İSTANBUL UNIVERSITY PRESS

Journal of Advanced Research in Health Sciences
Sağlık Bilimlerinde İleri Araştırmalar Dergisi,
JARHS 2025, 8 (2): 106–113

https://doi.org/10.26650/JARHS2025-1671717

Submitted: 14.04.2025
Accepted: 14.04.2025
Published Online 19.06.2025

Journal of Advanced Research in Health Sciences
Sağlık Bilimlerinde İleri Araştırmalar Dergisi

Research Article **Open Access**

The Role of Liquid Biopsy in Patients Treated with Gamma Knife Radiosurgery for Brain Metastasis

Beyin Metastazı için Gamma Knife Radyocerrahisi ile Tedavi Edilen Hastalarda Likit Biyopsinin Rolü

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Abstract

Objective: The treatment modality for brain metastases (BM) is surgical resection or radiotherapy. Gamma Knife radiosurgery (GKR) treatment prevents toxicity to healthy brain tissue; therefore, this modality is preferred for treating selected patients with BM. Our aim in this study was to investigate the role of cfDNA levels, exosome levels, and miR-208a expressions as biomarkers for diagnosis and predicting the outcome in patients with BM treated with GKR.

Material and Methods: We included nine patients with brain metastasis who received GKR in this study. Samples were taken from patients at different time intervals (before GKR, 1 month after GKR, and at recurrence). Eight age- and gender-matched eight controls were used as healthy individuals. cfDNA, exosome, serum miRNA, and serum exosomal miRNA were isolated from the patient and control groups.

Results: The levels of cfDNA and exosomes were higher in BM patients than in the healthy group. Exosome and cfDNA levels decreased after GKR and increased at recurrence. The expression of serum miR-208a and serum exosomal miR-208a was increased after GKR. The expression of serum miR-208a was significantly higher in patients with lung cancer than in healthy controls and non-lung cancer patients. The expression of serum exosomal miR-208a was found to be significantly higher in patients with lung cancer than in healthy controls.

Öz

Amaç: Beyin metastazlarının (BM) tedavi yöntemi cerrahi rezeksiyon ve/veya radyoterapidir. Gamma Knife radyocerrahisi (GKR) tedavisi, sağlıklı beyin dokusuna toksisiteyi önler. Bu nedenle, BM'li seçilmiş hastaların tedavisinde tercih edilen bir modalitedir. Bu çalışmada, GKR ile tedavi edilen BM hastalarında tanı koyma ve tedavi sonucunu öngörmede biyobelirteç olarak cfDNA seviyeleri, eksozom seviyeleri ve miR-208a ekspresyonunun rolünün araştırılması amaçlandı.

Gereç ve Yöntemler: Bu çalışmaya GKR uygulanan beyin metastazlı dokuz hasta dahil edildi. Hastalardan farklı zaman aralıklarında (GKR öncesi, GKR sonrası 1. ay ve nüks döneminde) örnekler alındı. Yaş ve cinsiyet açısından eşleştirilmiş sekiz sağlıklı birey kontrol grubu olarak kullanıldı. Hasta ve kontrol grubundan cfDNA, eksozom, serum miRNA ve serum ekzozomal miRNA izole edildi.

Bulgular: BM hastalarında cfDNA ve eksozom düzeyleri sağlıklı gruba kıyasla daha yüksek bulundu. GKR sonrası cfDNA ve eksozom düzeyleri azaldığı, ancak nüks döneminde tekrar arttığı tespit edildi. Serum miR-208a ve serum ekzozomal miR-208a ekspresyonu GKR sonrası artmış bulundu. Serum miR-208a ekspresyonu, akciğer kanseri hastalarında sağlıklı kontrollere ve akciğer kanseri olmayan hastalara kıyasla anlamlı derecede daha yüksek olduğu saptandı. Serum ekzozomal miR-208a ekspresyonu ise akciğer kanseri hastalarında sağlıklı kontrollere kıyasla anlamlı derecede yüksek bulundu.

Citation: Karaçam B, Khan I, Burce Elbasan E, Mahfooz S, Akdur K, Hatiboğlu MA. The Role of Liquid Biopsy in Patients Treated with Gamma Knife Radiosurgery for Brain Metastasis. Journal of Advanced Research in Health Sciences 2025;8(2):106-113. https://doi.org/10.26650/JARHS2025-1671717

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Conclusion: Our results showed that cfDNA and exosome levels might be used as diagnostic markers for BM patients.

