The Journal of Liquid Biopsy 4 (2024) 100142

Contents lists available at ScienceDirect

The Journal of Liquid Biopsy

journal homepage: www.sciencedirect.com/journal/the-journal-of-liquid-biopsy

Role of cell-free DNA and extracellular vesicles for diagnosis and surveillance in patients with glioma

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ARTICLE INFO

Keywords:
Liquid biopsy
Glioblastoma
cfDNA
Extracellular vesicles

ABSTRACT

Objectives: Liquid biopsy can be used to make the diagnosis, to screen treatment response, and to predict the prognosis. Extracellular vesicles (EVs) and cell-free DNA (cfDNA) sources are used as liquid biopsy biomarkers from body fluids such as serum, cerebrospinal fluid, urine, and mucosa. The purpose of this study was to investigate whether EVs and cfDNA are predictive for diagnosis and prognosis in patients with glioma.
Methods: cfDNA and EVs levels were measured from 17 glioma patients at three different time intervals (before surgery, 10–14 days after surgery, and at the time of recurrence) and 7 healthy individuals. We investigated whether their level increased in glioma patients. Also, the correlation between clinical outcome and their levels was analyzed.
Results: The mean serum cfDNA level in glioma patients was found to be higher compared to that in healthy controls. The difference between cfDNA level before surgery and that at 3 months follow-up was found to be statistically significant. Also, the mean serum EVs level in the glioma patients was found to be significantly higher compared to that in the control group.
Discussion: Our results suggested that cfDNA and EVs could be used as diagnostic biomarkers in patients with glioma. cfDNA could be also a possible biomarker for the surveillance of glioma patients. Further studies are warranted to confirm our findings.

1. Introduction

Gliomas are deadly brain tumors with poor outcomes and are known to be resistant to conventional therapies. Glioblastoma is the most common malignant primary brain tumor in adults, which represents almost %50 of gliomas. Despite surgical resection, chemotherapy with Temozolomide (TMZ), and radiotherapy the overall survival (OS) varies between 16 and 20 months for glioblastoma patients [1,2].

In this group of patients, it is crucial to make the right diagnosis before starting the treatment and also appropriate evaluation of the patients during their follow-ups, (recurrence vs. treatment response). For these purposes, magnetic resonance imaging (MRI) is mostly used, although the information may be limited and conflicting. Stereotactic tissue biopsy is the gold standard in the diagnosis of glioma [3,4]. Nonetheless, the invasiveness of this method, side effects after biopsy, and tumor heterogeneity are the disadvantages of tissue biopsy [5]. Therefore, researchers have started to search for alternative diagnostic approaches which would offer simplicity.

Liquid biopsy (LB) is a non-invasive, sensitive, and rapid technique that can overcome the disadvantages of standard diagnostic methods [6]. LB can be used to make the diagnosis, to screen treatment response, to predict the prognosis, to perform early detection of cancer, and to determine the progression of the disease [1]. The most common body fluid used in LB is blood. However, patients' serum, cerebrospinal fluid (CSF), urine, and mucosa samples have been used to detect biomarkers with LB [7]. Circulating tumor DNA, circulating tumor RNA, miRNAs, circulating tumor cells, extracellular vesicles (EVs), tumor-educated platelets, metabolites, and other analytes have been investigated during LB processes [6,8].

In systemic cancers, LB is also used as an informative tool on a patient's prognosis [9]. There are some challenges in the use of LB in central nervous system (CNS) malignancies. Due to the presence of the blood-brain barrier (BBB), it is challenging to investigate biomarkers for CNS tumors from blood [10]. Nevertheless, many studies are going on

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https://doi.org/10.1016/j.jlb.2024.100142
Received 10 November 2023; Received in revised form 18 January 2024; Accepted 18 January 2024

blood biomarkers in patients with CNS malignancies and it is a useful method due to the invasiveness of tissue biopsy and tumor heterogeneity [11].

EVs and cell-free DNA (cfDNA) sources are studied in different types of cancer. However, there is limited information on the role of cfDNA and EVs levels for diagnosis and surveillance in patients with glioma. Therefore, we aimed to investigate the role of cfDNA and EVs levels in diagnosis and evaluating the treatment response in glioma patients.

2. Materials and methods

2.1. Patient selection

Patients, who underwent surgical resection for glioma, were included in this study. Characteristics of the patient's including sex, age, tumor volume, Karnofsky performance status (KPS), and survival time were retrospectively reviewed. Matched healthy individuals were used as controls. The design of the study is summarized in Fig. 1.

This study was performed in line with the principles of the Declaration of Helsinki. This study was approved by the Ethical Committee of Bezmialem Vakif University (Ethical no: 2019–19037). All the patients signed an informed consent form to participate in the study.

