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ORIGINAL ARTICLE

Long noncoding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1): A molecular predictor of poor survival in glioblastoma multiforme in Egyptian patients

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KEYWORDS

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Abstract *Background:* Long noncoding RNAs (lncRNAs) are a recently discovered class of transcribed RNA molecules with a length of more than 200 nucleotides. Recent studies have shown that lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) could play an important role in carcinogenesis and cancer progression in several types of malignancies.

Objective: As little is known about the role and clinical significance of lncRNA MALAT1 in glioblastoma multiform (GBM) patients in Egyptian population, this study aimed to investigate the expressions of lncRNA-MALAT1 in human GBM samples and to correlate these expressions with the available clinicopathological features including patient survival data.

Subjects and methods: The relative expression of *MALAT1* was determined in 37 human glioblastoma formalin-fixed paraffin embedded (FFPE) tissue samples and 10 FFPE non-neoplastic brain tissues using quantitative reverse transcription polymerase chain reaction (qRT-PCR) technology.

Results: The current results revealed that lncRNA MALAT1 expression was down-regulated in all tumor specimens compared to normal tissues. A receiver operating characteristic (ROC) curve analysis showed high diagnostic performance; area under curve (AUC) = 0.925 ± 0.038 ($P < 0.001$), 95% CI = 0.850–1.00, with 94.6% sensitivity, and 72.7% specificity. Lower *MALAT1* expression was associated with poor prognosis; higher frequency of recurrence ($P < 0.044$), lower overall survival ($P < 0.005$), and shorter disease-free survival ($P < 0.004$).

Abbreviations: DFS, disease-free survival; FFPE, formalin-fixed paraffin embedded; GBM, glioblastoma multiform; lncRNAs, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NEAT2, nuclear-enriched abundant transcript 2; OS, overall survival; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROC, receiver operating characteristic; TBP, tata binding protein

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Conclusion: Taken together, we could postulate that MALAT1 might have a tumor-suppressive function in GBM in Egyptian population and this specific type of lncRNAs may be included in the lists of both potential prognostic biomarkers and the future therapeutic targets for glioblastomas.

1. Introduction

Glioma is by far the most common primary brain tumor associated with poor outcome and survival [1]. It has been classified into well-differentiated low grade astrocytomas (grade I–II), anaplastic astrocytomas (grade III), and glioblastoma multiforme (GBM; grade IV) according to World Health Organization (WHO) [2]. Despite standard treatment that typically includes neurosurgery, chemotherapy and, radiation, recent clinical trials have reported a median survival of only 14–16 months with a 26–33% 2-year survival rate [3]. It has been suggested that the WHO criteria to predict the patient clinical outcomes may not be sufficient alone to estimate patient prognosis [4]. More recently, improved understanding of glioma molecular genetics has led to identifying new potential biomarkers for early diagnosis, prognosis prediction, and novel therapeutic targets [5].

Long noncoding RNAs (lncRNAs) are a recently discovered class of transcribed RNA molecules with a length of more than 200 nucleotides. Similar to protein-coding genes, they have promoter structure, and are transcribed by RNA polymerase II, polyadenylated, and subjected to splicing. However, unlike mRNA, they do not encode proteins [6]. LncRNAs are thought to be important regulators of gene expression at transcription, translation and epigenetic levels [7,8]. An accumulating number of evidences suggested that lncRNAs may have critical roles in a wide range of biological processes [9]. Screening lncRNAs expression profile in glioma has revealed a significant contribution to pathogenesis [10], development and progression [11–13] by regulating cell growth and metastasis; indicating that lncRNAs play significant roles in glioma tumorigenesis [14].

