CIRCULATING PROTEIN BIOMARKERS OF GLIOBLASTOMA: METHODOLOGIC APPROACHES AND PERSPECTIVES OF DEVELOPMENT

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Glioblastoma multiforme (GBM) is the most aggressive primary brain tumor, characterized by an extremely poor prognosis. Difficulties in diagnostics and monitoring this disease stimulate the search for minimally invasive approaches. In this context liquid biopsy is considered as a promising approach. This review analyzes results of recent studies aimed at identifying circulating protein biomarkers of GBM in plasma and serum. These biomarkers include cell-free circulating plasma proteins and proteins of extracellular vesicles (EVs). Special attention is paid to the results obtained using both immunochemical methods and mass spectrometric approaches for identification of protein biomarkers, which have been summarized here as a list of identified potential diagnostic and prognostic biomarkers. Analysis of the literature demonstrates that proteomic analysis focused on the plasma EV fraction significantly expands the possibilities for identifying biomarkers for noninvasive GBM diagnostics and monitoring.

Keywords: glioblastoma; circulating biomarkers; extracellular vesicles; proteomics

DOI: 10.18097/PBMCR1637

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant brain tumor, accounting for 48.6% of primary malignant tumors of the central nervous system (CNS) and 14.5% of all CNS tumors [1]. Histologically, GBMs are malignant tumors of astrocytic origin. A distinctive feature of GBM is the heterogeneity of the tumor tissue structure: foci of necrosis alternate with dense regions of proliferating tissue, as well as with regions of cystic degeneration and hemorrhage. The tumors themselves are typically solitary, irregular in shape, and arise in the white matter of the brain [2]. GBM is currently characterized by an extremely poor prognosis and high mortality (median survival is 15 months) [1]. In most cases, a multimodal approach is used to treat GBM. This includes maximal safe surgical resection of the tumor, allowing (among other things) histological and genetic examination of the tumor tissue. The surgery is followed by radiotherapy, accompanied by a course of chemotherapy with temozolomide. Tumor treating fields (TTF) therapy can also be used as an adjuvant therapy [3]. Magnetic resonance imaging (MRI)-based methods are widely used in GBM diagnostics: for example, using perfusion MRI (perfusion weighted imaging) it is possible to detect increased blood flow in tumor tissue, while MR spectroscopy records an increase in the choline/N-acetylaspartate and choline/creatinine ratios, correlating with tumor progression [4]. However, these techniques have a number of limitations: MRI does not always distinguish GBM from other neoplasms (lower-grade gliomas, metastases from other tumors) or pathological conditions of brain tissue (inflammation, edema, hemorrhage), as well as tumor recurrence from pseudoprogression [5]. In this regard, molecular biology methods have become widely used for the search for markers of a specific disease and identification of the molecular mechanisms underlying disease occurrence and development [6]. The most relevant material for such studies is tumor tissue, but its collection is a highly invasive procedure [7].

Over the past 20 years, techniques collectively known as “liquid biopsy” have become widely used. This approach identifies tumor markers in body fluids, particularly plasma or serum, which can be sampled repeatedly with significantly reduced risk to the patient's health [8]. The tumor biomarkers detected in such material are commonly referred to as circulating biomarkers. These include, in particular, cell-free tumor DNA (cfDNA) and miRNA (microRNA), circulating tumor cells, as well as proteins and extracellular vesicles (EVs) [5, 9].

The presence of GBM markers in the blood is associated with increased permeability of the blood-brain barrier (BBB) in tumor tissue, observed in both GBM and benign brain tumors [10, 11]. Possible mechanisms causing increased BBB permeability in tumor tissue include: increased expression of vascular endothelial growth factor (VEGF), which stimulates the formation of “immature” vessels with highly permeable walls,

and physical disruption of the BBB integrity by tumor cells (in particular, due to the displacement of astrocytic endfeet) [12, 13]. In addition, a reduced content of tight junction proteins is observed in the endothelium of tumor vessels. The endothelium demonstrates structural heterogeneity: among the "microvascular populations", there are both capillaries of normal structure and fenestrated capillaries, as well as capillaries with gaps between endothelial cells [14]. It is also noteworthy that the results of studies of *in vitro* BBB models demonstrate an increase in BBB permeability during GBM cell differentiation [15]. Protein biomarkers are of interest to researchers because they are directly involved in most molecular processes and signaling pathways associated with tumor initiation and development.

1. FREE PROTEIN BIOMARKERS IN BLOOD

1.1. Search for GBM Protein Biomarkers Using Immunochemical Methods

Immunochemical methods have become widely used in the study of biological fluids due to their high sensitivity and specificity, as well as the relatively small amount of biomaterial required. Methods such as enzyme-linked immunosorbent assay (ELISA) are considered as the gold standard for protein quantification in clinical trials [16]. This explains the widespread use of such methods to search for GBM biomarkers in body fluids.

