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Molecular analysis of oncogene expressions in different grades of gliomas

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ABSTRACT

The aggressiveness of brain tumors is attributed to the expression of multiple oncogenes involved in proliferation, metabolism and therapeutic resistance whose potential correlation with tumor progression has not been well-studied. In this study, we aimed to investigate the relationship of oncotargets involved in pathogenesis with respect to glioma grades. Gliomas ($n=40$) were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and sequencing for the detection of epidermal growth factor receptor (*EGFR*) mutants. Expressions levels of *EGFR*, *EGFR variant III (EGFRvIII)*, Lck/Yes novel tyrosine kinase (*Lyn*), Spleen tyrosine kinase (*SYK*), insulin receptor substrate 1 (*IRS1*), phosphatidylinositol 3-kinase (*PI3K*), Src homology 2 domain-containing inositol 5-phosphatase 1 (*SHIP1*) and glucose transporter 3 (*GLUT3*) were studied using real-time PCR and compared against glioma grades via statistical methods. Protein expressions were analyzed using immunohistochemistry and western blotting. *EGFRvIII* was detected in 53% and exon 4 deletion (*de4 EGFR*) in 20% of gliomas. Importantly, the expressions levels of candidate oncogenes were significantly upregulated ($P<0.05$) and positively correlated with the glioma grades. Hence, these oncotargets require high surveillance during tumor progression and further investigations on larger patient cohorts can affirm their role as potential markers in the pathology of glioma, thereby aiding in the development of patient-specific multi-targeted therapy.

Keywords: Glioblastoma; EGFRvIII; exon 4 deletion variant; oncogenes, metabolism

1. Introduction

Glioblastomas are the most common, highly invasive and neurologically destructive tumors with the worst prognosis [1,2]. The standard of care consists of maximum safe surgical resection and radio-/chemotherapy, but these tumors are highly resistant to therapy owing to their diffuse infiltrative nature [3]. In order to develop efficacious treatments, the molecular pathology of glioma involving genetic abnormalities and aberrant signaling mechanisms activated by oncogenes involved in metabolism, proliferation and therapeutic resistance have to be investigated [4,5].

Epidermal growth factor receptor variant III (*EGFRvIII*), a genomic variant of *EGFR*, which is absent in normal tissues, is a marker signature often characterized in glioblastoma [6]. *EGFRvIII* exhibits constitutive signaling property as a result

of an in-frame deletion of 801 base pairs (bp) or exons 2-7 loss from the N-terminal extracellular domain of *EGFR* [7]. But the role of *EGFRvIII* in gliomagenesis and the precise molecular mechanism by which it acquires constitutive activity remains complex and unresolved [8]. Previous reports have shown that *EGFRvIII* expression is rapidly lost in primary cell cultures; furthermore, glioblastoma cell lines provide a limited understanding of *EGFRvIII*-driven signaling networks operating in tumors [9]. Nevertheless, some independent studies have shown direct or indirect involvement of *EGFRvIII* with specific downstream signaling molecules involved in metabolism, proliferation and resistance [10-19].

Based on the following collective evidences, molecular targets such as *EGFR*, *EGFRvIII* and their associated signaling partners, *Lyn*, *SYK*, *IRS1*, *PI3K*, *SHIP1* and *GLUT3* involved

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in metabolism, proliferation and therapeutic resistance associated with glioma progression were chosen for our analysis. EGFRvIII has been found to be associated with non-receptor tyrosine kinases like Lck/Yes novel tyrosine kinase (Lyn) involved in enhancing proliferation and migration in head and neck cancer [10]. Lyn is indeed an essential factor for cancer cells that rely on EGFR signaling [11]. It was also shown to activate insulin-independent glucose transport in adipocytes [12]. It phosphorylates the downstream effector, spleen tyrosine kinase (Syk) leading to the activation of receptor-associated adaptor proteins like insulin receptor substrate 1 (IRS1) and lipid kinases like phosphatidylinositol 3-kinases (PI3Ks) which are involved in metabolism and proliferation [13-15]. Activated PI3Ks have been shown to be involved in the translocation of glucose transporters from the cytosol to the plasma membrane [16]. The src homology 2 domain-containing inositol 5-phosphatase 1 (SHIP1) was shown to negatively regulate phosphoinositide 3-kinase effectors, thereby acting as a tumor suppressor [17]. EGFRvIII was also demonstrated to promote constitutive PI3K signaling enhancing glioblastoma cell proliferation [18]. Interestingly, the role of EGFRvIII has also been implicated in metabolic fueling in glioma wherein EGFRvIII mediated upregulation of heterogeneous nuclear ribonucleoprotein (*hnRNP*) A1 splicing factor and delta Max led to the increased expression of glycolytic genes like glucose transporter 3 (GLUT3) [19].

