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CLINICAL STUDY

Clinical impact of circulating oncogenic MiRNA-221 and MiRNA-222 in glioblastoma multiform

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Abstract

Background and aim Glioblastoma multiform (GBM); most fatal brain cancer, is incurable with molecular diversity hence identification of molecular targets that contribute to GBM tumorgenesis will be suitable for the development of diagnostic and treatment strategies. Micro-RNAs (miR); small RNA molecules, are stable in blood and play a crucial role in molecular processes in GBM. Thus it was aimed to investigate the clinical role of miR-221 and miR-222 among GBM cases as compared to healthy individuals and illustrate their role in patient's survival.

Materials and methods Blood samples were withdrawn from 20 GBM cases before and after treatment, a group of 20 healthy individuals were served as control. For all enrolled samples expression of miR-221 and miR-222 were detected using quantitative PCR (QPCR). Sensitivities, specificities of investigated miRs and their relation with GBM clinical characteristics and patient's outcome were analyzed using Kaplan Meir curve.

Results Expression of investigated miR- 221 and -222 were significantly increased in GBM cases as compared to healthy individuals ($F = 12.9$, at $P < 0.001$, $F = 28.78$, at $P < 0.0001$, respectively) and with absolute specificity for both and 90% sensitivity for miR-221 and 85% for miR-222. Among GBM patients ($n = 20$), mean expression level miR-221 reported significant increase with elder GBM (> 60 years) at $F = 5.7$, $P = 0.028$, while both miR-221 and -222 showed significant difference in performance status (ECGO) at $P = 0.036$ and 0.007, patients with primary lesion at $P = 0.001$ and 0.005, surgically treatment strategy at $P < 0.001$ and 0.004, respectively. Patients were grouped according to their outcomes into response (complete [CR] or partial [PR]), stable disease[SD] and progressive disease [PD], miR-221 and miR-222 showed increase expression with PD and patients with worse PFS and OS were those with high miRs expression.

Conclusion Detection of circulating miR-221 and miR-222 may be used as circulating molecular marker for diagnosis and prediction of outcome for patients with GBM. Further studies with large cohort of samples are encouraged.

Keywords MiRNA · Glioblastoma multiform · Prognosis · Response

Introduction

Gliomas are used to describe the primary brain tumors, and they were categorized according to assumed cell of origin [1]. Among them is glioblastoma multiform (brain tumor grade IV) (GBM) which is the most malignant and commonly occurring type. Although advances have been made in treatment strategies it is still with a poor prognosis and a median patient survival of approximately 18 months [1–3].

Recent advances in identification of molecular markers have been made for better specify the prognosis or to delineate new target therapy strategies for GBM patients. Among them are microRNAs; small non- protein-coding RNA sequences of about 19–26 nucleotides [4], which were reported to be stable in body fluids of many types of cancer

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with same level in tumor tissue [5]. MicroRNA (miRNA) expression profiling has been reported to affect the function of genes involved in gliomagenesis, tumor growth, proliferation, apoptosis and posttranscriptional regulation of anti-oncogenes [6, 7].

Many miRNA have been related to cancer among them GBM as previously reported [8, 9]. In the current study, the expression of two highly homologous miRNAs (miR-221, miR-222) was investigated. They are commonly acting as a gene cluster (miR-221/222 cluster), miR-221 and 222 have been extensively studied in human malignancies [10–14]. Study by Zhang and his colleagues [15] reported significant relation between miRNA221/222 expression in tissue samples and progression of GBM, Moreover, an up-regulation of miRNAs in brain tumor formalin-fixed paraffin – embedded samples have been reported previously [16].

In the current study, authors aimed to investigate the clinical significance of circulating miRNA-221 and miRNA-222 in blood samples as minimally invasive approach for management of GBM patients.