For example, Carlsson et al. investigated applicability of panels of single-chain variable fragment antibody (scFv) for protein profiling of blood plasma samples from GBM patients undergoing immunotherapy [17]. The authors identified proteins, vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8), which were upregulated in samples obtained from GBM patients compared to healthy individual. VEGF has been shown to be involved in such key processes in the development of GBM as angiogenesis and proliferation of tumor cells (including tumor stem cells); this determines the prevalence of therapeutic approaches aimed at suppressing the activity of this protein [18, 19]. IL-8 is a proinflammatory chemokine, which is involved in the development of certain types of malignant tumors via CXC chemokine receptors [20–22]. In addition, the same study found that the levels of IL-12 and IFN-γ cytokines, responsible for the development of the cellular immune response, were increased in the blood plasma samples from GBM patients undergoing immunotherapy with the longest survival (more than 400 days).

In the work of Elstner et al., the concentrations of 13 proteins selected by the researchers were measured in the blood serum of GBM patients and healthy individuals using ELISA [23]. Among the proteins with differences in concentration, BMP2, CXCL10, and HSP70 were identified. For these proteins the researchers determined threshold concentrations that allowed them to distinguish GBM patients from healthy individuals within the studied cohort with a sensitivity of 96% and a specificity of 89%. In addition, the serum concentrations of TSP1, HSP70, and IGFBP3 proteins were proposed for survival prediction; the longer survival (more than 15 months after tumor removal) was observed in two cases: 1) TSP1 ≥ 84.08 μg/ml; 2) HSP70 ≥ 0.351 ng/ml and IGFBP3 ≥ 4.122 mg/ml. Interestingly, TSP1, HSP70, and IGFBP3 proteins were also detected in tumor tissue based on immunohistochemical analysis, which could indicate their tumor origin.

Jung et al. demonstrated that the concentration of glial fibrillary acidic protein (GFAP) in the plasma of patients with GBM was significantly higher than in patients with other types of brain tumors and also in healthy donors. Furthermore, the concentration of this protein correlated with such GBM parameters as tumor volume and the volume of necrotic tumor area [24]. The increased concentration of this protein in serum of GBM patients as compared to healthy donors was confirmed by later publications, which also determined the threshold concentration of GFAP (≥ 0.01–0.014 ng/ml) distinguishing GBM patients with healthy individuals and patients with other brain tumors [25, 26].

Iwamoto et al. [27] and Bernardi et al. [28] studied possible relationship between concentrations of chitinase-3-like protein (CHI3L1; YKL-40) in the serum of glioma patients and key indicators, such as survival and the presence of radiographic signs of a tumor. Both research groups found an association between higher concentrations of the YKL-40 protein in the serum and shorter survival. In addition, higher concentrations of YKL-40 were associated with the presence of radiographic signs of a tumor in patients with GBM and anaplastic glioma. The authors proposed this protein was as a promising prognostic biomarker. YKL-40 plays a role in tumor tissue vascularization by stimulating angiogenesis and vasculogenic mimicry mediated by VEGF and its receptor (VEGFR 2) [29–31]. The close association of YKL-40 with tumor vascularization has attracted much interest in this protein as a potential prognostic factor and a therapeutic target. Elevated plasma concentrations of this protein are associated with decreased survival in patients treated with bevacizumab [32]. Furthermore, the use of anti-YKL-40 antibodies in combination with ionizing radiation has been shown to reduce tumor vascularization and proliferation and improve survival in animal models [33].

Using ELISA Lin et al. [34] assessed the diagnostic and prognostic value of TIMP-1 (tissue inhibitor of metalloproteinase 1) and MMP-9 (matrix metalloproteinase 9) proteins in plasma

of patients. Based on the results obtained, TIMP-1 was proposed as a potential biomarker of GBM, since its quantitative content in the blood plasma of patients was associated with such parameters as the stage of the disease and patient survival. Ramachandran et al. studied the content of MMP-2 and TIMP-1 proteins in GBM tissue biopsies using immunohistochemical methods [35]. The researchers showed that higher MMP-2/TIMP-1 ratios were associated with a less favorable prognosis, thus suggesting the diagnostic potential of TIMP-1 determination. However, it should be mentioned that Aaberg-Jessen et al. did not find significant differences between plasma concentrations of TIMP-1 in GBM patients and healthy controls [36]. In any case, TIMP-1 and related proteins have been shown to suppress GBM cell migration *in vitro*, suggesting a possible dysregulation of its activity during GBM development [37].

