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Research Paper

PBEF1/NAmPRTase/Visfatin

A potential malignant astrocytoma/glioblastoma serum marker with prognostic value

P. Sreekanth Reddy,[1] Srikantha Umesh,[5] Balaram Thota,[4] Ashwani Tandon,[4] Paritosh Pandey,[5] A. S. Hegde,[6] Anandh Balasubramaniam,[5] B. A. Chandramouli,[5] Vani Santosh,[4] M. R. S. Rao,[3,7] Paturu Kondaiah[2] and Kumaravel Somasundaram[1,*]

Departments of [1]Microbiology and Cell Biology; [2]Molecular Reproduction; Development and Genetics; [3]Biochemistry; Indian Institute of Science; Bangalore INDIA; Departments of [4]Neuropathology and [5]Neurosurgery; National Institute of Mental Health and Neuro Sciences; Bangalore INDIA; [6]Sri Satya Sai Institute of Higher Medical Sciences; Bangalore INDIA; [7]Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore INDIA

Abbreviations: GBM, glioblastoma multiforme; DA, diffuse astrocytoma; AA, anaplastic astrocytoma; PBEF1, pre-B-cell colony enhancing factor; NAmPRTase, nicotinamide phosphoribosyltransferase

Key words: glioma, astrocytoma, glioblastoma multiforme, microarray, PBEF1, serum marker, survival analysis, nampt, visfatin, prognosis

Malignant astrocytomas comprise anaplastic astrocytoma (AA; grade III) and Glioblastoma (GBM; grade IV). GBM is the most malignant with a median survival of 10–12 months in patients. Using cDNA microarray based expression profiling of different grades of astrocytomas, we identified several fold increased levels of PBEF1 transcripts in GBM samples. Pre-B-cell colony enhancing factor 1 gene (PBEF1) encodes Nicotinamide phosphoribosyltransferase (NAmPRTase), which catalyses the rate limiting step in the salvage pathway of NAD metabolism in mammalian cells. Further validation using real time RT-qPCR on an independent set of tumor samples (n = 91) and normal brain samples (n = 9), GBM specific higher expression of PBEF1 was confirmed. Immunohistochemical staining for PBEF1 on a subset of the above samples largely reinforced our finding. We carried out ELISA analysis on serum samples of astrocytoma patients to determine whether this protein levels would correlate with the presence of tumor and tumor grade. PBEF1 serum levels were substantially elevated in many of the AA and GBM patients. Statistical analysis of these data indicates that in patients with astrocytoma, serum PBEF1 levels correlate with tumor grade and is highest in GBM. Immunohistochemical analysis of an independent set of 51 retrospective GBM cases with known survival data revealed that PBEF1 expression in the tumor tissue along with its co-expression with p53 was associated with poor survival. Thus, we have identified PBEF1 as a potential malignant astrocytoma serum marker and prognostic indicator among GBMs.

*Correspondence to: Kumaravel Somasundaram; Department of Microbiology and Cell Biology; Room No. 302; Indian Institute of Science; Bangalore, Karnataka 560 012 India; Tel.: 91.80.22932973; Fax: 91.80.23602697; Email: skumar@mcbl.iisc.ernet.in

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Introduction

The most common of the brain cancers is astrocytomas, which occurs at a rate of almost 12 in 100,000 people.[1] Astrocytomas may be either circumscribed (graded as Grade I/II) or diffuse. The diffuse type is being further divided into low-grade diffuse astrocytoma, (DA; Gr. II), anaplastic astrocytoma (AA; Gr. III) and glioblastoma multiforme (GBM; Gr. IV)[2] with order of increasing malignancy. Patients with AA have an average survival of 3 years, whereas patients with grade DA have the best prognosis, with approximately 5 years median survival. GBM patients have the worst prognosis with a median survival of approximately 12 months, even after surgical resection, radiation therapy and chemotherapy.[3] GBMs are histologically characterized by nuclear atypia, high mitotic activity, microvascular proliferation and/or necrosis.