Materials and methods

Enrolled individuals and sample collection

The study was conducted on individuals admitted to Faculty of Medicine, Ain Shams University from 2016 to 2018. After obtaining the approval from the Ethical Committee and individuals signed their informed consents, blood samples were collected from newly adult diagnosed glioblastoma (GBM) patients (n = 20) whom fulfill the inclusion criteria (newly diagnosed glioblastoma multiform cancer patients with age more than 18 and before they have received any treatment strategies with performance less than or equal 2 according to the ECOG (Ester Clinical Oncology Group); which assesses disease progression affecting on patient's daily living abilities and determine the appropriate treatment and prognosis [17], any GBM patients whom did not fulfill the inclusion criteria were excluded from the study. Then after they have received their standardized treatment protocol which involve radiotherapy (total dose of 60 Gy, given in 30 fractions over 6 weeks) with concomitant temozolomide (TMZ) chemotherapy (100 mg/day for 45 days), followed by six cycles of TMZ treatment at a dose of 150 mg/sq. m body surface area. After words another blood sample were collected from them and they were followed up at regular intervals and evaluated clinically and radiological by MRI with contrast. As a control group, 20 healthy individuals were enrolled in the study and they were of matched ages and gender.

Blood samples were collected from all enrolled individuals in tubes with polymer gel and clot activator (Greiner Bio-One GmbH, Kremsmünster, Austria) then allowed to clot at room temperature for 30 min and all samples were centrifuged at 10,000 × *g* for 10 min at 4 °C (3-18KS, Sigma, Germany). Separated sera were liquated in RNase free tubes and stored at – 80 °C for further processing of miRNA expression.

MiRNA extraction

MicroRNA extraction was performed using miRNeasy Mini kit (Catalogue # 217,004, Qiagen, USA). Five volumes of lysis Reagent were added to a volume of the thawed serum sample then lysates were left for 5 min at room temperature, then an equal volume of chloroform was added to the tubes. After vortex, samples were centrifuged at 12,000×*g*for 15 min at 4 °C (13-18KS, Sigma, Germany). The upper aqueous phase was transferred to a new collection tube. Afterwards, 1.5 volumes of 100% ethanol were added to the aqueous phase followed by pipetting up and down several times. The sample (up to 700 µl) was transferred into an RNeasy Mini spin column in a 2 ml collection tube and then centrifuged for 15 s at ≥ 8000×*g* for 15 min at room temperature. The RWT buffer (700 µl) was added to the RNeasy Mini spin column. After centrifugation, the flow-through was discarded and RPE buffer (500 µl) was added and the column was centrifuged and flow-through was discarded. RNase-free water (30 µl) was directly added onto the RNeasy Mini spin column membrane and centrifugation was done for 1 min at ≥ 8000 × *g*. The purity and the concentration of the purified miRNA was detected using spectrophotometer Nano-drop (Quawell, Q-500, Scribner, USA) and stored at – 80 °C till further assessments.

Reverse transcription and cDNAsynthesis

Reverse transcription of miRNA was carried out using MiScript II reverse transcription kit (Cat number # 218,160, Qiagen, USA). As recommended in the manufacturer's instructions by using a total volume of 20 µl of reverse transcription reaction components as follows: 4 µl MiScriptHiFlex buffer, 2 µl nucleic mixture, 2 µl MiScript RT mixture, and RNase-free water and template of purified miRNA with adjusted concentration 100 ng/reaction. The polymerase chain reaction (PCR) tubes were then placed in thermal cycler (SureCycler 8800, Agilent, USA) and the transcription profile was adjusted for 60 min at 37 °C. Complementary DNA (cDNA) purity and concentration was detected using spectrophotometer Nano-drop (Quawell, Q-500, Scribner, USA) and stored at – 20 °C till performing QPCR.