An antibody-array-based proteomic approach employing 656 antibodies was also used to identify candidate protein biomarkers of GBM in plasma [38]. In plasma samples of GBM patients, the researchers identified three proteins with higher levels (FTL, GNAO1, and S100A9) and eight proteins with lower levels (FADD, CDKN1B, ICAM1, MLH1, MMP11, POLG, SKP1, ST8SIA1) than in healthy donors. Functional annotation of the identified proteins has shown that they are involved in processes such as T-cell signaling, immune response, cell adhesion and migration, cell cycle control, and apoptosis. Among the identified proteins, an increased concentration of GNAO1 (the α-subunit of the guanine nucleotide binding protein G(o)) was significantly associated with longer patient survival (> 365 days). Interestingly, an association between increased GNAO1 concentrations in tumor tissue and a more favorable prognosis has been shown for hepatocellular carcinoma [39]. A possible mechanism of action for this protein may be the induction of neuronal differentiation of tumor stem cells, as demonstrated by Sun et al. [40].

1.2. Search for GBM Protein Markers Using Mass Spectrometric Approaches

One of the characteristic features of mass spectrometric (MS) methods differing them from immunochemical approaches is the ability to perform a shotgun proteomic analysis without limiting the researcher to a specific set or panel of proteins studied; this introduces an additional degree of freedom in the search for potential disease biomarkers [41, 42].

Such analysis of serum samples from GBM patients, conducted by Gollapalli et al. [43], revealed quantitative differences for 55 proteins between samples obtained from GBM patients and healthy controls. The most significant quantitative differences recognized in this set were confirmed by Western blot analysis for four proteins: HP, CP, RBP4, and HPX. Functional annotation using DAVID, PANTHER, and IPA tools has shown that the quantitatively different proteins are involved in the complement and coagulation cascades, as well as immune signaling.

A search for proteomic markers of GBM in patient plasma samples was conducted by Miyauchi et al. [44] using shotgun SWATH-MS and targeted MS analysis for absolute protein quantification. SWATH-MS analysis was performed using a spectral library generated from the results of data-dependent acquisition (DDA) analysis of whole and fractionated plasma samples, tumor tissue, and cyst fluid obtained from GBM patients. This search resulted in identification of five proteins (LRG1, C9, CRP, SERPINA3, APOB) characterized by increased content in the blood plasma samples of GBM patients and three proteins (GSN, IGHA1, APOA4) with lower content in the GBM patients than in healthy controls. According to the results of targeted MS analysis, the statistically significant quantitative differences were confirmed for the proteins LRG1, C9, and SERPINA3, as well as for GSN, IGHA1, and APOA4. In addition, the researchers showed a positive correlation between the concentrations of proteins LRG1, C9, and CRP in plasma and tumor size, as well as between the concentration of GSN and overall survival. In subsequent studies, the researchers focused on leucine-rich α2-glycoprotein 1 (LRG1). The results of the studies showed that the content of this protein significantly increased in GBM tissue compared to gliomas of lower grades [45]. Furthermore, LRG1 suppressed tumor cell invasion *in vitro* and it was also associated with peritumoral inflammation, which correlated with tumor pseudoprogression [46].

Kumar et al. detected elevated levels of the α2 chain of haptoglobin (HP) in the serum of GBM patients compared to healthy individuals. This protein influenced glioma cell migration *in vitro*, and its increased levels stimulated tumor growth and reduced survival in mouse models [47]. Considering its diagnostic value in GBM, some researchers pay particular attention to the quantitative content of various proteoforms of this protein; in their viewpoint, one of the proteoforms of unprocessed HP (zonulin) is particularly interesting as a potential biomarker [48, 49].

Studies conducted on serum samples from patients with various types of gliomas have shown that reduced concentrations of the fetuin (AHSG) in patient serum are associated with decreased survival, and therefore this protein can be used to predict the course of the disease. Although AHSG is involved in signaling pathways involved in GBM growth and invasion [50], the authors note that AHSG alone cannot be considered a GBM-specific biomarker, since its serum concentration is reduced in patients with other brain pathologies, as well as in patients with neoplasms not originating from the central nervous

system [51]. Petushkova et al. considered AHSG, identified in plasma samples of GBM patients and healthy individuals in the context of post-translational modifications [52]. The researchers showed that one of the products of AHSG tryptic hydrolysis, a peptide containing phosphorylated threonine-158, was detected in samples obtained from GBM patients, but was not detected in the blood plasma samples of healthy controls.

However, it should be noted that the discussed above proteins HB, YKL-40, and AHSG were also investigated by van Linde et al., and these authors did not confirm any relationship between blood protein concentrations and disease prognosis [53].