2.2. Isolation and quantification of cfDNA

cfDNA samples were extracted from a 1 ml serum sample with ChargeSwitch gDNA Serum Kit (Invitrogen, Life Technologies) according to the manufacturer's guidelines.

One ml of serum was treated with lysis buffer, proteinase K, and then incubated at room temperature. After treatment with purification buffer, the sample was mixed with magnetic beads and incubated in EasySep Magnet (StemCell Technologies, Canada). The washing steps were completed. The DNA was eluted with elution buffer and stored at −20 °C. cfDNA samples were measured with Qubit® 2.0 fluorometer using Qubit dsDNA High Sensitivity Assay (Invitrogen, Life Technologies). Quantification was done according to the manufacturer's guidelines.

2.3. Isolation and quantification of EVs

EVs were isolated from a 500 µl serum sample with Total Exosome Isolation Reagent (Invitrogen™, Massachusetts, USA) according to the manufacturer's guidelines. 100 µL of EVs isolation reagent was added to the serum sample and incubated at 4 °C for 30 min. The sample was centrifuged at 10.000 rpm for 10 min. The supernatant was removed and the pellet was resuspended in PBS. Samples were stored at −20 °C. The EVs sample were quantified with the EXOCET Exosome Quantitation Kit (System Bioscience, CA, USA) according to the manufacturer's guidelines.

2.4. Statistical analysis

IBM SPSS 22 was used for statistical analysis. The levels of cfDNA and EVs in the patient group were compared with the control group using the Mann-Whitney-U test. Levels of cfDNA and EVs in the patient group for different time intervals were compared using the Wilcoxon Signed Rank test. The correlation between the levels of cfDNA and EVs and clinical data was analyzed with a Pearson Correlation test. Statistical significance was set at $p < 0.05$.