Keywords Brain metastasis · cfDNA · exosome · miRNA

Sonuç: Bulgularımız, cfDNA ve eksozom düzeylerinin BM hastaları için tanısal belirteç olarak kullanılabileceğini göstermektedir.

Anahtar Kelimeler Beyin metastazı · cfDNA · eksozom · miRNA

INTRODUCTION

The process of metastasis consists of sequential and interrelated steps during the migration of malignant tumour cells to distant microenvironments (1). The occurrence of metastasis requires malignant cells to spread in the bloodstream and new discontiguous niches, use angiogenesis for nutrient requirements and evade immune cells in the receiving tissue (1, 2). The metastasis of systemic cancers-frequently lung, breast, melanoma, and gastrointestinal tumors-to the brain is more common than primary brain tumours. The preferred treatment for brain metastases (BM) is surgical resection or radiotherapy (3, 4). After surgery alone, the average survival ranges from 4 to 6 months; with surgery and radiation, the average survival time can reach 12 months. An important cause for the poor outcome is the local and distant recurrences of the tumours (5).

Researchers have shown that concomitant therapy with tumour resection and whole-brain radiation therapy improved the survival time of patients with BM. However, the Gamma Knife radiosurgery (GKR) treatment modality prevents toxicity to normal brain tissue. Therefore, GKR limits the development of radiation-induced neurocognitive diseases and decreases the performance status (4, 6, 7).

In some cases, there are difficulties in distinguishing BMs from other brain tumours, such as glioblastoma and lymphoma. Appropriate diagnosis is of the utmost importance in effectively treating patients with BM. The challenges in diagnosing and monitoring BM patients directed researchers to find new non-invasive methods such as liquid biopsy (LB). LB provides information about the molecular structure of the tumour through the analysis of samples taken from the body fluids (blood, saliva, urine, and cerebrospinal fluid) of patients. LB is more applicable to monitor the response to treatment following the initial diagnosis and treatment regimens. Furthermore, analysis of circulating tumour cells (CTCs), extracellular vesicles (EVs) such as exosomes, and circulating cell-free DNA (cfDNA) can potentially reflect the tumour genome and transcriptome (8). In addition, the molecular examination of cfDNAs obtained by LB and the exosomal content including DNA, RNA, proteins, and micro-RNAs (miRNAs) can be performed (9, 10).

However, in the literature, molecular studies conducted on patients' peripheral blood for diagnostic purposes are very limited in patients with BM (10). There is no study showing the role of exosome and cfDNA levels in diagnosis and their changes after GKR treatment. Therefore, we aimed to investigate the role of exosome levels, cfDNA levels, and miR-208a expression as biomarkers for diagnosis and predicting the outcome in patients with BM treated with GKR.

MATERIAL AND METHODS

Patient selection

We included nine patients who had brain metastasis (primary tumour type; five lung cancer, two breast cancer, one colon cancer, and one ovary cancer) who received hypofractionated GKR in this study. The radiation treatment was applied in three fractions in all patients. The patients' characteristics, including the type of primary cancer, sex, age, and survival time, were reviewed retrospectively. Gender- and age-matched controls were used as healthy individuals. The samples were collected at three different time periods: 1) Before GKR; 2) 1 month after GKR; and 3) At recurrence. This study was conducted in line with the principles of the Declaration of Helsinki. The Ethical Committee of Bezmialem Vakif University approved this study (Date: 13.01.2020, No: 2020-538). All patients signed the informed consent form to participate in the study.

cfDNA Isolation and Measurement

One ml of the serum sample of BM patients and healthy individuals was used to extract cfDNA using the ChargeSwitch gDNA Serum Kit (Invitrogen, Life Technologies) according to the manufacturer's guidelines using the EasySep Magnet (StemCell Technologies, Canada). Isolated DNA samples were stored at –20°C. Qubit® 2.0 fluorometer device was used with a Qubit dsDNA High Sensitivity Assay (Invitrogen, Life Technologies) according to the manufacturer's guidelines to measure cfDNA levels.