There is enough evidence in the literature about the presence of unrecognized clinically relevant subclasses of astrocytomas both with respect to their gene signature and their clinical response to therapy.[4] It has also been shown that histologically identical tumors behave differently for a given treatment protocol.[4] While several genetic markers have been reported to be associated with different grades of astrocytoma, these markers do not allow identifying clinically relevant factors predictive for outcome or response to therapy.[5] Detailed investigation leading to complete understanding of mechanisms and their relevance in the cancer progression will allow us to develop cancer specific therapy by targeting deregulated pathways and design future treatment protocols tailored according to the biology of the individual tumors.[6]

Expression profiling using microarrays offers the option of unbiased, quantitative and reproducible tumor assessment by simultaneously evaluating thousands of individual gene expression measurements. To identify markers of prognostic and therapeutic utility in malignant astrocytomas, in particular GBM, we performed expression profiling of several samples of diffusely infiltrating astrocytomas comprising different grades and four normal brain tissue samples (pooled) as control using cDNA microarrays. Data analysis revealed PBEF1 to be a highly expressed gene among GBM samples. Initially PBEF1 was

identified as a cytokine namely pre-B-cell colony-enhancing factor 1.[7] Later, it was found that the protein encoded by PBEF1, namely Nicotinamide phosphoribosyltransferase (NAmPRTase) also functions as an enzyme involved in the NAD biosynthesis. PBEF1 has also been recognized as a hormone named Visfatin as it was found in high levels in visceral fat.[8] Although PBEF1 has been shown to be overexpressed in colon cancer, the role of PBEF1 in human cancers, in particular astrocytomas is largely not known.[9] We have confirmed the GBM specific expression of PBEF1 by real time RT-PCR and immunohistochemical analysis on an independent set of tumor samples. We have shown that increased PBEF1 expression is indicative of poor prognosis amongst GBM patients. More importantly, by estimation of serum PBEF1 levels in astrocytoma patients, we show that increase in serum PBEF1 levels correlate with tumor grade. These preliminary data suggest that PBEF1 levels may be an efficient and accurate indicator of tumor presence and grade and a potential prognostic marker in high grade neoplasms. To our knowledge, this is the first report, which clearly demonstrates the possible involvement of PBEF1 in cancer development, particularly astrocytoma.

Results

PBEF1/NAmPRTase/Visfatin is over expressed in malignant astrocytomas. cDNA microarray based expression profiling of mRNA derived from 25 diffusely infiltrating astrocytomas comprising different grades and four normal brain tissue was carried out. Analysis of microarray data identified several differentially regulated genes between normal brain tissue and different grades of astrocytoma (data not shown). Of the genes found to be expressed highly in GBMs, we found PBEF1 to be very interesting for variety of reasons. While PBEF1 was originally isolated as a presumptive cytokine named pre-B-cell colony-enhancing factor, it has been now shown to encode Nicotinamide phosphoribosyltransferase (NAmPRTase), which catalyses the rate limiting step in the salvage pathway of NAD metabolism in mammalian cells and regulates the function of the Sir2 ortholog, Sirt1, in mammalian cells.[7,11] To further validate the grade specific expression of PBEF1, we measured the relative levels of PBEF1 transcripts by real-time RT-qPCR on an independent (different from those used for microarray analysis) set of tumor and normal samples (five DAs, thirty one AAs and fifty five GBMs and nine normal brain samples). We found that PBEF1 transcript levels were upregulated more than three fold (Log2 ratio = 1.585) in majority of GBMs (63.60%; 35/55 with a mean log2 ratio of 2.48) in comparison to AAs (9.60%; 3/31 with a mean log2 ratio of 0.44), DAs (0.00%; 0/5 with a mean log2 ratio of 0.50) and normal brain samples (0.00%; 0/9) with a p value <0.001 (Fig. 1). Further, to confirm the above results as well as correlate the RNA levels with protein levels in tumor samples, we analyzed tissue sections derived from a subset of above samples for which paraffin blocks were available. The staining pattern of PBEF1 was generally found to be granular and diffuse cytoplasmic. We found a vast majority of GBMs (72.22%; 26/36) found to be positive for PBEF1 staining (Fig. 2E and F). As expected, reduced percentage of samples among AAs (40.00%; 4/10) and DAs (20.00%; 2/10) showed PBEF1 positive staining (Fig. 2B and C). Further, the average percent positive tumor cells was found to be high among GBMs (37.50%) as against 22.00% and 8.50% among AAs and DAs respectively suggesting the fact that PBEF1 is expressed in very high levels among GBMs. The staining of normal brain revealed that the glial cells are negative for PBEF1 staining (Fig. 2A).