The functional lncRNA-MALAT1 (metastasis-associated lung adenocarcinoma transcript 1); encoded by *MALAT1* gene, which is located at chromosome 11q13.1. [15], and also known as nuclear-enriched abundant transcript 2 (NEAT2), was one of the first lncRNAs found to have a pathogenic role [16] and it has been linked to various cancers besides lung adenocarcinoma [17]. Its expression profile has been found to be dysregulated and correlated with clinical parameters and prognosis in several types of human cancer, such as hepatocellular carcinoma [18], osteosarcoma [19], lung cancer [20], bladder cancer [21], and glioblastoma multiforme [22].

As there are no previous studies, up to the researchers' knowledge, on the expression of this type of lncRNAs in GBM patients among the Arab population, this study for the first time will aim to determine the expression levels of *MALAT1* in a sample of GBM patients and correlate these expressions with the available clinicopathological features including patient survival data in a sample of Egyptian population.

2. Subjects and methods

2.1. Patients and tissue samples

The present study included 37 formalin-fixed paraffin embedded (FFPE) glioblastoma samples, fulfilling the WHO criteria of GBM and 10 FFPE non-neoplastic brain tissue specimens. GBM patient samples (9 females and 28 males, aged 35 to 60 years old) have been assessed retrospectively from the archive of the Pathology Departments, Mansoura University Hospitals and Suez Canal University Hospitals, Egypt, from 2010 to 2013. Detailed patients' data were retrieved from their medical follow up records. All patients had GBM (i.e. grade IV), undergone surgical removal and post-operative irradiation, and followed for more than 3 years. The work has been carried out in accordance with the code of ethics of the world medical association (Declaration of Helsinki) for experiments on humans. All patients gave written informed consent, except for deceased individuals or patients who provided archived tissue samples and can't be traced.

2.2. Total RNA extraction

Following deparaffinization in xylene and washing with alcohol, the total RNA was extracted from tumor and control FFPE tissue sections (4–5 μm) collected in sterile eppendorf tubes. Qiagen RNeasy FFPE Kit (Cat. No. 73504, Qiagen, Hilden, Germany) has been used following the protocol supplied by the manufacturer. Extracted total RNA concentration and purity at the absorbance ratio 260/280 nm were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA). In addition, RNA degradation and contamination were assessed by 1.5% agarose gel electrophoresis.

2.3. Reverse transcription

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, P/N 4368814) was used for reverse transcription (RT) reaction. For each 20 μl RT reaction, 10 μl (10 ng) RNA sample was combined with 10 μl of 2× RT reaction mix containing 2 μl of 10× RT Buffer, 0.8 μl of 25× dNTP Mix (100 mM), 2 μl of 10× RT random primers, 1 μl of MultiScribe™ Reverse Transcriptase, 1 μl of RNase inhibitor, and 3.2 μl of nuclease-free water. RT was carried out in a T-Professional Basic, Biometra PCR System (Biometra, Goettingen, Germeny) at 25 °C for 10 min, followed by 37 °C for 120 min, and finally 85 °C for 5 min, then held at 4 °C. Appropriate negative controls were included in each experiment.

2.4. Gene expression analysis

Real-time polymerase chain reaction (PCR) was performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. Expression of *MALAT1* and the endogenous control *TBP* (Tata binding protein) were quantified using TaqMan® assays (Applied Biosystems, assay ID Hs00273907_s1, and Hs00427620_m1, respectively) and Taqman® Universal PCR master mix II, No UNG (2×) (Applied Biosystems, P/N 4440043). Apart for *TBP*, none of the other conventionally used housekeeping genes in GBM studies, were found to be suitable as they showed variation in RNA expression [23]. Hence, *TBP* expression was used for normalization of qPCR data in the current work. The PCR reactions were carried out in a final volume of 20 µl, including 1.33 µl RT product, 10 µl 2 × TaqMan® Universal PCR Master Mix, 1 µl TaqMan® assays. All reactions included a non-template control (with water instead of cDNA), and a no-reverse transcriptase control. The PCR was performed on StepOne™ Real-Time PCR System (Applied Biosystems) as follows: 95 °C for 10 min followed by 40 cycles of 92 °C for 15 s and 60 °C for 1 min.