Quantitative MiRNA expression

Quantitative real-time PCR was performed using miScript primer assay (Cat number 218,300, Qiagen, USA) for miRNA-221 (Hs_miR_221_2 miScript Primer Assay, MS00003857) and miRNA 222 (Hs_miR_222_2p miScript Primer Assay, MS0007609), the reaction was carried out using MiScript SYBR Green PCR kit (Cat number 218,073, Qiagen, USA). Also, RNU6–2 (Hs_RNU6-2_11 miScript Primer Assay, MS00033740) was used as an endogenous control to normalize the expression levels of the investigated miRNAs. The reaction for miScript primer assays were carried out using cDNA with concentration adjusted to 2 ng/ml and a total volume of 20 µl, whereas thermal reaction conditions were as follows: 95 °C for 15 min followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 34 s, in which fluorescence was acquired and detected by Stratagene Real-time PCR system (Max3005P QPCR system, Stratagene, Agilent biotechnology, USA). The relative expression levels of the investigated miRNAs were evaluated using the $2^{-\Delta\Delta Ct}$ method [18]. The cycle threshold (*Ct*) value is the number of qPCR cycles required for the fluorescent signal to cross a specified value. ΔCt was calculated by subtracting the *Ct* values of RNU6–2 from miRNAs. $\Delta\Delta Ct$ was calculated by subtracting the ΔCt of the control samples from the ΔCt of the GBM samples.

Statistical analysis

Data were analyzed using SPSS (version 10 SPSS, Inc, Chicago USA) and P-value were two-tailed and considered significant if < 0.05. The fold change in investigated miRNAs was calculated using the equation of $2^{-\Delta\Delta CT}$. The association between the clinicopathological and demographic factors with investigated miRNAs was determined by ANOVA analysis. Receiver operating characteristic (ROC) curve was blotted between GBM patients and healthy individuals to detect the sensitivities and the specificities for the miRNAs and their clinical efficacy [19]. Progression free survival (PFS) was reported as the time from the first receiving neoadjuvant treatment strategy to distal, regional or local recurrence, while overall survival (OS) was measured from the date of first diagnosis to the date of last follow-up or death of the patients, both were analyzed using Kaplan–Meier statistical method and compared by long-rank test.

Results

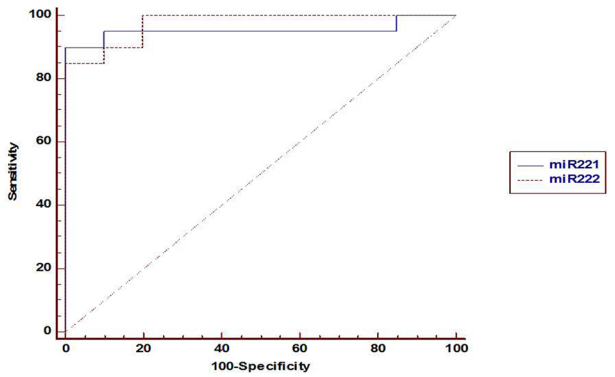
A total of forty individuals were enrolled in the current study they were divided into control cases (n = 20) and GBM cases (n = 20), their clinical and demographic characters were summarized in Table 1. There was no significant difference between their ages among the two investigated groups as both were of matched ages, also no significant was reported among their gender.

Table 1 Demographic and clinical data for GBM cases and control

Factor	GBM cases (n = 20) (n, %)	Control group (n = 20) (n, %)
Age (years)		
< 60 years	11 (55%)	17 (85%)
≥ 60 years	9 (45%)	3 (15%)
Gender		
Male (n = 29)	14 (70)	15 (75)
Female (n = 11)	6 (30)	5 (25)
Surgery type		
Resection	9 (45)	–
Biopsy	11 (55)	–
Site of primary lesion		
Frontal	10 (50)	–
Partial/temporal	8 (40)	–
Posterior forsa	2 (10)	
ECGO		
< 2	10 (50)	–
= 2	10 (50)	–
Response		
Complete response	1 (5%)	–
Partial response	3 (15%)	–
Stable disease	8 (40%)	–
Progressive disease	8 (40%)	

Detection of investigated miRNAs was carried out for all samples as they were applied in triplicates. To determine the sensitivity and specificity for the investigated miRs, ROC curve was plotted as shown in Fig. 1, the specificities for both were absolute and both revealed an AUC higher than 0.9. Significant difference was reported between miRs expression among groups as increased miR-221 and miR-222 expression in GBM cases (mean ± standard error of mean [SEM] = 39.9 ± 0.6 and 217 ± 5.9) as compared to healthy control cases (5.6 ± 0.2 and 4.1 ± 0.4) respectively at ($P < 0.001$) as shown in Fig. 2a, b.