Exosome Isolation and Measurement

Five hundred µL of serum sample was used to isolate exosomes with Total Exosome Isolation Reagent (Invitrogen™, Massachusetts, USA) according to the manufacturer's protocols. The pellet was dissolved in PBS and stored at –20°C. EXOCET Exosome Quantitation Kit (System Bioscience, CA, USA) was used according to the manufacturer's guidelines to measure the exosome numbers.

miRNA Isolation and Quantification

The exosome samples were pelleted at 110.000 x g for 70 minutes with a Beckman Coulter Allegra 25R centrifuge (CA, USA). The miRNeasy serum/plasma kit (Qiagen, Hilden, Germany) was used to isolate miRNAs from 200 µL of serum and 30 µL of pelleted exosome samples according to the manufacturer's protocol. The extracted RNA samples were stored at -80ºC.

miRNA cDNA libraries were prepared using the miRNA All-In-One cDNA Synthesis Kit (ABM, Richmond, Canada) according to the manufacturer's guidelines. The qPCR method was used to detect the expression level of miR-208a by using specific primers, and normalisation was done with U6 snRNA. The SensiFAST SYBR No-Rox Kit (Meridian Bioscience, Ohio, USA) was used for qPCR in the conditions 95°C 3 min, 95°C 30 sec, 55°C 30 sec, 72°C 30 sec.

Statistical analysis

IBM SPSS 22 (IBM SPSS Corp., Armonk, NY, USA) was used for the statistical analysis. The Wilcoxon Signed Rank test was used to compare exosome and cfDNA levels and the expression of miRNA in the patient group for different time intervals. The Mann-Whitney-U test was used to compare exosome and cfDNA levels and the expression of miRNA in the patient group versus the control group. The Pearson Correlation test was used to analyse the correlation between the clinical data and the experimental results. Statistical significance was set at $p<0.05$.

RESULTS

Patient characteristics

Nine patients who underwent GKR for brain metastasis and eight healthy controls were included in the study. The median age of the patients was 58 years (range: 43-72 years). The median age of the healthy controls was 57 years (range: 41-70 years). The median tumour volume was 11.26 cm^3 (range: 3.18-28.54 cm^3). The median overall survival was 9.5 months (range: 2.8-36.8 months). The median total radiation dose was 25.5 Gy (range: 22.5 and 27 Gy). GKR was applied in 3 fractions for all patients. The characteristics of the patients are presented in Table 1. There was no correlation between the clinical data and the experimental results.

cfDNA levels

The serum cfDNA levels of BM patients and healthy controls were compared to investigate the role of serum cfDNA in diagnosing BM patients. The mean serum cfDNA levels of BM patients (n=9) (518.7 ng/mL) were higher than those of healthy controls (n=8) (325.9 ng/mL), although the difference was not significant. When the patients were grouped as lung cancer and non-lung cancer patients, lung cancer patients (n=5) (620.5 ng/mL) had a higher level of cfDNA compared to non-lung cancer patients (n=4) (391.6 ng/mL) and controls (n=8) (325.9 ng/mL) (Figure 1).

Table 1. Characteristics of the patients who underwent GKR treatment

Characteristic	Value
Cases (F/M), n	5/4
Median age (range), years	58 (43-72)
Median tumour volume, cm3 (range)	11.26 (3.18-28.54)
Median total radiation dose, Gy (range)	25.5 (22.5-27)
Primary Tumour Type, n (%)	
Lung	5 (56)
Breast	2 (22)
Colon	1 (11)
Ovary	1 (11)
Chemotherapy, n (%)	
Yes	6 (67)
No	3 (33)
Radiation therapy, n (%)	
Yes	9 (100)
No	0 (0)
Median overall survival time, months (range)	9.5 (2.8-36.8)

In addition, serum cfDNA levels were measured at different time intervals (before GKR, one month after GKR, and at recurrence) to elucidate the effect of GKR on the cfDNA level of BM patients. The serum cfDNA level was reduced 1 month after GKR (373.8 ng/mL) compared with the cfDNA level before GKR (518.7 ng/mL) in BM patients. Additionally, the serum cfDNA level was the highest at recurrence (647.9 ng/mL) among all time intervals. There was a decreasing trend for cfDNA in non-lung cancer patients after GKR (before GKR 391.6 ng/mL, one month after GKR 343.8 ng/mL, at recurrence 257.8 ng/mL). However, the cfDNA level was increased at recurrence for lung cancer patients (before GKR 620.5 ng/mL, one month after GKR 397.9 ng/mL, at recurrence 882 ng/mL) (Figure 1).