2.5. Statistical analysis

Statistical Package for the Social Sciences (SPSS) for Windows software (version 20.0) was used for statistical graphics and analyses. The following parameters were considered besides the expression values of the *MALAT1* analyzed: age at diagnosis, gender and tumor site. Categorical variables were compared using the chi-square (χ^2) or Fisher's exact tests where appropriate, while Student's t test or One way ANOVA were used to compare continuous variables between two groups or more than two groups, respectively in case the data distribution was concordant with normal distribution (Shapiro–Wilk test) and after checking variance homogeneity (Levene's test). If the data did not meet the criteria mentioned above, the non-parametric Mann–Whitney-*U* test or Kruskal–Wallis tests were used for comparison between groups with subsequent Bonferroni's post hoc test for multiple testing. The receiver operating characteristic (ROC) curves were derived and area-under-the curve (AUC) analysis performed to get the best cut-off value of *MALAT1* for discriminating GBM patients from controls. *P*-values < 0.05 were considered statistically significant. Disease-free survival is defined as the time between diagnosis of disease and recurrence or distant metastasis. Overall survival is defined as time from diagnosis of disease to death of patients with brain cancer [24]. Median follow-up time was computed by the Kaplan–Meier method. For analysis of disease-free and overall survival, frontal and fronto-temporal tumors were combined for comparison with those with parietal and temporo-parietal. Log rank (Mantel–Cox) was used for survival curves comparison. The fold change of *MALAT1* expression was calculated using Livak's method based on the threshold cycle (C_T) value with the following equation: relative quantity $= 2^{-\Delta\Delta CT}$ [25].

3. Results

3.1. Baseline characteristics of the study population

The main patient characteristics have been summarized in Table 1. In addition, the mean ± SD of the GBM patients overall survival (OS) and disease-free survival (DFS) were 15.4 ± 5.17 months and 14.7 ± 5.34 months with a range of (8–27) and (6–27) months, respectively. Twenty three patients (62.2%) showed short (⩽1 year) disease free survival (DFS) and only 12 (32.4%) showed a higher overall survival rate (> 1 year). As shown in Table 1, male gender was significantly associated with shorter DFS than females ($P < 0.04$).

3.2. Expression of MALAT1 in glioblastoma multiform

Compared with normal brain tissues, all GBM patients showed low expression levels of lncRNA MALAT1

Table 1 Baseline patient characteristics ($n = 37$) and survival.

	Mean ± SD or Number (%)	OS (mo)	*P* value	DFS (mo)	*P* value
Age (years)	51.5 ± 5.9				
Age, categories					
35-	16 (43.2)	17.3 ± 7.08	0.241	17.0 ± 6.89	0.123
50-	21 (56.8)	13.9 ± 2.25		13.09 ± 2.98	
Gender					
Female	9 (24.3)	18.5 ± 6.9	0.086	18.5 ± 6.9	**0.040***
Male	28 (75.7)	14.3 ± 4.13		13.5 ± 4.20	
Tumor site					
Frontal	18 (48.6)	14.4 ± 5.09	0.179	13.5 ± 5.14	0.133
Fronto-temporal	4 (10.8)	19.0 ± 3.46		19.0 ± 3.46	
Parietal	1 (2.7)	11.0		11.0	
Temporo-parietal	14 (37.8)	19.5 ± 5.52		15.4 ± 5.72	
Recurrence					
Non-recurrent	30 (81.1)	15.5 ± 5.25	0.719	15.3 ± 5.26	0.259
Recurrent	7 (18.9)	15.0 ± 5.22		12.4 ± 5.47	

OS, overall survival; DFS, disease-free survival; mo, months. Student's *t* and ANOVA tests were used.
* Statistically significant, $P < 0.05$.

