LIQUID BIOPSY OF GLIOMAS WITH DETECTION OF EXTRACELLULAR TUMOR NUCLEIC ACIDS

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ABSTRACT

Gliomas are the reason of fatal outcomes in an overwhelming number of patients with oncology diseases located in the central nervous system. The diagnostics of such neoplasms requires using stereotaxic biopsy, which cannot be performed in a certain percentage of the patients. Besides, this disease is characterized by high recurrence rates, despite the advances in developing resection and chemotherapy — based technologies. The early detection of oncological diseases located in the central nervous system and the differential diagnostics of tumor pseudo progression, not affecting the survival of the patient, represents a challenge for modern Medicine. Liquid biopsy is a minimally invasive diagnostic method based on the analysis of tumor derivatives (such as extracellular tumor DNA and RNA), contained within the biological fluids of the organism. For the purpose of defining the presence of the tumor component, the tests are used to detect the so-called hot-spot mutations and the patterns of epigenetic regulation, found in specific types of tumors. The technology can be used for detecting tumor recurrences and for the differential diagnostics of space-occupying mass lesions in patients, in which stereotaxic biopsy is contraindicated. The review contains a discussion on modern advances of fluid biopsy based on the analysis of the extracellular tumor DNA and RNA levels in blood plasma and in the cerebrospinal fluid of glioma patients.

Keywords: *circulating tumor DNA; microRNA; liquid biopsy; glioma; central nervous system; central nervous system malignancies; screening.*

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BACKGROUND

Despite the fact that gliomas represent only 18–19% of all the brain neoplasms, they are the cause of fatal outcomes in an overwhelming number of patients with oncology diseases affecting the central nervous system. Its most widespread type is glioblastoma, in which the 5-year survival rate does not exceed 7% [1]. Low-grade gliomas are characterized by the relative 5-year survival of more than 80%, however, the majority of them ultimately show a tendency to further malignization [2]. At the present moment, the first stage of treating the brain tumors, the recommendations include performing the maximal possible resection of the neoplasm. Sadly, but the infiltrating growth type of gliomas prevents its total resection. Moreover, due to the severity of the patient status and due to the risk of possible complications, the total resection of such tumors can be switched to partial resection or the resection itself can be cancelled, which decreases the efficiency of the conducted treatment even more [3].

In order to increase the survival rate of the patients, adjuvant therapy is being used, however, despite its use, within a year and a half after setting the diagnosis, in about 70% of the glioblastoma patients and in 20% of low-grade glioma patients, the development of recurrences is observed, requiring repeated surgical intervention [3–5]. For the purpose of their timely detection, every 3–6 months the patients undergo examinations using the method of magnetic resonance imaging (MRI) [3]. The changes in the brain tissues, such as radiation-induced necrosis, swelling or decreased contrasting, caused by the therapy, in 36% of the glioblastoma cases lead to the findings similar to the manifestations of tumor recurrence — pseudoprogression [6]. Despite the fact that the median of progression-free survival for low-grade gliomas is approximately 5 years, in 20% of the cases they are also characterized by the presence of pseudoprogression [7]. This event, apparently, does not affect the overall survival of the patients and requires using separate therapy. The use of specific anti-relapse therapy at this stage, on the contrary, can worsen the patient status [3, 6, 7]. When using classical MRI modes (T1-weighed MRI with contrast enhancement and T2-FLAIR), it is not always possible to determine the presence of true progression of the tumor, which results in untimely

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ЖИДКОСТНАЯ БИОПСИЯ ГЛИОМ С ВЫЯВЛЕНИЕМ ВНЕКЛЕТОЧНЫХ ОПУХОЛЕВЫХ НУКЛЕИНОВЫХ КИСЛОТ

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АННОТАЦИЯ

Глиомы являются причиной гибели подавляющего числа больных с онкологическими заболеваниями центральной нервной системы. Диагностика таких новообразований требует использования стереотаксической биопсии, которая может быть проведена далеко не у всех пациентов. Кроме того, данное заболевание характеризуется высокой частотой рецидивов, несмотря на успехи в развитии резекционных и химиотерапевтических технологий. Раннее выявление онкологического заболевания центральной нервной системы и дифференциальная диагностика с псевдопрогрессией опухоли, не влияющей на выживаемость пациента, представляет актуальную задачу для современной медицины. Жидкостная биопсия является малоинвазивным методом диагностики, основанным на анализе опухолевых дериватов (таких как внеклеточная опухолевая ДНК и РНК), находящихся в биологических жидкостях организма. Для определения опухолевого компонента используют анализ так называемых hot-spot мутаций и паттернов эпигенетической регуляции, присущих определённому типу опухоли. Технология может быть использована для выявления рецидивов опухоли и дифференциальной диагностики объёмных образований у пациентов, которым противопоказана стереотаксическая биопсия. В обзоре обсуждаются современные достижения жидкостной биопсии на основе анализа внеклеточной опухолевой ДНК и РНК в плазме крови и спинномозговой жидкости пациентов с глиомами.

Ключевые слова: *циркулирующая опухолевая ДНК; микроРНК; жидкостная биопсия; глиомы; центральная нервная система; злокачественные новообразования ЦНС; скрининг.*

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use of therapy and a decrease in the survival rate. In both cases, clinicians receive corrupted data on the prognosis and on the efficiency of conducted therapy.

At the present moment, there is a development going on in the field of perfusion and radio-isotopic methods of diagnostics, allowing for more precise defining the tumor status, however, their wide spreading is still pending [6].