Exosome levels

The serum exosome levels of BM patients and healthy controls were compared to investigate the role of exosomes in diagnosing BM. The mean serum exosome levels of BM patients (n=9) (6507.9 U/mL) were higher than those of healthy controls (n=8) (5744.4 U/mL), although the difference was not significant. Patients with lung cancer (n=5) (8680.2 U/mL) had the highest level of exosomes compared to non-lung cancer patients (n=4) (3792.4 U/mL) and healthy controls (n=8) (5744.4 U/mL) (Figure 2).

The serum exosome levels were measured at different time intervals (before GKR, one month after GKR, and at recurrence) to elucidate the effect of GKR on the exosome level of BM patients. The serum exosome levels decreased one month after GKR (5655.5 U/mL) compared with those before GKR (6507.9 U/mL) in BM patients. Besides, the serum exosome level was highest at recurrence (8269.4 U/mL) among all time intervals. A similar trend was observed in lung cancer patients in different time intervals (before GKR 8680.2 U/mL, one month after GKR 6031.8 U/mL, and at recurrence 11203.9 U/mL). The serum exosome level of non-lung cancer patients fluctuated during the following time (before GKR 3792.4 U/mL, one month after GKR 5185.1 U/mL, at recurrence 3378.7 U/mL) (Figure 2).

The expression of miR-208a in serum and serum exosomes

The expression of serum miR-208a was higher in BM patients compared with the healthy control group. The expression of serum miR-208a was significantly higher in patients with lung cancer (n=5) than in healthy controls (p=0.008) and non-lung cancer patients (n=4) (p=0.05) (Figure 3).

Serum miR-208a expression increased 1 month after GKR and decreased at recurrence. Serum miR-208a expression increased one month after GKR and decreased at recurrence in both lung and non-lung primary cancer patients (Figure 3).

The expression of serum exosomal miR-208a was higher in the BM patient group than in the healthy ones. The expression of serum exosomal miR-208a was found to be significantly higher in lung cancer patients (n=5) than in healthy controls (n=8) (p=0.04). The expression of serum exosomal miR-208a was higher in lung cancer patients than in non-lung cancer patients, although the difference was not significant (Figure 4).

The expression of serum exosomal miR-208a was increased one month after GKR compared with that before GKR. The expression of serum miR-208a and serum exosomal miR-208a was decreased at recurrence compared with that at one month after GKR. The expression of serum exosomal miR-208a was increased one month after GKR and decreased at recurrence in patients with primary lung cancer. The expression of serum exosomal miR-208a fluctuated during the follow-up time in non-lung primary cancer patients (Figure 4).