Proteomic approaches using MS methods generally allow for the search and quantitative evaluation of potential biomarkers among thousands of identified proteins in biological samples. However, they have significant limitations in case of analysis of such complex biosamples as serum and plasma. These limitations are related to the low concentration of clinically significant proteins combined with a wide dynamic range of the plasma proteome (more than ten orders of magnitude [54]), partly due to the presence of so-called major proteins in the analyzed biomaterial. One of the most technically simple methods for solving this problem is the depletion of major proteins from plasma or serum samples by means of commercially available kits. A similar method was used in the study by Gautam et al. [55], in which shotgun analysis of pooled plasma samples obtained from GBM patients and healthy donors identified a set of 61 quantitatively different proteins. Functional annotation revealed the presence of acute phase proteins, as well as components of the complement and coagulation cascades, in the set. In addition, significant quantitative differences in three proteins (FTL, S100A9, and CNDP1) were confirmed using ELISA in individual plasma samples obtained from GBM patients and healthy donors. Among these proteins, the calcium-binding protein S100-A9 (S100A9) is of particular interest; it stimulates migration and proliferation of GBM cells *in vitro* and mediates migration of anti-inflammatory M2 macrophages, which contribute to the formation of the immunosuppressive tumor microenvironment [56]. A list of potential circulating biomarkers of GBM identified in the considered publications is presented in Table 1. The predominance of participants in the complement, coagulation, and immune signaling cascades among the quantitatively distinct proteins given in these studies may indicate specific molecular interactions between the tumor and key body systems. However, these results, combined with the relatively small number of identified proteins in these publications (approximately 200–300), may also point to the problem of limited sensitivity of MS methods for plasma and serum analysis, which still remains relevant even with depletion of major proteins.

2. PROTEIN BIOMARKERS IN EVs

To address the above problem, researchers are increasingly enriching the study material with low-copy proteins by isolating EVs. EVs are lipid bilayer-bound structures secreted by all living cells of the body; they represent two subpopulations: microvesicles separated from the plasma membrane, with a diameter of 100–1000 nm, and exosomes formed by invagination of the endosomal membrane, with a diameter of 40–150 nm [57]. EVs represent an attractive target for searching for protein profiles associated with various diseases; they are secreted by all living cells of the body, reflect their molecular composition, and are also present in biological fluids [58]. Good evidence exists in the literature, that EVs are involved in such processes as the cellular response to stress, tissue regeneration, and immune reactions [59–61]. The role of EVs in the occurrence, development and metastasis of tumors has also been demonstrated [62–65]. Furthermore, it has been shown that even in healthy brain tissue, EVs are able to penetrate the BBB formed by vascular endothelial cells, pericytes, and astrocyte endfeet [66, 67], while malignant tumor cells are characterized by increased EV secretion compared to healthy cells of the body [68]. These factors make EV a promising biological material for studying the molecular processes underlying various diseases. In light of the increased interest of the scientific community in EV as a promising material for studying proteomic profiles associated with various diseases, special attention in the world literature is paid to methods for EV isolation and applicability of these methods to different types of biological material [69–73]. Currently, the most relevant methods for EV isolation include precipitation using hydrophilic polymers, ultrafiltration, differential ultracentrifugation, density gradient ultracentrifugation, size-exclusion chromatography (gel filtration), and immunoaffinity methods [74].

2.1. Search for EV-associated GBM Markers In Vitro

In GBM studies, cell lines used for isolation of the EV fraction and characterization of its proteome often serve as model objects. For example, Lane et al. [75] used for EV isolation from culture medium various GBM cell lines representing different tumor cell types (in particular, LN18, U118, U87), as well as tumor stem cell lines G166 and GS090. According to results of proteomic EV analysis, the studied cell lines could be subdivided into two groups including LN18, U87, U118 cells in one group and G166 and GS090 cells in the other group. In the tumor stem cell lines G166 and GS090, proteome enrichment analysis identified functional groups of proteins associated with normal metabolism, while for other cell lines, groups related to cellular signaling associated with GBM progression predominated. Moreover, in the EV fraction isolated *in vitro* from the most

Table 1. Potential free protein markers of GBM identified in human plasma and serum. Proteins identified in several independent studies are shown in bold

Proteins		Material	Methods of the study	References
VEGF, IL-8	Associated with the presence of GBM	Plasma	Immunofluorescence analysis (*scFv* panels)	[17]
IL-12, IFN-γ	Associated with longer survival			
BMP2, CXCL10, HSP70,	Associated with the presence of GBM	Serum	ELISA	[23]
TSP1, IGFBP3, HSP70	Associated with survival			
GFAP	Associated with the presence of GBM	Plasma	ELISA	[24–26]
HP	Associated with the presence of GBM	Serum	Mass spectrometry (MALDI-TOF), ELISA	[47]
YKL-40	Associated with lower survival	Serum	ELISA	[27, 28]
AHSG	Associated with longer survival	Serum	Mass spectrometry (MALDI-TOF), ELISA	[51]
TIMP-1	Associated with disease stage and survival	Plasma	ELISA	[34]
FTL, GNAO1, **S100A9**	Upregulated in GBM patients *GNAO1* associated with longer survival	Plasma	ELISA	[38]
FADD, CDKN1B, ICAM1, MLH1, MMP11, POLG, SKP1, ST8SIA1	Downregulated in GBM patients			
HP, CP, RBP4	Upregulated in GBM patients	Serum	Mass spectrometry (MALDI-TOF), immunoturbidimetry, immunoblot	[43]
HPX	Downregulated in GBM patients			
LRG1, C9, SERPINA3	Upregulated in GBM patients	Plasma	Mass spectrometry (SWATH-MS, QTAP)	[44]
GSN, IGHA1, APOA4	Downregulated in GBM patients			
LRG1, C9, CRP	Associated with tumor size			
GSN	Associated with survival			
The set of 61 proteins, including **FTL**, **S100A9**, CNDP1		Plasma	Mass spectrometry (iTRAQ DDA), ELISA	[55]