Due to the fact that the histopathological examination of surgical material still remains the main method for the differential diagnostics of space-occupying neoplasms in the brain, the absence of surgical intervention encumbers not only the process of fighting the disease, but also setting the correct diagnosis [3]. In such cases, the decisive diagnostic procedure is the stereotaxic biopsy of the neoplasm [6]. Despite this method demonstrating high sensitivity and specificity, it is characterized by relatively high rate of complications (up to 17%), as well as by high requirements in terms of the qualification of the medical staff and of the visualization equipment quality [8]. For this same reason, the stereotaxic biopsy, probably, can not be used for routine regular detection of recurrences. Moreover, such factors as the involvement of the brain stem, the presence of serious concomitant diseases in the patient or progressive worsening in the neurological status, may ultimately become the reason for avoiding such a manipulation [9]. Due to the fact that MRI signs of some non-oncological diseases may match to those of gliomas (and vice versa), the absence of precise diagnosis shall impose a risk of using incorrect therapy and shortening the life expectancy of the patient [10, 11].

For the purpose of increasing the survival rate and the quality of life in the patients, it is necessary to develop new methods for diagnosing gliomas. Currently, the potential of fluid biopsy is being actively studied — the method for analyzing the cellular and molecular tumor derivatives within various biological fluids of the organism. Special attention in this field is paid to the analysis of extracellular tumor DNA and RNA, the levels of which are informative regarding the volume and the mutation burden of the investigated tumor [12, 13].

The aim of this review is to summarize the data from the research works evaluating the diagnostic and prognostic potential of fluid biopsy of glioblastoma based on the analysis of the extracellular tumor DNA and RNA.

GENETIC CHARACTERISTICS OF GLIOMAS (DNA AND RNA OF THE TUMOR TISSUE)

Initially, the analysis of molecular markers as an important component of diagnosing gliomas was recommended in the classification of the central nervous system tumors, issued by the World Health Organization (WHO) in 2016. According to this classification, the key mutations of gliomas, associated with better survival of the patients, are the mutations in genes *IDH1*, *IDH2*, *TP53*, the deletion of *ATRX* gene and the co-deletion of 1p/19q. In the later classification issued in 2021, as well as in the Clinical recommendations from the European Association of Neuro-Oncology and from the Korean Association of Oncology, a relation was reported for the mutations in genes *CIC* and *FUBP1* with better survival of patients, while the mutations of *TERT* (*pTERT*) promoter, of the *NOTCH1* and *EGFR* genes, the deletion of *CDKN2A/B*, as well as the alteration of the number of chromosomes 7/10 — with worse survival. These mutations are most commonly found in cases of gliomas and they have the most influence on the clinical signs. Their presence is considered a justification for referring gliomas to one of three main histotypes, defined by the WHO classification issued in 2021 [3]. At the same time, the mutation of genes *VEGF*, *ARF*, *PTEN*, *NF1*, *RTK/ RIS* and others were not used for typological classification of gliomas, but they also can be found in a significant part of glioma patients, being the negative prognostic markers [14]. The list of commonly found molecular changes having a prognostic value, is provided in table 1.

The characteristics of glioma, besides the genetic changes, are also affected by alterations in the epigenetic regulation of the cells. In glioblastoma cells, generally, hypermethylation is shown for chromosomes 1, 2, 3 and 17 with hypomethylation of chromosomes 11, 16, 19 and 20. Most commonly, the hypermethylated gene promoters include *pLRRC4*, *pANKDD1A*, *pGAD1*,

Table 1

List of mutations that have the greatest impact on the prognosis of patients with adult type diffuse glioma

Molecular alterations	Positive prognosis	Negative prognosis
Nucleotide substitutions	*IDH1*$^{R132H, R132C}$, *IDH2*^{R172*} *CIC*$^{R1124W, R1110W, R1111W}$ etc. *FUBP1*$^{X83\_splice, I443Rfs*47, X314\_splice}$ etc. *ATRX*$^{R1426*, R907*}$ etc.	*pTERT*$^{C228T, C250T}$ *VEGF* *p14*^{ARF}/*p16*^{INK4A} *EGFR*$^{G598V, A289V}$ etc. *TP53*$^{R273C, R175H, R248Q}$ etc. *PTEN*$^{R130*, R233*, R335*, R173H}$ etc. MUC16$^{T11587M, T11535M, T4653K}$ etc. *PIK3R1*$^{G376R, N564D, X583\_splice}$ etc. *NF1*$^{F1247Ifs*18, R2450*, C167Qfs*10}$ etc. *PIK3CA*$^{H1047R, R88Q, G118D}$ etc. *RB1*$^{S318Nfs*13, R552*, X445\_splice}$ etc. *PDGFRA*$^{E229K, N468S, V309F}$ etc. *RTK/RIS* *NOTCH1*$^{F357del, A465T, D338del}$ etc.
Deletion of genome areas	Deletion of *ATRX* Co-deletion of 1p/19q	Loss of chromosome 10 Deletion of *CDKN2A/B* Deletion of *MTAP*
Duplication and amplification of genome areas	-	Duplication and amplification of 7th chromosome Amplification *MDM2/MDM4* Amplification *EGFR* Amplification *MYC*
Methylation etc.	Hypermethylation of *pMGMT*, *CDKN2A*, *RASSF1A* Microsatellite instability	Hypermethylation *pPARP-1*, *pSHP-1*, *pDAPK-1 и pTIMP-3*
Increase in the number of extracellular tumor RNA	*miRNA-1-3p*, *26a-1-3p*, *487b-3p*, *342-3p* etc. cRNA *CM21D*, *circPTK2*, *circSERPINE2* etc. lnRNA *CASC2*, *MEG3*, *PDCD4-AS1*, *GSCAR*, *SPRY4-IT1* etc.	*miRNA-454-3p*, *21*, *17-5p*, *125b*, *221*, *128*, *342-3p* etc. кRNA *circSKA3*, *CircXPO1*, *circENTPD7* etc. lnRNA *HOTAIRM1*, *STEAP3-AS1*, *CASC2c*, *HOXA11-AS*, *ASLNC22381*, *ASLNC20819*, *CRNDE* etc.