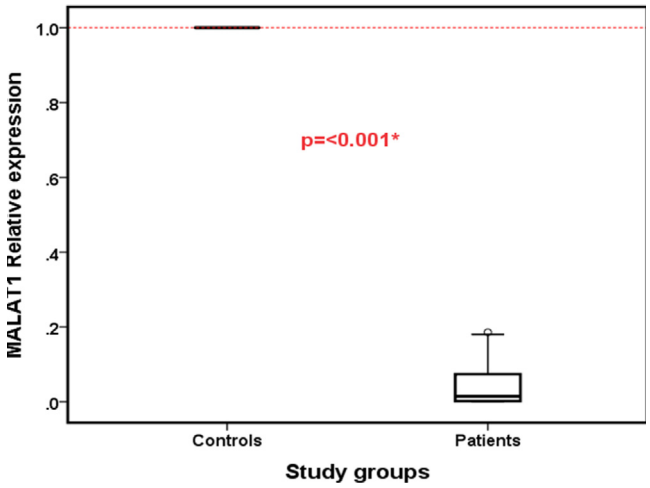
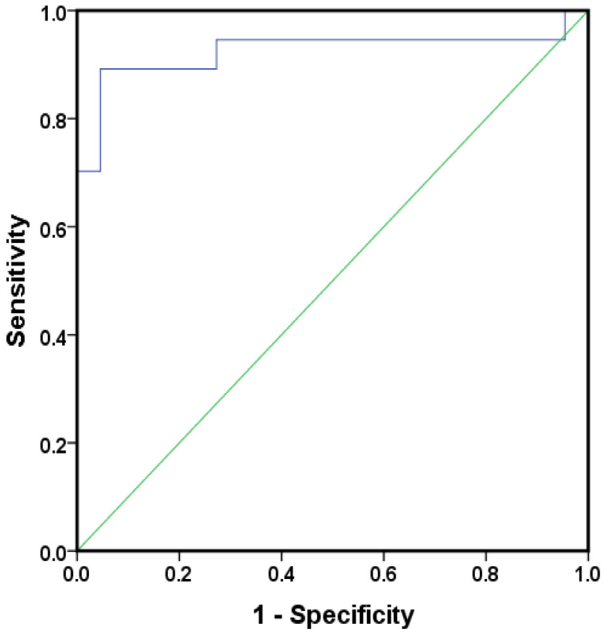


Figure 1 LncRNA-MALAT1 expression levels in glioblastoma. Data are represented as medians. The box defines upper and lower quartiles (25% and 75%, respectively) and the error bars indicate upper and lower adjacent limits. Mann–Whitney *U* test was used; *Statistically significant.

($P < 0.001$, Fig. 1) with a median expression value (quartile) = 0.015 (0.001–0.086).

3.3. ROC curve analysis

Relative expression levels were used to determine *MALAT1* sensitivity and specificity at the cutoff fold change value for discriminating GBM patients from controls. The curve showed high diagnostic performance with an AUC = 0.925 ± 0.038 ($P < 0.001$), 95% CI = 0.850–1.00, with 94.6% sensitivity and 72.7% specificity (Fig. 2).



Area	Std. Error	Asymptotic Sig.	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.925	0.038	.000	0.850	1.000

Figure 2 Receivers operating characteristic curve of MALAT1. AUC: area under curve; CI: confidence interval; SE, standard error; SEN, sensitivity; SP, specificity.

3.4. LncRNA MALAT1 relative expression and clinicopathological features in GBM patients

Median *MALAT1* relative expression was not associated significantly with age (< 50 *vs.* ⩾50, $P = 0.387$) or gender (females *vs.* males, $P = 0.196$), however fronto-temporal brain tissue samples showed a significant lower expression in comparison to other sites, ($P = 0.025$); Fig. 3. Lower *MALAT1* expression, in addition, was associated with poor prognosis; higher frequency of recurrence, lower overall survival, and shorter disease-free survival (Fig. 3).

As summarized in Table 2, *MALAT1* expression was directly correlated with shorted overall survival ($r = 0.359$, $P = 0.029$) and disease-free survival ($r = 0.361$, $P = 0.028$) and was inversely correlated with recurrence ($r = -0.334$, $P = 0.044$).