The impact of miR-221 on demographic characteristics reported no significant difference between gender status but significant difference was reported with patients' age above 60 years old (median 55.7) *versus* below 60 years old (median 23.2) at (F = 5.7, $P = 0.028$). For miR-222; no significant difference was reported between its expression for either gender or age. The level of investigated miRs with clinical features are summarized in Table 2, increased the mean levels of miR-221 and miR-222 were reported with GBM patients having surgical biopsy samples with non-frontal sites (tempo-partial and posterior-forsa) as sites of



MiRNA	Sensitivity	Specificity	AUC (SD)	95%CI
MiR-221	90%	100%	0.925 (0.035)	0.834 – 0.993
MiR222	85%	100%	0.957 (0.025)	0.868 – 0.996

Fig. 1 Receiver operating characteristic curves that discriminate between GBM and healthy control with area under curve (AUC), sensitivities and specificities of miR-221 (straight line) and miR-222 (dashed line)

lesion as compared with other frontal mass, possessing ECGO equals 2 and having progression of the disease.

All patients underwent standardized treatment protocol and the levels of investigated miRs were measured before and after treatment. Expressions of miRs as fold change were significantly decreased after treatment as plotted in Fig. 3a, b, as the mean ± SEM for miR-221 before treatment was 39.8 ± 6.4 and after 7.8 ± 2.1, while for miR-222 was 217 ± 59 and after was 55 ± 13.4.

Survival analyses were carried out using Kaplan Meier curve to examine the expression of miR-221 and miR-222 with both PFS and OS. High miR-221 expression was significantly increased with worse PFS as the estimated time for high miR-221 to report PFS was 7.3 months compared to those with low miR-221 as they reported better PFS (10.4 months) (Fig. 4a), similarly increased expression of miR-221 was reported with worse OS as 12.6 months compared to decrease expression of miR showed better OS at 18.5 months as shown in (Fig. 4b). Similarly, elevated miR-222 was significantly increased with worse PFS (7.6 months) compared to low expression of miR-222 with better PFS (10.4 months) as plotted in (Fig. 5a), also increased miR-222 was reported with worse OS (12.9 months) as compared low miR-222 expression at better OS (19.3 months) as reported in (Fig. 5b).

Discussion

Glioblastoma multiform genomic features reported the extension of its genetic and epigenetic appearances to controlling mechanisms encompassing several molecular mechanisms asposttranscriptional alterations, chromosomal