Hence, as an initial step, the glioma cases selected for the analysis were screened for *EGFR* mutants. The incidence of *EGFRvIII* in glial tumors has been reported in previous studies [20,21], but the detection of a novel *EGFR* variant, exon 4 deletion (*de4 EGFR*) mutation possessing enhanced proliferation and invasiveness has been reported only once in glioblastoma [22]. Therefore, the present study was undertaken to determine the prevalence of *EGFRvIII* and *de4 EGFR* mutations in the patient cohort and to analyze the expression levels of *EGFR*, *EGFRvIII*, *LYN*, *SYK*, *IRS1*, *PI3K*, *SHIP1* and *GLUT3* with respect to glioma grades. In this pilot study, correlation analysis was performed to determine the significance of *EGFRvIII* expression with that of these selected molecular targets and evaluated the expression levels against the clinical grades of glioma.

2. Material and Methods

2.1 Tumor Samples

Tumor tissues were collected from glioma patients who underwent surgery at the Amrita Institute of Medical Sciences, India from 2009 to 2013. The study protocol and consent were approved by the Institutional Ethics Committee and were performed in accordance with the ethical standards laid down in the Declaration of Helsinki. All tumors were histologically confirmed by pathologists and graded according to the World Health Organization (WHO) classification of tumors of the central nervous system [23].

2.2 Ribonucleic acid (RNA) Extraction and Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from tumor samples ($n=40$) were extracted from snap-frozen tissues ($< 25\text{mg}$) according to the trizol method of RNA extraction (Sigma-Aldrich, MO, USA). The concentration and purity (260/280 and 260/230 ratios) were checked using Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, MA, USA) and quality of the preparation was analyzed on a 1% agarose gel. RNA obtained was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using PrimeScript 1st strand cDNA synthesis kit (Takara, CA, USA) as per manufacturer's protocol. The reaction mixture with a total volume of 20 μl contained template RNA (total RNA $< 5 \mu\text{g}$), 5x primescript buffer, deoxynucleotide (dNTP) mixture (10 mM), Oligo dT primer (50 μM), primescript RTase (200 U/ μl), RNase inhibitor (40 U/ μl) and RNase-free water. The cDNA synthesis reactions were carried out at 50°C for 50 minutes followed by 70°C for 15 minutes in a G-storm GS4 thermal cycler (Life Science Research, Somerset, UK).

2.3 Conventional PCR and Sequencing analysis

The amplifications of cDNAs were performed in 25 μl reaction volumes containing 1 μl product from the RT reaction according to the manufacturer's protocol (PrimeStar Max DNA Polymerase, Takara, CA, USA). *EGFR* and *EGFRvIII* were amplified using forward and reverse primers (Supplementary Data 1) spanning the beginning of exon 1 and within exon 8 respectively. These RT-PCR primers generate a 180 bp truncated product for *EGFRvIII* whereas 846 bp and 981 bp amplification products for *de4 EGFR* and full-length *EGFR* respectively. PCR amplification products from U87 cell line (American Type Culture Collection - ATCC, VA, USA) and plasmid DNA (Plasmid# 20737- MSCV-XZ066EGFRvIII, Addgene, MA, USA) served as positive controls for *EGFR* and *EGFRvIII* respectively. PCR cycling conditions were: initial denaturation at 98°C for 10 seconds, 35 cycles of 98°C denaturation (10 seconds), 64°C annealing (15 seconds) and 72°C extension (10 seconds) and a final extension at 72°C (5 minutes). PCR reaction products (10 μl) were electrophoresed in 1% agarose gels and stained with ethidium bromide. The *EGFRvIII* amplification product was extracted from the agarose gel (QIAquick gel extraction kit, Qiagen, MD, USA) and verified by Sanger sequencing using the RT-PCR primers [24].