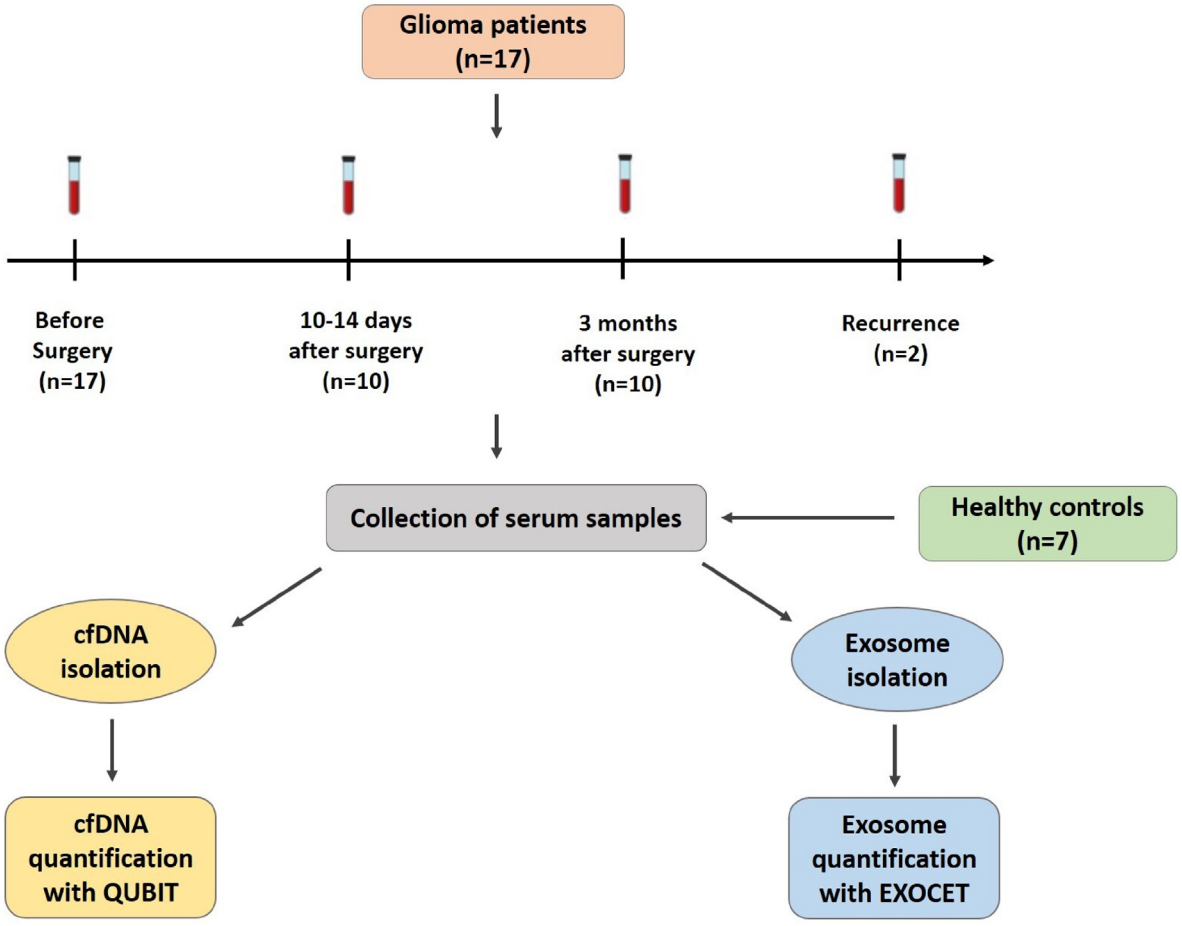

Fig. 1. Summary of the methods.

3. Results

3.1. Patient characteristics

Seventeen patients, who underwent surgical resection for glioma, and seven healthy controls were included in the study. The median age of the patients was 61 years (range: 31–81 years). The median age of the healthy controls was 61 years (range: 50–70 years). The median tumor volume was 56.1 cm^3 (range 3.8–182.7 cm^3). The median overall survival time was 9.4 months (range: 0.4–29.03 months). An overview of patients' characteristics is presented in Table 1.

3.2. cfDNA analysis in glioma patients

To elucidate whether cfDNA level in serum could be an indicator for diagnosis in patients with glioma, cfDNA levels were measured in patients' serum during various time intervals. The mean serum cfDNA level in the glioma patients (n = 17) (643.4 ng/ml) was found to be higher compared to that in healthy controls (n = 7) (287.9 ng/ml), although the difference was not statistically significant (Fig. 2a). There was a trend for the increase in different grades of glioma patients, and those with glioblastoma had the highest level of cfDNA among others (Fig. 2b).

Also, to examine whether cfDNA level in serum could be an indicator for treatment response, cfDNA levels were measured before surgical resection, 10–14 days after surgery, 3 months after surgery, and at the time of recurrence. Ten patients were included in the statistical analysis because seven patients were lost to follow-up or we were not able to obtain serum samples. There was a trend for a decrease in the cfDNA levels during the follow-up period after surgical resection. cfDNA levels were 600.2 ng/ml before surgery, 368 ng/ml at 10–14 days after surgery, and 181.4 ng/ml at three months after surgery. The difference between cfDNA level before surgery and that at 3 months follow-up was found to be statistically significant (p = 0.007). Also, the difference between cfDNA level 10–14 days after surgery and at 3 months follow-up was found to be statistically significant (p = 0.037) (Fig. 3a).

Further, we investigated whether cfDNA could be an indicator of tumor recurrence in patients with glioma. For this, we looked at two glioblastoma patients, who had a recurrence, and found that cfDNA levels were 349.2 ng/ml before surgery, 300.2 ng/ml at 10–14 days after surgery, 93.1 ng/ml at 3 months after surgery and 177.6 ng/ml at the time of recurrence. Although this was not statistically significant, the cfDNA level increased at the time of recurrence (Fig. 3b).

Table 1
Characteristics of patients who underwent surgical resection for glioma.

Characteristic	Value
Cases (F/M), n	6/11
Median age (range), years	61 (31–81)
Median KPS (range)	80 (60–90)
Median tumor volume, cm^3 (range)	56.1 (3.8–182.7)
IDH status, n (%)	
Wild	13 (76)
Mutant	4 (24)
Grades, n (%)	
2	1 (6)
3	1 (6)
4	15 (88)
Median radiological follow-up time (range)	13.1 (0.03–38.5)
Median clinical follow-up time (range)	17.2 (0.3–39.2)
Radiation therapy after surgery, n (%)	
Yes	10 (59)
No	7 (41)
Chemotherapy, n (%)	
Yes	9 (53)
No	8 (47)
Extend of tumor resection, n (%)	
Gross total	9 (53)
Subtotal	8 (47)
Median overall survival time, months (range)	9.4 (0.4–29)

3.3. EVs level

To elucidate whether EVs levels in serum could be an indicator for diagnosis in patients with glioma, EVs levels were measured in patients' serum. The mean serum EVs level in the glioma patients (n = 17) (12998.5 U/ml) was found to be significantly higher compared to that in the control group (n = 7) (5135.3 U/ml) (p = 0.002) (Fig. 4a). There was a trend for the increase among different grades of glioma patients, and grade 4 glioma patients had the highest level of EVs (Fig. 4b).

