

Exosomes as a biomarker platform for detecting epidermal growth factor receptor–positive high-grade gliomas

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OBJECTIVE High-grade glial brain tumors are often characterized by an elevated expression of the tumorigenic epidermal growth factor receptor variant III (*EGFRvIII*). The authors sought to establish a clinically adaptive protocol as a noninvasive diagnostic tool for *EGFRvIII* detection through serum exosomes.

METHODS Purity of serum exosome/RNA was confirmed by electron microscopy and flow cytometry and through an RNA bioanalyzer profile. *EGFRvIII* amplification was initially established by semiquantitative polymerase chain reaction in tumor tissues and exosomes. Diagnostic performance of *EGFRvIII* transcript in tissue versus exosome was determined using a 2 × 2 clinical table approach. Overall survival was determined using Kaplan-Meier analysis.

RESULTS The *EGFRvIII* transcript was detected in 39.5% of tumor tissue samples and in 44.7% of their paired serum exosome samples; 28.1% of biopsy tumors coexpressed wild-type *EGFR* and *EGFRvIII*. Tissue *EGFRvIII* amplification served as the reference-positive control for its paired serum expression. The overall clinical sensitivity and specificity of semiquantitative exosome *EGFRvIII* polymerase chain reaction detection assay in serum were 81.58% (95% CI 65.67%–92.26%) and 79.31% (95% CI 66.65%–88.83%), respectively. Age, sex, tumor location, and side of the body on which the tumor was located had no effect on the detection rate of exosomal *EGFRvIII* transcript. *EGFRvIII* expression either in exosomes or tissue correlated with poor survival.

CONCLUSIONS The authors established a serum-based method for detection of *EGFRvIII* in high-grade brain tumors that might serve as an optimal noninvasive method for diagnosing *EGFRvIII*-positive high-grade gliomas.

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KEY WORDS exosomes; *EGFRvIII*; liquid biopsy; circulatory biomarkers; oncology

GLIOMAS account for approximately 80% of all malignant brain tumors, of which 54% are glioblastomas (GBMs).¹⁵ Primary GBMs develop rapidly and have a very short clinical history with a median survival of approximately 1 year, even after the standard of care treatment.^{9,27} Pathologically, GBMs are the most invasive and aggressive of the brain tumors, with marked nuclear atypia, mitosis, neovascular proliferation, and necrosis,

often occurring in focal areas and contributing to tumor heterogeneity and difficulties in pathological diagnosis.¹⁵

Although neuroimaging may be an important modality in the diagnosis of high-grade tumors, its usage is compromised, as GBMs share common radiological features with other primary tumors, abscesses, and metastases. Moreover, despite the recent advances in surgical care, there is a small but significant (5%–10%) morbidity associated with

ABBREVIATIONS *EGFR* = epidermal growth factor receptor; *EGFRvIII* = epidermal growth factor receptor variant III; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; GBM = glioblastoma; NPV = negative predictive value; OS = overall survival; PCR = polymerase chain reaction; PPV = positive predictive value; RT = reverse transcription; TCGA = The Cancer Genome Atlas; TEM = transmission electron microscopy.

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invasive tumor biopsy.³⁶ Therefore, there is a critical need for the development of blood-based biomarkers that can be used not only in diagnosis, but also for monitoring disease progression after surgery.

The Cancer Genome Atlas (TCGA) has divided GBMs into 4 distinct subgroups: proneural, classical, neural, and mesenchymal, based on genetic, epigenetic, and transcriptional variations (<https://cancergenome.nih.gov>).²⁸ Classical GBM is the most common TCGA subgroup and is characterized by the presence of elevated levels of expression of epidermal growth factor receptor (*EGFR*) and its variants.³⁵ In approximately 50%–60% of these classical tumors, point mutations and/or *EGFRvIII* (a result of the deletion of exons 2–7 of wild-type *EGFR*) are prevalent.³³ *EGFRvIII* is exclusively present in patients with malignant tumors and not in healthy individuals,⁵ making it an attractive target for diagnosis. *EGFRvIII* signaling has been proven to promote tumorigenesis and angiogenesis, and even confer resistance to radiation therapy and chemotherapy.²⁴ A tumor-specific origin of *EGFRvIII* is highlighted by its downregulated expression in patients after surgical tumor removal.³² Thus, detection of *EGFRvIII* is indicative of a high-grade glioma and can define GBM diagnosis even in the absence of histological criteria,¹² and hence it can play an important role in clinical diagnosis.³

Exosomes are nanovesicles ranging from 30 to 100 nm in diameter that are formed by plasma membrane invagination of late endosomes.⁶ Exosomes are present in all major body fluids.¹⁷ It has been shown that exosomes play important roles not only in cell signaling and signal transduction but also in transporting intracellular cargo, including protein, DNA, RNA, and other metabolites.³⁴ Exosomal components represent the tumor cellular transcriptome and can be used for molecular profiling in real time.²³ The use of exosomal cargo as a source of biomarkers may circumvent limitations and challenges associated with current histological diagnosis of gliomas.²¹ Recent studies have demonstrated the presence of *EGFRvIII* transcripts in cell culture supernatants of primary GBM cultures and plasma components of GBM patients,³² as well as platelet RNA,²⁶ that host GBM oncosomal RNA.

Presently, clinical validation of *EGFRvIII* detection through liquid biopsy methods is lacking. In this study we sought to establish a serum-based method of detecting exosome *EGFRvIII* that might help in the diagnosis of high-grade gliomas, as per recommended guidelines for tumor marker prognostic studies (REMARK),² taking into consideration the possible impact of sex, age, and anatomical location of the tumors.

Methods

Sample Collection and Assay Establishment

The Apollo Hospitals ethics committee granted approval for this prospective and blinded study. Written informed consent was obtained from all study participants. Tumor biopsies and paired serum samples from 96 adult patients with histologically confirmed high-grade glioma who had undergone craniotomy were obtained from Apollo Hospital, Hyderabad. Sera obtained from patients with other neurological diseases (meningioma, tuberculous abscess,

brain injury, and stroke) were used as controls. High-grade breast cancer samples were used as a positive source for detection of *EGFRvIII*.⁸ U87MG (a high-grade glioblastoma cell line; a kind gift from Dr. E. SreeKumar, Rajiv Gandhi Centre for Biotechnology, Kerala, India) was used as a positive control for wild-type *EGFR*. This cell line was procured initially from ATCC (lot no. 59681610; purchased on October 19, 2012); no additional authentication was performed on the cell line after obtaining it from ATCC. *EGFRvIII* 4.12 clone cell line, a positive cell line for *EGFRvIII*, was procured from Celther, Polska, and was used as a positive control for *EGFRvIII* assay establishment. No additional authentication was performed on the cell line after obtaining it from the company.

