

RNU6-1 in circulating exosomes differentiates GBM from non-neoplastic brain lesions and PCNSL but not from brain metastases

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Abstract

Background. Glioblastoma (GBM) is the most common malignant primary brain tumor in adults. Circulating biomarkers may assist in the processes of differential diagnosis and response assessment. GBM cells release extracellular vesicles containing a subset of proteins and nucleic acids. We previously demonstrated that exosomes isolated from the serum of GBM patients had an increased expression of *RNU6-1* compared to healthy subjects. In this exploratory study, we investigated the role of this small noncoding RNA as a diagnostic biomarker for GBM versus other brain lesions with some potential radiological similarities.

Methods. We analyzed the expression of *RNU6-1* in circulating exosomes of GBM patients ($n = 18$), healthy controls ($n = 30$), and patients with subacute stroke ($n = 30$), acute/subacute hemorrhage ($n = 30$), acute demyelinating lesions ($n = 18$), brain metastases ($n = 21$), and primary central nervous system lymphoma (PCNSL; $n = 12$) using digital droplet PCR.

Results. Expression of *RNU6-1* was significantly higher in GBM patients than in healthy controls ($P = .002$). *RNU6-1* levels were also significantly higher in exosomes from GBM patients than from patients with non-neoplastic lesions (stroke [$P = .05$], hemorrhage [$P = .01$], demyelinating lesions [$P = .019$]) and PCNSL ($P = .004$). In contrast, no significant differences were found between patients with GBM and brain metastases ($P = .573$). Receiver operator characteristic curve analyses supported the role of this biomarker in differentiating GBM from

subacute stroke, acute/subacute hemorrhage, acute demyelinating lesions, and PCNSL ($P < .05$), but again not from brain metastases ($P = .575$).

Conclusions. Our data suggest that the expression of *RNU6-1* in circulating exosomes could be useful for the differentiation of GBM from non-neoplastic brain lesions and PCNSL, but not from brain metastases.

Key Points

Increased levels of RNU6-1 in GBM patients than in healthy controls.

RNU6-1 in circulating exosomes could be useful for the differentiation of GBM from non-neoplastic brain lesions and PCNSL, but not from brain metastases.

Importance of the Study

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults. Although neuroimaging may suggest the diagnosis, histological examination of the tumor tissue remains mandatory for definitive diagnosis and further specific treatment. The identification of diagnostic and prognostic biomarkers for GBM in more accessible fluid specimens might be helpful for assisting in the process of differential diagnosis in those patients in whom surgery is contraindicated or with inconclusive histopathological results and in monitoring response to treatment. In this work, we

evaluated the role of *RNU6-1* in circulating exosomes isolated from the serum of patients with GBM and other brain lesions that might potentially exhibit some radiological similarities: subacute stroke, acute/subacute hemorrhage, acute demyelinating lesions, brain metastases, and PCNSL. We observed that the expression of *RNU6-1* was higher in patients with GBM compared to the remainder pathologies except for brain metastases, concluding that *RNU6-1* might allow differentiating GBM from nontumoral brain lesions and PCNSL but not from brain metastases.

Glioblastoma (GBM) is the most common malignant primary brain tumor in adults,¹ with an estimated incidence of about 3 cases per 100 000 people per year.² The current standard of care consists of maximal safe resection when feasible, followed by radiotherapy with concomitant and adjuvant temozolomide.^{3,4} Despite such multimodal approach the prognosis of patients with this diffusely infiltrating disease remains dismal, with median overall survival of 14.6 months and 5-year survival rates of less than 10%.⁵

Although magnetic resonance imaging (MRI) often suggests its diagnosis, other enhancing tumors and brain lesions such as acute demyelinating plaques, subacute ischemic stroke, or intraparenchymal hemorrhages might exhibit similar radiological features.⁶ Apart from the therapeutic and prognostic role of surgical resection, histological examination of tumor tissue is required for definite diagnosis and further specific treatment. The identification of a blood-based diagnostic biomarker for GBM would be clinically helpful, particularly in the process of differential diagnosis in those patients in whom surgery is contraindicated or with inconclusive histopathological results^{7,8} and in monitoring response to treatment.