2.4 Real-time PCR

Real-time PCR analysis of *EGFRvIII* positive ($n=21$) and negative ($n=4$) glioma cases were done on Real-time PCR instrument (7300 Real-time PCR system, Applied Biosystems, CA, USA) using SYBR method. Normal appearing brain tissues (non-malignant and non-traumatic

brain tissues) were used as control samples for gene expression analysis. The cDNA products obtained from the RT-PCR reaction (2 μ l) were used as template in 20 μ l PCR reaction containing 10 μ l of SYBR green mixture (Power SYBR green PCR master mix, Applied Biosystems, CA, USA), 0.4 μ M of each primer (Supplementary Data 1) and 6.4 μ l of distilled water. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as internal control. All reactions were done in duplicate. Amplification conditions were: 95°C for 10 minutes, 40 cycles of 95°C/15 seconds, 60°C/1 minute and a dissociation stage of 95°C/15 seconds, 60°C/15 seconds and 95°C/15 seconds. The threshold cycle number (C_T) was automatically determined by SDS 2.0 software (Applied Biosystems, CA, USA).

2.5. Data Analysis of Real-time PCR

The comparative C_T method was used to compute the fold change in gene expression. The mean C_T number of duplicate run was used for data analysis and the average value for C_T of GAPDH was used as the reference gene C_T number. The relative expression of each gene compared with the reference gene was calculated as ΔC_T , by subtracting the C_T number of reference gene from that of each target gene. This was further normalized to calculate $\Delta\Delta C_T$, i.e. difference in the ΔC_T values between experimental and control samples. The fold change in expression of each target gene was then determined using the formula: $2^{-\Delta\Delta C_T}$.

2.6. Immunohistochemistry (IHC) analysis

Immunohistochemical staining was performed on formalin-fixed paraffin embedded (FFPE) sections of the tumor cases. Briefly, as per manufacturer's protocol (Super sensitive polymer-HRP detection system, Biogenex, CA, USA), the four- μ m-thick sections were deparaffinized and rehydrated using varying concentrations of ethanol (100%-70%) and washed in phosphate buffered saline (1X PBS). For heat-induced antigen retrieval, the sections were heated in a microwave oven (700W for 5 minutes) in Tris-Ethylenediaminetetraacetic acid (EDTA) buffer (1X, pH 9). The blocking steps were performed using blocking solutions - power block (15 minutes incubation) and hydrogen peroxide block (15 minutes incubation). The sections were then incubated with a primary antibody targeting EGFR and EGFRvIII [25,26], (monoclonal antibody 528 / mAb 528; immunoglobulin G/ IgG purified from Hybridoma-HB8509, ATCC, VA, USA) at 1:100 dilution for 2 hours at 4°C. For the analysis of GLUT3 expression, the sections were incubated with the anti-GLUT3 antibody (Abcam, MA, USA) at 1:50 dilution for 2 hours at 4°C. On the other hand, the sections incubated with mouse negative control - HK119, non-immune serum or immunoglobulins, (Super sensitive polymer-HRP detection system, Biogenex, CA, USA) served as negative controls. After incubation, the sections were washed in PBS followed by incubation with

super-enhancer reagent for 30 minutes and then treatment with a secondary antibody (Polymer-Horse radish peroxide reagent) for 30 minutes. The sections were stained using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) for 1-5 minutes and counter stained using hematoxylin. The sections were mounted and then visualized using a bright-field microscope (Leica DMI3000 B, IL, USA).

2.7. Western blotting

Tissue samples (< 25 mg) were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) containing 1X protease and phosphatase inhibitors (Cell signaling technologies, MA, USA). The lysates were normalized for protein concentrations and resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). β -actin was used as the loading control. The proteins were then transferred from the gel onto the polyvinylidene difluoride (PVDF) membrane (Immobilon - P, Merck Millipore, MA, USA) and blocked for one hour using blocking buffer [5% bovine serum albumin (Sigma-Aldrich, MO, USA) in TBST (tris-buffered saline and 0.2% Tween-20)]. The blots were incubated with primary antibodies at 1:5000 dilution (anti-EGFR and anti-Lyn antibodies, Cell signaling technologies, MA, USA; anti-GLUT3 and anti- β -actin antibodies, Abcam, MA, USA) for three hours at 4°C and then washed using TBST. Followed by probing with horseradish peroxidase-linked secondary IgG for 45 minutes at 1:5000 dilution and washed using TBST. The bound antibodies were detected by enhanced chemiluminescence reaction (Clarity™ Western ECL Blotting Substrate, Bio-Rad, CA, USA) and the resulting chemiluminescent signal was detected on the Bio-Rad ChemiDoc XRS+ System using Image Lab™ Software, version 4.0 (Bio-Rad, CA, USA).