Note. p — gene promoter; RNA — ribonucleic acid; miRNA — micro ribonucleic acid; cRNA — circular ribonucleic acid; lnRNA — long non-coding ribonucleic acid.

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pSIX3, *pSST*, *pPHOX2B*, *pPCDHA8*, *pHIST1H3E* and *pPCDHA13*, while the hypomethylated ones include *pF10*, *pPOTEH*, *pCPEB1*, *pLMO3*, *pELFN2* and *pPRDM16* [14]. One of the most studied markers is the hypermethylation of *MGMT* (*pMGMT*) promoter, which occurs in more than half of glioma cases and which is associated with better survival [3]. Additionally, data is available on more than 160 genes, the expression of which in glioma cells is decreased under the effects of hypermethylation in their promoters [15]. At the same time, there is a point of view stating that the features of glioma course are affected not by methylation of individual genes, but by the change in the epigenetic regulation pattern of the whole cell genome in general. For example, there is a well known interrelation between the G-CIMP glioma methylation profile and the presence of *IDH1/IDH2* mutations, associated with better survival among the patients [14].

The epigenetic regulation of gene expression includes not only the changes in the methylation of their promoters, but also the interaction with a broad set of non-coding RNA. Among them, the most studied is the microRNA, consisting of 20–22 nucleotides. In glioma cases, modified expression is found in more than 300 microRNA, the most prominent representatives of which are the *microRNA-21*, *221*, *222*, *26-a*, *10-b* and *182*, the hyperexpression of which is often observed in glioma tissues, as well as *microRNA-181a*, *181b* and *181c*, *34a*, the expression of which in gliomas is reduced [14]. Minor RNA also include the circular RNA, of which the role is the regulation of microRNA and matrix RNA activity, which results in changes in the expression of the key genes, such as *PAQR3*, *MKP1*, *GLUT1* etc. In glioma tissues, more than 400 abnormally expressed circular RNA were found [16]. The characteristics of the tumor are also affected by long-chain non-coding RNA, consisting of more than 200 nucleotides. The most comprehensively described are the *ASLNC22381*, *ASLNC20819*, *CRNDE* and *HOTAIRM1* long-chain non-coding RNA, which become significantly activated in glioblastoma tissues (the high levels of which, in turn, are considered as a negative prognostic sign), as well as *CASC2*, *PDCD4-AS1*, *GSCAR*, *MEG3* and others, which prevent the development of tumors and the levels of which are decreased in gliomas comparing to normal tissues [14, 17].

EXTRACELLULAR TUMOR DNA

The extracellular tumor DNA is a component of the total extracellular DNA, which, generally, consists of DNA fragments with a length of 80–200 base pairs, which corresponds to about one volution of the nucleosome. Its main source is believed to be the dead cells, as well as the cells that actively produce the extracellular DNA. Besides the immune system cells, releasing extracellular DNA during the NETosis, such sources include the tumor cells, apparently, using these molecules as intercellular messengers [18]. In physiological conditions, the blood concentration of extracellular DNA does not exceed 40 ng/ml, however, in cases of cancer processes, it can increase by a factor of tens [19]. The increase of DNA concentration is associated not only with the secretion by tumor cells or their necrosis, but also with the death of cells surrounding the tumor caused by the Warburg effect [20].

Upon the analysis of the extracellular tumor DNA, information can be obtained on the mutation pattern and on the changes of the epigenetic regulation pattern in the tumor cells. These may be important for non-invasive diagnostics of neoplasms. It is worth noting that extracellular tumor DNA have a half-life period of less than 1.5 hours, which also allows for using them for dynamic monitoring of the treatment efficiency [18].

Genetic changes of the extracellular tumor DNA

Most commonly, the fluid biopsy of gliomas reveals mutations in the *pTERT* (~65%), *TP53* (40–60%), *H3F3A* (~50%), *IDH1* (30%), *CDKN2A/B* (25%), *NF1* (~24%), *EGFR* (20–25%), *ATRX* (10–20%), *MET* (~18%), *APC* (~15%), *PDGFRA* (10–14%) and *FAT1* (<10%) genes (table 2, 3) [12–52]. Reports were also provided on

Table 2

Studies on the analysis of cell-free tumor DNA and RNA in the diagnosis of adult type diffuse glioma

Research	Research sample	Substrate (tested volume); Test method (marker tested)	Analysis outcome, %		
			Se	Sp	AUC
Analysis of mutations in the extracellular tumor DNA					
[21]	57, glioma	CSF (3 ml) + serum (3 ml) + tissue (n/d); NGS (Panel of 68 genes)	91.9	-	-
[22]	85, glioma (46 cases of brain glioblastoma — BGB); 7, control	Serum (3.5 ml) + CSF (3.5 ml); NGS (Panel of 410 genes)	49.4	-	-