Circulating vesicles released by tumor cells have recently emerged as promising reservoirs of diagnostic biomarkers in GBM.^{9–12} These extracellular vesicles are

composed of a lipid bilayer containing transmembrane proteins and enclosing cytosolic proteins and nucleic acids such as DNA, mRNA, miRNA, and long noncoding RNA. They constitute biologically active molecules that mediate both surrounding and distant intercellular communication, thus favoring immune evasion and tumor growth and dissemination.^{13–17} According to their origin, content, and size, extracellular vesicles can be classified in shedding microvesicles (microvesicles, ectosomes, and microparticles) and exosomes.¹³ Exosomes are 30–100 nm diameter vesicles formed by inward budding of endosomal compartments and secreted into the environment when these compartments fuse with the plasmatic membrane.¹⁸ They are very stable vesicles that express different surface markers such as CD9, CD63, CD81, TSG101, and different types of integrins.¹⁹ Several groups have described an increased release of exosomes from GBM cells, and the potential of their molecular cargo for facilitating the diagnosis and predicting both response to treatment and prognosis.^{11,20–22}

In a previous study, we found a significantly higher expression of RNU6-1 in exosomes isolated from the serum of GBM patients compared with healthy controls, thus hypothesizing its potential role as a diagnostic biomarker for GBM.⁹ RNU6-1 is a small noncoding RNA (sncRNA)

involved in RNA processing and cellular growth rate regulation.²³⁻²⁵

Based on our previous results, we conducted this study to assess the role of RNU6-1 isolated from circulating exosomes as a diagnostic biomarker for GBM and its accuracy for distinguishing other tumors and brain lesions that may mimic GBM on neuroimaging.

Methods

Study Population

Between 2016 and 2018, a total of 159 patients exhibiting different brain lesions or non-glioma malignancies that can share some radiological features with GBM, and 18 patients with newly diagnosed GBM were prospectively included in the current study. Nonmalignant brain lesions consisted of subacute ischemic non-lacunar hemispheric stroke (from 7 to 28 days after onset) in 30 patients, acute or subacute hemispheric intraparenchymal hemorrhage

(from onset to up to 28 days) in 30 patients, and acute enhancing demyelinating plaques in 18 patients with multiple sclerosis. Brain tumors consisted of primary central nervous system lymphoma (PCNSL) in 12 patients and intraparenchymal brain metastases in 21 patients. **Figure 1** illustrates the potential radiological similarities between GBM and the other brain disorders considered in this study. In addition, 30 healthy subjects with no recent history of head trauma or symptoms of an intracranial lesion served as controls.

The local ethics committee approved the study protocol and informed written consent was obtained from all patients and healthy controls.

Serum Sample Collection

For patients with GBM and non-glioma brain tumors, blood samples were taken before surgery and any other specific antitumor treatment. Additionally, in patients with PCNSL peripheral blood was drawn prior to receiving any