2.8. Statistics

The Statistical Package for the Social Sciences (SPSS version 20, IBM Inc., IL, USA) was used for statistical analysis of the real-time PCR data. The data were summarized using mean, standard deviation, standard error for each group. In order to test the differences in mean, Kruskal-Wallis test was applied. When the differences were significant ($P < 0.05$), a Dunn-Bonferroni test was performed for multiple comparison. Spearman's rank correlation (Spearman's Rho) test was used for calculating the correlation of the genes with the different clinical grades of glioma. In addition, Pearson's correlation coefficient (r) was calculated to measure the correlation between the candidate genes. The correlation between oncogenes was also verified by the linear regression (r^2) plot.

3. Results

3.1. Incidence of *EGFRvIII* and *de4 EGFR* mutations in glioma patients

The clinical details and summary of the RT-PCR analysis of glioma patients are listed in Table 1. The histological features of the tumors sections of the patients are depicted in the representative Figure 1. A total of 40 glioma cases were used in this study for the detection of *EGFRvIII*, *de4 EGFR* and *EGFR* transcripts. Figure 2A and B are representative agarose gel images depicting the RT-PCR detection of amplicons for *EGFRvIII* (180 bp), *de4 EGFR* (846 bp) and *EGFR* (981 bp) from glioma patients. Amplification products from MSCV-XZ066-*EGFRvIII* plasmid and U87 cell line served as positive controls for *EGFRvIII* and *EGFR* respectively. Figure 2C depicts the sequence chromatogram of the 180 bp *EGFRvIII* amplicon from a glioblastoma patient. As summarized in Table 1, 21/40 cases (53%) showed the presence of *EGFRvIII* mutation and 8/40 cases (20%) were positive for *de4 EGFR* mutation. Furthermore, the incidence of *EGFRvIII* and *de4 EGFR* mutations was observed to be higher in malignant tumors or high-grade gliomas when compared to low-grade tumors.

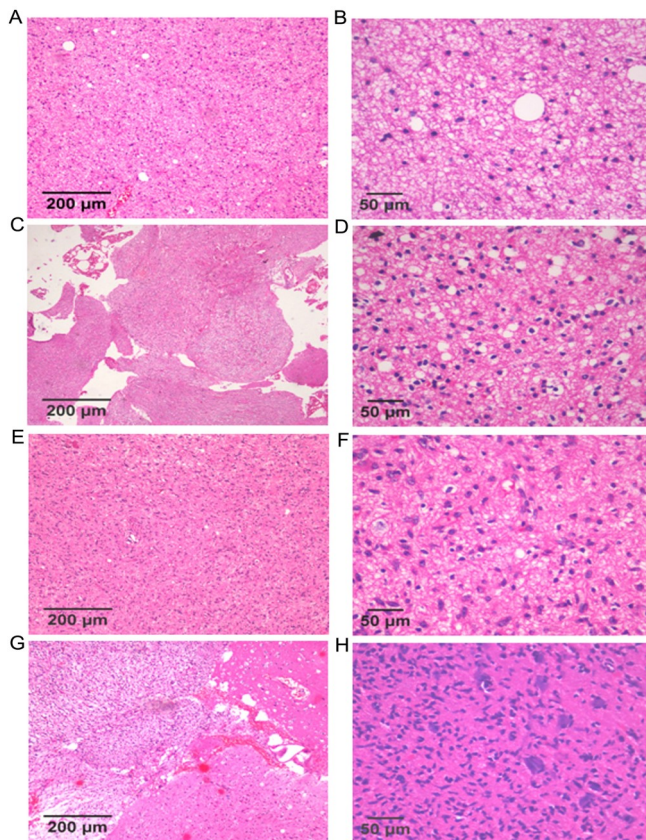


Figure 1. Representative pathological hematoxylin and eosin analysis of tumor sections - A, B) Diffuse fibrillary astrocytoma (IDH-mutant) C, D) Oligodendroglioma (NOS) E, F) Diffuse astrocytoma (IDH-mutant) G, H) Anaplastic astrocytoma (IDH-mutant).