invasive GBM lines LN18 and U87, the authors identified the proteins WNT5a, TGFBI, SERPINE1, and GDF-15, which could be considered as potential markers of malignancy. Other studies have shown that increased WNT5a content in primary GBM cell lines was associated with more infiltrative cell behavior [76]. Keratoepithelin (TGFBI) plays a role in inducing proliferation and migration of GBM cells *in vitro* and *in vivo*, as well as in the development of resistance to temozolomide [77–79]. Plasminogen activator inhibitor (SERPINE1) is one of the proangiogenic proteins; its content in GBM increases in response to hypoxia [80]. Growth and differentiation factor 15 (GDF-15) is associated with increased migration and invasion of GBM cells, as well as resistance to radiotherapy [81, 82].

Naryzhny et al. [83] performed a study similar in design; however, the main goal of their study was to search for potential protein markers common for several studied GBM cell lines: Glia-Sh, Glia-L, A172, Glia-R, and Glia-Tr. Among 896 identified proteins, 133 were common to all five cell lines. Fifteen of these proteins were proposed by the authors as "potential exosomal markers of GBM" (ANXA1, ANXA2, ENOA, G3P, HS90B, KPYM, PRDX1, TPIS, TERA, VIME, 1433E, COF1, NPM, CD44, TNC). Interestingly, 13 of them were previously identified by the authors in the whole-cell proteome of GBM cell cultures and distinguished them from healthy cells, (human fibroblast cultures) [84].

2.2. Search for EV-Associated GBM Markers in Animal Models

Despite the convenience of using *in vitro* GBM models, the results of studies using *in vivo* models are more relevant. Therefore, a number of studies have been carried out using mouse models. For example, Anastasi et al., performed a study using a mouse model of GBM [85]. In EVs isolated from serum samples of mice with different stages of GBM, they identified 274 proteins. A significant proportion of the proteins that distinguished GBM-bearing mice from healthy animals were involved in binding of antigens, proteases, integrins, and other proteins. The researchers identified 25 proteins whose content significantly differed between samples obtained from mice with GBM. In particular, these include the proteins Lrp1, Cpn1, Mhy9, and Tln1, involved in tumor invasion and metastasis, and Angpt1, Vtn, and Vcan, involved in cell adhesion, as well as in the induction of angiogenesis, proliferation, and cell survival via the PI3K/Akt signaling pathway.

Later, this research group performed proteomic analysis using three types of biological material obtained from model animals at different time points (including the period before GBM induction and during disease development): serum, the EV fraction isolated from serum, and cerebrospinal fluid (CSF). The comparison of the serum and serum EV proteomes resulted in identification of 99 proteins in the serum EV fraction that were not detected in the serum proteome. The levels of 19 proteins identified in EV samples increased with disease progression. These proteins participate in processes associated with cancer development: cell motility, invasion, proliferation, and angiogenesis. Six of these proteins (Tln1, Myh9, Thbs1, Flna, Vcan, Lamb1) were previously identified in EVs isolated from human GBM cell lines [86].

2.3. Search for EV-Associated GBM Markers in Clinical Material

Despite the widespread use of MS methods in studying the EV protein composition, immunochemical methods are also used to search for EV-associated GBM biomarkers. For example, Chandran et al. used HPLC-MS analysis and immunoprofiling of plasma EVs to identify potential biomarkers that could distinguish GBM from lower-grade gliomas. They found that the syndecan 1 (SDC1) protein could be a potential EV-associated marker, distinguishing grade IV and grade II gliomas [87]. In other studies, SDC1 was identified as a marker of unfavorable prognosis [88], and its involvement in the formation of radioresistance of GBM cells was also demonstrated [89, 90].