3.5. MALAT1 expression and survival of patients with different clinical variables

To explore the prognostic value of the lncRNA-MALAT1 expression in GBM patients, Kaplan–Meier analysis with the log-rank test was used in comparing survival of patients with different clinical variables (Fig. 4). Poor survival has been shown in older patients [median (95% CI); 14 (12.5–15.4) and 16 (8.81–23.1) in patients ⩾50 year-old and < 50 year-old, respectively] and among males [median (95% CI); 14 (12.2–15.7) and 22 (13.6–30.3) in males and females, respectively]. Otherwise, there were no significant differences of lncRNA MALAT1 expressions and survival of patients regarding the tumor location or recurrence rate (Fig. 4).

4. Discussion

Human transcriptome analysis has been revealed that the majority of human genome transcripts are non-coding RNAs, including lncRNAs that previously were considered as transcriptional noises [26]. However, an accumulating number of evidences have revealed that lncRNAs could have important functions and may interact with a broad range of RNA molecules through competitively binding with miRNAs, suggesting their vital roles in a wide range of biological processes and human diseases [27]. More specifically, they have been found to be involved in brain development and in the pathogenesis of gliomas, and could serve as novel biomarkers for early diagnosis, prediction of prognosis, and therapeutic targets in gliomas [28,29].

The lncRNA *MALAT1* gene produces a highly abundant and ubiquitously expressed transcript of > 8000 nucleotides that is retained in the nucleus where it is thought to form molecular scaffolds for ribonucleoprotein complexes [30]. MALAT1 was found to act as decoys that bind to and interfere with the function of other RNAs or proteins (e.g. miRNAs, transcription factors, or RNA-binding proteins),