Table 2

Continued

Research	Research sample	Substrate (tested volume); Test method (marker tested)	Analysis outcome, %		
			Se	Sp	AUC
[26]	34, glioma	CSF (1–3 ml); ddPCR (*IDH1*, *pTERT*, *H3F3A*)	87	-	-
[27]	42, glioma *TERT-mut*; 9, glioma *TERT-wt*; 23, control	Serum (1 ml); ddPCR (*pTERT*)	52.38	90.91	-
[28]	45, glioma	Serum (1 ml) + tissue (n/d); RtPCR (*IDH1*)	11.54	-	-
[32]	395, BGB	Serum (n/d) + tissue (n/d); NGS (*pTERT*)	75	-	-
[34]	4, recurrence of BGB; 111, glioma; 111, control	CSF (10 µl) + tissue (n/d); NGS (panel of 68 genes)	-	-	94.4
The analysis of changes in the methylation patterns of extracellular tumor DNA					
[35]	149, glioma	Serum (1.2–9.3 ml); bisulfite conversion + NGS (panel of 100 epigenetic variants)	100	97.78	-
[36]	17, glioma	Serum (3 ml) + tissue (n/d); bisulfite conversion + PCR in the agarose gel (*pMGMT*, *pRASSF1A*, *p15INK4B*, *p14ARF*)	70.58	-	-
[38]	20, astrocytoma; 20, oligodendroglioma; 10, control	Serum (1 ml) + tissue (n/d); bisulfite conversion + PCR in the agarose gel (*pCDKN2A*)	75	-	-
[39]	41, astrocytoma; 29, oligodendroglioma	Blood (5 ml) + serum (200 µl) + tissue (n/d); bisulfite conversion + RtPCR (*pPTEN*, *pMGMT*); RtPCR (loss of heterozygosity in 10q, 19q, 1p)	Astrocytoma 59%; oligodendroglioma 58%	Astrocytoma 100%; oligodendroglioma 94%	-
[40]	89, glioma	CSF (4–5 ml) + tissue (n/d) + serum (n/d); bisulfite conversion + PCR + chromatography (*pMGMT*)	65	100	-
Analysis of the extracellular tumor RNA levels					
[12]	7, BGB; 4, glioma stage II	Serum (200 µl); ddPCR (*miRNA-320e*, *223*, *23a*, *21*)	100	97.8	98
[43]	111, BGB; 84, control (non-oncological diseases)	CSF (1 ml); RtPCR (*miRNA-21*, *218*, *193b*, *331*, *374a*, *548c*, *520f*, *27b*, *130b*)	80	67	75
[44]	30, glioma stage II–IV; 10, adenoma of the hypophysis; 10, meningioma; 10, control	Serum (400 µl); RtPCR (*miRNA-21*, *128*, *342-3p*)	90	100	93
[47]	23, BGB; 5, glioma stage III; 10, control	Serum (n/d); ddPCR (*circHIPK3*, *circSMARCA5*)	-	-	90.1
[48]	25, BGB; 20, control	Serum (n/d); RtPCR (*miRNA-17-5-p*, *125b*, *221*)	96	95	98.8
[49]	30, EGFRvIII positive; 10, EGFR wild type; 14, control	Serum (2 ml) + tissue (n/d); ddPCR of tissues and serum, RtPCR of tissue samples (*mRNA-EGFRvIII*, *mRNA-EGFR wild type*)	72.77	97.67	-

Note. Se — sensitivity; Sp — specificity; AUC — area under the ROC curve; BGB — brain glioblastoma; CSF — cerebrospinal fluid; NGS — next generation sequencing; RtPCR — real-time PCR; ddPCR — digital droplet PCR; RNA — ribonucleic acid; miRNA — micro ribonucleic acid; cRNA — circular ribonucleic acid; lnRNA — long noncoding ribonucleic acid; *p* — gene promoter; n/d — data is not available.

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Table 3

Studies on the relationship between cell-free tumor DNA and RNA in the prognosis of adult type diffuse glioma

Research	Research sample	Substrate (test sample); Test method (investigated marker)	Analysis outcome	
			Positive prognosis	Negative prognosis
Analysis of mutations in the extracellular tumor DNA				
[13]	370, glioma (222 BGB)	Serum (n/d); NGS (panel of >54 genes)	-	*TP53* ↑ *NF1* ↑ *EGFR* ↑ *PIK3CA* ↑
[19]	122, BGB; 55, adenocarcinoma; 130, control	Serum (n/d); fluorimetry (ecDNA)	-	*ecDNA* ↑
[23]	30, glioma (TISF); 14, glioma (CSF)	TISF (n/d) + CSF (n/d) + serum (n/d); NGS (panel of 68 genes)	-	etDNA ↑
[24]	42, BGB; 42, control	Serum (1 ml); RtPCR (ecDNA)	-	etDNA ↑
[25]	240, glioma; 25, control	Serum (n/d) + tissue (n/d); RtPCR (*IDH1*)	*IDH1* ↑	-
[28]	45, glioma	Serum (1 ml) + tissue (n/d); RtPCR (*IDH1*)	*IDH1* ↑	-
[29]	49, BGB	Serum (1–5 ml); ddPCR (*pTERT*)	The marker level is not a prognostic sign	
[31]	60, BGB	Serum (n/d) + CSF (n/d) + tissue (n/d); ddPCR (*pTERT*)	-	*pTERT* ↑
[32]	395, BGB	Serum (n/d) + tissue (n/d); NGS (*pTERT*)	-	*pTERT* ↑
Analysis of changes in the methylation patterns of extracellular tumor DNA				
[25]	240, glioma; 25, control	Serum (n/d) + tissue (n/d); bisulfite conversion + RtPCR (*pPARP-1*, *pSHP-1*, *pDAPK-1*, *pTIMP-3*, *pMGMT*)	*pMGMT* ↑	*pPARP* ↑ *pSHP* ↑ *pTIMP* ↑
[33]	124, glioma; 58, control	Serum (n/d); bisulfite conversion + Sanger sequencing (Alu, *pMGMT*, *pRASSF1A*, *pCDKN2A*)	-	Alu ↑ *pMGMT* ↑
[35]	149, glioma	Serum (1.2–9.3 ml); bisulfite conversion + NGS (Panel of 100 epigenetic signs)	-	High level of the tumor methylation scale
[34]	4, recurrence BGB; 111, glioma; 111, control	CSF (10 μl) + tissue (n/d); NGS (Panel of 68 genes)	*pFLRT2* ↑ *pETV1* ↑ *pNTRK3* ↑ *pC1orf226* ↑	*NKD1* ↑ *GNB5* ↑ *COMMD1* ↑ *CHI3L2* ↑
[37]	66, glioma; 20, control	CSF (n/d) + serum (n/d) + tissue (n/d); Immunoprecipitation of methylated DNA + RtPCR (*pMGMT*, *pTIMP-3*, *pP16INK4a*, *pTHBS1*)	-	*pMGMT* ↑ *pTIMP-3* ↑ *pP16INK4a* ↑ *pTHBS1* ↑
[40]	89, glioma	CSF (4–5 ml) + tissue (n/d) + serum (n/d); bisulfite conversion + PCR + chromatography (*pMGMT*)	-	*pMGMT* ↑

