition to regulating the actin cytoskeleton, formins bind to microtubules through the FH2 domain and regulate the acetylation and stability of microtubules and their alignment with actin filaments [28].

Members of the human diaphane formin subfamily, which include Diaph1-3, are regulated by the interaction of the diaphane inhibitor domain (DID) at the N-terminal end and the diaphane autoregulatory domain (DAD) in the C-terminal region. The transition between closed/inactive and open/active states is mediated by the interaction of ATPases from the Rho family with DID, which releases its interaction with DAD. Other forms with similar regulation are associated with Disheveled activators of morphogenesis 1 and 2, formin-like 1-3 and FH1/FH2 domain-containing 1 and 3 [28].

The functions of Diaph3 are largely characteristic of most of the proteins in this family, as its physiological role is to mediate the polymerization of actin molecules, stabilize microtubules, and also has a role in contact with profilins - membrane proteins, thus participating in the structural formation of mobile cell projections such as kinocilia and pseudopods [29]. Although less well studied, the protein also plays a role in cell division, participating in the stabilization of the mitotic spindle and the cytoskeleton as a whole [18].

Functional changes in the inactivation of Diaph3 in stem cells during embryonic and fetal development are associated with mitotic errors, leading to depletion of neuronal progenitors and disturbances in the formation of epithelial cell cilia [29,30]. At this stage, there is limited data on the role of the protein in diseases of the nervous system, the strongest of which exist for the autistic spectrum of diseases in loss of expression and autosomal dominant auditory neuropathy in overexpression [31].

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 acquire plastic properties, which allows them to move in the extracellular matrix without the need to destroy it (by proteolysis or induced necrosis) or adhere to it, thus malignant cells more easily and quickly enter the lymph and blood vessels, which favors the metastatic potential of tumors [21,32]. In a large group of histogenetically different tumors, the loss of Diaph3 is a time-related process - observed as a heterogeneous phenomenon with tumor advancement [21].

Although it is associated with tumor progression and favors metastatic potential, the phenomenon of Diaph3 loss may have a potential prognostic effect on the pharmacological response of tumors, given its stabilizing role in the cell's cytoskeleton. Despite conflicting data on the role of Diaph3 in malignant tumors - high expression is associated with a favorable prognosis in some and unfavorable in other

groups, most likely due to multifaceted mechanisms of tumor progression, with some playing a pivotal role, while others are a side participant in tumor progression [33].

One of the proposed mechanisms is the role of Diaph3 in the mTOR signal transduction pathway, where the loss of expression is associated with the inactivation of this cascade [33]. mTOR is a 289 kDa serine/threonine-protein kinase located in two structurally and functionally distinct multiprotein complexes known as mTORC1 and mTORC2.

The role of mTORC2 in the processes of gliomagenesis was demonstrated by a model in *Drosophila*, where tumorigenesis was induced by hyperactivation of EGFR, RAS and PI3K [34]. RICTOR and mSIN1 function loss in this model were observed to prevent tumor formation. In addition to its role in tumor induction, mTORC2 activation is also responsible for the growth and progression of glioblastoma multiforme, with Gulati describing that treatment of glioblastoma multiforme cell lines with rapamycin not only results in a time-dependent reduction in S6K phosphorylation but also thus causing a paradoxical increase in AKT phosphorylation on serine 473, which is known to be responsible for cell proliferation [35].

mTORC2 is also involved in inducing the effect of Warburg, a metabolic process by which tumor cells metabolize glucose by aerobic glycolysis, also in the presence of sufficient oxygen levels to provide macromolecular demand for fast-growing cells [36,37]. Indeed, by stimulating the phosphorylation of AKT on serine 473, mTORC2 triggers the expression of the glucose transporter type 4 (GLUT4) and the activation of the glycolytic enzyme hexokinase 2 (HK2) and phosphofructokinase-1 (PFK-28). It has also been found that acetyl coenzyme A (acetyl-CoA), derived from glucose and lactate metabolism, is used by GBM cells to induce RICTOR acetylation, which activates mTORC2; this mechanism creates an autoactivation chain through which mTORC2 triggers cell proliferation and growth, bypasses growth factor-activated upstream signaling and makes tumors resistant to receptor tyrosine kinase inhibitors [37]. In addition, mTORC2 has been shown to confer resistance to the alkylating agent cisplatin through an activated B-cell kappa-light-chain-enhancer (NF- κ B) enhancer in an AKT-independent manner [37,38]. In the same study, Tanaka demonstrated that NF- κ B signaling was enhanced in rapamycin-treated glioblastoma multiforme cell lines and that inhibition of both mTORC1 and mTORC2 with the mTOR kinase inhibitor PP242 sensitized EGFRvIII mutant tumors to cisplatin-induced cellular death [38].

Results published by Wan demonstrate that high Diaph3 expression is positively correlated with mTOR, and cell culture analysis showed that decreased Diaph3 levels decreased the expression of mTOR-related proteins p-AKT, mTOR and p-p70s6k and increased PTEN expression - tumor suppressor cascade [33].