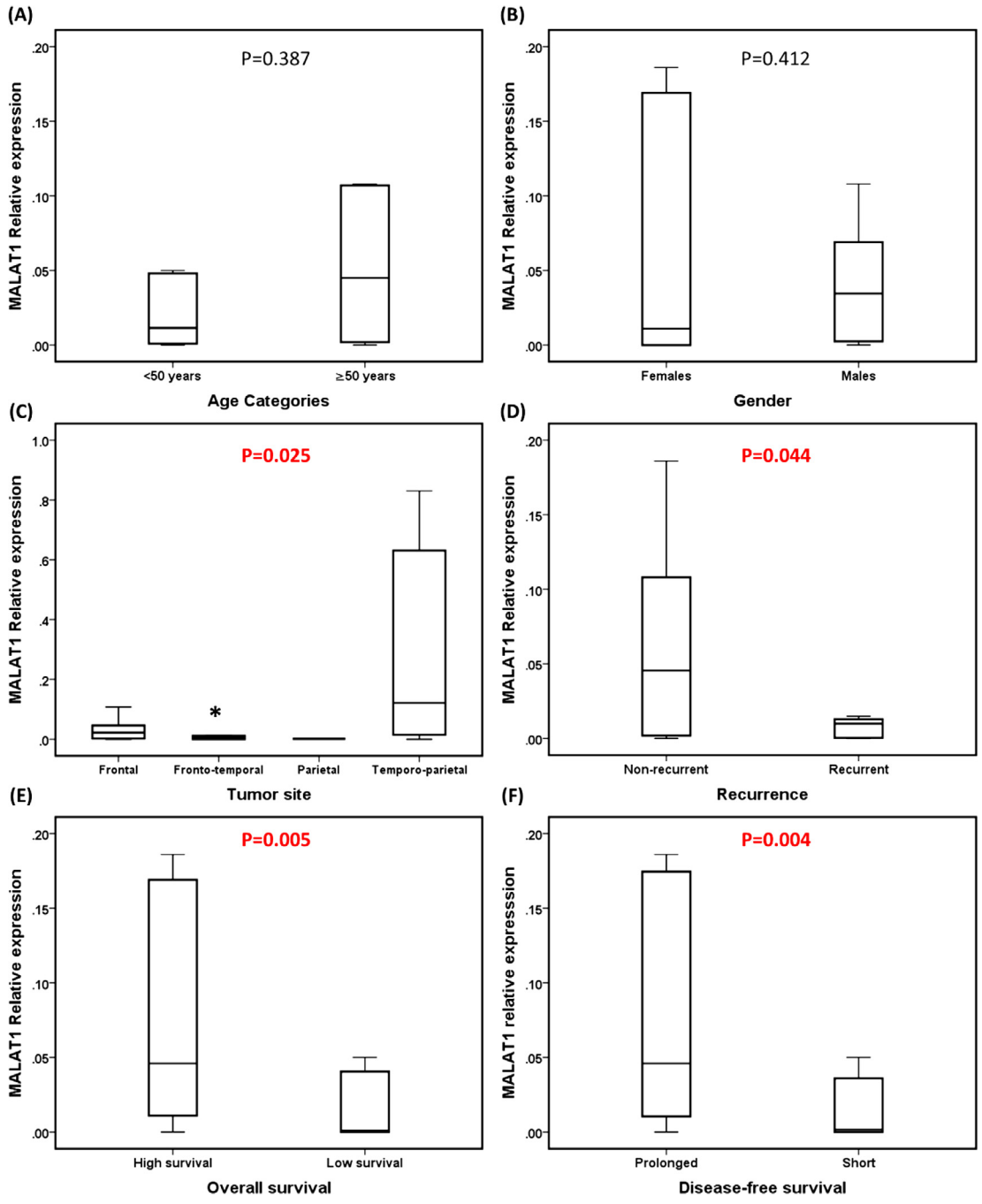


Figure 3 LncRNA MALAT1 relative expression and clinicopathological features in GBM patients. Data are represented as medians. The box defines upper and lower quartiles (25% and 75%, respectively) and the error bars indicate upper and lower adjacent limits. *N.B. In posthoc test parietal tumor group was merged with tempero-parietal one as test could not be performed by single sample.

especially those involved in cell cycle regulation, cell migration, and cancer metastasis [31].

Initially, *MALAT1* was identified by Ji and co-workers in (2003) [32], through subtractive hybridization in a screen for genes associated with metastasis in non-small cell lung cancer. Subsequently, several studies have associated *MALAT1* expression with various cancers and metastasis [33–35]. In the current study, *MALAT1* showed significant lower expression levels in all GBM tissues compared with normal brain tissues. This is consistent with a recent study finding conducted by Han et al. [22] who found that lncRNA MALAT1 could have a tumor-suppressive function in glioma. This inhibitory effect could be due to suppression of both growth and cell invasion. At the molecular level, they suggested that this could be mediated via regulation of the ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase) pathway and expression of MMP2 (matrix metalloproteinase 2). The ERK/MAPK pathway is one of the most important signal transduction pathways, and *MALAT1* upregulation in glioma cells inhibits the growth and invasion of tumor by

Table 2 Spearman's rho correlation analysis between brain tissue MALAT1 relative expression and the clinicopathological features in GBM patients ($n = 37$).

	Correlation coefficient	*P* value
Age	0.104	0.539
Gender	0.142	0.402
Tumor site	0.050	0.768
OS	0.359	**0.029***
DFS	0.361	**0.028***
Recurrence	−0.334	**0.044***

OS, overall survival; DFS, disease-free survival.
* Statistically significant, $P < 0.05$.

reducing the phosphorylated ERK1/2 expression rather than affecting the total ERK1/2, leading to inactivation of this signaling cascade [22]. In earlier studies, it has been proved that knockout of *MALAT1* in lung cancer cells could significantly reduce the expression of several metastasis-related genes, including Glypican 6 (*GPC6*) [36] and C-X-C motif chemokine 5 (*CXCL5*) [37], leading to MAPK pathway inactivation. Hence *GPC6* or *CXCL5* expression down-regulation could be one of the underlying mechanisms by which *MALAT1*inactivate ERK/MAPK pathway in glioma which require further future validation.