Also, to examine whether EVs levels in serum could be an indicator of treatment response, EVs levels were measured before surgical resection, 10–14 days after surgery, 3 months after surgery, and at the time of recurrence. Ten patients were included in the statistical analysis because the remaining seven patients were lost to follow-up or we were not able to obtain serum samples. There was a trend for the increase in EVs levels among different time intervals, although this change was not statistically significant. EVs levels were 13056.4 U/ml before surgery, 15921.9 U/ml 10–14 days after surgery, and 15707.3 U/ml 3 months after surgery (Fig. 5a).

Further, we investigated whether EVs level could be an indicator of tumor recurrence in patients with glioblastoma. However, the level of EVs did not increase as we expected at the time of recurrence. EVs levels were found to be 19557.7 U/ml before surgery, 13226.3 U/ml at 10–14 days after surgery, 13124.7 U/ml at 3 months after surgery and 8006.1 U/ml at the time of recurrence (Fig. 5b).

cfDNA level decreased after surgery and increased at the time of recurrence in one out of two patients. EVs level decreased after surgery but there was no trend for the time of recurrence. The demonstration of cfDNA and EVs levels of two patients is seen in Fig. 6.

3.4. Correlations between cfDNA and EVs levels and clinical outcome

The correlation between the data of cfDNA and EVs and the data of clinical information was investigated. There was a correlation between the cfDNA level before surgery and the tumor volume (p = 0.001). There was an association between the surgical technique and the cfDNA level 10–14 days after surgery (patients who underwent subtotal resection had higher cfDNA levels at 10–14 days post-surgery) (p = 0.012)).

4. Discussion

We tested the hypothesis that cfDNA and EVs would be diagnostic biomarkers in patients with glioma. Our results showed that cfDNA and EVs were found to be higher in glioma patients compared to healthy individuals, and cfDNA could be an indicator of treatment response in glioma.

Researchers have started to study liquid biopsy to find new diagnostic biomarkers in the cancer field. In the study by Husain et al., the authors investigated the role of cfDNA level in the diagnosis of glioma in 25 adult patients with diffuse glioma. They showed that serum levels of cfDNA were higher in glioma patients compared to healthy controls [12]. Similar to this study and our findings, Bagley et al. assessed 42 patients with glioblastoma and reported that cfDNA levels were higher in patients compared to healthy controls [13]. Also, as expected we found that cfDNA levels decreased during the follow-up period after surgical resection of the tumor. Similarly, Nørøxe et al. [14] found that the level of cfDNA decreased after surgery in 8 glioblastoma patients. This is possibly due to a decrease in the size of the tumor bulk. Also, our results showed that cfDNA levels increased at the time of recurrence. Similar to our findings, the elevation in the cfDNA level of glioblastoma patients at the time of progression is shown in other studies [13,15]. Early detection of the tumor recurrence/progression is crucial to initiate the appropriate treatment as early as possible since glioblastomas are aggressive tumors. Serum cfDNA might be a possible biomarker for the detection of tumor recurrence/progression in glioblastoma patients. Due to the limitations of tissue biopsy and MRI, measuring the cfDNA level of patients can be