The work by Osti et al. [91] attracts much attention because of biomaterial used in this study: besides plasma samples from GBM patients and healthy controls the authors analyzed samples obtained from patients with other types of brain tumors (secondary tumors, adenomas, meningiomas, neurinomas). The results of the study have shown that the amount of EVs in the material obtained from GBM patients was significantly higher than that in healthy individuals and patients with other types of brain tumors. It is important to note that the amount of EVs in blood plasma did not depend on the tumor size, but did depend on the degree of GBM tissue necrosis. The source of elevated blood EV levels is tumor tissue, as evidenced by the decrease in EV levels in samples from patients after tumor removal demonstrated in the same study. This is also confirmed by the results of orthotopic transplantation of GBM stem cells into laboratory animals conducted by the authors; after transplantation, approximately half of the EVs circulating in the blood of the laboratory animals were tumor-derived EVs. MS proteomic analysis identified protein sets with quantitative differences between samples obtained from GBM patients and healthy individuals. The analysis revealed a set of 19 proteins exhibiting elevated levels in GBM patient samples and involved in the development of inflammation, immune responses, and cell growth and migration. To determine the impact of GBM tissue on the plasma EV proteome, a comparative analysis of samples obtained before and after tumor removal was conducted. This analysis resulted in identification of a set of 102 quantitatively different proteins. Based on the comparison of these sets, 11 common proteins (vWF, APCS, C4B, AMBP, APOD, AZGP1, C4BPB, SERPIN3, FTL, C3, APOE) involved in the complement system (whose members are considered as potential therapeutic targets and prognostic factors [92]), coagulation cascades, and regulation of iron metabolism were identified.

Hallal et al. [93] conducted a search for potential GBM markers in the plasma EV from patients. The researchers identified 463 proteins that differed quantitatively in different histological subtypes of gliomas (GBM, astrocytoma, oligodendroglioma), and 310 proteins with differences in their content in gliomas of different grades (II–IV). Moreover, 11 proteins were detected exclusively in samples obtained from GBM patients: AIDA, ARHGEF10, BNIP3L, FYB1, KMT2D, MAP7, MAST4, PDE8A,

POLR2D, RENBP, and SLC25A17. This set of proteins, as well as CETN3, PPP1R11, and SYT7 proteins, were identified exclusively in samples obtained from patients with grade IV glioma. Comparative analysis of samples obtained from GBM patients and patients from the control groups (healthy donors and meningioma patients) revealed quantitative differences in 68 proteins; the content of 2 proteins (EBNA1BP2 and FAM129A) differed in all histological subtypes of gliomas. The authors note that the identified set of proteins demonstrates overlap with a previously reported set of 145 "signature EV-associated GBM proteins" identified in several GBM cell lines [94]. Among these "signature" proteins, 10 were quantitatively different in samples obtained from GBM patients compared with the control groups: PSAP, CALR, PLOD3, HSPA4, GANAB, LGALS3BP, CCT2, PPIA, C3, and KRT10. Results of more recent studies have shown that the Niban 1 (FAM129A) protein plays a role in the self-renewal of tumor stem cells and stimulates their migration and invasion through the regulation of the Notch signaling cascade [95]. The results of studies considered in this chapter demonstrate the intersection of various approaches used for identification of potential protein markers of GBM associated with EV. Potential markers identified in these studies are listed in Table 2.

3. PROSPECTS FOR FREE AND EV-ASSOCIATED PROTEINS AS BIOMARKERS

The search for potential EV-associated protein markers of diseases in body fluids is a more complex task compared to the detection of freely circulating biomarkers. At the same time, this approach has a number of significant advantages. The key one is the increased sensitivity of proteomic analysis that makes it possible to increase the detectable segment of the proteome by including low-copy proteins. Previous studies conducted in our laboratory have shown that using MS analysis of the EV fraction isolated from serum it is possible to identify 1.8-fold more proteins (141) compared to whole serum samples (78) [96]. This expansion of the range of detectable proteins may increase the specificity of the identified biomarkers. According to the definition of the National Cancer Institute, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process or condition, or disease" [97]. Thus, both molecules produced by tumor cells and molecules synthesized by the body in response to the presence of a neoplasm can be considered markers. Isolation of EVs expands the possibilities for searching for markers specifically produced by a tumor and makes an approach that includes a preliminary search for such proteins in cellular or animal models more justifiable. For GBM, this approach was implemented in a number of studies considered previously [91, 93]. Previously, our laboratory conducted studies of a similar design aimed at searching for EV-associated protein markers of lung adenocarcinoma and colorectal cancer [98–100]. One of these studies resulted in identification of 28 EV-associated proteins produced by the lung adenocarcinoma cell lines NCI-H23 and A549 [98]. Among them, talin-1 (TLN1), tubulin α-4A (TUBA4A), and 70 kDa heat shock protein 8 (HSPA8) were identified as proteins with the greatest diagnostic potential. The TLN1 protein content was increased in plasma samples from patients with lung neoplasms compared to the control group, and the TUBA4A and HSPA8 proteins were identified exclusively in samples obtained from patients with neoplasms. Furthermore, the obtained proteomic signature distinguished samples obtained from patients with lung adenocarcinoma and squamous cell carcinoma (AUC ≥ 0.88). A similar set of proteins was identified in the study of EV-associated biomarkers of colorectal cancer *in vitro* and in clinical plasma samples. In this study the proteins TLN1, ITGB3, and TUBA4A were downregulated, while perlecan (HSPG2) was increased in samples obtained from patients with colorectal cancer compared to the control group samples [99].