Blood samples were collected from 50 healthy volunteers in the Apollo Hospital community and served as negative controls to detect *EGFRvIII* expression. Biopsy tissue was flash frozen/collected in RNAlater (ThermoFisher Scientific). All tissue sample aliquots were stored in liquid nitrogen. All serum samples were stored as multiple aliquots at -80°C .

Study Design

Samples obtained from 96 high-grade glioma patients, 15 patients with other neurological diseases, and 50 age- and sex-matched healthy volunteers were used in this blinded study (Fig. 1). Tumor tissue and paired serum samples were collected from the operating room directly at the time of surgery. Tumor tissue was stored in RNAlater, and serum was stored at -80°C . After an initial optimization of the assay using U87 and U87-*EGFRvIII* cell lines for wild-type *EGFR* and *EGFRvIII*, the PCR assay was run on the 96 tumor and exosome RNA samples along with the healthy and neurological controls. All experiments were repeated at least 3 times.

The overall study design is shown in Fig. 1. Statistical analysis for estimating the diagnostic performance of the exosome *EGFRvIII* PCR assay was performed. Overall survival (OS) was calculated. The effects of covariates (e.g., age, sex, and anatomical tumor location and side) were also analyzed.

Exosomal Handling and Storage

Paired serum samples were stored as multiple aliquots at -80°C . Serum samples were thawed at 37°C in a water bath and centrifuged in 3 steps, 600g/10 min, 2000g/20 min, and finally at 10,000g/20 min, to acquire a cell/pellet-free clarified serum. Exosomes were precipitated from the clarified serum using the Total Exosome Isolation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The typical serum volume used for RNA isolation was around 2–3 ml per sample. Serum samples were incubated with the precipitating reagent at 4°C for 30 minutes and then centrifuged at room temperature at 10,000g for 10 minutes. Supernatant was discarded, and the exosome pellet was suspended in the exosome resuspension buffer and immediately used for assays. Protein quantification was performed using a conventional bicinchoninic acid assay (BCA) kit (ThermoFisher Scientific).

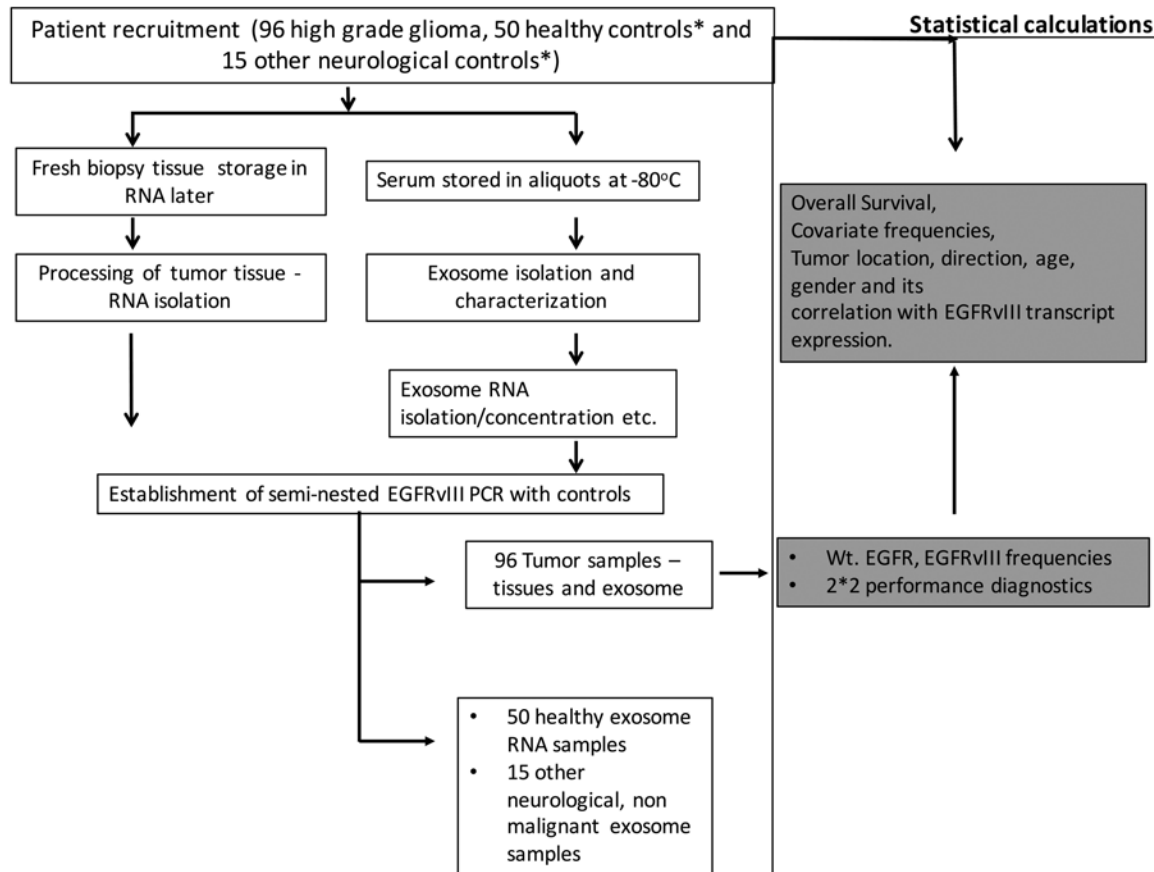


FIG. 1. Study design. Representative study design. Wt. = wild type. *Only serum samples.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed at the Center for Cellular and Molecular Biology, Hyderabad. Freshly isolated exosomes were dispensed on a 200-mesh copper grid (carbon coated), followed by glow discharge for 30 seconds using a Polaron carbon evaporator (Electron Microscopy Sciences). Five microliters of exosome samples was loaded on the glow-discharged grids and allowed to dry for 5 minutes. In addition, 5 μ l of uranyl acetate was loaded onto the grid after a 45-second incubation. Excess uranyl acetate was wicked off using filter paper and air dried for 30 minutes. Samples were loaded on the TEM single tilt Holder (Gatan), loaded onto a JEM-2100Plus Transmission Electron Microscope (JEOL), imaged, and analyzed at 120 kV.