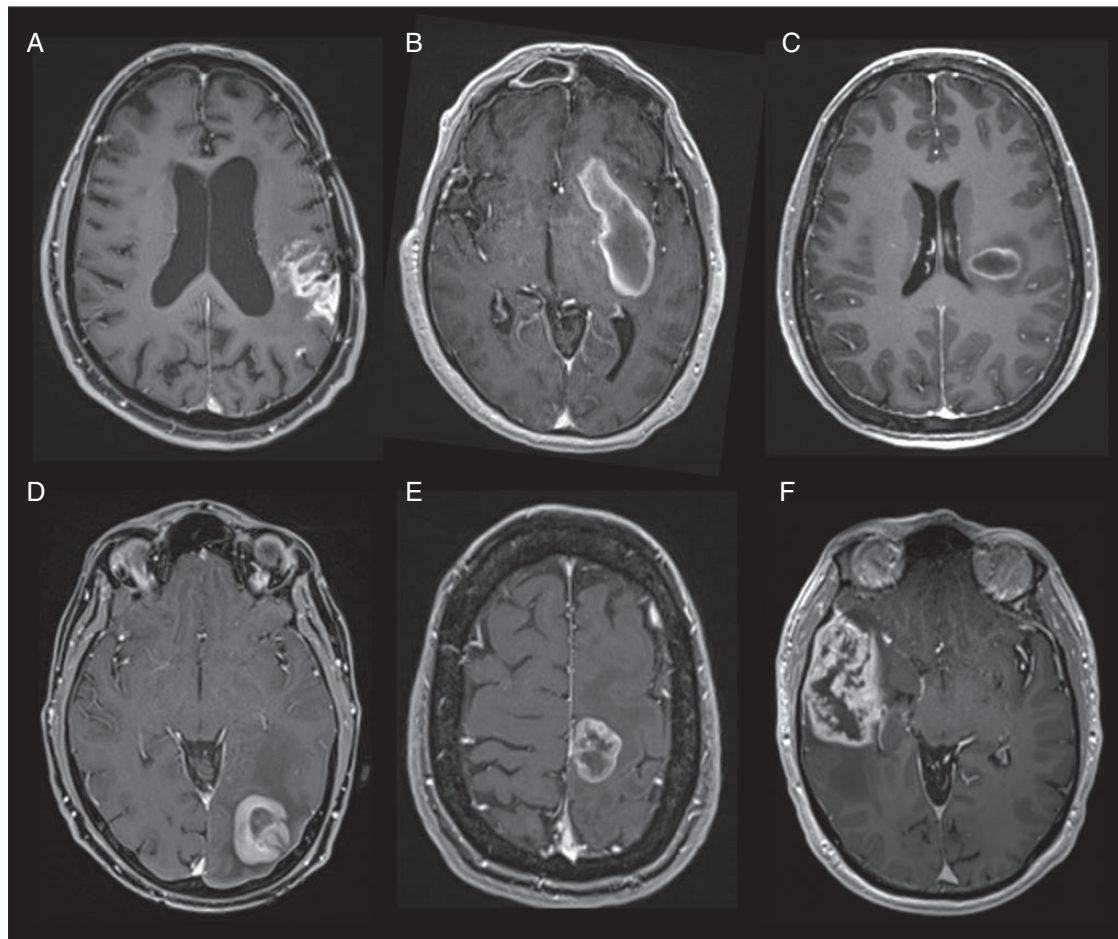


Figure 1 Examples of potential radiological similarities between glioblastoma and other types of brain lesions included in the study: (A) subacute stroke, (B) subacute hemorrhage, (C) acute multiple sclerosis lesion, (D) primary central nervous system lymphoma, (E) brain metastasis, and (F) glioblastoma.

corticotherapy. Lack of previous corticosteroid treatment before blood sampling was also required for patients with acute multiple sclerosis lesions. Samples from volunteer subjects were collected in the absence of a concurrent inflammatory illness at the time of peripheral blood sampling.

Venous blood was drawn into Vacutainer tubes containing sodium citrate solution as anticoagulant, inverted 4 to 6 times, and allowed to clot at 4°C for 1 h. Samples were then centrifuged at 2400 rpm for 10 min to separate the serum. The supernatant fluid was collected and aliquoted into 2-mL cryotubes and stored at -80°C until subsequent analysis.

Exosome Isolation and Characterization

After filtering 250 µL of serum through a 0.22 µm filter, microvesicles were isolated using the Exoquick precipitation solution (System Biosciences) according to the manufacturer's instructions. Then, the obtained pellet was treated with RNase (Ambion) and DNase I (New England Biolabs) for 30 min at room temperature and at 37°C, respectively.

Dynamic light scattering

Particle number and exosome size were analyzed using the Zetasizer Nano ZS (Nanosizer, Malvern Panalytical) equipped with a blue laser (405 nm), according to the manufacturer's instructions. Distilled water (MilliQ) was used for the measurements and samples were diluted 1:125.

Western blot

Protein extracts from exosomes were prepared using a 100 µL lysis buffer (PBS + 0.1% Triton) and were kept 30 min on ice. Samples were then centrifuged at 10 000× *g* for 15 min at 4°C and supernatant was reserved for further analysis. All protein extracts were quantified using Protein Assay Dye Reagent Concentrate (Bio-Rad) following the manufacturer's indications; 30 µg of protein were then loaded and separated in a 10% polyacrylamide gel under denaturing conditions. Afterward, proteins were transferred to a nitrocellulose membrane and incubated with the respective antibodies: ADAM 10 (Cell Signaling), Alix (Cell Signaling), Calnexin (Cell Signaling), CD63 (Sigma-Aldrich), CD9 (Millipore), Syntenin-1 (Abcam), and TSG101 (Sigma-Aldrich). Protein bands were detected by enhanced chemiluminescence (GE Healthcare) measured by densitometry and quantified using ImageJ software.

RNA Extraction

Total RNA was extracted from the isolated exosomes using the Trizol reagent (Invitrogen). Quantity and quality of the obtained RNA were determined with Nanodrop 1000 Spectrophotometer (Thermo Scientific). Forty nanograms of RNA were further retro-transcribed using GoScript™ Reverse Transcription System to obtain cDNA.