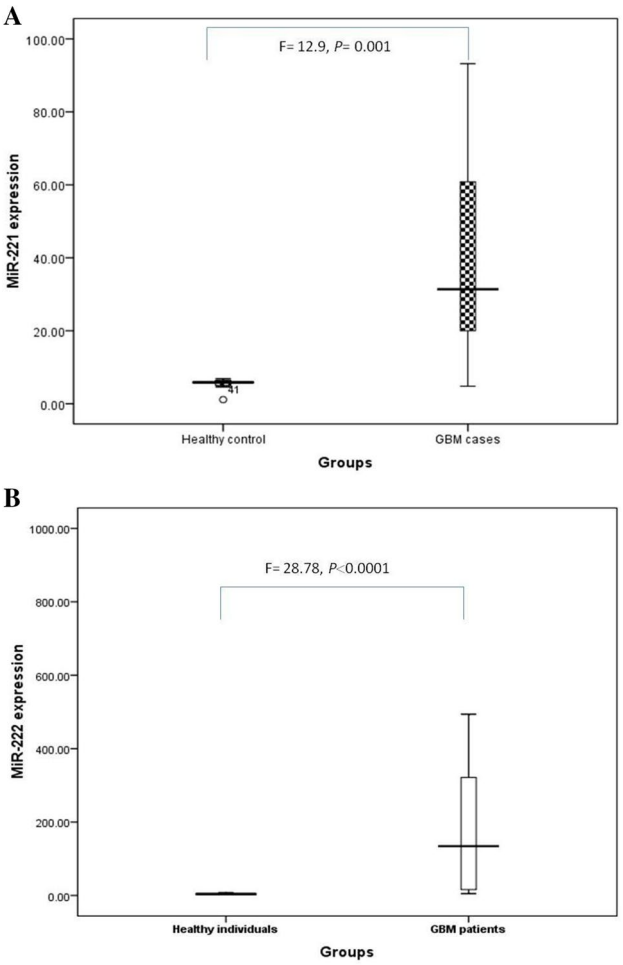


Fig. 2 Expression level of miR-221 and miR-222 among GBM *versus* healthy control expressed as fold change. **a** Expression level of miR-221 reported significant difference between GBM cases (39.9 ± 0.6) *versus* healthy control group (5.6 ± 0.2), and **b** Expression level of miR-222 among GBM cases (217 ± 5.9) versus healthy control group (4.1 ± 0.4)

transformation and small RNAs as micro RNA (miR). Previously the stability of circulating miRs under sever conditions [13] has been reported, for GBM combined research attempts have focused on the role of miRs as diagnostic markers taking into consideration their stability in cerebrospinal fluid and blood [20] hence studying their clinical impact on GBM diagnosis, survival and clinical impact is a goal of interest.

In the current study, the two studied groups were of matched ages as no significant was reported between their ages. Authors investigated the difference between expression of miR-221 and miR-222 among healthy control individuals and GBM cases which revealed significant increase in miRs expression in GBM cases by 8.57 folds for miR-221 and 53 folds for miR-222. These results were in agreement with previous reports indicating the increased expression of

Table 2 Relation between median level of investigated miRs and clinical features for GBM patients

Characteristic factors	MiR-221	MiR-222
Surgery type		
Resection	21	14
Biopsy	55	259
Statistics	F = 18, $P < 0.001$	F = 10.5, $P = 0.004$
Site of primary lesion		
Frontal	22	18
Non-frontal	60	321
Statistics	F = 5.1, $P = 0.001$	F = 10.07, $P = 0.005$
ECGO		
< 2	22	16
= 2	49	305
Statistics	F = 5.1, $P = 0.036$	F = 9.1, $P = 0.007$
Response		
Complete response	6.8	4.78
Partial response	9.73	10.1
Stable disease	24	28.3
Progressive disease	73.74	389.2
Statistics	F = 19.6, $P < 0.0001$	F = 9.07, $P = 0.002$

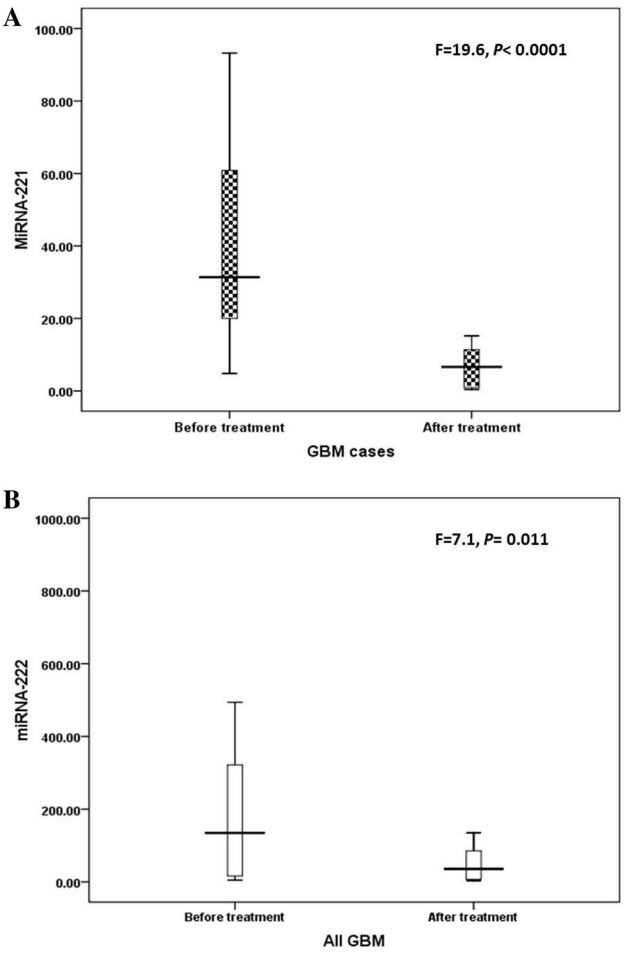


Fig. 3 Relation between before treatment and after-treatment for investigated miRs. **a** miR-221 expression level was (39.8 ± 6.4) before treatment and (7.8 ± 2.1) after treatment at F = 19.6, $P < 0.0001$, and **b** miR-222 expression level was (217 ± 59) before treatment and (55 ± 13.4) after treatment at F = 7.1, $P = 0.011$