Exosome Flow Cytometry

Direct quantification of exosomes through traditional flow cytometers is a challenge because of their small size (30–200 nm). Therefore, in this study the protocol was slightly modified to indirectly quantify exosomes through quantification of the most common exosome surface marker, CD81. Briefly, 20 μ g of exosome protein was conjugated with 25 μ l of 4- μ m latex beads (ThermoFisher Scientific) in 100 μ l MES buffer and incubated overnight on a rotary shaker. The following day, the binding reaction

was stopped by adding 100 mM glycine for 30 minutes. Exosome-conjugated beads were washed in 0.1% bovine serum albumin wash buffer and conjugated with CD81 (BioLegend) and its corresponding isotype control (Mouse IgG1 κ Isotype Control, BD Biosciences). Solutions were passed through a 0.2- μ m filter, and the fluorescence-activating cell sorter (FACS) instrument was cleaned twice to eliminate background noise. Exosome conjugated beads were acquired using BD FACSDiva (BD Biosciences) and analyzed with FlowJo (10.07, FlowJo LLC).

RNA Handling and Storage

Polymerase chain reaction (PCR) run conditions were designed to keep aerosol contamination to a minimum. RNase-free protocols were followed throughout all procedures, starting from PCR setup to PCR preparations. Dedicated, separate UV hoods were used for exosome and tissue RNA isolations. A dedicated laminar airflow cabinet was used for all PCR reaction preparation workups. All surfaces were thoroughly cleaned with 0.2% sodium dodecyl sulfate followed by 3% H_2O_2 . All pipettes and the hood floor were cleaned with RNase-zap (Sigma) to decrease contamination with external RNA. Furthermore, the laminar airflow cabinet is located in a Class 10,000 sterile facility. Double-gloving, regular changes, and clean laboratory coats were used. All reagents used in the PCR workup were RNA-free certified reagents.

RNA Isolation

Tissue was homogenized, and then RNA was isolated using an RNAeasy Easy Lipid Tissue Kit (Qiagen) according to the manufacturer's instructions. Any DNA contamination was removed using the DNA-free RNA Kit (Zymo research). RNA was isolated from exosomes by using Total Exosome RNA and a protein isolation kit (ThermoFisher Scientific) as per the manufacturer's instructions. Ten micrograms of glycogen (ThermoFisher Scientific) was added during the RNA precipitation step after overnight incubation. RNA was eluted in 10–15 μ l of nucleic acid-free water supplied along with the aforementioned kit followed by quantification with a RiboGreen (ThermoFisher Scientific) assay according to the manufacturer's instructions. The exosome RNA quality, yield, and size were analyzed using capillary electrophoresis (2100 Bioanalyzer, Agilent Technologies). One microliter of exosome RNA was used with the Agilent RNA 6000 Pico Kit (catalog no. 5067–1513) according to the manufacturer's protocol. Electropherograms were analyzed using the Agilent 2100 Expert B.02.07 software.

Reverse Transcription PCR

One to 2 μ g of RNA isolated from either tissue or exosomes was reverse-transcribed using a high-capacity complementary DNA Reverse Transcription Kit (Life Technologies), according to the manufacturer's instructions. Around 0.5 μ l of cDNA was used as a template in the PCR reaction containing 0.2 μ M of each primer. Forward and reverse primer sequences for *EGFR* and *EGFRvIII* (primer set 1) were 5'-CTTCGGGGAGCAGCGATGCGAC3' and 5'-ACCAATACCTATTCCGTTACAC3'.²² PCR was performed using a Master Cycler Pro S model (Eppendorf). From another set of primers (primer set 2), forward primer 5'-ATGCGACCCTCCGGGACGGC3' was used, retaining the reverse primer mentioned above. PCR cycling conditions were the same for both primer set combinations: an initial denaturation step at 95°C for 5 minutes, followed by 37 cycles of denaturation at 95°C for 30 seconds, annealing at 58.5°C for 30 seconds, and extension at 68°C for 80 seconds. PCR products were analyzed on a 1.5% agarose gel. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control (forward 5'-GAAGGTGAAGGTCTCGGAGTC3', reverse 5'-TCAGAAGATGGGATTTTC3').

Kaplan-Meier Survival Curves

Tumors were graded by the pathology team according to WHO guidelines.²⁰ OS (Grade III vs Grade IV) was calculated at the last follow-up or from the date of surgery to death. Death was the only defined event after surgery. Comparison of survival curves of patients with the *EGFRvIII* messenger RNA transcript in tissues and exosomes was done using the log-rank test (Mantel-Cox); $p < 0.05$ was considered statistically significant. The prognostic relevance with regard to OS of these parameters (e.g., grade, age, sex, *EGFRvIII* [tissue and exosome], and transcript presence or absence) was assessed. To compare the correlations between different groups, we performed a log-rank test (Mantel-Cox) of equality of survival dis-

TABLE 1. Clinicopathological characteristics of the 96 patients with high-grade gliomas

Characteristics	Grade III (n = 23)	Grade IV (n = 73)
Frequency	24.0%	76.0%
Median age in yrs*	44 (35–55)	53 (40–61)
Sex		
Male	19 (82.6%)	48 (65.8%)
Female	4 (17.4%)	25 (34.2%)

* Presented with the IQR.

tributions for the different tumor grades and the effect on OS of age, sex, and the presence or absence of *EGFRvIII* in tissues and exosomes.

Statistical Analysis

Statistical analysis was performed using SPSS (version 16.0, SPSS Inc.) and GraphPad (Prism). All continuous variables were assessed for normality using the Shapiro-Wilk's test. All values are expressed as mean $\pm$ SD if they followed a gaussian distribution. All categorical variables are expressed as percentages. Comparisons of normally distributed continuous variables were performed using an independent sample t-test. Comparisons of categorical variables were performed using the chi-square test or Fisher's exact test based on the number of observations. Sensitivity, specificity, positive predictive values (PPVs), and negative predictive values (NPVs) with 95% confidence intervals were computed. Kaplan-Meier survival curves were plotted, and differences in survival between various groups were compared using the log-rank test. To measure the level of agreement, the kappa value was computed; $p < 0.05$ was considered statistically significant.