Digital Droplet PCR

Digital droplet PCR (ddPCR) was performed as described previously.²⁶ Briefly, 20 µL of ddPCR assay mix was loaded into the wells of a disposable DG8 cartridge (Bio-Rad) with 70 µL of droplet generation oil for probes (Bio-Rad). The cartridge was then placed into the QX200 Droplet Generator (Bio-Rad). Around 15 000 highly uniform nanoliter-sized droplets were generated in each well and transferred to a 96-well PCR plate (Eppendorf, Germany). PCR amplification was performed in a thermal cycler (Bio-Rad) at 95°C for 10 min, then 40 cycles of 94°C for 30 s and 52°C for 1 min (ramping rate reduced to 2%), and a final inactivation step at 98°C for 10 min. After PCR, the plate was loaded into the QX200 Droplet Reader (Bio-Rad) for automatic reading of positive and negative droplets in each sample. All samples were run in duplicate, and a positive control and a blank (BLK) were included in every assay. Obtained data were analyzed using the QuantaSoft software™ (Bio-Rad). Discrimination between negative and positive droplets was achieved by setting manually a fluorescence amplitude threshold for the RNU6-1 assay based on results from BLK wells. The absolute amount of RNU6-1 was calculated by counting the number of positive droplets per well. The corrected number of targets, determined by Poisson statistical analysis, was multiplied by the corresponding dilution factor to obtain the total copy number per microliter of PCR mixture.

Statistical Analysis

Continuous data are expressed as means with SDs and medians with 25th and 75th percentiles. The assumption of normality was checked using the Shapiro-Wilk test. Differences in serum RNU6-1 expression levels between patients with GBM, patients with other brain lesions, and healthy controls were conducted using the Wilcoxon rank-sum test. The area under the receiver operator characteristic (ROC) curve was calculated to explore the diagnostic role of RNU6-1. The area under the curve (AUC) ranges from 0.5 to 1, with 0.5 meaning no predictive ability and 1 meaning perfect predictive ability. Two-tailed *P* values ≤ 0.05 were considered statistically significant. All analyses were performed using Stata 14 (StataCorp. 2015).

Results

Characteristics of the Study Population

Gender and age characteristics of the study population are summarized in [Table 1](#). Among patients with brain metastases, primary tumors consisted mainly of lung and breast cancers and were as follows: 8 patients (36.36%) had lung cancer, 6 patients (27.27%) had breast cancer, 4 patients (18.18%) had colorectal cancer, and 4 patients had each (18.18%) a melanoma, pancreatic cancer, gastroesophageal cancer, and bladder cancer metastases. Eleven of these patients (52.38%) had a single brain metastasis whereas 10 patients (45.45%) had multiple metastatic brain lesions.

Characterization of Exosomes Isolated from Patients' and Healthy Controls' Sera

First, exosomes were isolated from the serum of patients with GBM and with the other brain pathologies that can mimic GBM on neuroimaging, as well as from healthy controls. In order to confirm the nature of the obtained microvesicles, their size and the expression of several protein markers were evaluated. Interestingly, the proportion of exosomes, that is, microvesicles with a size ranging from 30 to 120 nm, was higher in patients with GBM compared to the other brain disorders and healthy controls. In addition, healthy controls exhibited the lowest proportion of circulating exosomes (Figure 2A). The expression of several exosomal markers and the lack of detection of the endoplasmic reticulum marker calnexin confirmed that resulting samples from all groups of patients and controls were enriched with exosomes (Figure 2B). Importantly, the number of circulating exosomes did not significantly differ between the group of patients with tumoral lesions and the group of patients with non-neoplastic lesions. Although we

observe an increasing tendency in the group of patients with neoplastic lesions (Supplementary Figure S1).

RNU6-1 Expression in Circulating Exosomes from GBM Patients, Patients with Other Brain Lesions, and Healthy Subjects

In order to accomplish the aim of the study, RNA from the isolated exosomes was extracted and the expression of RNU6-1 was determined by ddPCR. Obtained results are given in Table 2.

Statistically significant differences in the expression levels of this sncRNA were found between all groups ($P = .006$). Particularly, the highest expression of RNU6-1 was observed in the group of patients with GBM (412 copies/20 μ L [189–611]) followed by the group of patients with brain metastases (325 copies/20 μ L [65–617]) (Figure 3A).

Importantly, the expression of RNU6-1 was significantly higher in GBM patients than in healthy subjects (93

Table 1 Characteristics of the Study Population

	Healthy Controls	Stroke	Hemorrhage	Multiple Sclerosis	PCNSL	Metastasis	GBM
<i>N</i>	30	30	30	18	12	21	18
Sex <i>n</i> (%)							
Female	17 (56.7)	11 (36.7)	9 (30)	13 (68.4)	4 (33.3)	10 (47.61)	8 (44.4)
Male	13 (43.3)	19 (63.3)	21 (70)	6 (31.6)	8 (66.7)	11 (52.38)	10 (55.6)
Age, years							
Median (range)	47 (21–77)	70.8 (44–93)	65.6 (28–91)	40.6 (20–61)	66.2 (47–84)	60.3 (45–82)	63 (20–72)

PCNSL, primary central nervous system lymphoma; GBM, glioblastoma.