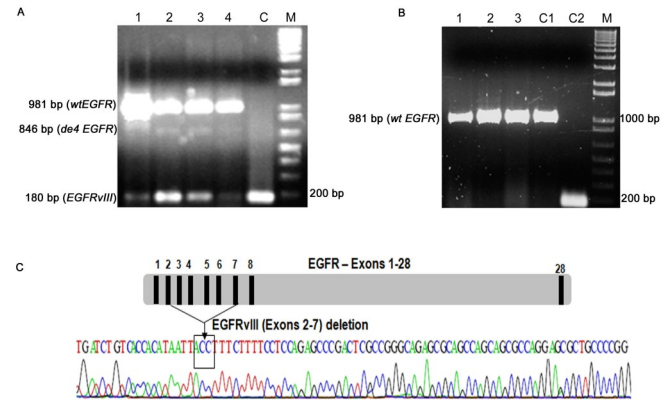


Figure 2. *EGFRvIII* and *de4 EGFR* mutation analysis in glioma patients - (A) RT-PCR products visualized by ethidium bromide staining of a 1% agarose gel: Lanes 1 - 4 - glioblastoma patients with *EGFRvIII* (180 bp), *de4 EGFR* (846 bp) and *EGFR* (981bp), lane C - positive control for *EGFRvIII*, lane M - 1 kb marker. (B) Lanes 1-3 - RT-PCR products from glioma patients with only *EGFR* (981 bp), lanes C1 and C2 - positive controls for *EGFR* and *EGFRvIII* respectively. (C) Sequence chromatogram of a glioblastoma patient depicting *EGFRvIII* deletion.

3.2. Oncogene expressions are significantly upregulated and positively correlated with glioma grades

Using the real-time PCR assay, the fold change in gene expressions of *EGFR*, *EGFRvIII*, *Lyn*, *SYK*, *IRS1*, *PI3K*, *SHIP1* and *GLUT3* in glioma patients were computed by the comparative C_T method of relative quantification and normalized to control. The gene expressions of all candidate oncogenes in the *EGFRvIII* RT-PCR positive glioma cases ($n=21$) were compared with that of the *EGFRvIII* negative cases ($n=4$).

Low-grade glioma patients without *EGFRvIII* expression (RT-PCR negative cases), showed decreased expression levels of all selected oncogenes (Figure 3). On the other hand, *EGFRvIII* positive (RT-PCR positive) grade I-IV patients showed a significant increase in gene expression levels of all candidate oncogenes ($P < 0.05$) whereas *SHIP1* levels were consistently downregulated ($P < 0.05$) when compared to control (Figure 3). The molecular level gene expression profiles of these candidate oncogenes were compared with the clinical grades of glioma using Spearman's correlation test. The gene expressions of *EGFR* (Spearman's coefficient, $r = 0.617$; $P < 0.01$), *EGFRvIII* ($r = 0.838$; $P < 0.01$), *LYN* ($r = 0.573$; $P < 0.01$) and *GLUT3* ($r = 0.771$; $P < 0.01$) showed significant positive correlation with the clinical grades of glioma (Figure 3). The strong positive correlation between expressions of *EGFRvIII* and *GLUT3* in all patients is indicated by the Pearson's coefficient, $r = 0.865$ ($P < 0.01$) and linear regression value, $r^2 = 0.75$ (Figure 4A and B). The oncogenes analyzed for the expression levels in this study are represented in the schematic Figure 4C.