Table 3

Continued

Research	Research sample	Substrate (test sample); Test method (investigated marker)	Analysis outcome	
			Positive prognosis	Negative prognosis
[41]	58, glioma	Serum (n/d) + tissue (3–5 samples with a thickness 10 μm); bisulfite conversion + RtPCR (*pMGMT*)	*pMGMT* ↑	-
Analysis of the extracellular tumor RNA levels				
[12]	7, BGB; 4, glioma stage II	Serum (200 μl); ddPCR (*miRNA-320e*, *223*, *23a*, *21*)	-	*miRNA-320e* ↑ *miRNA-223* ↑ *miRNA-21* ↑
[43]	111, BGB; 84, control (non-oncological diseases)	CSF (1 ml); RtPCR (*miRNA-21*, *218*, *193b*, *331*, *374a*, *548c*, *520f*, *27b*, *130b*)	-	*miRNA-21* ↑
[44]	30, glioma stage II–IV; 10, adenoma of the hypophysis; 10, meningioma; 10, control	Serum (400 μl); RtPCR (*miRNA-21*, *128*, *342-3p*)	*miRNA-128* ↑ *miRNA-342-3p* ↑	*miRNA-21* ↑
[45]	15, BGB; 4, low grade glioma; 7, control	Serum (n/d); RtPCR (panel of 84 mRNA)	-	*mRNA-GZMB* *mRNA-HLA-A*
[46]	25, glioma; 25, control	Serum (n/d) + tissue (n/d); RtPCR (*circMMP1*, *miRNA-433*, *HMGB3*)	-	*circMMP1* ↑ *miRNA-433* ↑ *HMGB3* ↑
[47]	23, BGB; 5, glioma stage III; 10, control	Serum (n/d); ddPCR (*circHIPK3*, *circSMARCA5*)	-	*circSMARCA5* ↓ *circHIPK3* ↓
[48]	25, BGB; 20, control	Serum (n/d); RtPCR (*miRNA-17-5-p*, *125b*, *221*)	-	*miRNA-17-5-p* ↑ *miRNA-125b* ↑ *miRNA-221* ↑
[49]	30, EGFRvIII positive; 10, EGFR wild type; 14, control	Serum (2 ml) + tissue (n/d); ddPCR from the tissues and serum, RtPCR from the tissues (*mRNA-EGFRvIII*, *mRNA-EGFR wild type*)	-	*mRNA-EGFRvIII* ↑
[50]	50, astrocytoma; 60, control	Serum (100 μl); RtPCR (*9 miRNA*)	-	*miRNA-19a-3p* ↑ *miRNA-106a-5p* ↑ *miRNA-181b-5p* ↑
[51]	15, glioma (8 IDH-wt and 7 IDH-mut); 15, control	Serum (200 μl); ddPCR (*10 miRNA*)	-	*miRNA-1-3p* ↓ *miRNA-26a-1-3p* ↓ *miRNA-487b-3p* ↓
[52]	106, BGB	Serum (n/d); RtPCR (*miRNA-222-3p*, *20a-5p*, *106a-5p*, *182*, *145-5p*)	*miRNA*-182 ↑ *miRNA*-145-5p ↑	*miRNA*-222-3p ↑ *miRNA*-20a-5p ↑ *miRNA*-106a-5p ↑

Note. BGB — brain glioblastoma; CSF — cerebrospinal fluid; NGS — next generation sequencing; RtPCR — real-time PCR; ddPCR — digital droplet PCR; DNA — desoxyribonucleic acid; RNA — ribonucleic acid; mRNA — messenger RNA; miRNA — micro RNA; cRNA — circular RNA; lnRNA — long noncoding RNA; *p* — gene promoter; n/d — data is not available; ↑ or ↓ — identification of elevated or decreased level of marker in groups of patients in comparison with control groups.

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the elevation of the total extracellular DNA levels in glioma patients being 1.3–30 -fold higher comparing to the individuals in the control group [19, 24]. At the same time, in healthy volunteers, no mutation-related changes were observed in plasma and in the cerebrospinal fluid, which was expected [22, 25]. The sensitivity of droplet digital polymerase chain reaction (PCR) when searching for extracellular tumor DNA in the cerebrospinal fluid when diagnosing gliomas reaches up to 87%, with a specificity of 100%. The use of next generation sequencing (NGS) allows for increasing the sensitivity up to 91.9% [21, 26, 27]. However, testing the serum samples for extracellular tumor DNA using digital droplet PCR is characterized by the sensitivity of only 52.38% [27]. The sensitivity of PCR performed in the real time mode when searching for extracellular tumor DNA in the serum samples, is only 11.54% [28]. The results of determining the diagnostic efficiency of fluid biopsy are provided in table 2.