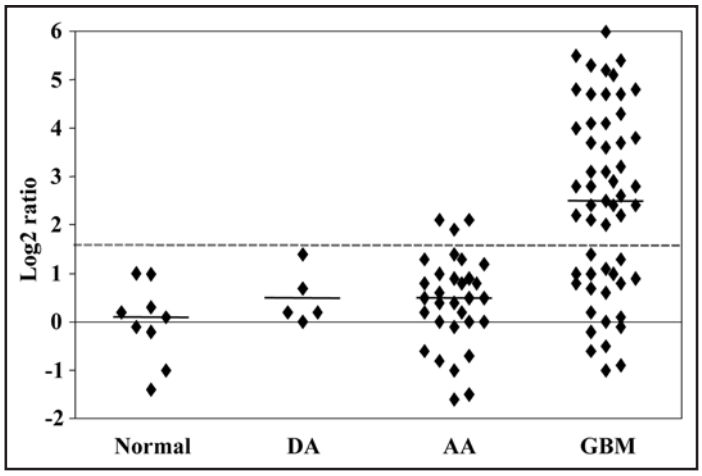

Figure 1. Scatter plot of transcript levels of PBEF1 in different grades of astrocytoma. Log2-transformed gene expression ratios obtained from real-time quantitative PCR analysis are plotted for PBEF1. Each dot represents a data derived from one sample. In each sample, fold change in gene expression is calculated over its mean expression in normal brain samples. Three fold (Log2 ratio = 1.585) up regulation was considered significant.

Serum PBEF1 levels in astrocytoma patients correlate with grade. The concentration of Visfatin in plasma has been shown to increase during the development of obesity and in patients with type 2 diabetes.[12] On the basis of the above information and our results that PEBF1 is overexpressed in GBMs, PBEF1 appears to be a promising candidate serum marker for GBMs. To test whether PBEF1 could be used as a clinical serum marker correlating with astrocytoma grade, we measured the levels of PBEF1 protein in available serum samples belonging to different grades and normal controls. Serum levels of PBEF1 were measured in 47 patients with GBM, 27 patients with AA, 6 patients with DA and 9 controls (Fig. 3). Comparisons of serum PBEF1 levels were made between different grades and normal as well as between different grades. The results of these comparisons are given in Table 1. Statistically significant differences were detected in PBEF1 serum levels in patients with different grades compared to controls: GBMs Vs controls ($p < 0.001$); AAs Vs controls ($p = 0.043$) and DAs Vs controls ($p = 0.018$). Further, the serum PBEF1 levels in patients with GBMs in comparison to that of AAs or DAs approached significance (AAs Vs GBMs, $p = 0.066$; DAs Vs GBMs, $p = 0.066$). No significant differences were detected between DAs and AAs.

Survival value of PBEF1. To analyze the survival value of PBEF1, we subjected a different set of 51 retrospective GBM cases where follow up was available, for expression of PBEF1. In GBM patients, while the prognostic significance of clinical variables in predicting survival have been clearly defined, altered protein expression of the well known genetic alteration found in these tumors, like overexpression of p53 and EGFR expression, have individually failed to give a clear cut prognostic significance, with confounding results in different studies.[13-15] Therefore, for the purpose of multivariate analysis, we immunostained the sections to study the expression of p53 and EGFR in order to analyse the significance of their co-expression with PBEF1 with respect to patient survival.