Due to the potential for pharmacological effects of these mechanisms, several preclinical studies have investigated the effect of rapamycin (trade name sirolimus) in treating patients with histologically verified glioblastoma [39–41]. Using cell cultures and xenographic models of glioblastoma, Arcella reported a reduction in tumor volume of up to 95% and almost twice as prolonged survival with rapamycin therapy through induction of autophagy [42]. A clinical study on the effect of rapamycin in the treatment of patients with glioblastoma multiforme published by Cloughesy showed a good response in some

patients, with a significant reduction in cell proliferation, no significant side effects, with adequate intratumoral drug concentration [43]. No effect was observed in almost half of the patients, as well as no development of resistance to therapy over time [43]. As seen by the dichotomous separation into low and high expression groups of GBM in our study, this may indicate a role for Diaph3 expression levels as predictors of tumor response to rapamycin treatment. Given the substantial heterogeneity of Diaph3 expression in our cohort and the mechanisms described so far, it follows that only patients with a high percentage of antibody-positive tumor cells would benefit from rapamycin. Additional evidence for this correlation is available from data on patients with tuberous sclerosis who are predisposed to developing multiple tumors, including astroglial ones, in which sirolimus therapy has a preventive and antitumor effect [44].

Another important proposed role of Diaph3 in pharmacological therapy of malignancies is related to low levels of Diaph3 and sensitivity to taxanes and other drugs acting on microtubules due to cytoskeletal destabilization [19,22,30]. According to data published by Morley, low-expression Diaph3 cells show increased sensitivity to these taxanes, while other members of the Diaph1 and Diaph2 families do not show such a correlation, with Diaph1 showing an inverse correlation - increased resistance with decreased expression [22].

Analysis, again by Morley, shows that several randomized clinical trials for treating mammary carcinoma with taxanes report that low levels of intratumoral expression or loss of Diaph3 are associated with a better prognosis in taxane therapy [22]. Similarly, data from the same report show that low levels of DIAPH3 mRNA correlate with a longer time to relapse with the same therapy [22].

Following the mechanisms described so far, it follows that taxanes would benefit patients resistant to rapamycin therapy in loss of expression of Diaph3.

Study limitations and future directions

Small cohort size

The current study includes a small number of patients with a relatively low survival rate. Furthermore, there is also a need for comparison between GBM cases and other CNS tumors with astroglial differentiation to establish a Diaph3 expression correlation pattern regarding the nosological unit and WHO CNS grade.

Need for molecular analysis verification

As our study focuses on expression patterns of Diaph3 on immunohistochemistry, future studies into the topic will need molecular verification of the expression pattern observed as well as molecular correlation with the discussed intracellular mechanisms with implications for pharmacological tumor treatment.

Conclusion:

As seen by our results, Diaph3 is expressed heterogeneously in GBM, with a specific expression pattern distinguishable from that of the normal brain parenchyma and reactive gliosis. Furthermore, based on its intracellular interactions, Diaph3 can be used as a clinical marker not only on the differential diagnosis between normal brain parenchyma, reactive gliosis, and tumor proliferation but also as a marker for prediction to rapamycin and taxane treatment, clinical studies into which have shown promising, if varied results.

Declarations:

Financing: Medical University - Varna Scientific Fund, grant number 19010 and National scientific fund - young researchers (Republic of Bulgaria) grant number 2990/07.06.2021

Ethical approval: Medical University - Varna committee on scientific ethics approval protocol number 93/21.05.2020

Conflict of interest: the authors declare no conflicting interests

Author contributions: GSS conceptualized the study, analyzed preliminary findings, performed the statistical analysis and wrote the initial draft with figure design; EL collected patient data, revised findings and statistical results; RG analyzed preliminary findings and assisted with statistical analysis and figure design; DD and LP assisted in patient selection and data analysis; AK and BDI assisted in statistical analysis and finding interpretation; PG revised the findings, manuscript and figures as well as approved the final version of the manuscript; all authors have read and approve the final version of the manuscript

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Figures

Figure 1

Expression of Diaph3 in normal brain parenchyma.

A: Low to medium-intense cytoplasmic reaction in cortical cells with astrocytic morphology, original magnification x200; B: lack of reaction in the white matter, original magnification x400; C: Low-intensity cytoplasmic reaction in vascular endothelial cells, original magnification x400; Low-intensity cytoplasmic reaction in meninges, original magnification x100.

Figure 2

Expression of Diaph3 in reactive astrogliosis, original magnification x200.

Figure 3

Expression of Diaph3 in GBM.

A: high-intensity expression, original magnification x200; B: low intensity to lack of expression, original magnification x200; C: high-intensity expression in cells surrounding pseudopalisadic necrosis (primary Scherer figures), original magnification x20; D: high intensity expression of tumor cells surrounding glomeruloid vascular proliferation, with a slightly higher than the physiological expression observed in vascular endothelial cells, original magnification x200; E: gradient towards loss of expression in the tumor periphery, original magnification x40; F: moderate to high expression in perivascularly spreading tumors cell in the border zone, original magnification x400; G: moderate to high expression in periaxonal (tractal) spreading tumors cell in the border zone, original magnification x200; H: gradient towards loss of expression in the submeningeal aggregation, tumor periphery, original magnification x20; I: high-intensity expression in large vessel perivascular aggregation of tumor cells, original magnification x100.

Figure 4

Correlation between Diaph3 expression levels in GBM.

A: Kaplan-Meier survival analysis, $p > 0.05$; B: heatmap for expression levels and tumor size, $p > 0.05$.