The level of mutation burden is related to the tumor tissue volume, decreasing after the tumor resection or after chemotherapy with an increase during the recurrence [27, 29], however, no correlation was observed between the extracellular DNA levels in plasma and the radiologically determined tumor volume [24]. In cases of recurrence, the number of observed alterations in the mutated genes or in the signaling pathways associated with them, can increase up to 3 times then the levels in the primary tumor [22, 23]. Probably, the increase of genetic variability of the tumor is due to the effect of post-therapeutic evolution, in which, under the effects of therapy, there occurs the selection of subclones having a dysregulated reparative system responsible for being more prone to mutagenesis, which, in turn, can explain their resistance to therapy. This phenomenon can be observed in about 78% of gliomas, representing worse prognosis and the risk of developing remote recurrences. As it was found by G. Liu et al. [30], after the conducted therapy and further tumor recurrence, the tumors show significantly more aggressive mutational phenotype.

The patients with high-grade gliomas are characterized by the presence of higher levels of extracellular tumor DNA [13]. Respectively, higher levels of extracellular tumor DNA (>15 ng/ml) are associated with lesser progression-free survival (p <0.0001, Spearman's rank correlation coefficient ρ=-0.844) and lesser total survival rate of the patients (the overall survival in patients with low levels of extracellular tumor DNA is about 2 times higher than in patients with high levels of extracellular tumor DNA) [19, 22–24]. However, in some cases such an interrelation was not observed [29]. The variety of mutations, apparently, does not correlate with the progression-free survival [23], but it is closely related to worse total survival rates (median of overall survival — 15.4 months [95% CI 11.6–19.2] in a group showing low variability of mutations compared to the values of 8.3 [95% CI 2.3–14.4] in a group with high variability of mutations) [31]. Despite this fact, the presence of *IDH1* mutations in patients is the positive prognostic sign (at an average, the patients with mutant *IDH* have at least 3 months higher overall survival) [25, 28]. The *pTERT* gene mutation is a negative prognostic factor (median of overall survival — 13.8 months for patients with mutated *pTERT* comparing to 37.6 for wild type *pTERT*; p <0.0022), while the patients with *EGFR* amplification show 2 times lesser overall survival comparing to patients not having such alterations [32]. Table 3 shows the results of studying the prognostic efficiency of fluid biopsy.

Epigenetic alterations of the extracellular tumor DNA

When evaluating the epigenetic changes, most commonly by means of real-time PCR and digital droplet PCR, the alterations of Alu-repeats methylation are being studied, as well as the alterations of the *MGMT*, *RASSF1A*, *pPARP-1*, *pSHP-1*, *pDAPK-1*, *CDKN2A* and *TIMP-3* gene promoters (see table 2, 3). M. Gong et al. [33] have used Sanger sequencing for the evaluation of the methylation of Alu-repeats, as well as for *pMGMT*, *pRASSF1A* and *pCDKN2A*. L. Dai et al. [34] have studied the methylation of extracellular tumor DNA from the cerebrospinal fluid using NGS with further use of the UCSC RefSeq data bases to determine the differentially methylated genome areas in glioma patients and in healthy individuals. These areas were then analyzed to search the most differentially expressed genes and for compiling the diagnostic and prognostic models. T. Sabedot et al. [35] have proposed the use of NGS to investigate the methylation of extracellular tumor DNA in serums and glioma tissues. After bisulfite conversion and performing the sequencing procedures using the Illumina Human EPIC set for serum and Illumina Human 450K (HM450K) set for tumor tissues, the authors have isolated 476 genome sites, differentially methylated in glioma patients and in healthy individuals. Using this information, a scale was compiled for evaluating the DNA methylation, which could allow for differentiating the samples obtained from glioma patients (the scale

value that is close to 100%) and from healthy individuals (close to 0%). An increase of the scale value in each patient means that, in his plasma, extracellular tumor DNA was found, methylated in a similar manner to the set of 476 sites used for creating the scale. As a result of evaluating the data from the test cohort, after using the machine learning, the threshold level for the scale intended for the differentiation of individuals with gliomas and without them, was set as being equal to 49%.

The methylation of Alu-repeats is significantly lower in glioblastoma patients (46–47%) comparing to the control group (approximately 60%) [33], with the mean level of *MGMT*, *CDKN2A* and *RASSF1A* methylation, on the contrary, being significantly higher in glioma patients, than in healthy individuals [33, 36, 37]. The occurrence rate of *p16* hypermethylation varies among the patient cohorts with gliomas of different histological types: in 9/20 patients with astrocytomas and only in 1/20 patients with oligodendrogliomas (p <0.05) [38]. L. Dai et al. [34] have stated that *pFLRT2*, *pETV1*, *pNTRK3* and *pC1orf226* are hypomethylated in tumor cells, while the *pNKD1*, *pGNB5*, *pCOMMD1* and *pCHI3L2* are hypermethylated.

T. Sabedot et al. [35] have found a decrease of the genome methylation scale value after successful therapy. During the primary diagnostics, the median scale value among the patients was 78.41%, in cases of remission or pseudoprogression, the scale value had decreased below 49%, while in cases of recurrences it was increasing (for the first recurrence, the median was 61.1%, for the second — 56.1%). It is also probable that, during the abovementioned post-therapeutic tumor evolution, the tumor mass was accumulating cell subclones, the methylation DNA in which differed from the initial set of 476 sites, due to which, the scale value during the recurrence did not return to previous values. Nevertheless, for all the glioma recurrence patients, the scale value exceeded 49%, which allowed for clearly differentiating them from the patients with pseudo-recurrence. The sensitivity of the test was 100%, the specificity was 97.78% [35].