Results

Study Population

A total of 96 patients with high-grade glioma, 50 age- and sex-matched healthy controls, and 15 controls with neurological diseases (nonglioma) were recruited from Apollo Hospitals, Hyderabad, India. Table 1 summarizes the clinicopathological features of tumor patients used in the study. Healthy controls were sex and age matched to the tumor patients. All samples were anonymized for this study. All tissue specimens acquired at initial diagnosis and resection were classified morphologically and graded according to WHO criteria by the histopathologists. Investigators establishing the assay were blinded to the tumor grade and the patient's outcome. The patients ranged in age from 20 to 75 years old at the time of primary surgery (median age 53 years for patients with Grade IV tumors and 44 years for those with Grade III tumors); 24% of patients harbored a Grade III glioma, and 76% harbored a Grade IV glioma.

Characterization of Microvesicles Isolated From Serum From High-Grade Glioma Patients

To detect *EGFRvIII* in serum exosomes of high-grade

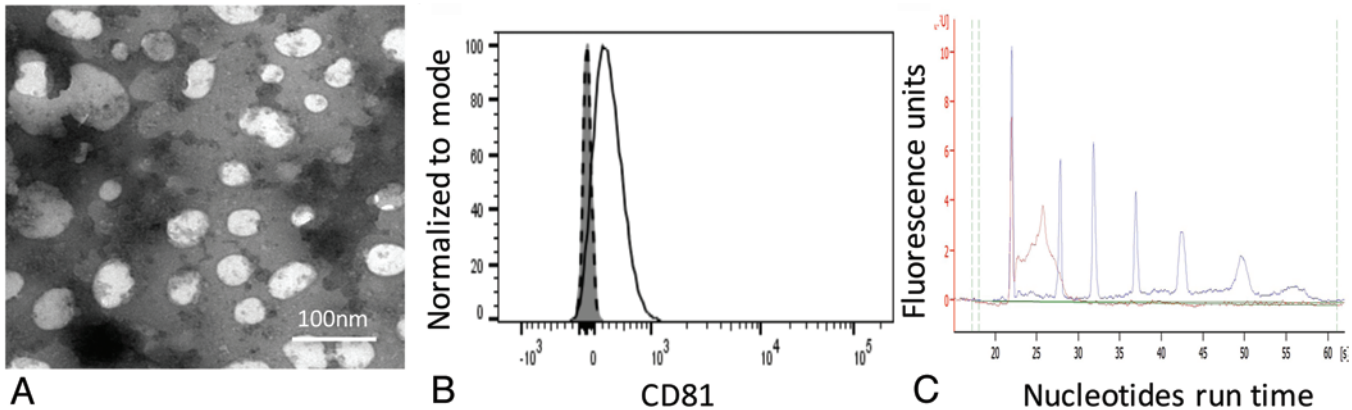


FIG. 2. Characterization of exosomes/exosomal RNA from serum exosomes. **A:** Representative TEM image of an exosome obtained from a high-grade glioma patient and suspended in 1% glutaraldehyde ($n = 6$). **B:** Representative flow cytometry overlay of CD81 fluorescence (solid line) on exosomes overlaid with isotype control (dashed line) and solid bead fluorescence (solid peak) ($n = 20$). **C:** Representative overlay of total exosomal RNA isolated from serum obtained in a high-grade glioma patient. An exosomal RNA electrophoretogram is overlaid with RNA standard for size estimation ($n = 15$). Figure is available in color online only.

glioma patients, we first isolated and characterized exosomes. Exosomes were isolated using commercial kits. The morphology of exosomes was confirmed by TEM (Fig. 2A). Double-membrane, round, intact structures in the range of 50–200 nm were observed. Furthermore, we performed flow cytometry to quantify the expression of the pan-exosome-specific marker CD81. Figure 2B clearly shows the expression of CD81 when compared with bead autofluorescence and isotype control staining. After morphological and biophysical/biochemical characterization of exosomes, we isolated RNA from these exosomes under RNase-free conditions using commercially available kits. Exosomal RNA was in the size range of 20–250 nucleotides (Fig. 2C). On the basis of these results, we confirmed the isolation and purity of exosome/exosomal RNA as per the parameters described in the published literature about the nature of exosomes and their RNA.

Establishment of *EGFRvIII* and Wild-Type *EGFR* Semiquantitative Reverse Transcription PCR

Given that exosomes are very small and the amount of isolated RNA was very low, we used semi-nested PCR (2–4 μ l of the first-step PCR product is used as a template for the second-step PCR amplification) to amplify the *EGFR* variant.

EGFR primers (spanning the 5' untranslated region and the beginning of exon 1 and within exon 9) were used to amplify both the wild-type *EGFR* and *EGFRvIII* variants in a semiquantitative reverse transcription (RT)–PCR. With the use of primer set 1, these primers generated a 1044-bp PCR product for the wild-type *EGFR*, compared with a 243-bp PCR product for its splice variant *EGFRvIII* (a difference of 801 bp) (Fig. 3). For primer set 2, wild-type *EGFR* yielded a band at 1029 bp, while *EGFRvIII* yielded a band at 228 bp (Supplementary Figure 2). RNA from the U87 cell line was used as a positive control for wild-type *EGFR* expression. U87-MG and U87MG-*EGFRvIII* cell lines and breast cancer samples were used as positive controls in establishment of the PCR assay.

RNA from primary breast cancer patients was used as a positive control for *EGFRvIII* expression. The amplified products were further sequenced to confirm the specific expression of wild-type *EGFR* and *EGFRvIII*.

Assessment of Wild-Type *EGFR* and *EGFRvIII* in Biopsy Tissue and Paired Exosomes

Once we optimized exosome isolation and PCR amplification of wild-type *EGFR* and *EGFRvIII*, we proceeded to test the expression of *EGFRvIII* in exosomes isolated from patients with high-grade gliomas. Analyses were performed in 96 patient samples. Exosomal expression of the *EGFRvIII* transcript was found in 43 of 96 (44.79%) tumor patients, whereas it was expressed in 38 of 96 (39.6%) tumor tissue samples. Among the tumor samples, 72 of 96 (75.0%) were positive for wild-type *EGFR* expression. In 27 of 96 (28.12%) patients with tumors, the tumor tissue samples coexpressed wild-type *EGFR* and *EGFRvIII*. The wild-type *EGFR* transcript was not detected in any of our exosome fractions, which may be due to the nature of small RNA present in the exosomes.³¹ Sex, age, and side of the body where the tumor was located (right or left) did not significantly affect detection of exosome *EGFRvIII* expression. Frequency and tissue expression of wild-type *EGFR* and *EGFRvIII* for Grade III and IV gliomas are depicted in Table 2. Representation of *EGFRvIII* expression in both tissue and exosome fractions are represented in Fig. 3. GAPDH was used as an internal control. All experiments were repeated 3 times for confirmation.