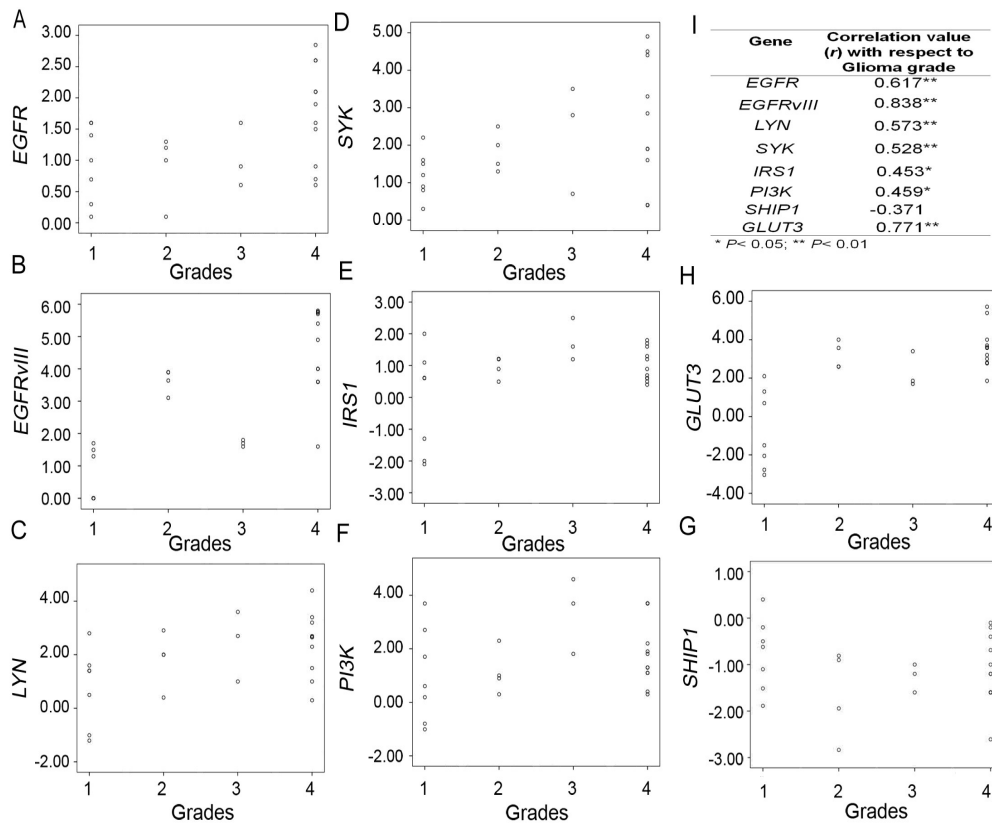


Figure 3. Correlation analysis of gene expressions with respect to glioma grades using Spearman’s rank test - A, H) Scatter plots of gene expressions of *EGFR*, *EGFRvIII*, *LYN*, *SYK*, *IRS1*, *PI3K*, *SHIP1* and *GLUT3* with respect to different grades of glioma patients I) Correlation values (*r* values) of all genes with respect to glioma grades analyzed using Spearman’s test.

3.3. Expressions of EGFR, EGFRvIII, Lyn and GLUT3 are elevated at the protein level in patient tumors

Immunohistochemical staining was performed to evaluate the presence of EGFR, EGFRvIII and GLUT3 protein expressions in the tumor sections. The regions of receptor expression (EGFR and EGFRvIII) were indicated by the brown staining by mAb 528, on the other hand, there was no staining with the negative control antibody (Figure 5A and B). Similarly, the expressions of GLUT3 protein in the glioblastoma FFPE sections were indicated by the intense

brown staining or reactivity with anti-GLUT3 antibody when compared to the negative control antibody treated sections with no reactivity (Figure 5C and D). The protein expressions of EGFR (170 kilodalton or kDa), EGFRvIII (145 kDa), Lyn (56/ 53 kDa isoforms) and GLUT3 (49 kDa) were verified on a western blot (Figure 5E). The levels of protein expression were elevated in the higher grades of the tumors when compared to control. This further confirms the increased expressions of cell surface receptors (EGFR and EGFRvIII), Lyn and GLUT3 at the protein level with respect to the degree or malignant grade of the tumor.

Table 1. Clinical details and summary of RT-PCR analysis of glioma patients for detection of *EGFR*, *EGFRvIII* and *de4 EGFR*.

Glioma grades	Total cases (n)	Age Range (years)	Median Age (years)	Treatment			RT-PCR Analysis		
				Tumor resection (n)	Radio-Therapy (n)	Chemotherapy (Temozolo-mide) treatment (n)	Patients with <i>EGFR</i> (n)	Patients with <i>EGFR-vIII</i> (n)	Patients with <i>de4 EGFR</i> (n)
Grade I	15	15-55	32	15	13	None Received	15	3	1
Grade II	11	20-51	33	11	11	None received	11	4	1
Grade III	3	30-52	37	3	3	3	3	3	2
Grade IV	11	26-62	43	11	11	11	11	11	4
Total	40						40	21	8

RT-PCR, Reverse transcriptase polymerase chain reaction; *EGFR*, Epidermal growth factor receptor; *EGFRvIII*, epidermal growth factor receptor variant III; *de4 EGFR*, epidermal growth factor receptor exon 4 deletion variant.