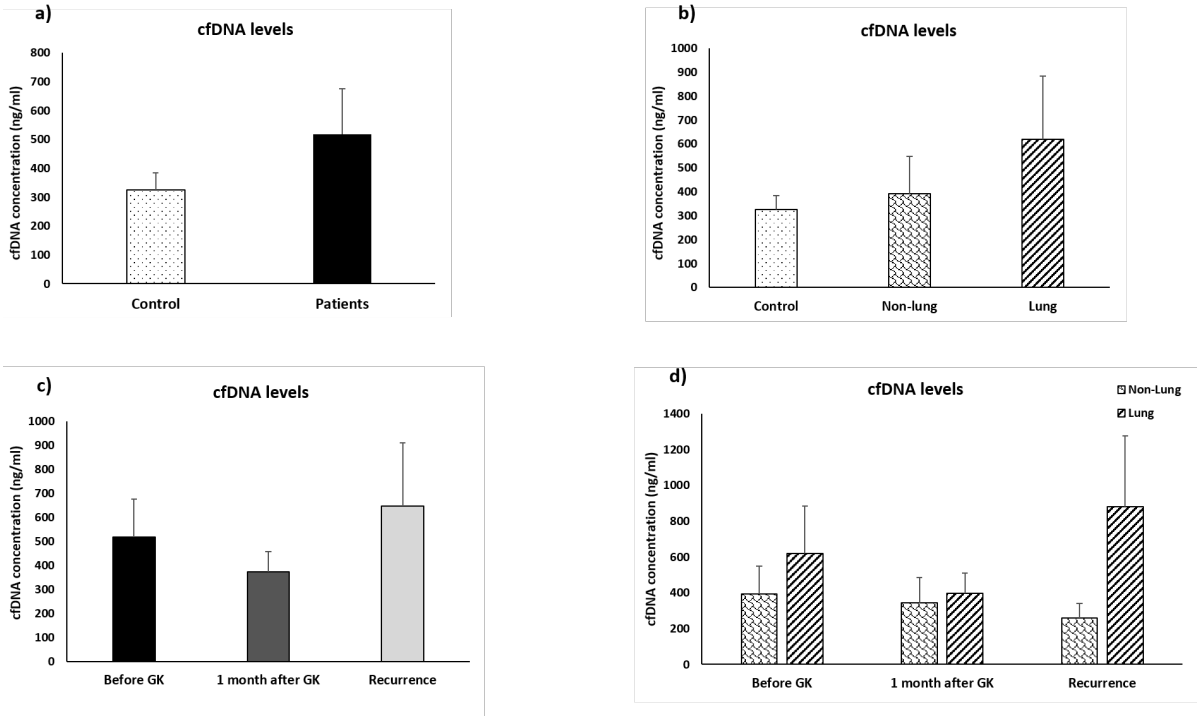

Figure 1. cfDNA levels in BM patients and controls. a) cfDNA levels are higher in BM patients (n=9) compared to the control group (n=8). b) Lung cancer patients had higher levels of cfDNA than non-lung cancer patients and controls. c) cfDNA levels decreased 1 month after GKR compared to before GKR and increased at recurrence in BM patients. d) The level of cfDNA decreased over time in non-lung cancer patients. The cfDNA levels decreased after GKR and increased at recurrence in patients with lung cancer