circulating miRs in cancer as compared to non-cancer cases [14, 16], moreover the diagnostic efficacy for investigated miRs was assessed suing ROC curve as both showed AUC equals 0.9 with slight increase in miR-222 (0.957) than miR-221 (0.925) these results indicates that the upregulation of miRs -221 and -222 is linked to tumorgenesis of GBM [21]. In concordance with previous researches on miRs -221 and -222 which reported their upregulation in FFPE BM samples versus non-cancerous brain tissue [9, 16].

Patients with GBM are grade IV and their clinical characteristics were reported in Table 1, the mean level of miR-221 expression reported to be significant increase in patients above 60 years, similarly for miR-222 as its median expression level was high in elder GBM patients although no significant difference was reached. A link between GBM and aging has been reported by others [22, 23]. Thus the concordance between increased miRs expression with elder cases postulates their link with aggressiveness of GBM patients. In the current study, there was an increase of both miRs expression in GBM male patients as compared to females although no statistical difference was reported, the relation between gender and GBM is being extensively investigated and it may referred to different molecular signature of GBM between male and female as reported by Yang and his colleagues [24]. A study on sizeable GBM number is in progress to detected the link between miRs and gender status. GBM patients with ECGO equal 2 reported significant increase in miRs expression indicating their usefulness as prognostic markers. The association between tumor location either in frontal, temporal or occipital and aggressiveness of GBM has been reported by other researchers previously [25], in the present study investigated miRs were significantly decrease in the 10 cases with frontal mass versus other sites of GBM lesions. Expression of miR-221 and miR-222 were increased in patients underwent biopsy resection of the mass as compared to others ($P < 0.01$) indicating the link between their elevation and risk of GBM cases.

The standard of care (SOC) for GBM patients involved in the current study was surgical resection followed by concomitant chemotherapy with adjuvant TMZ [26]. The expression levels of miRs were detected at diagnosis and after treatment, and they were significantly decreased after treatment. These preliminary findings may direct to the clinical applicable usefulness of these miRs for targeted therapy [20], however further supporting studies are encouraged to be carried out. MiRs expression were elevated significantly