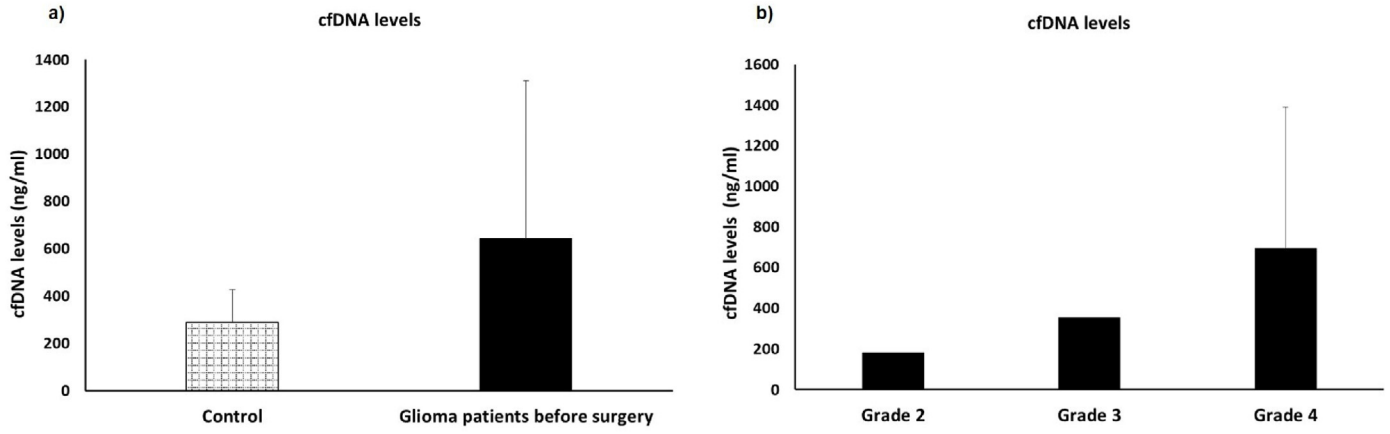

Fig. 2. The levels of cfDNA in glioma patients. a) cfDNA level is higher in glioma patients (n = 17) compared to the control group (n = 7) (control group 287.9 ng/ml (± SD 138.3), glioma patients 643.4 ng/ml (± SD 667)). b) cfDNA levels increased as the grade of the glioma increased (n = 17) (183 ng/ml in grade 2, 353.5 ng/ml in grade 3, 693.5 ng/ml in grade 4 (± SD 696.1). However, the difference did not reach statistical significance.

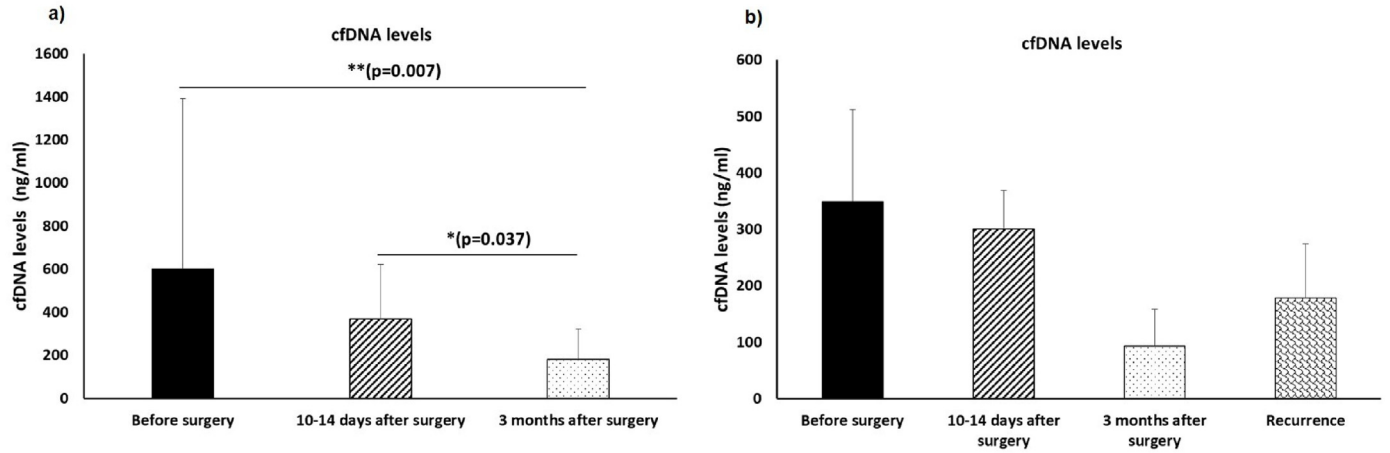

Fig. 3. The levels of cfDNA in patients with glioma during the follow-up periods a) cfDNA levels in glioma patients with three different time intervals (n = 10). CfDNA levels of glioma patients 3 months after surgery significantly decreased compared to the level before surgery (p = 0.007). CfDNA levels of glioma patients at 3 months after surgery significantly decreased compared to those at 10–14 days after surgery (p = 0.037) (before surgery 600.2 ng/ml (± SD 789.2), 10–14 days after surgery 368.05 ng/ml (± SD 255), and 3 months after surgery patients 181.4 ng/ml (± SD 138.8)). b) Despite this was not statistically significant, cfDNA levels increased at the time of recurrence (n = 2) (349.25 ng/ml (± SD 162.988) before surgery, 10–14 days 300.2 ng/ml (± SD 68.2) after surgery, 93.13 ng/ml (± SD 64.8) 3 months after surgery, and 177.66 ng/ml (± SD 96.3928) at the time of recurrence). (*p < 0.05, **p < 0.01).