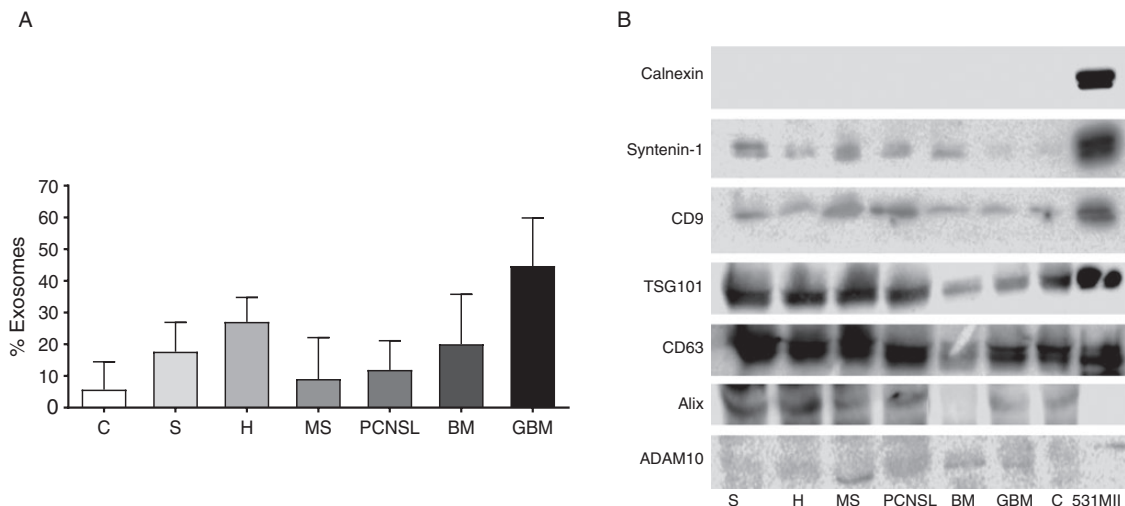


Figure 2 The proportion of circulating exosomes in the serum of patients with GBM and other brain pathologies and healthy controls (A). Characterization of circulating exosomes by Western blot (B). C, controls; S, stroke; H, hemorrhage; MS, multiple sclerosis; PCNSL, primary central nervous system lymphoma; BM, brain metastasis; GBM, glioblastoma.

Table 2 RNU6-1 Expression Levels in Circulating Exosomes

		Healthy Controls	Stroke	Hemorrhage	Multiple Sclerosis	PCNSL	Metastasis	GBM
<i>N</i>		30	30	30	18	12	21	18
RNU6-1 copies/ 20 μ L	Mean (SD)	194.9 (224.3)	432.9 (709.8)	203.1 (195.3)	214.6 (250.4)	160.5 (245.8)	529.6 (632.1)	546.2 (550.5)
	Median (p25–p75)	93 (20–409)	223 (44–390)	135 (67–277)	111.5 (82–273)	18.1 (6.4–241)	325 (65–617)	412 (189–611)
<i>P</i> value of comparison with GBM		.002	.05	.01	.019	.004	.573	—

PCNSL, primary central nervous system lymphoma; GBM, glioblastoma.