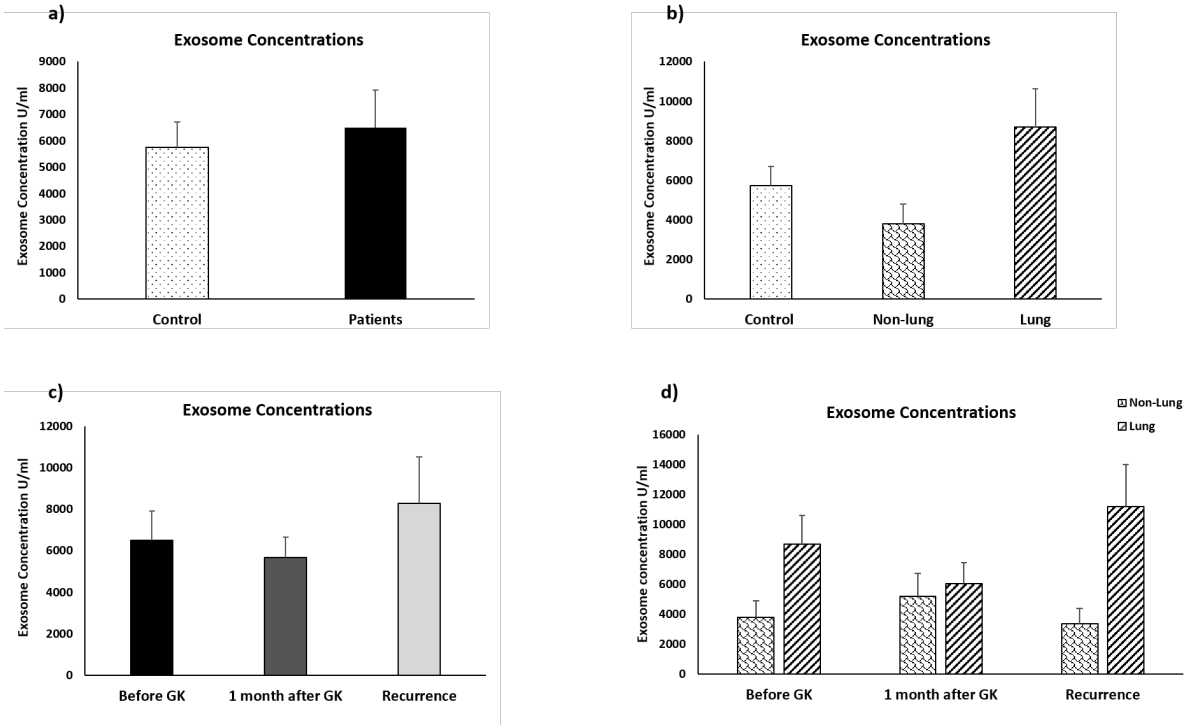

Figure 2. The levels of exosomes in BM patients. a) Exosome levels are higher in BM patients (n=9) compared to healthy individuals (n=8), and controls. b) Primary lung cancer patients had the highest level of exosomes compared to non-lung primary patients and controls. c) Exosome levels decreased 1 month after GKR compared to before GKR and increased at recurrence in BM patients. d) The exosome levels fluctuated during the follow-up time in non-lung cancer patients. The exosome levels decreased after GKR and increased at recurrence in primary lung cancer patients

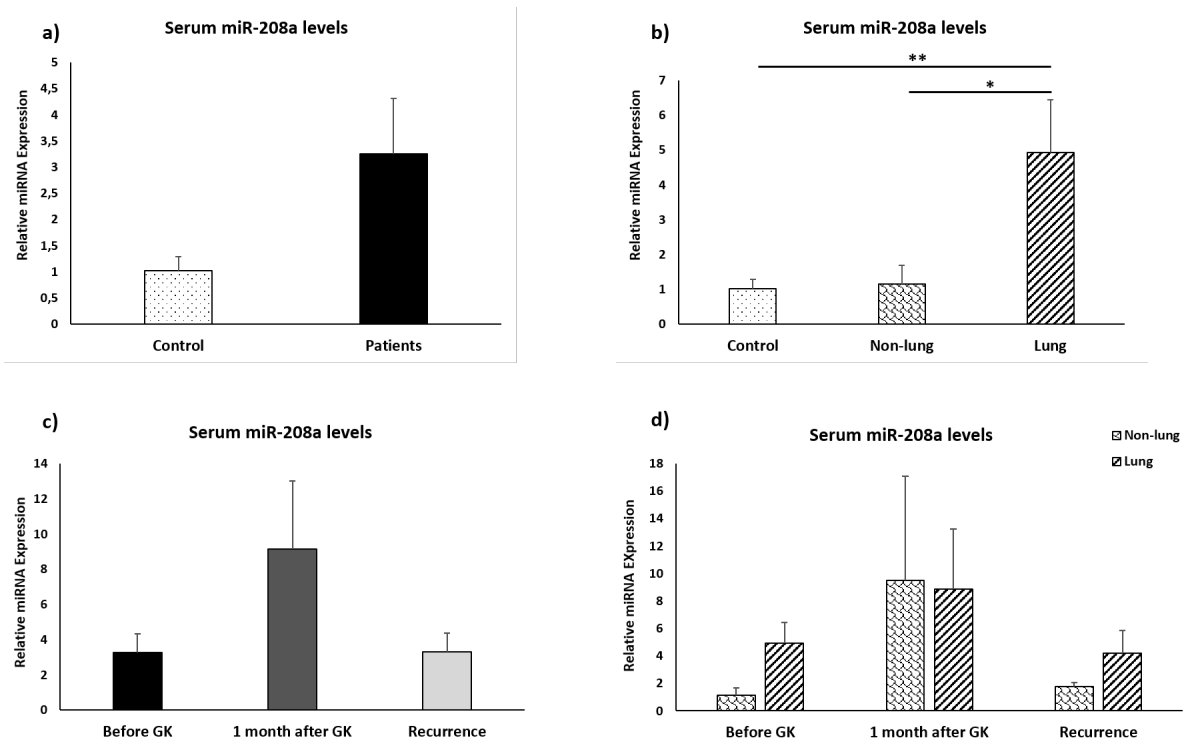

Figure 3. The expression of serum miR-208a in BM patients and controls. a) The expression of serum miR-208a was higher in BM patients than in healthy controls. b) Serum miR-208a levels were significantly higher in primary lung cancer patients than in non-lung primary patients (p=0.05) and controls (p=0.008). c) Serum miR-208a expression was increased 1 month after GKR and decreased at recurrence. d) Serum miR-208a expression was increased one month after GKR and decreased at recurrence in both lung and non-lung primary cancer patients (*p<0.05, **p<0.01)