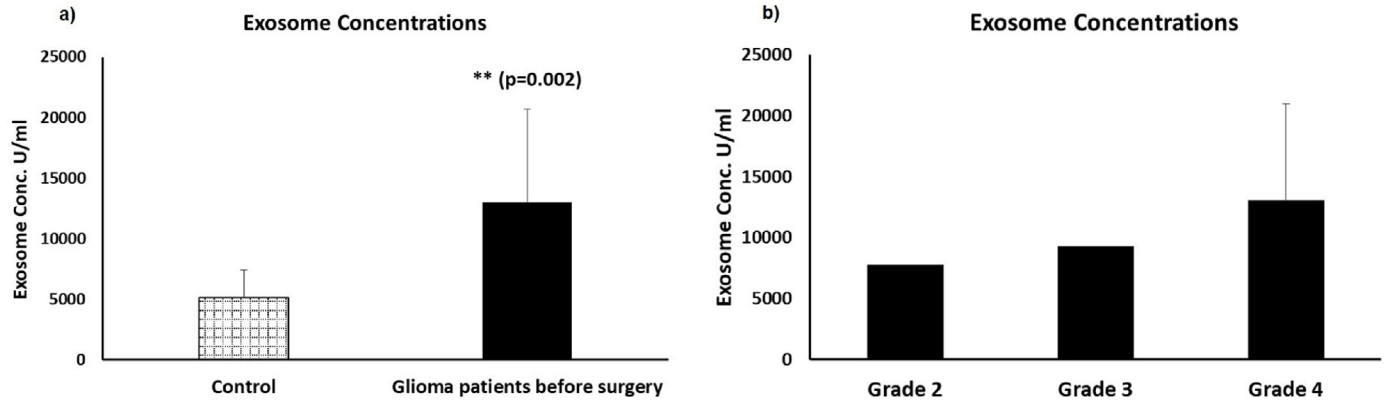

Fig. 4. The levels of EVs in glioma follow-up patients. a) EVs level is significantly higher in glioma patients (n = 17) compared to the control group (n = 7) (p = 0.002) (control group 5135.3 U/ml (± SD 2299), glioma patients 12998.5 U/ml (± SD 7699.7)). b) EVs level increased as the grade of the glioma increased (n = 17) (7750.88 U/ml in grade 2, 9284.9 in grade 3, 13030.5 U/ml in grade 4 (± SD 7973.1)). (**p < 0.01).

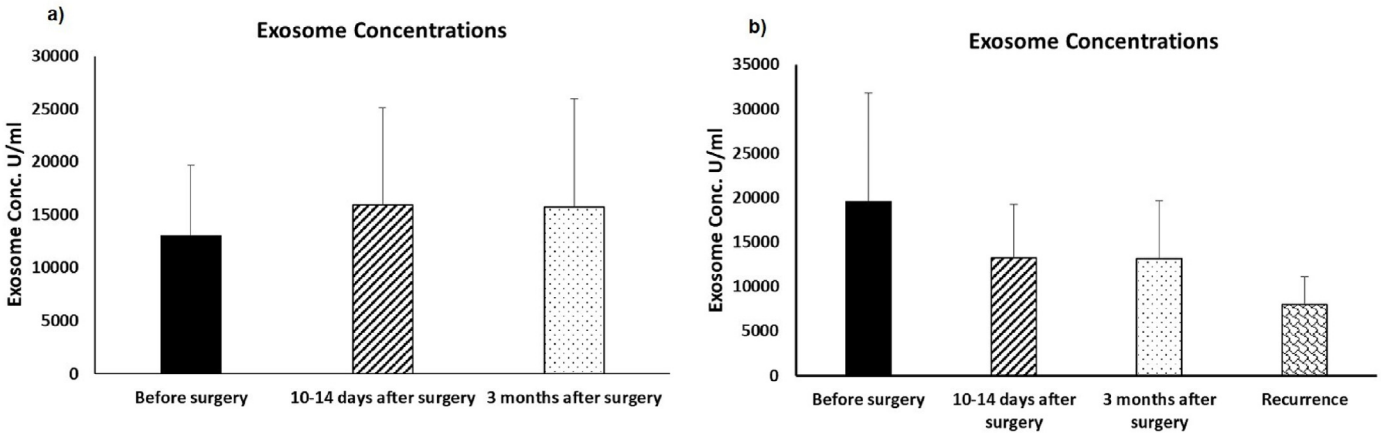

Fig. 5. The levels of EVs in glioma patients during the follow-up periods. a) There was a trend for an increase in EVs levels after surgery, however, this increase was not statistically significant (n = 10). (before surgery 13056.4 U/ml (± SD 6579.2), 10–14 days after surgery 15921.9 U/ml (± SD 9180.1), and 3 months after surgery 15707.3 U/ml (± SD 10248.9)). b) EVs levels in glioma patients with various time intervals (n = 2). (19557.7 U/ml (± SD 12266.5) before surgery, 13226.3 U/ml (± SD 6041.6) 10–14 days after surgery, 13124.7 U/ml (± SD 6516.1) 3 months after surgery, 8006.1 U/ml (± SD 3092.6) and at the time of recurrence).