The sensitivity of differentiating glioma patients from the healthy volunteers for the *pMGMT*, *pRASSF1A*, *p15INK4B*, *p14ARF*, *pPTEN*, *pCDKN2A* methylation test using the PCR method was 58–75% with the specificity of 94–100% (see table 2). The diagnostic model by L. Dai et al. [34] has allowed for differentiating the patients and healthy volunteers with an AUC 94.4%.

The patients with high methylation levels of Alu, *NKD1*, *GNB5*, *COMMD1*, *CHI3L2* and *pMGMT* have greater overall survival than the patients with low methylation level (the mean survival after setting the diagnosis is approximately 23 months in patients with pronounced methylation of Alu comparing to 11 months in patients with no methylation; p <0.05) [34, 41], at the same time, high methylation levels of *pPARP-1*, *pSHP-1*, *pFLRT2*, *pETV1*, *pNTRK3*, *pC1orf226*, *pP16INK4a*, *pTHBS1 and pTIMP-3* for serum extracellular tumor DNA are associated with lesser survival [25, 34, 37]. Besides, the methylation degree of *pPARP-1*, *pSHP-1* and *pTIMP-3* promoters in tumor samples and in the serum is, to a significant extent, related to the malignancy grade of glioma (the mean methylation levels for the stated genes is 0.18–0.30 in patients with grade I gliomas and up to 0.4–0.6 in patients with malignancy grade IV gliomas) [25]. In the research by L. Dai et al. [34], the high level of *pNKD1*, *pGNB5*, *pCOMMD1* and *pCHI3L2* methylation is associated with negative prognosis, and the high level of *pFLRT2*, *pETV1*, *pNTRK3* and *pC1orf226* methylation is a positive prognostic sign. It is worth noting that, at the present moment, the test of *pMGMT* methylation is already being used for predicting the course of the disease, though the test substrate is limited to using only the tumor tissue [3]. The analysis of methylation for other markers, as of today, is not being widely used in practice. The results of studying the prognostic efficiency of fluid biopsy with testing the methylation of extracellular tumor DNA are summarized in table 3.

EXTRACELLULAR TUMOR RNA

The research works show that both the tumor and the normal cells release high quantities of RNA into the environment, with its release taking place not only from the dying cells, but also by means of secretory mechanisms, such as exosome-mediated transfer of signals by live cells. The concentration of the total extracellular RNA in blood plasma samples from oncology patients has an average level of 7.9 ng/ml, which is comparable to the release of this substance in healthy individuals [42]. At the same time, the concentration of separate RNA in patients may differ from those in healthy individuals by a factor of tens [43, 44]. The analysis of them allows for judging not only on the presence of a neoplasm, but also on its characteristics.

The majority of the research works devoted to the analysis of extracellular tumor RNA, evaluates the levels of microRNA, among which the most attention is paid to *microRNA-21* and *221* (see table 2, 3). Some studies evaluate the levels of circular RNA *circHIPK3*

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and *circSMARCA5*, long-chain non-coding RNA — *HOTAIR*, *SOX21-AS1* and *STEAP3-AS1*, as well as levels of expressed matrix RNA [45–47]. *MicroRNA-21*, *218, 198b* and other, as well as long-chain non-coding RNA *HOTAIR*, *SOX21-AS1* and *STEAP3-AS1* are significantly (100–10000 -fold; p <0.05) increased in glioblastoma patients comparing to the control group [43, 44]. *MicroRNA-17-5p*, *125b*, *21*, *221* and *222*, as well as *circMMP1*, were found to be elevated in glioma patients comparing to the control by a factor of 2–10 (p <0.05) [46, 48], however, the levels of *microRNA-128* and *342-3p*, as well as the *circSMARCA5* and *circHIPK3* circular RNA, on the contrary, were found to be lower in glioma patients than in healthy individuals, 2–10 -fold (p <0.05) [44, 47]. Based on the results of the analysis of circulating matrix RNA, glioma patients are characterized by overexpression of *BCL2L1*, *GZMB*, *HLA-A*, *IRF1*, *MYD88*, *TLR2* and *TP53* genes, while the *BCL2*, *CCR2*, *CXCL9*, *CXCR3*, *GBP1*, *HIF1A* and *IL23A* genes are insufficiently expressed (with a 2–10 -fold difference; p <0.05) [45].

The sensitivity of differentiating glioma patients and healthy individuals using the Real time PCR by the presence of such RNA as *microRNA-10b*, *17-5p*, *125b* and *221*, is 30–96% with the specificity reaching up to 95% (see table 2).

The levels of detectable *microRNA-21*, *128*, *342-3p* and some others decrease after resection or chemotherapy, though increasing during the recurrences [43, 44, 49], with the elevation of *microRNA-320e* levels being associated with higher progression risk than the tumor volume according to data from MRI [12].

The levels of detectable *microRNA-21*, *17-5p*, *125b* and *221* are higher in glioma patients with higher malignancy degree and with more aggressive histological type (2–10 times higher in high malignancy degree gliomas comparing to low-grade gliomas; p <0.05), while the levels of *microRNA-128 and 342-3p* decrease 2–3 -fold with higher tumor grade (see table 3) [44, 48]. High expression levels of *microRNA-17-5p*, *125b* etc., as well as of the *HOTAIR* and *STEAP3-AS1* long-chain non-coding RNA is associated with worse total survival rate and progression-free survival [48, 50], while the high expression of *microRNA-1-3p*, *26a-1-3p*, *487b-3p* and *342-3p* is a positive prognostic factor [44, 51, 52]. The plasma levels of *microRNA-1-3p*, *26a-1-3p* and *487b-3p* are decreased in patients with wild type IDH, which is associated with lower survival among these patients (odds ratio, OR, 0.24; 95% CI 0.12–0.47; p <0.05) [51].