Thus, both the literature and own results obtained by our laboratory demonstrate the potential for studying EV as a potential source of protein biomarkers for oncological diseases, particularly tumors of the nervous system.

CONCLUSIONS

Analysis of existing data suggests that the search for circulating protein biomarkers of GBM is a dynamically developing area attracting the attention of researchers. The results of the considered studies are graphically presented in Figure 1. The data obtained in these studies suggest the theoretical applicability of plasma and serum proteomic profiling for solving diagnostic problems, prognosing the course of the disease, and possibly monitoring GBM response to therapy. Furthermore, the analysis of EV-associated proteins appears to be a promising branch of this research line: using this approach it is possible (at least partially) to overcome the limitations associated with the low concentrations of clinically significant proteins and the high dynamic range of the plasma proteome. Studies aimed at EV analysis resulted in identification of protein sets, many of which are involved in key processes of oncogenesis (invasion, angiogenesis) and the immune response [85, 91, 93]. However, the lack of uniform standards for EV isolation and biomarker validation in large independent cohorts remains a serious challenge. Further research is needed to clarify the specificity of the identified protein markers and their diagnostic value. Nevertheless, the integration of proteomic approaches into the liquid biopsy paradigm holds significant potential for probable implementation in clinical practice.

Table 2. Potential EV-associated protein markers of human GBM. Proteins identified in several studies are highlighted in bold; proteins identified in both animal models and human samples are highlighted in color

Proteins		**Studied objects**	**Methods of the study**	**References**
WNT5a, TGFBI, SERPINE1, GDF-15	Associated with tumor invasiveness	Human cell lines (LN18, U87, and others)	Mass spectrometry	[75]
ANXA1, **ANXA2**, **ENOA**, **G3P**, **HS90B**, **KPYM**, **PRDX1**, **TPIS**, **TERA**, **VIME**, **1433E**, **COF1**, **NPM**, CD44, TNC	Common protein markers for several GBM cell lines	Human cell lines (Glia-Sh, Glia-L, A172, and others)	Mass spectrometry	[83, 84]
Thbs1, Apoc4, **C4bpa**, **C1ra**, **C1qa**, **C1sa**, **Tfrc**, **Lrp1**, **Itih1**, Apoc3, Angpt1, **Uba52**, Pm20d, **Itga2b**, **Myh9**, Cpn1, **Vtn**, Itih3, **Glul**, **Tln1**, **Fbln1**, **Vcan**, **Flna**, **Lamb1**, **Pcyox1**	Associated with the presence of GBM in model animals	Mouse models (EVs from serum)	Mass spectrometry (DDA)	[85]
Thbs1, **C4bpa**, **C1ra**, **C1qa**, **C1sa**, **Tfrc**, **Lrp1**, **Itih1**, **Uba52**, **Itga2b**, **Myh9**, **Vtn**, **Glul**, **Tln1**, **Fbln1**, **Vcan**, **Flna**, **Lamb1**, **Pcyox1**	Associated with disease development in model animals	Mouse models (serum, EVs from serum, CSF)	Mass spectrometry (DDA)	[86]
SDC1	A marker distinguishing GBM from lower-stage gliomas	Clinical samples (plasma)	Mass spectrometry, ELISA, and immunofluorescence analysis	[87]
vWF, APCS, C4B, AMBP, APOD, AZGP1, C4BPB, SERPIN3, FTL, C3, APOE	Associated with the presence of GBM; distinguish GBM patients from patients with other brain tumors	Clinical samples (plasma)	Mass spectrometry (DDA)	[91]
AIDA, ARHGEF10, BNIP3L, FYB1, KMT2D, MAP7, MAST4, PDE8A, POLR2D, RENBP, SLC25A17	Found exclusively in biomaterial obtained from GBM patients	Clinical samples (plasma)	Mass spectrometry (SWATH-MS)	[93]
PSAP, CALR, PLOD3, HSPA4, GANAB, LGALS3BP, CCT2, PPIA, C3, KRT10	Their quantities differed in samples from GBM patients compared to healthy donors			