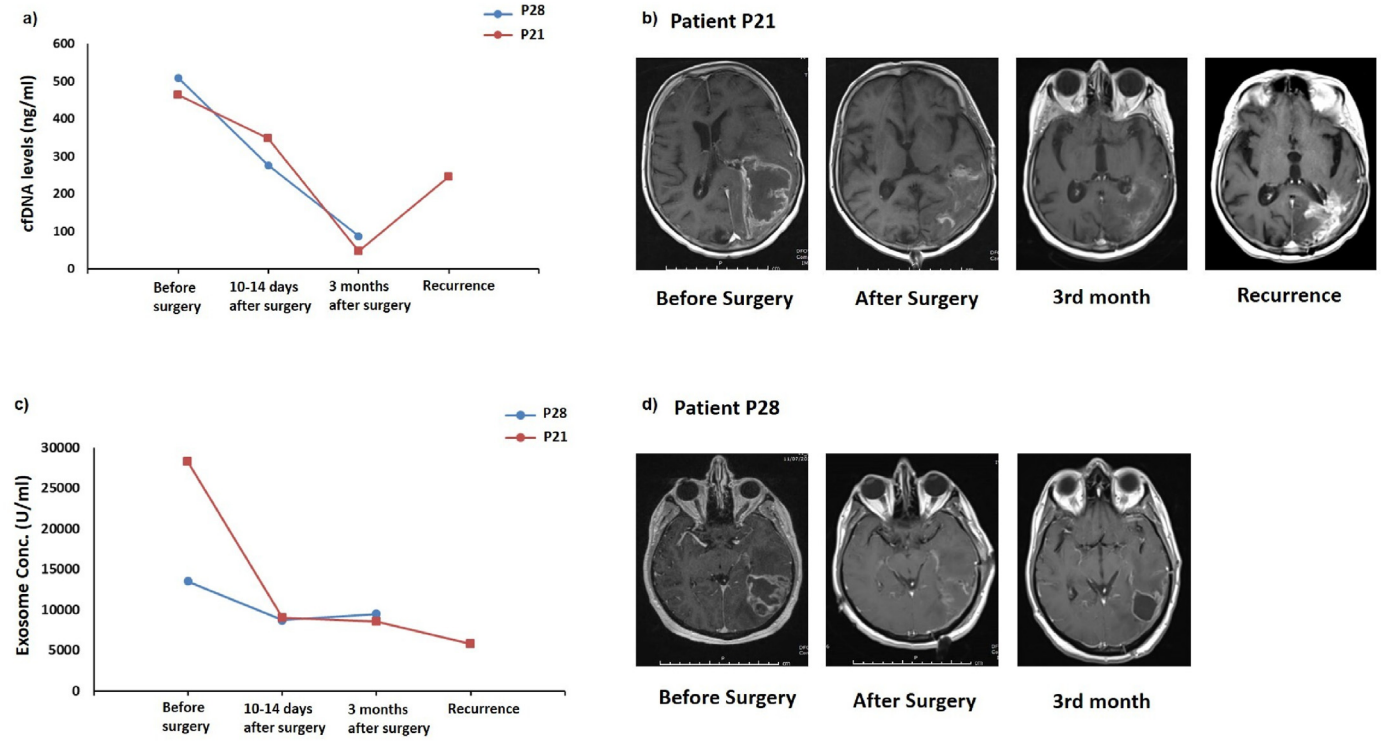

Fig. 6. Demonstration of cfDNA and EVs levels in patients P21 and P28 and their MRI images. a) cfDNA levels of patient P21 and P28, b) MRI images of patient P21, c) EVs levels of patient P21 and P28, d) MRI images of patient P28.

more applicable and non-invasive.

We found a significant correlation between the tumor size and the cfDNA level before surgery. Similarly, Bagley et al. demonstrated the relationship between increasing tumor volume and cfDNA concentration in glioblastoma patients [13]. Also, there was a significant association between the subtotal resection (STR) and cfDNA levels 10–14 days after surgery. The elevation of cfDNA level in patients with STR after surgery might be due to the remaining cancerous tissue in the brain. Also, during STR of the tumor, the rupture of the tumor capsule possibly leads to increased release of cfDNA from tumor cells into the microenvironment and bloodstream. However, previous studies have not investigated the correlation between the type of surgical resection and the level of cfDNA.

EVs are used as an LB tool for different types of cancers [16–19]. The association between EVs and treatment response, tumor size, and status of mutation has been investigated in several studies [20]. In our study, the level of EVs in glioma patients before surgery was significantly higher than in the healthy controls. Similarly, Osti et al. documented that the concentration of plasma EV was higher in 43 glioblastoma patients than in the healthy controls [21]. Also, the elevation in EV concentration compared to the paired healthy group was shown higher in other types of cancers such as prostate cancer [16], and non-small-cell lung cancer [17]. However, it has been rarely investigated in glioma patients.