In addition, Bernard et al. [38] have found that *MALAT1* expression in B-cell malignancies was decreased compared with solid tumors. Another study demonstrated significantly lower *MALAT1* expression levels in the plasma of multiple

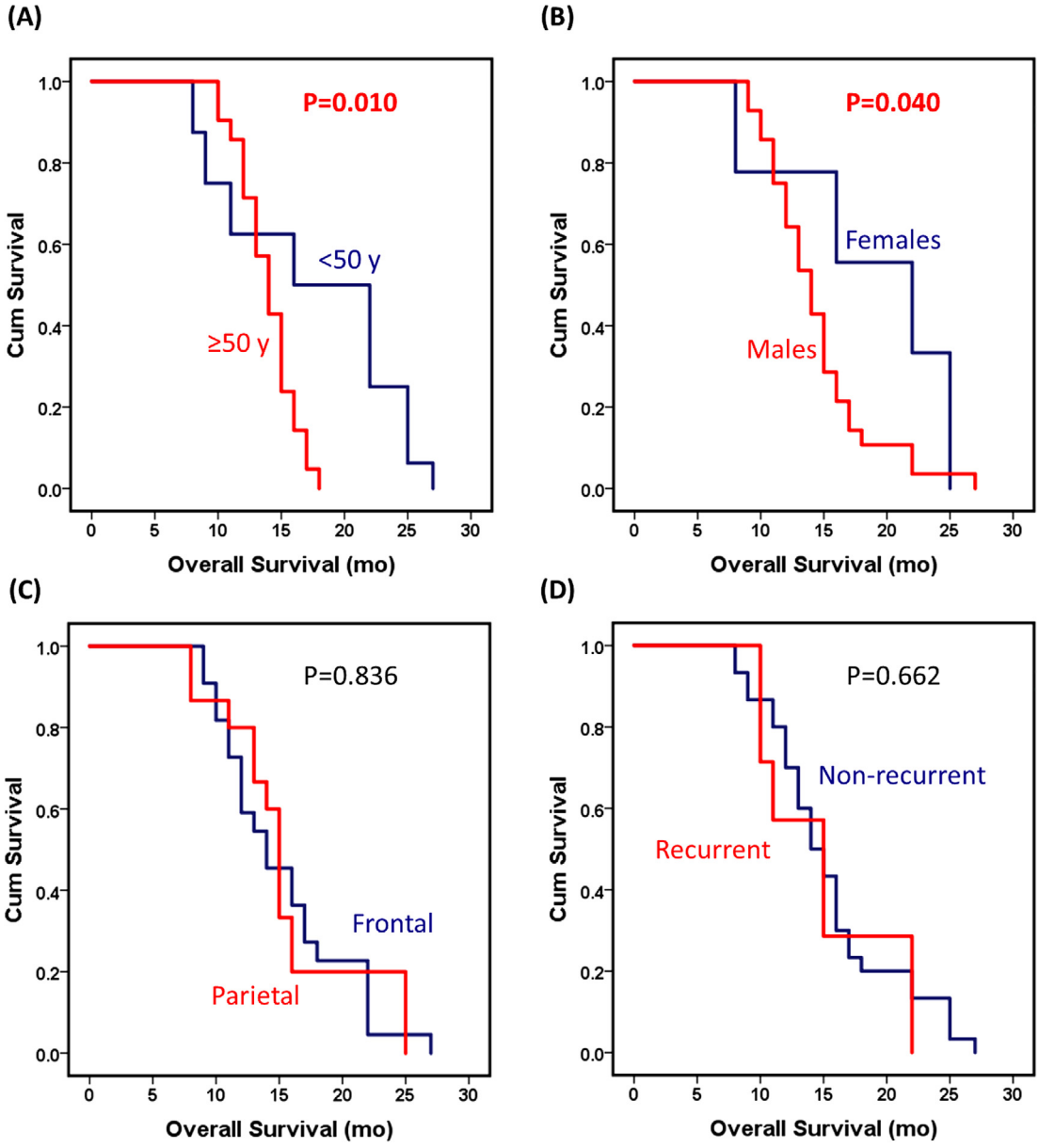


Figure 4 Kaplan–Meier curves and the log-rank (Mantel–Cox) test for comparison of the patients' survival with different clinical variables. Cum. Cumulative; mo, months.