DISCUSSION

The capability of fluid biopsy to qualitatively and quantitatively determine the levels of markers in various biological fluids allows for not only diagnosing the presence of glioma, but also for differentiating the true tumor recurrence from the pseudorecurrence. Besides, fluid biopsy demonstrates the capability to define the malignancy degree of the tumor and to predict the survival of the patients after conducted therapy (see table 3). The optimal markers for this instrument are the extracellular tumor nucleic acids, such as DNA and RNA, with the most significant genetic and epigenetic alterations, such as mutations in genes *TERT*, *TP53*, *H3F3A*, *IDH1*, *CDKN2A/B* etc., aberrant methylation patterns of *MGMT*, *RASSF1A*, *pPARP-1*, *pSHP-1*, *pDAPK-1*, *CDKN2A* and *TIMP-3*, as well as *microRNA-21* and *microRNA-221*, detectable in the cerebrospinal fluid using digital droplet PCR. With the introduction of NGS into wide clinical practice, most probably, spreading of the analysis of large gene panels could be observed, capable of precisely diagnosing oncological diseases when testing the serum samples. However, this list will probably be expanded during further studies of fluid biopsy.

The highest diagnostic potential in the diagnostics of gliomas, probably, belongs to testing the cerebrospinal fluid for markers using NGS and digital droplet PCR, which are characterized by high (up to 90–100%) sensitivity and specificity [12, 21, 26, 34]. On the other hand, testing the serum for markers using the abovementioned methods or testing the cerebrospinal fluid with using Real time PCR shows significantly lesser values (up to 50–75% and up to 90%, respectively) [22, 27, 39]. To a great extent, this is due to the ability of the blood-brain barrier to hamper the penetration of marker substances into blood from the cerebrospinal fluid and to prevent its detection in serum [40]. Probably, for the reason that the extracellular tumor RNA has lesser size, its capabilities of passing through the blood-brain barrier is significantly higher comparing to the extracellular tumor DNA. Thus, detecting the given marker in serum using Real time PCR shows the sensitivity and specificity of up to 90–100%, unlike the testing for extracellular tumor DNA [44, 48]. Besides, low sensitivity and specificity of detection can be resulting from the features of the testing methods themselves. As it is known, Real time PCR has a lower resistance to PCR inhibitors comparing to digital droplet PCR, along with the limitations when operating in the range of low concentrations of nucleic acids (characteristic for plasma extracellular tumor DNA of gliomas), which can

negatively affect the testing results [53]. NGS allows for simultaneously testing multiple genomic loci, detecting the exact sequence alterations, which may determine its higher sensitivity and specificity [54]. Thus, for example, in the research by T. Sabedot et al. [35], testing the extracellular tumor DNA for methylation in serum samples using NGS had a sensitivity of 100% with a 97.78% specificity, which is significantly higher comparing to Real time PCR in similar settings.

Thus, fluid biopsy shows a significant diagnostic and prognostic potential. Despite this, it is characterized by a number of limitations (one of the main being the absence of validated approaches to testing the marker substances), preventing its routine application. For example, in the majority of the research works, DNA isolation is performed out of 1–4 ml of the substrate, while the RNA — from 100–400 µl (see table 2, 3), with the reagent kits used by the authors, which allow for processing up to 5 ml and 900 µl of biological fluids, respectively, without losing the extraction efficiency [55, 56]. As a result, there is a potential loss of 20–90% of nucleic acids present, which may negatively affect the test outcome. Moreover, in most part of the research works, the volume of the test material is not disclosed, which hampers even more the evaluation of the efficiency of the proposed approach to fluid biopsy. This problem can be solved by arranging the large scale multicenter research, which could allow for univocal defining the set of markers and the approach to testing them, which could be optimal for routine clinical application of fluid biopsy. Besides, the analysis of the epigenetic regulation of gliomas is encumbered by using bisulfite conversion, which may lead to the degradation of 50–90% of nucleic acids, resulting in a decrease in the sensitivity of this method. Apparently, the most part of the fragments after the conversion represent up to 80–90 base pairs, which sometimes affects the capabilities of analyzing them [57]. Probably, in order to detect the genome methylation aberrations, it could be practical to use methylation-sensitive restrictases, which, to a lesser degree, may result in the non-specific degradation of DNA [58]. However, the limitation of this approach is that, by no means all the perspective DNA foci, intended for testing and showing an altered methylation pattern, carry the restriction sites matching the currently available enzymes [59].

The promising methods of DNA methylation analysis include also the enzymatic conversion. This procedure, just like the bisulfite conversion, transforms the non-methylated cytosine into uracil, however, not resulting in massive degradation of the genetic material, which increases the test sensitivity and makes it possible to analyze also the damaged DNA, for example, obtained from the paraffin-embedded sample slices. The benefits of enzymatic conversion can also include the capability of operating with low levels of DNA content (ranging from 100 pg), which is oftentimes observed in liquid biopsy substrates. Due to its novelty, this technology has still not gained wide spreading, however, in future, probably, more and more research groups shall prefer this method, rather than bisulfite conversion [60].

CONCLUSION

The present review has estimated the role of fluid biopsy with sampling plasma and cerebrospinal fluid containing extracellular tumor nucleic acids in glioma patients. This promising method has demonstrated its efficiency in the diagnostics and predicting the course of this disease, however, it requires further development. Upon its implementation in clinical practice, the medical community may gain access to wide possibilities in the diagnostics, treatment efficiency control and therapy selection in cases of oncological diseases.

ADDITIONAL INFORMATION

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