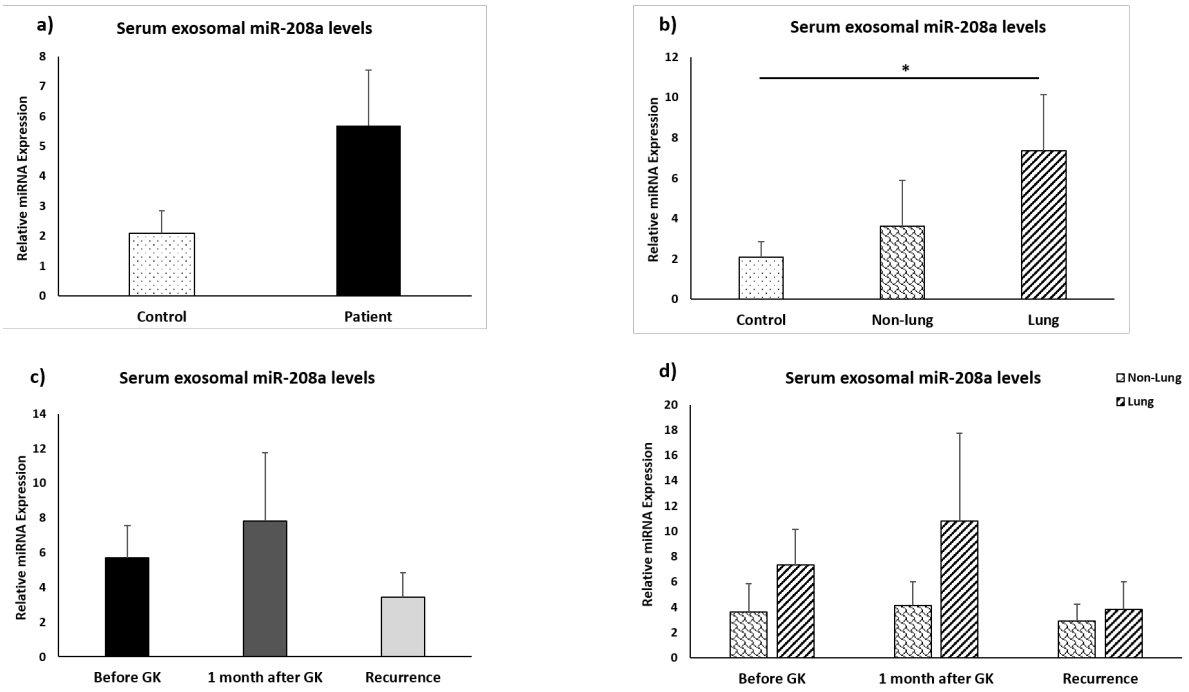

Figure 4. The expression of serum exosomal miR-208a in BM patients and controls. a) The expression of serum exosomal miR-208a was higher in BM patients than in healthy controls. b) The expression of serum exosomal miR-208a was significantly higher in primary lung cancer patients than in controls (p=0.04). The expression of serum exosomal miR-208a was higher in primary lung cancer patients compared with non-lung primary cancer patients, although the difference was not significantly significant. d) The expression of serum exosomal miR-208a was increased one month after GKR and decreased at recurrence in primary lung cancer patients. The expression of serum exosomal miR-208a fluctuated during the follow-up time in non-lung primary cancer patients (*p<0.05, **p<0.01)

DISCUSSION

We tested the hypothesis of whether cfDNA, exosome, and miR-208a could be diagnostic biomarkers in patients with BM. The results showed that the expression of serum miR-208a was significantly higher in patients with lung cancer compared to those with non-lung cancer and healthy controls. Additionally, exosome and cfDNA levels were higher in BM patients compared with the control group. The cfDNA and exosome levels decreased after GKR and increased at recurrence, although there was no statistical significance.