Diagnostic Evaluation Test

The performance of the exosome *EGFRvIII* assay was statistically determined through clinical sensitivity, specificity, PPV, NPV, and *EGFRvIII* frequency. For diagnostic evaluation, we tested 96 tumor samples, 50 serum samples from healthy controls, and 15 serum samples from patients with nonmalignant neurological disease. Only the tumor and exosome *EGFRvIII* transcript data were obtained for statistical calculations. We used a clinical calculator

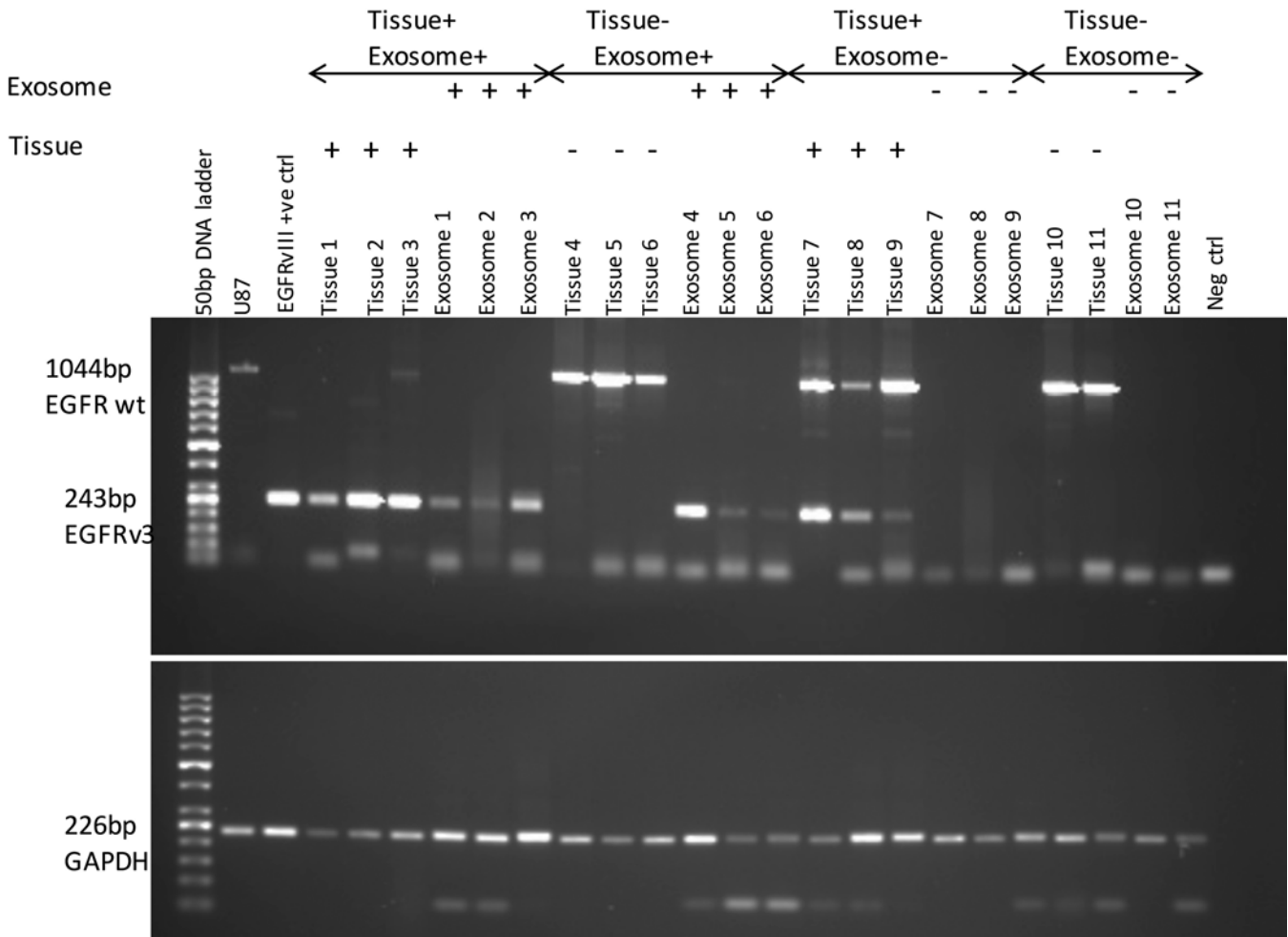


FIG. 3. *EGFR/EGFRvIII* expression in tissue and exosomal fractions. RT-PCR was used to detect *EGFRvIII* messenger RNA in the tumor samples, and semi-nested RT-PCR was used to detect *EGFRvIII* from its paired serum exosome RNA fraction. The wild-type *EGFR* PCR product appears as a band at 1044 bp and its splice variant, *EGFRvIII*, at 243 bp. Lane 1 represents a 50-bp ladder, Lane 2 represents positive control for wild-type *EGFR* (U87 cell line), and Lane 3 represents positive control for *EGFRvIII* (high-grade glioma), confirmed by sequencing. The first group (Samples 1–3) represents positive *EGFRvIII* expression, both in tissues and exosomes. The second group (Samples 4–6) includes negative *EGFRvIII* expression in tissues and positive in exosomes. The third group (Samples 7–9) includes positive *EGFRvIII* expression in tissues and negative *EGFRvIII* expression in exosomes. The fourth group (Samples 10 and 11) includes double negative for *EGFRvIII* expression in tissues and exosomes. RT-PCR of GAPDH messenger RNA was included as a loading control (226 bp). Figure is representative of 96 glioma samples, repeated 3 times each. ctrl = control; wt = wild type; + = present; – = absent.