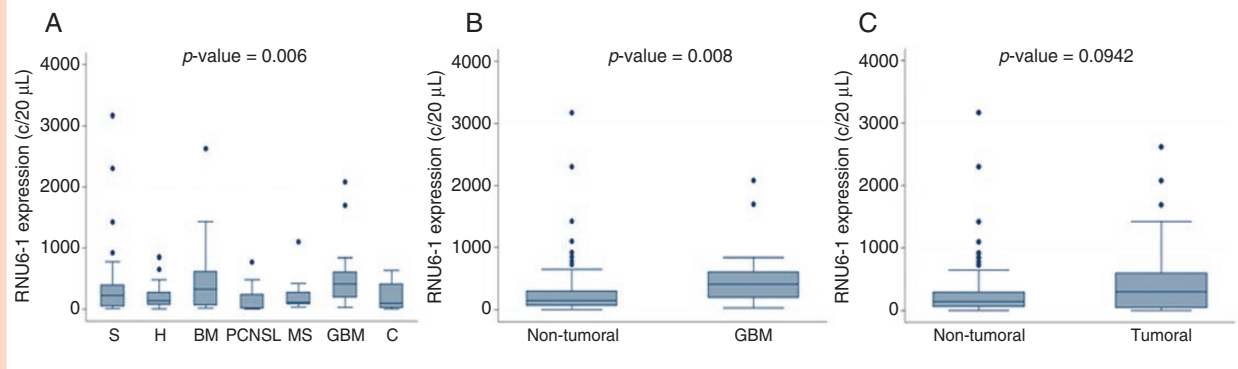


Figure 3 Box-plot figures of RNU6-1 expression in circulating exosomes from GBM patients, patients with other brain pathologies, and healthy controls (A), GBM patients and patients with nontumoral lesions (B), and patients with nontumoral lesions and tumoral lesions (C). S, stroke; H, hemorrhage; BM, brain metastasis; PCNSL, primary central nervous system lymphoma; MS, multiple sclerosis; GBM, glioblastoma; C, controls.

copies/20 μ L [20–409]; $P = .002$), thus corroborating the results of our previous study.⁹

Mean levels of RNU6-1 were also significantly higher in patients with GBM compared with patients with nonmalignant brain lesions altogether (143 copies/20 μ L [62–298]; $P = .008$) (Figure 3B). Differences retained statistical significance when comparisons were made individually between GBM patients and stroke patients (412 copies/20 μ L [189–611] vs 223 copies/20 μ L [44–390]; $P = .05$), brain hemorrhage patients (412 copies/20 μ L [189–611] vs 135 copies/20 μ L [67–277]; $P = .010$) and acute multiple sclerosis lesions' patients (412 copies/20 μ L [189–611] vs 111.5 copies/20 μ L [82–273]; $P = .019$).

Regarding non-glial tumor etiologies, the difference was also found to be statistically significant between patients with GBM and those with PCNSL (412 copies/20 μ L [189–611] vs 18.1 copies/20 μ L [6.4–241]; $P = .004$). On the contrary, differences did not reach significance between GBM patients and patients with brain metastases (412 copies/20 μ L [189–611] vs 325 copies/20 μ L [65–617]; $P = .573$).

No statistically significant differences were observed when etiologies were grouped altogether as tumoral versus nontumoral ($P = 0.0942$) (Figure 3C).

RNU6-1 as a Predictive Biomarker for the Diagnosis of GBM

Finally, ROC curve analyses were performed to further analyze the usefulness of RNU6-1 for distinguishing GBM patients from healthy controls and from patients with other radiologically similar brain lesions. In general, this analysis afforded an AUC of 0.700 [95% CI, 0.576–0.824, $P = .001$] for such differentiation (Figure 4A). More concretely, the expression levels of RNU6-1 in circulating exosomes were found to be a helpful biomarker for discriminating patients with GBM from healthy subjects (AUC 0.759 [95% CI, 0.621–0.897], $P < .001$), as well as from those with subacute stroke (AUC 0.695 [95% CI, 0.501–0.817] $P = .048$), acute or subacute hemorrhage (AUC 0.724 [95% CI, 0.563–0.884] $P = .006$), and acute multiple sclerosis plaques (AUC 0.728 [95% CI, 0.552–0.904] $P = .011$) (Figure 4B–E). Importantly, the discriminative role of this circulating biomarker was maintained when patients with all 3 nontumoral pathologies were grouped altogether (AUC 0.700 [95% CI, 0.558–0.841] $P = .006$) (Figure 4F). Concerning the neoplastic lesions, RNU6-1 was also able to differentiate GBM patients from those with PCNSL (AUC 0.814 [95%