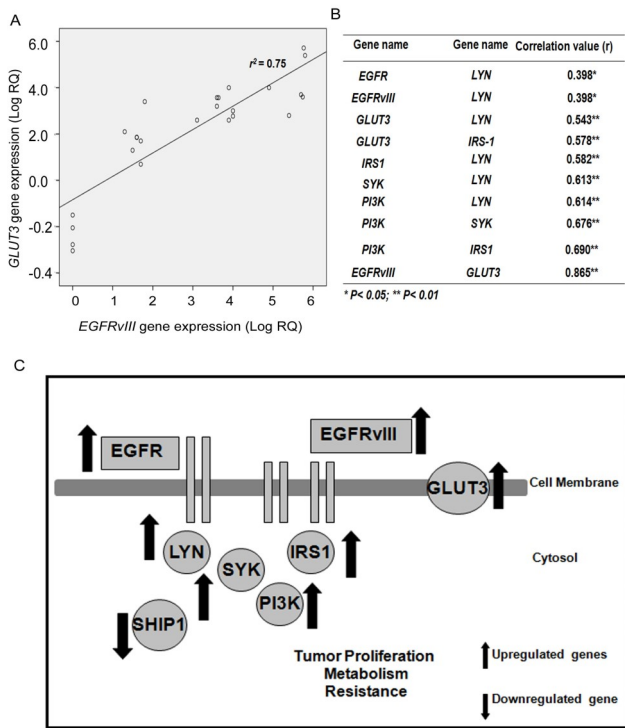


Figure 4. Correlation analysis of EGFRvIII and GLUT3 using Pearson’s statistical method - A) Scatter plot depicting correlation between EGFRvIII and GLUT3 gene expressions in all glioma patients using Pearson’s test (r^2 - linear regression value) B) Correlation values (r values) of all genes analyzed in this study using Pearson’s test C) Schematic representation of the targets analyzed in this study for gene expression levels in glioma patients involved in tumor progression/ pathogenesis [10-19].

4. Discussion

The molecular heterogeneity and complexity of gliomas are governed by multiple oncogenic factors [1,4]. This study was undertaken as a pilot to evaluate the potential correlation of *EGFRvIII* expression with that of the downstream oncogenic targets associated with the progression of glioma. As a first step, we have analyzed the frequency of *EGFRvIII* mutation in glioma subjects (n=40) and detected 21/40 cases (53%) with *EGFRvIII* mutation (Figure 2). The frequency of *EGFRvIII* detection was higher in malignant gliomas when compared to low-grade patients (Table 1). This report is supported by previous evidences on the detection of *EGFRvIII* in high-grade [20,27,28] as well as low-grade gliomas [20]. Interestingly, along with *EGFRvIII*, we have observed a rare variant of *EGFR*, *de4 EGFR* (8/40 cases - 20%) whose frequency also increased with tumor grade (Figure 2, Table 1). But long-term follow-ups on larger patient cohorts are required to decipher the relation of *EGFRvIII* and *de4 EGFR* detection with patient outcomes. Till now, only one group has reported the detection of *de4 EGFR* in glioblastoma and ovarian cancer [22,29]. *De4 EGFR* showed similar properties as that of *EGFRvIII* in promoting metastasis, ligand-independent autophosphorylation and

self-dimerization properties by interactions with downstream molecules such as mitogen-activated protein kinase (MAPK)/ extracellular signal-regulated kinase (ERK), protein kinase B (AKT), Jun, Src, E-cadherin, focal adhesion kinase (FAK) and matrix metalloproteinase-9 (MMP9) in glioma and ovarian cancer [22,29]. But the detailed molecular mechanisms of *de4 EGFR* and *EGFRvIII*-mediated capacity for tumor proliferation and invasion require further investigations [22].