TABLE 2. Wild-type *EGFR* and *EGFRvIII* expression status in tumor patients

Characteristic	No. of Patients (%)	
	Grade III (n = 23)	Grade IV (n = 73)
Exosome <i>EGFRvIII</i>	9 (39.1)	34 (46.6)
Tissue biopsy <i>EGFRvIII</i>	9 (39.1)	29 (39.7)
Tissue wild-type <i>EGFR</i>	21 (91.3)	51 (69.9)
Tissue biopsies coexpressing wild-type <i>EGFR</i> & <i>EGFRvIII</i>	8 (34.8)	19 (26.03)

based on a 2×2 -table format for testing (https://www.medcalc.org/calc/diagnostic_test.php). All cases of detectable *EGFRvIII* messenger RNA in both exosome fraction and tissue biopsies were considered to be true positives; all cases of undetectable *EGFRvIII* in tissues and all cases of detectable *EGFRvIII* in exosomes were considered to be false positives; all cases of detectable *EGFRvIII* in tissues and all cases of undetectable *EGFRvIII* in exosomes were considered to be false negatives; all cases of undetectable *EGFRvIII* both in exosomes and tissue biopsy were considered to be true negatives (Table 3). The performance of the assay is shown in Table 4.

The sensitivity and specificity of the data (96 high-grade glioma samples) were 81.58% (95% CI 65.67%–92.96%) and 79.31% (95% CI 66.65%–88.83%), respectively, while

TABLE 3. The 2 × 2 diagnostic matrix for defining the 4 diagnostic groups

Exosome Transcript	Tissue Biopsy Transcript	
	<i>EGFRvIII</i> (+)	<i>EGFRvIII</i> (–)
<i>EGFRvIII</i> (+)	True positive (A) (n = 31)	False positive (C) (n = 12)
<i>EGFRvIII</i> (–)	False negative (B) (n = 7)	True negative (D) (n = 46)

+ = present; – = absent.

Letters A–D are used in Table 4.

the PPV and NPV were 72.09% (95% CI 56.33%–84.67%) and 86.79% (95% CI 76.87%–92.86%), respectively. *EGFRvIII* prevalence (Table 4) in the data set was 39.58% (95% CI 29.75%–50.08%, $p = 0.0001$).

Accuracy of the test was calculated using the following formula: (true positive + true negative) divided by (sum of all *EGFRvIII* positive + negative cases). Accuracy of *EGFRvIII* detection was $80\% \pm 0.2\%$. A statistically significant ($p < 0.0001$) association between exosome and tissue biopsy *EGFRvIII* expression was observed on chi-square tests. In addition, there was a moderate agreement ($\kappa = 0.548$) between exosome and tissue biopsy *EGFRvIII* expression ($p < 0.0001$). The confidence intervals represented were “exact” Clopper-Pearson confidence intervals. In 12 patients *EGFRvIII* and GBM were detected in the serum exosome fraction and were undetectable in the tissue fraction. In addition, in 7 of the patients, *EGFRvIII* was undetectable in the serum exosome fraction even though it was detected in the tissue biopsy samples.

Impact of *EGFRvIII* on OS

No statistically significant difference in OS was observed between the Grade III and IV glioma patient groups (Fig. 4A). However, a clear trend toward worse prognosis was evident in the Grade IV glioma cohort. The mean survival durations for patients with Grade IV and Grade III tumors were 22.5 and 26.5 months, respectively ($p = 0.200$, log-rank test).

In line with the previous findings, we observed that the patients with *EGFRvIII* present in the tumors had a worse prognosis.¹³ The mean OS durations for patients with and without exosome *EGFRvIII* transcript expression were 21.1 and 28.6 months, respectively ($p = 0.005$; Fig. 4B). The mean OS durations for patients with and without tumor tissue *EGFRvIII* expression were 22.1 and 26.20 months, respectively ($p = 0.032$ [Fig. 4C]). The expression of *EGFRvIII* correlated with OS, suggesting that detection of *EGFRvIII* expression in either of these compartments would be sufficient for accurate diagnosis of GBMs (Fig. 4D). This effect was more pronounced when analysis was made through exosomes rather than tissues because of the increased detection rate. A multivariate survival model (Table 5) for OS was established that included pathology (Grades III and IV), age, sex, and *EGFRvIII* expression (tissue and exosome).

Discussion

It is well known that high-grade glioma cells use tumor exosomes as vehicles to transfer genetic information into blood and CSF.¹⁸ The internal cargo of the exosome consists of RNA, DNA, proteins, and other metabolites, which makes exosomes prime targets for interrogation in clinical diagnostics and therapeutics.²⁹ *EGFRvIII* expression is unique to certain cancer types and serves as a good marker for their prognosis, diagnosis, and therapy monitoring and as a form of targeted therapy.¹⁹

Most of the protocols used for characterizing exosomes are research based and are not standardized for clinical adaptation.³⁷ We established a robust method incorporating 2 different primer sets to detect exosome *EGFRvIII* through serum exosomes (Fig. 3 and Supplementary Figure 1). We used a commercially available exosome-precipitating reagent to precipitate serum exosomes and ascertained their purity. Typically, the RNA we found in our exosome preparations was very small (25–200 nucleotides). Incidentally, we could only detect *EGFRvIII* and not the wild-type *EGFR*, which is in line

TABLE 4. Diagnostic performance of the exosome *EGFRvIII* assay

Diagnostic Parameter	Formula*	Performance Characteristic (95% CI)†
Sensitivity‡	A/A + B	81.58% (65.67–92.26%)
Specificity‡	D/C + D	79.31% (66.65–88.83%)
Positive likelihood ratio	Sensitivity/100 – specificity	3.94 (2.33–6.67)
Negative likelihood ratio	100 – sensitivity/specificity	0.23 (0.12–0.46)
<i>EGFRvIII</i> prevalence	(A + B)/(A + B + C + D)	39.58% (29.75–50.08%)
PPV‡	A/A + C	72.09% (56.33–84.67%)
NPV‡	D/B + D	86.79% (76.87–92.86%)

Sensitivity: probability that a test result will be positive when the *EGFRvIII* expression is present in both tissues and exosomes; specificity: probability that a test result will be negative when the expression is not detectable in both tissues and exosomes; positive likelihood ratio: how much more likely an individual with *EGFRvIII* expression is to have a positive test result than a person without it; negative likelihood ratio: how much more likely an individual without *EGFRvIII* is to have a negative test result, compared to a person with *EGFRvIII* expression; PPV: probability that *EGFRvIII* is detectable when present; NPV: probability that *EGFRvIII* is not detectable when absent.

* See Table 3 for definitions of Letters A–D.

† Confidence intervals are “exact” (Clopper-Pearson) confidence intervals.

‡ Sensitivity, specificity, PPV, and NPV are expressed as percentages for ease of interpretation.