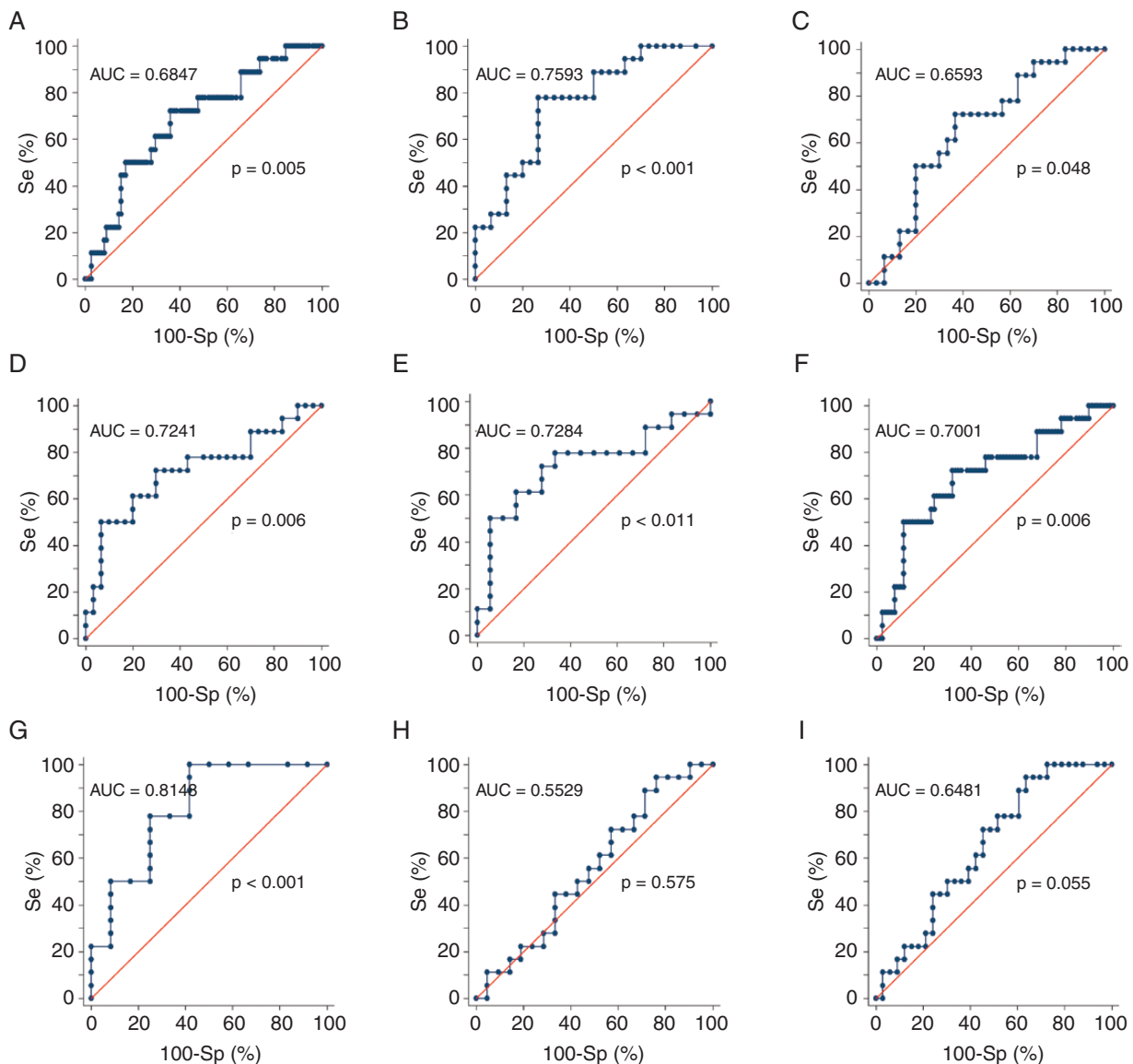


Figure 4 Receiver operator characteristic curves for the differentiation of GBM from other radiologically similar brain lesions and healthy controls (A), GBM from healthy subjects (B), GBM from subacute stroke (C), GBM from acute or subacute hemorrhage (D), GBM from acute multiple sclerosis plaques (E), GBM from nontumoral lesions grouped together (F), GBM from PCNSL (G), GBM from brain metastases (H), and GBM from other tumoral lesions grouped together (I). AUC, area under the curve; Se, sensitivity; Sp, specificity.

CI, 0.646–0.983] $P < .001$) but not from patients with brain metastases AUC 0.552 [95% CI, 0.368–0.737] $P = .575$) (Figures 4G–H). Indeed the ROC analysis did not yield a significant result for the differentiation of patients with GBM from those with non-GBM tumors overall (AUC 0.648 [95% CI, 0.496–0.799] $P = .055$; Figure 4I).

Discussion

The identification of a diagnostic marker for GBM in an accessible specimen such as blood would be helpful in the

clinical setting, especially for assisting in the differential diagnosis process in those patients in whom surgery is not feasible or in whom the histological diagnosis is inconclusive after a biopsy. In the current exploratory study, we investigated the diagnostic role of RNU6-1 isolated from circulating exosomes and its accuracy for distinguishing GBM from other brain lesions that might, in some cases, mimic GBM on neuroimaging.

In agreement with previous reports, we found a higher amount of circulating exosomes in patients with GBM compared with healthy subjects,^{11,20–22} reflecting the increased release of these vesicles from GBM tumor cells and their potential usefulness as reservoirs of biomarkers.