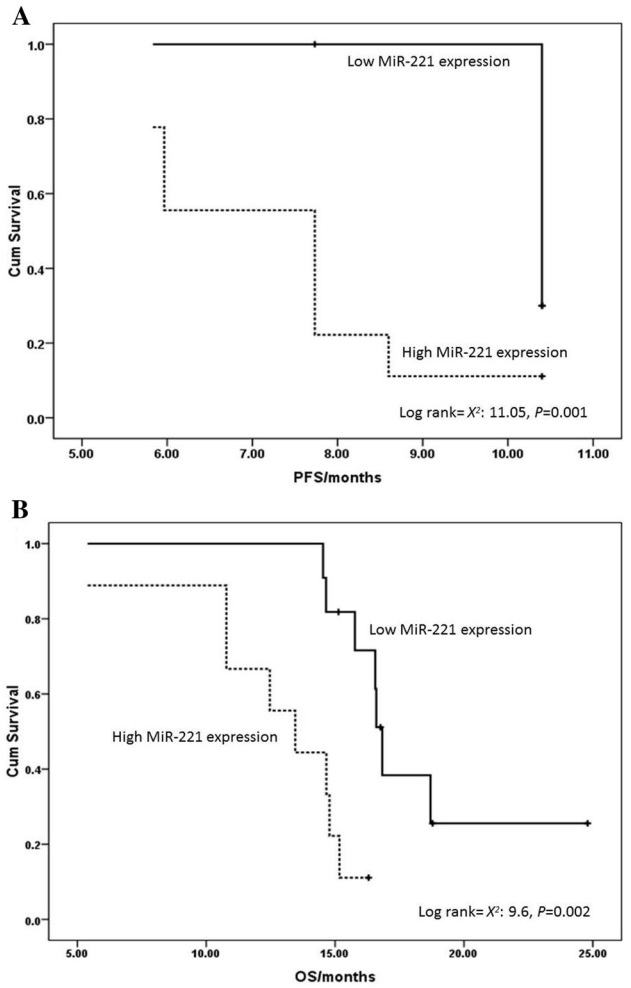


Fig. 4 Survival analysis of miR-221 fold expression level. **a** Relation between PFS and miR-221 fold expression level reported significant as PFS reported mean of 10.4 months with low miR-221 fold expression level while PFS reported mean of 7.3 months with high miR-221 fold expression level. **b** Relation between OS and miR-221 fold expression level reported significant as OS reported mean of 18.5 months with low miR-221 fold expression level while OS reported mean of 12.6 with high miR-221 fold expression level

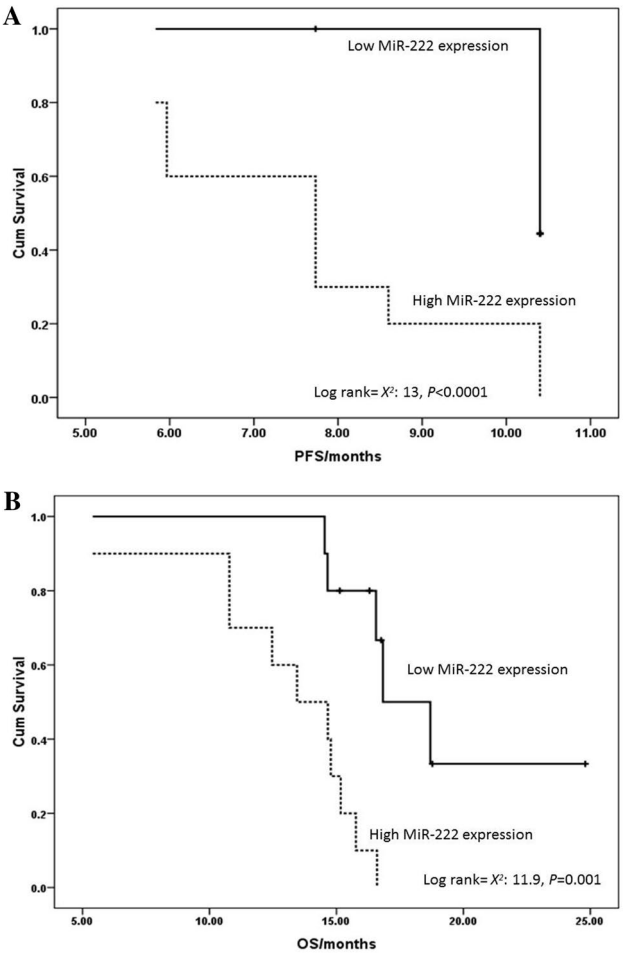


Fig. 5 Survival analysis of miR-222 fold expression level. **a** Relation between PFS and miR-222 fold expression level reported significant as PFS reported mean of 10.4 months with low miR-221 fold expression level while PFS reported mean of 7.6 months with high miR-222 fold expression level. **b** Relation between OS and miR-222 fold expression level reported significant as OS reported mean of 19.3 months with low miR-222 fold expression level while OS reported mean of 12.9 with high miR-222 fold expression level

among patients with progression disease and survival analysis reported the worse PFS and OS in patients with increased miRs expression. These results emphasize the association between increased miR-221 and miR-222 expression and worse survival patterns of GBM cases.

In conclusion, to our knowledge this is the first study to address the role of circulating miR-221 and miR222 in GBM cases as compared to healthy individuals and examine their role in patient's survival analysis, although the limitation in the study is the limited number of GBM cases enrolled, our findings were comparable to previous reports pointing the usefulness of miRs role in diagnosis and predicting of cancer and their role with GBM patients outcome is in progress with a large number of cohort.

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Compliance with ethical standards

Conflict of interest Authors report no conflicts of interest in this work.

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