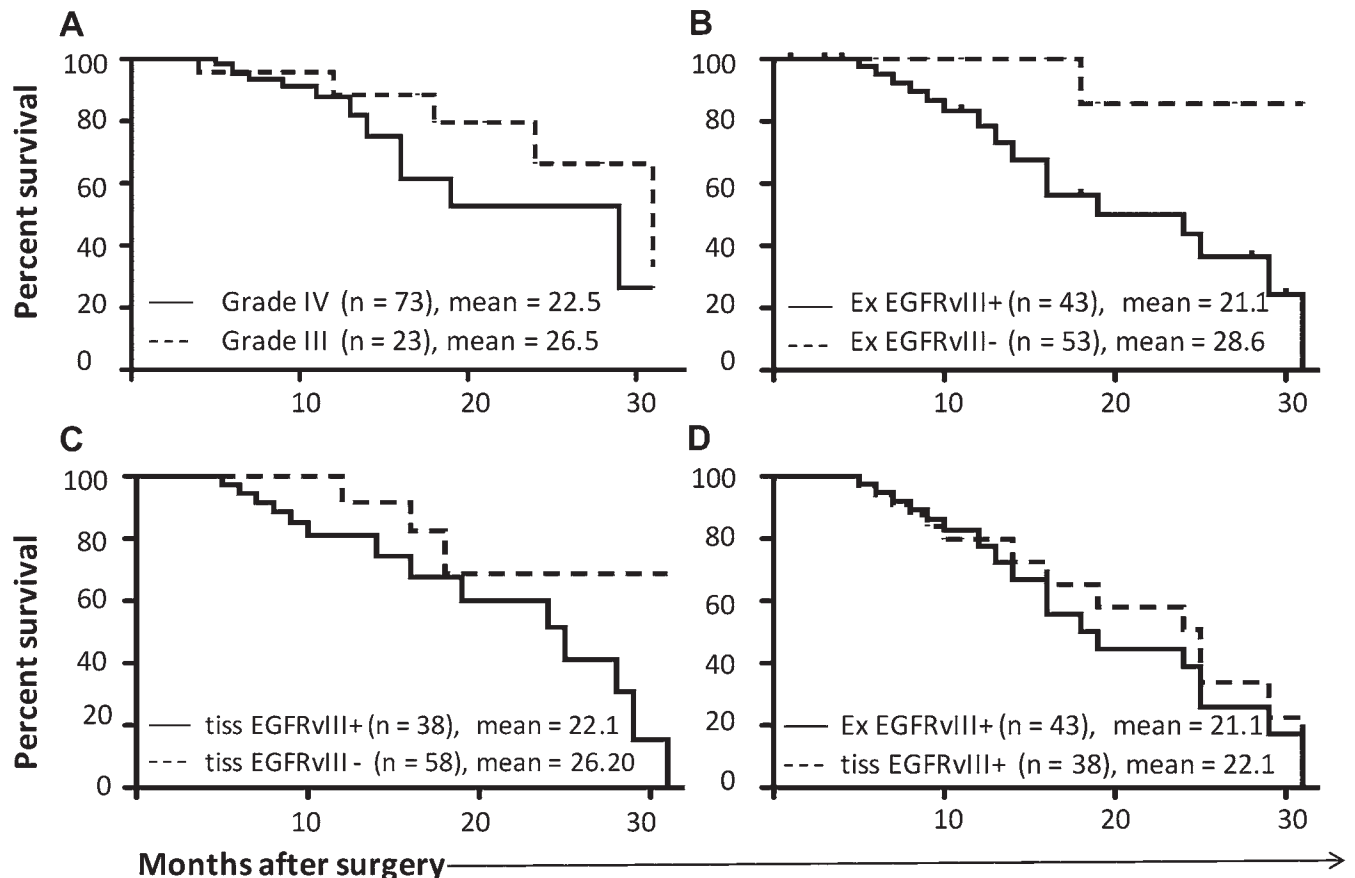


FIG. 4. Kaplan-Meier estimates of the OS of tumor patients in various *EGFRvIII* cohorts. **A:** OS of patients with Grade IV ($n = 73$) and Grade III ($n = 23$) tumors. Mean OS times were 22.5 and 26.5 months, respectively ($p = 0.200$). **B:** OS of tumor patients with exosomal *EGFRvIII* transcript ($n = 43$) and patients devoid of exosomal *EGFRvIII* expression ($n = 53$). Mean OS times for the exosomal *EGFRvIII*-positive versus exosomal *EGFRvIII*-negative patient groups were 21.1 and 28.6 months, respectively ($p = 0.005$). **C:** OS of tumor patients with *EGFRvIII* transcript in tissues ($n = 38$) and patients devoid of tissue *EGFRvIII* ($n = 58$). Mean OS times for the tissue *EGFRvIII*-positive versus tissue *EGFRvIII*-negative patient groups were 22.1 and 26.2 months, respectively ($p = 0.032$). **D:** OS of tumor patients with *EGFRvIII* transcript in tissues ($n = 38$) and patients with exosomal *EGFRvIII* transcript ($n = 43$). Mean OS durations for the exosomal *EGFRvIII* and tissue *EGFRvIII* patient groups were 21.1 and 22.1 months, respectively ($p = 0.2556$). Ex = exosomal; tiss = tissue.

with previous findings that wild-type *EGFR* is undetectable in exosomes probably due to the larger size of the transcript.³¹ Accuracy of *EGFRvIII* detection through exosomes was 80% for tissue *EGFRvIII* expression with an overall sensitivity and specificity of 81.58% and 79.31%, respectively.

In this study, the tumor tissue *EGFRvIII* expression frequency was 39.58%. These results are in agreement with earlier studies that have reported *EGFRvIII* tissue expression in about 24%–67% of GBMs.¹³ Additionally, the PPV and NPV were 72.09% and 86.79%, respectively. PPV and NPV are considered good indicators of the accuracy of diagnostic procedures. The present study builds on previous findings¹¹ with significant modifications in the PCR analytical methodology, associated with exosome RNA processing/handling for biomarker analysis. Glycogen addition during exosome RNA precipitation, exosomal RNA concentration, exosomal DNA contamination removal, and introduction of a semi-nested PCR to amplify exosome *EGFRvIII* expression during analysis were the

major changes that were incorporated into the *EGFRvIII* detection protocol. In addition, extensive care was taken to decrease aerosol contamination by working in different UV hoods throughout the entire procedure and working with multiple serum aliquots. To overcome the problem of complementary DNA degradation in exosome preparations, we made multiple aliquots of complementary DNA to avoid repeated freeze-thaw procedures.