In this sense, we also confirmed our previous finding of increased expression of RNU6-1 in exosomes isolated from the serum of GBM patients in comparison to healthy controls.⁹

The expression of the sncRNA RNU6-1 in circulating exosomes was also significantly higher in patients with GBM than in patients with nonmalignant brain lesions, including subacute ischemic non-lacunar hemispheric strokes, subacute hemispheric intraparenchymal hemorrhages, and acute demyelinating multiple sclerosis lesions. Regarding the neoplastic brain lesions, RNU6-1 expression levels were also significantly higher in GBM patients than in patients with PCNSL. However, differences did not reach statistical significance and thus did not allow a clear-cut distinction between patients with GBM and patients with metastatic brain lesions.

These results might be relevant from a clinical perspective, particularly with regard to nontumoral lesions. Although clinical characteristics and advanced neuroimaging techniques are usually enough for diagnosing such disorders, there are some particular cases in which this issue still results challenging. This is especially true for patients with pseudotumoral multiple sclerosis²⁷ or patients with spontaneous intraparenchymal hemorrhage and no risk factors for bleeding. Indeed, in these latter patients, serial MRI exams are frequently performed to ensure, once the hematoma has resolved, that there is no underlying brain neoplasm.²⁸ Parenchymal enhancement is also a frequent radiological finding few days after stroke onset,²⁹ and consequently this consideration necessarily needs to be taken into account when new enhancement develops in the first weeks following GBM surgery.³⁰

RNU6-1 is located in the spliceosome of cells with 4 other sncRNA³¹ and is transcribed by RNA polymerase III. This sncRNA is involved in RNA processing and cell growth rate regulation,^{23–25} and its enhanced activity has been shown essential for tumorigenesis.^{32,33} The transcription of this sncRNA is negatively regulated by the phosphatase and tensin homolog (*PTEN*) tumor-suppressor gene via one of the transcription factors of the *BRF2* (BRF2 RNA polymerase III transcription initiation factor subunit) oncogene.³⁴ Therefore, it can be expected that tumors with significant *PTEN* pathway alterations, such as GBM,³⁵ might exhibit overexpression of the sncRNA RNU6-1. Accordingly, the different prevalence of these genetic pathway alterations in PCNSL and other metastatic systemic cancers might explain, at least in part, the results observed in this study. Indeed genomic alterations at this tumor-suppressor gene are usually rare in PCNSL, whereas they occur more frequently in other types of systemic tumors.³⁵

The heterogeneity of the group of patients with brain metastases and the limited number of patients included in the groups of patients with GBM, brain metastases, and specially PCNSL constitute a limitation of the current study and require further validation in more homogeneous and larger series. In addition, the lack of differences among GBM and brain metastases patients limits the usefulness of this biomarker, although in an appropriate clinical setting a negative whole-body computed tomography scan might favor the former diagnosis. Regarding patients with brain metastases, it remains unclear whether circulating exosomes are released by brain metastatic cells, by

systemic tumor cells, or by both types of cells. Because brain abscesses are frequently included in the radiological differential diagnosis of GBM, further studies incorporating a group of patients with this type of ring-enhancing lesions would be of interest. This issue seems particularly challenging because of the low prevalence of these infectious disorders and the common lack of further histological confirmation. In fact, during the study period, we only managed to include one patient with a single pyogenic brain abscess, in which the RNU6-1 expression in circulating exosomes was 76 copies/20 μ L.

In summary, in the current study, the expression of the sncRNA RNU6-1 was found to be significantly higher in GBM patients compared with healthy controls and also allowed for the differentiation of patients with GBM from those with PCNSL and other nontumoral lesions that might share some radiological features with GBM. These results suggest that RNU6-1 isolated from circulating exosomes could serve as a differential biomarker for GBM versus non-neoplastic brain lesions and PCNSL. However, further studies on independent and larger series are needed to confirm these findings and to explore the utility of RNU6-1 for monitoring response to treatment and its mechanistic role in the pathogenesis of GBM.

Supplementary Data

Supplementary data are available at *Neuro-Oncology Advances* online.

Keywords

biomarkers | exosomes | glioblastoma | miRNA | RNU6-1 | serum

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Conflict of interest statement. The authors have no conflict of interest to declare.

Authorship Statement. The conceptualization and methodology were carried out by M.M.A., J.G.P.-L., and M.P. Supervision was carried out by M.M.A. and J.G.P.-L. The investigation was carried out by M.P., M.G.H., M.G.M., and N.M.V. The selection of the samples and its collection were carried out by J.G.P.-L., I.E.V., J.B., B.Z., M.M., G.P., S.T.S., R.D.V., J.G.C., E.M.V., and A.P.G. Formal analysis was carried out by J.N.C., and writing of the original draft was handled by M.P., M.M.A., and J.G.P.-L. The draft was reviewed by all the authors and funding was acquired by M.M.A., J.G.P.-L., and M.P.

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