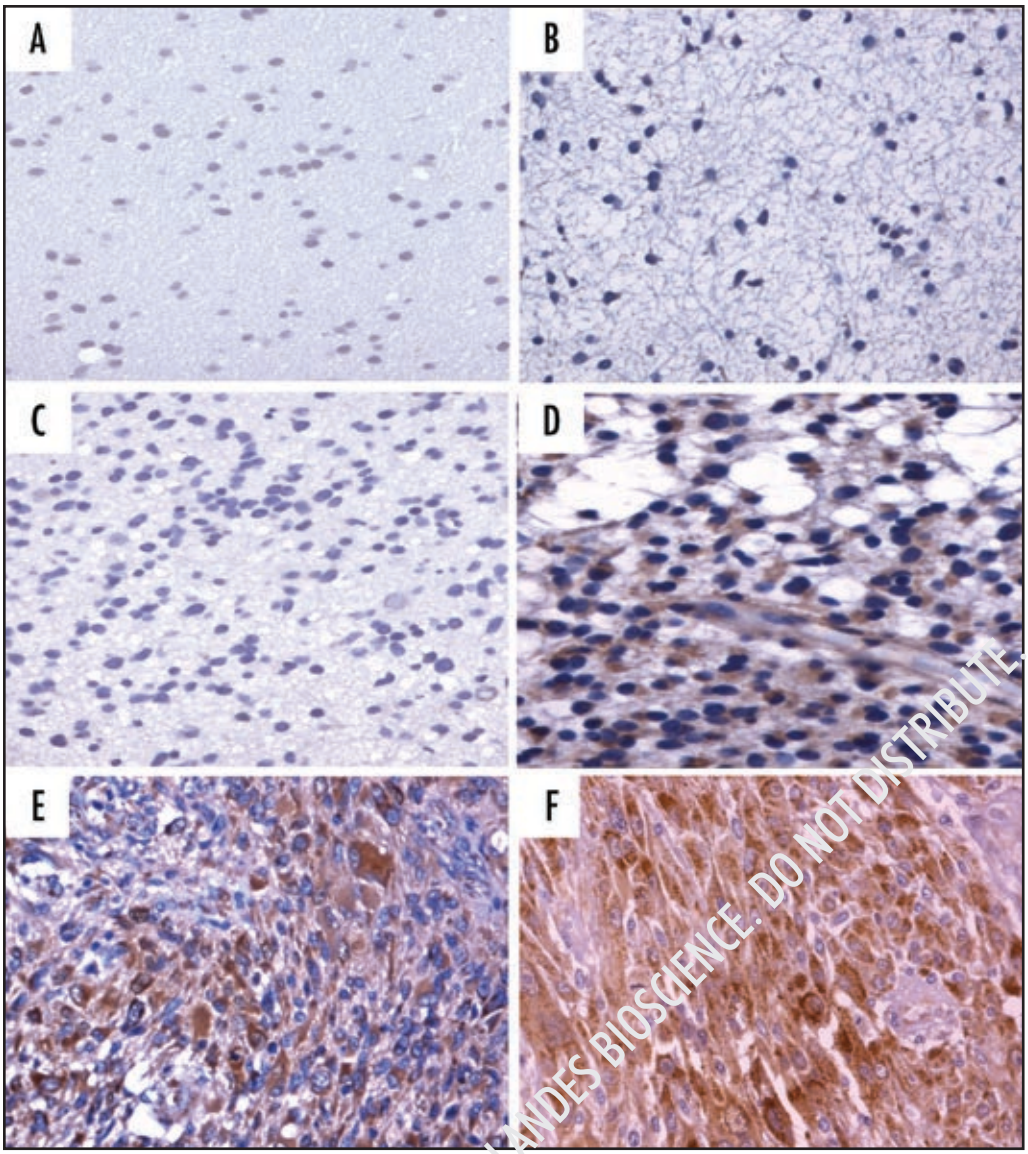

Figure 2. Immunohistochemical validation of PBEF1 overexpression in astrocytoma. Sections from normal brain—negative for staining (A), DA—negative for staining (B), AA—negative for staining (C), AA—positive for staining (D), GBMs—positive for staining (E and F) were stained for PBEF1.