Although the LB is a highly common technique in the monitoring of several types of cancers, the LB research for BM patients is highly limited and challenging due to the short survival time of these patients. Cheok et al. investigated the mutations present in the CSF, blood, and brain metastasis tissue samples of BM patients. Mutations of selected patients matched 80% in CSF and tissue samples and 20% in blood and tissue samples. They suggested that LB from CSF can be an alternative method to tissue biopsy (11). Similarly, Wu et al. compared the genomic status of DNA from primary tumour and BM tissue, CSF, and plasma of BM patients. As a consequence, they showed that CSF cfDNA was more representative of the mutational burden of BM tissue than plasma. Therefore, CSF is a highly reliable source for LB research in patients with BM (12). However, there are drawbacks to the collection of CSF with lumbar puncture that can cause neurological complications and side effects. There are few reports regarding the usage of blood and especially serum samples in the usage of LB in BM patients. However, serum cfDNA is a reliable material in the diagnosis and monitoring of different types of cancers (13-15). We aimed to use more applicable material to obtain from cancer patients and during the follow-up time. Additionally, the decrease after GKR may represent the shrinkage of tumour tissue in the brain, and its increase at recurrence may represent the recurrence of tissue intracranially.

Exosomes are extracellular vesicles that play a role in intercellular communication by carrying DNA, mRNA, miRNA, protein, and lipid particles. Researchers proposed that tumour-derived exosomes play various roles in the metastasis of primary tumours by contributing to angiogenesis, tumour growth, and invasion (16). The feature of exosomes contributing to intercellular communication between primary tumour-derived exosomes and receiving brain cells plays a key role in the formation of BM (17). Therefore, exosomes can be used as prognostic and diagnostic markers for BM because of their existence in different body fluids, contribution to metastasis, and acting as cargo (18). In our study, we found that the number of exosomes was higher in BM patients compared to the healthy control group in

their serum samples. Although exosome levels have not been studied in BM patients, there are several papers regarding the number of exosomes in other malignancies such as breast cancer, ovarian cancer, and hepatocellular carcinoma (19-21). Exosome levels decreased after GKR, which may indicate the effectiveness of the GKR, and increased at recurrence, which may be related to the regrowth of tumour bulk.

Although there are a limited number of studies regarding exosomes in BM, Li et al. collected plasma samples of five patients with BM before and after radiotherapy and performed miRNA-sequencing from exosomes for the first time. They found 35 differentially expressed miRNAs between before and after radiotherapy specimens, which can be defined as non-invasive biomarkers (22). Similarly, Catelan et al. investigated the expression of serum exosomal miR-21, miR-124-3p, and miR-222 in BM and high-grade glioma patients before and after surgical/radiological operations. The expression of miR-21 was increased and miR-124-3p was decreased in patients with BM compared with the control group. Additionally, the expression of miR-21 significantly declined after surgical/radiosurgical operations (23).

Tang et al. demonstrated the up-regulated expression of miR-208a after radiotherapy in patients with lung cancer using an miRNA microarray. They concluded that radiation may induce the increase of miR-208a expression and play a role in the radiosensitivity of lung cancer (24). As Tang et al. showed the effect of radiation on the expression of miR-208a, we also found that serum miR-208a expression was increased after GKR in lung cancer patients and non-lung cancer patients and decreased at recurrence. Additionally, the serum miR-208a expression was significantly higher in patients with lung cancer than in controls and non-lung patients. The serum exosomal miR-208a expression was found to be significantly higher in lung cancer patients compared with the healthy controls. Therefore, we showed that miR-208a could be a biomarker related to the treatment response after radiation in patients with BM. Besides, the small size of the patient group is the limitation of the study.

CONCLUSION

Our results revealed that LB using patients' serum cfDNA, exosome, and miRNAs might be a non-invasive method for diagnosis in patients with BM. However, further investigations are warranted.

Ethics Committee Approval	This study was approved by Bezmialem Vakif University (Date: 13.01.2020, No: 2020-538).
Informed Consent	Written informed consent was obtained from all the participants of the study.
Peer Review	Externally peer-reviewed.
Author Contributions	Conception/Design of Study- I.K., M.A.H.; Data Acquisition- B.K., E.B.E.; Data Analysis/ Interpretation- K.A., S.M., B.K.; Drafting Manuscript- B.K., M.A.H.; Critical Revision of Manuscript- M.A.H.; Final Approval and Accountability- M.A.H., I.K.
Conflict of Interest	The authors declare that there is no conflict of interest.
Financial Disclosure	This research was funded by Bezmialem Vakif University, Turkey (Grant number BAP-20200209).

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