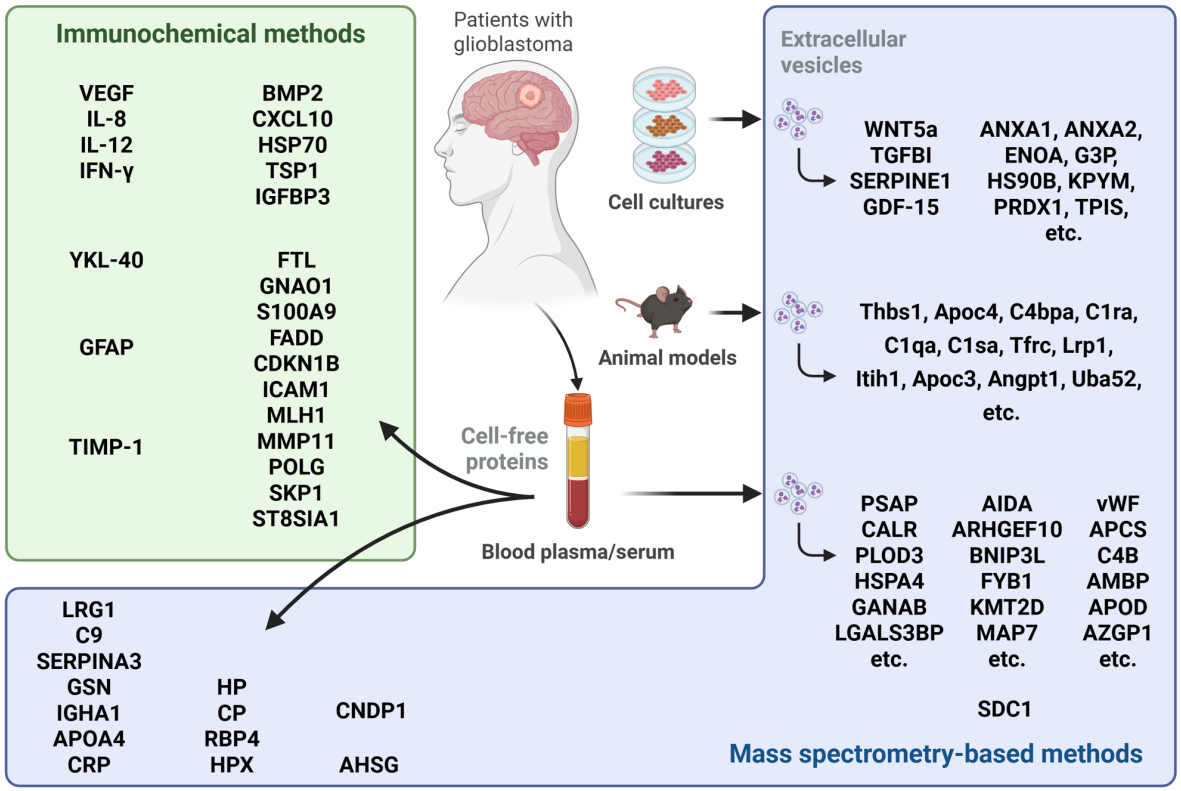

Figure 1. The main approaches used for identification of circulating protein markers of glioblastoma and the results obtained to date. Protein names are given according to the gene nomenclature.

ACKNOWLEDGMENTS

Own results, considered here, have been obtained using the equipment of the "Human Proteome" Core Facility at the Institute of Biomedical Chemistry.

FUNDING

The work was performed within the framework of the Program for Basic Research in the Russian Federation for a long-term period (2021–2030) (no. 122030100168-2).

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any research involving humans or the use of animals as objects.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Received: 12.11.2025.
Revised: 04.12.2025.
Accepted: 05.12.2025.

ЦИРКУЛИРУЮЩИЕ БЕЛКОВЫЕ БИОМАРКЕРЫ ГЛИОБЛАСТОМЫ: МЕТОДОЛОГИЧЕСКИЕ ПОДХОДЫ И ПЕРСПЕКТИВЫ РАЗВИТИЯ

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Мультиформная глиобластома (ГБМ) — наиболее агрессивная первичная опухоль головного мозга, характеризующаяся крайне неблагоприятным прогнозом. Трудности в диагностике и мониторинге данного заболевания создают необходимость поиска минимально инвазивных подходов, среди которых перспективным направлением считается жидкостная биопсия. Данный обзор посвящен анализу результатов современных исследований, направленных на поиск циркулирующих белковых биомаркеров ГБМ в плазме и сыворотке крови. В качестве биомаркеров рассматриваются свободно циркулирующие белки плазмы крови и белки, находящиеся в составе внеклеточных везикул (ВнВ). В обзоре обобщены результаты работ, использующих для поиска белковых биомаркеров как иммунохимические методы, так и масс-спектрометрические подходы, а также представлен перечень выявленных потенциальных диагностических и прогностических биомаркеров. Анализ представленных в литературе работ показывает, что протеомный анализ, сосредоточенный на фракции ВнВ плазмы крови, существенно расширяет возможности поиска биомаркеров для неинвазивной диагностики и мониторинга ГБМ.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: глиобластома; циркулирующие маркеры; внеклеточные везикулы; протеомика

Финансирование. Работа выполнена в рамках Программы фундаментальных научных исследований в Российской Федерации на долгосрочный период (2021–2030 годы) (№ 122030100168-2).

Поступила в редакцию: 12.11.2025; после доработки: 04.12.2025; принята к печати: 05.12.2025.