In order to better understand the correlation of oncogene expressions with respect to the clinical grades of glioma, we analyzed the gene expression levels of molecules which may play potential roles in metabolism, proliferation and therapeutic resistance [10-19]. We have observed that the increased expression profiles of all candidate oncogenic molecules (*EGFR*, *EGFRvIII*, *Lyn*, *SYK*, *IRS1*, *PI3K*, and *GLUT3*) depicted marked similarities to the clinical grading of glioma and this has not been previously reported (Figures 3 and 4). Importantly, the gene expressions of *EGFRvIII* displayed high levels of positive correlation with that of *GLUT3* in all glioma patients (Figure 4). Further, the protein level expressions of EGFR, EGFRvIII, Lyn and GLUT3 were also elevated in high-grade gliomas as confirmed by immunohistochemical evaluation and western blot analysis (Figure 5). This observation supports the previous findings on the role of EGFRvIII in malignant transformation and enhancing tumorigenicity of glioblastoma [30,31], and that of GLUT3 (but not GLUT1) in neoplastic transformation [32]. Our observations also support the previous evidences of the associations of EGFRvIII with the downstream oncogenic molecules involved in metabolic and proliferative pathways. For instance, the role of EGFRvIII in glucose metabolism has been implicated wherein limiting levels of glucose enhanced Src-induced mitochondrial localization of EGFRvIII promoting survival and proliferation of glioma cells [33]. Earlier reports have shown that EGFR signaling regulates functional GLUT3 and non-receptor Src-family

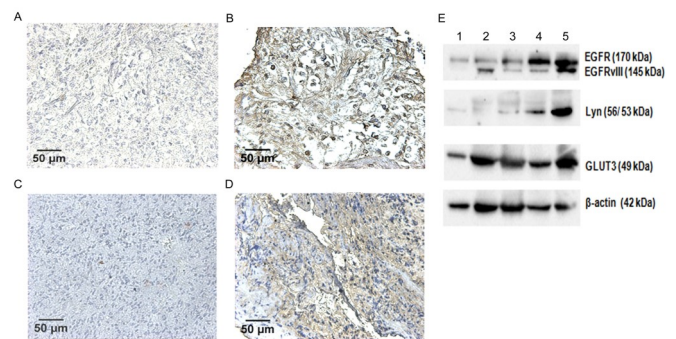


Figure 5. Protein expressions of EGFR, EGFRvIII, Lyn and GLUT3 in glioma patients – Immunohistochemical staining of FFPE tumor sections A) with control antibody B) with monoclonal antibody 528 for EGFR and EGFRvIII expression C) with control antibody D) with anti-GLUT3 antibody E) Western blotting analysis of EGFR, EGFRvIII, Lyn and GLUT3 expressions with respect to β -actin in normal tissues (lane 1) and glioma cases (lanes 2-5: Glioma grades I to IV respectively).

kinase, Lyn [34,35]. Moreover, previous findings have demonstrated that EGFRvIII expressing head and neck cancer displayed enhanced Lyn-mediated proliferation and invasiveness [10]. Lyn was further shown to activate Syk, IRS1 and PI3K through phosphorylation of these downstream effectors [12,13,36]. In contrast to the metabolic and proliferative effects of these oncogenic molecules, the tumor suppressor, SHIP1 regulates or downmodulates the phosphoinositide 3-kinase effectors [18]. Hence, the increased expression profiles of oncogenes observed in the present study in glioblastoma patients is in line with these previous findings with respect to their involvement in metabolism and tumor progression.

Earlier reports have demonstrated that EGFRvIII possessed advantage over EGFR in radioresistant tumors by conferring stronger cytoprotective response to radiation [37]. The importance of EGFR and EGFRvIII inhibition as a therapeutic strategy for radiosensitizing carcinomas has been described [38,39]. Likewise, it was demonstrated that temozolomide resistant astrogloma cells exhibited increased GLUT3 expression and inhibition of selected components of glycolytic pathway (like GLUTs) may represent a promising therapy in order to overcome drug resistance in glioblastoma [40]. Hence, from the data presented here the molecular level expression analysis of the candidate oncogenes could be correlated with the glioma grades and these oncogenes require high surveillance during tumor progression. Therefore, with further studies on larger patient data sets, the analysis of correlations between expression profiles of oncogenes and glioma aggressiveness can serve as a rationale for stratification of patients for EGFRvIII and GLUT3 targeted therapies for sensitizing radio-/chemoresistant gliomas.

5. Concluding Remarks

In conclusion, our study demonstrated the increased incidence of *EGFRvIII* and *de4 EGFR* mutations in malignant gliomas. The molecular analysis displayed a significant positive correlation of *EGFRvIII*, *GLUT3* and candidate oncogenic messages with glioma grades which has not been reported before in the clinical setting. This may have potential prognostic significance when expanded to larger patient cohorts. In addition, further investigations on the evaluation of downstream oncogenic targets involved in the pathology of glioma are warranted for the identification of potential molecular markers which would aid in the development of tailored multi-targeted therapies for patients.

6. Supplementary material

Supplementary Data S1 – Primers used in the study

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