myeloma patients [39]. The results of the present study, however, were a little different from others. It has been found that *MALAT1* expression was increased in glioma tissues compared with paired adjacent brain normal tissues by Ma et al. [14]. *MALAT1* was shown, in addition, to act as oncogene in several types of human cancer, such as lung cancer [40], breast cancer [41], cervical cancer [42], bladder urothelial carcinoma [21], gastric cancer [43], colorectal cancer (CRC) [44], hepatocellular carcinoma [34], pancreatic duct adenocarcinoma [45] and melanoma [46]. However, *MALAT1* was downregulated in the cell culture, with the cells exhibiting high metastatic potential for ovarian cancer metastasis [47]. This discrepancy of results among some studies and ours could be due to the fact that *MALAT1* function may vary with various cancer types and context; the same gene may play opposite roles in different cancer types or in different stages of cancer progression [48]. Additionally, emerging evidence has suggested that lncRNAs can potentially interact with other classes of non-coding RNAs including miRNAs [49], or are regulated by transcription factors [50], indicating that lncRNAs may have regulatory roles in a wide range of cellular processes at various levels. In addition, the recent molecular sub-classification of GBM includes *IDH1* (isocitrate dehydrogenase 1) mutation, *p53* mutation, *EGFR* (epidermal growth factor receptor) amplification and *MGMT* (O-6-methylguanine-DNA methyltransferase) promoter methylation status, all of which have proved to be partly associated and are useful in clinical practice [13]. Their aberration may result in significant epigenetic changes, including DNA methylation level, mRNA expression level [51], and lncRNA expression level [52]. Therefore, it is recommended to investigate whether there are some associations between *MALAT1* signature and these well-recognized genetic biomarkers by an intriguing multi-dimension analysis that can give useful explanations for the potential result discrepancies in GBM molecular research.

In the present study, we further found that lower *MALAT1* expression was associated with poor prognosis (i.e. higher frequency of recurrence, lower overall survival and shorter disease-free survival). Several reports indicate that *MALAT1* contributes to the complex molecular mechanisms involved in the control of cell growth, differentiation and motility that could predict the overall prognosis and survival [21]. For example, the expression of *MALAT1* was associated with prostate cancer progression and prognosis [53], GBM survival [13], tumor progression and survival in lung, liver and breast cancer [54], invasion and metastasis of CRC cells [55], proliferation and metastasis of gallbladder cancer cells [56] and tumor recurrence of hepatocellular carcinoma after liver transplantation [57]. All these findings indicate that lncRNA MALAT-1 could be considered as an important novel candidate for future therapeutic intervention. However, the applicability and epigenetic regulation of MALAT-1 targeted strategies for the clinical treatment of GBM requires additional studies.

5. Conclusion

In summary, the current study does confirm the lncRNA MALAT1 association with the glioblastoma and its potential role as prognostic biomarker. The study was limited by the relatively small sample size, and the fact that all patients were grade IV gliomas. We recommend further studies of a larger scale with different glioma grades, to explore the correlation of *MALAT1* expression with different WHO grades and to confirm its possible role as prognostic biomarker. Further functional investigations of this type of lncRNAs on tumor cell lines and xenograft models may increase our outstanding of its detailed molecular roles in GBM pathogenesis and progress. Hence, this may be an interesting target for future therapeutic interventions in GBM patients.

Declaration of conflict of interest

The authors declare that they have no conflict of interest.

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