In total, we observed 12 cases of false positives, for which *EGFRvIII* was not detected in tissues but could be detected in the exosomes. Given that high-grade glial tumors exhibit considerable inter- and intratumoral heterogeneity,²⁵ it is possible that the representative sample from which tissue RNA was isolated may not reflect the true molecular pathophysiology of the patient. Furthermore, some of the tumor tissue fractions from which we had extracted the RNA had considerable amounts of necrosis, which also might impact the detection ability of *EGFRvIII* in tissues. Thus, blood-based detection of *EGFRvIII* from the exosomal fraction could potentially mitigate the di-

TABLE 5. Multivariate survival model for OS

Parameter	No. of Patients	No. of Events	No. Censored (%)	Mean OS (mos)*	p Value†
All patients	96				
Grade					0.200
III	23	5	18 (78.3)	26.538	
IV	73	11	62 (84.9)	22.532	
Tissue <i>EGFRvIII</i>					0.032
Present	38	12	26 (68.4)	22.09	
Absent	58	4	54 (93.1)	26.20	
Exosome <i>EGFRvIII</i>					0.005
Present	43	14	29 (67.4)	21.13	
Absent	53	2	51 (96.2)	28.57	
Age (yrs)					0.317
<50	48	7	41 (85.4)	25.5	
≥50	48	9	39 (81.2)	21.1	
Sex					0.883
Male	67	10	57 (85.1)	23.53	
Female	29	6	23 (79.3)	24.94	

* Estimation is limited to the longest survival time if censored.

† Log-rank test of equality of survival distributions.

agnostic hindrances associated with tumor heterogeneity. Seven cases of false negatives were observed, wherein *EGFRvIII* was not detected in the exosomes, but was detected in tissue biopsies. Although exosomes were discovered a while ago, the recognition of their clinical potential as biomarker shuttles is very recent.¹⁶ Exosomes, being very small, contain a minute amount of RNA and therefore pose significant challenges in detection of their specific markers. Furthermore, the limited availability of standardized protocols for exosome isolation and subsequent RNA isolation is also a matter of concern.³⁷ It has been recently reported that diurnal variations influence exosome secretion into the peripheral circulation, and factors such as diet, exercise, and circadian variations do have an effect on their detectability.⁷

It is known that the sensitivity of the detection of exosomes or any circulatory biomarker may depend on various tumor intrinsic and extrinsic factors, such as tumor size, location, serum volume, RNA extraction method, and complementary DNA conversion procedures. However, in our study, age, sex, and tumor location did not show any impact on exosome *EGFRvIII* detection (Supplementary Figure 3).

The expression of *EGFRvIII* transcript in exosomes mirrored the OS pattern. Moreover, though not significant, the OS pattern correlated with the aggressive nature of Grade IV tumors over Grade III tumors (Fig. 4A). OS curves indicated worse prognosis in patients who harbored *EGFRvIII* (either in exosomes or tissue fractions) than in those patients who did not. Furthermore, OS curves of patients with *EGFRvIII* in their tissue biopsies and those with exosomal *EGFRvIII* mirrored each other, suggesting

that either tissue- or serum-derived exosomes can be reliably employed for diagnosis (Fig 4D). Age and sex did not have significant effects on OS in our cohort, which is consistent with previous findings that these factors are not predictors of OS in patients harboring high-grade tumors.¹⁴

This study could provide a platform for blood-based diagnosis of high-grade glial tumors by detection of *EGFRvIII* alone or as a part of a host of biomarkers. This protocol can also be used in patient stratification for *EGFRvIII*-targeted therapies like mAB806, rindopepimut, and other small peptides targeting *EGFRvIII*. It is known that *EGFRvIII* mutation is associated with the “classical” GBM subtype³⁵ and is an active target for various immuno-oncology drugs and immunotherapeutic agents.¹¹ As the downstream signaling components of *EGFRvIII* are different from the wild-type *EGFR*, *EGFRvIII* detection may also be used for predicting the therapeutic response to targeted therapies to *EGFR*.¹⁰

Taken together, our results indicate that exosome-based biomarker diagnosis is a promising tool to circumvent tissue biopsy in patients who carry a very high surgical risk. Although these results are promising and substantiate the utility of exosomes as a source of biomarkers for detection of brain tumors, we recognize that this study does have some limitations. The sample size requires verification in a larger cohort of patients, with longitudinal controls to confirm the clinical utility of this assay technology. Furthermore, to increase the sensitivity of the assay, purification of glioma-specific exosomes may be a viable option, as demonstrated in Alzheimer’s disease biomarkers through plasma neuronal exosomes.¹ Our analysis procedure needs to be extended to incorporate digital PCR methodology, which would further increase the sensitivity of detection, as has been shown in the case of detection of *IDH1* through CSF exosomes.⁴ In addition, a molecular signature/panel of biomarkers would add more value in establishing liquid biopsy than a single biomarker (*EGFRvIII*) and aid in the identification of TCGA subgroups.³⁰ Thus, detection of other circulatory biomarkers (e.g., cell-free DNA, circulatory tumor cells, and microRNA) can complement medical imaging and add more specificity to the overall detection capability.

Our inclusion of a control group of patients with other CNS complications (e.g., abscesses, stroke, meningitis, and brain injury) that may mimic GBM on neuroimaging strongly substantiates the relevance of our study. Furthermore, we tested 50 healthy volunteers who did not have *EGFRvIII* expression (Supplementary Figure 2), which is in line with previous studies.¹⁰

Conclusions

Using exosome-based liquid biopsy to measure *EGFRvIII* expression for diagnosis of high-grade gliomas is highly promising and may help differentiate high-grade gliomas from infective and demyelinating diseases that may have a similar radiological picture. Exosomal biomarker evaluation may provide an effective means of deciphering a tumor’s genetic profile and progression. Establishment of the clinical utility of this technology and

validation of its diagnostic accuracy may lead to a redefinition of the standard of care in glioma patients.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Ray, Manda, Ranjan. Acquisition of data: Kataria, Tatireddy. Analysis and interpretation of data: Ray, Manda, Kataria, Ratnam, Lath. Drafting the article: Ray, Manda. Critically revising the article: Ray, Manda. Approved the final version of the manuscript on behalf of all authors: Ray. Statistical analysis: Ramakrishnan. Administrative/technical/material support: Kataria, Tatireddy, Ratnam, Lath. Study supervision: Manda.

Supplemental Information

Online-Only Content

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