We found that the level of EVs increased after surgical resection. We speculate that since there were different types of tumor resection (piecemeal vs. en bloc resection), the level of EVs might not be standard in all patients. The tumor bulk was disrupted who underwent piecemeal

resection during surgery in some cases, due to this reason EVs might be released in larger amounts from tumor cells. This disruption can cause fluctuation in EVs levels. Of note, we were not able to assess the role of tumor resection type on the EVs level due to the retrospective nature of the study. On the other hand, since the patients had radiotherapy and chemotherapy with TMZ, there might be an effect of treatments on EVs levels. There is no relevant study evaluating the EVs level during the follow-up period with glioma in the literature.

In our study, we used serum to investigate the cfDNA and EVs in the bloodstream. Although plasma was used in several studies, serum is also used as a reliable source for LB applications. Plasma carries non-tumoral cell-released DNA and EVs particles, therefore serum can be preferred over plasma to prevent their presence in the analysis; and also serum contains a higher level of cfDNA and EVs compared to plasma [22]. In the study by Sabedot et al. [23], the authors investigated the DNA methylation status from serum cfDNA in glioma patients. Methylation status was found to be more reliable when used serum compared to plasma. There are several studies on the examination of cfDNA from serum in glioma patients [24,25]. EVs are secreted to the bloodstream from tissues and cells. Li et al. reported that tissue-related EVs are in the minority in plasma [26]. Also, there are several studies investigating Evs using serum in glioma patients [27–29]. Overall, serum can be considered a useful and safe option for liquid biopsy to investigate cfDNA and EVs in glioma patients.

LB studies consider body fluids such as blood, CSF, urine, and saliva as sources of biomolecules. In brain tumor research, CSF is a promising material for LB due to its closeness to the tumor region [30]. CSF has the advantages of being adjacent to the tumor, carrying larger amounts of tumor-related genetic material, and containing a lower number of molecules released from non-neoplastic cells compared to blood [31,32]. Miller et al. investigated genomic alterations in CSF samples of 85 glioma patients. 1p/19q codeletion, IDH1 mutation, and IDH2 mutation were common in DNA derived from tumor tissue and CSF [33]. Also, CSF has been used in order to obtain cfDNA and EVs in several studies [34–36]. Although CSF is a reliable material, there are several drawbacks for studying CSF from patients. Lumbar puncture is a risky procedure due to its invasiveness and post-procedure complications such as headache, bleeding, infection, and cerebral herniation [2,37]. On the other hand, blood collection is less invasive and can be repeated at different time intervals. In our study design, we did not include CSF, and we only obtained serum for investigating cfDNA and EVs.

This is the first report in the literature that both cfDNA and EVs levels were studied in the same group of glioma patients. However, there are some limitations of the study: 1) Limited number of patients, 2) missing data from follow-ups. However, this was a preliminary study, and further investigation with a larger patient group is in progress.

The use of EVs has been recently becoming popular in various diseases, such as cancer, neurodegenerative, and metabolic diseases [38–40]. In particular, for glioma research EVs have several promising clinical applications: 1) Diagnosis: As we presented in this research, the use of EVs has potential for diagnostic purposes [41]. 2) Monitoring the treatment response and outcome prediction: EVs, as we suggested, can be used to predict the outcome of the patient, facilitate the providing the optimal treatment for patients, and monitor the treatment response [42]. 3) Drug delivery: EVs are promising vehicles to carry drugs (i.e. chemotherapy and vaccines) into the tumor [43]. EVs have the advantage of having a small size across the BBB. The use of these applications needs to be confirmed with preclinical and clinical research.

cfDNA and EVs are known sources for liquid biopsy. The studies regarding sequencing of cfDNA from different sources such as urine, plasma, tumor in situ fluid, and cerebrospinal fluid revealed that cfDNA is an applicable biological source for liquid biopsy. Also, these studies showed detectable mutation rates in cfDNA of glioma patients [44–46]. Sequencing of cfDNA and EVs biomarkers such as miRNA, DNA, and proteins are used for the prediction, diagnosis, and screening of disease. Therefore, expanding our knowledge on liquid biopsy would lead to more extensive research such as sequencing of cfDNA and investigating the potential biomarkers in EVs (non-coding RNAs, DNA, proteins, metabolomics, etc.) in the future.

5. Conclusions

Our results suggested that cfDNA and EVs could be used as diagnostic biomarkers in patients with glioma. CfDNA could be also a possible biomarker for the surveillance of glioma patients. Further studies are warranted to confirm our findings.

Ethics

This study was performed in line with the principles of the Declaration of Helsinki. This study was approved by the Ethical Committee of Bezmialem Vakif University (Ethical no: 2019–19037).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors thank Bezmialem Vakif University for its infrastructure and support. This research was funded by Bezmialem Vakif University, Turkey, (Grant number BAP-6.2019/15).

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