Correlating the expression of PBEF1 with survival among GBM patients, in univariate analysis, the median survival of the group which was positive for PBEF1 (black line) was lesser than that of the group negative for PBEF1 (grey dotted line), albeit with lack of statistical significance (12 months vs. 16 months respectively, p = 0.16; Fig. 4A). In multivariate analysis, while PBEF-1 expression by itself did not correlate with survival, its co-expression with p53 showed a trend towards poorer survival. The median survival of the group positive for both the markers was 8 months (black line), as compared to 14 months (grey dotted line) of the group negative for both or either of them (p = 0.08; Fig. 4B). We also noted that radiotherapy, KPS at presentation and p53 expression were significant independent predictors of survival at all steps in the multivariate analysis (data not shown). The data put together suggest that PBEF1 is a potential prognostic marker amongst GBM patients particularly in combination with aberrant p53 expression.

Discussion

In this study, we found that both transcript and protein levels of PBEF1 are overexpressed in GBMs. Survival analysis indicated that GBM patients expressing higher level of PBEF1 in their tumors had a shorter survival. We show that the levels of PBEF1 protein can be reproducibly measured in serum of patients with malignant astrocytomas. Our data suggest that serum PBEF1 levels are very low in controls and that high levels are detected in astrocytoma patients. Furthermore, we demonstrate that increasing levels of serum PBEF1 in patients corresponds to the grade of malignancy. The levels of serum PBEF1 in all the grades of astrocytoma was significantly different from that of normal controls. In addition, the patient serum belonging to malignant astrocytoma (AAs and GBMs) had substantially higher levels of PBEF1 than the lower grade patients (DAs) with difference approaching statistical significance.

Pre-B cell colony-enhancing factor 1 (PBEF)/Visfatin/Nicotinamide phosphoribosyl transferase (NAmPRTase) is a multifunctional protein having phosphoribosyl transferase, cytokine and adipokine activities.[7,11] PBEF1 has been shown to inhibit apoptosis

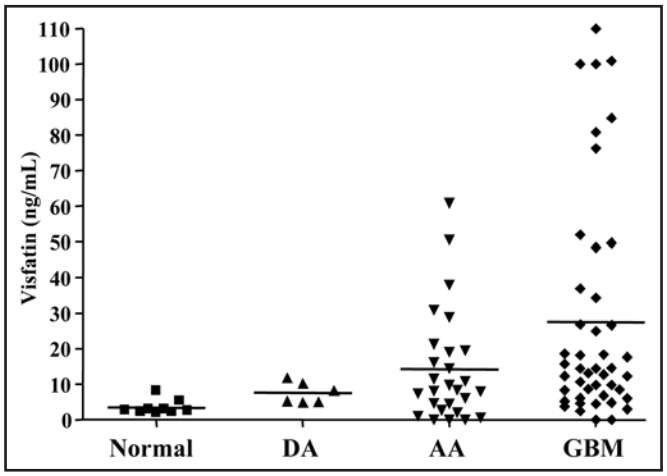

Figure 3. Scatter plot of serum PBEF1 levels. Serum levels of PBEF1/Visfatin (ng/mL) in normal controls (n = 9), DAs (n = 6), AAs (n = 27) and GBMs (n = 45) are plotted. Horizontal line—designates the mean value.[8]

Table 1 **Statistical analysis of serum PBEF1 levels**

Comparison categories (Group I vs Group II)	Group I				Group II				p value	Significance[c]
	Mean	SEM[a]	SD[b]	Median	Mean	SEM	SD	Median		
Normals Vs GBMs	3.49	0.68	2.06	2.70	27.54	4.68	31.43	14.50	<0.001	Significant
Normals Vs AAs	3.49	0.68	2.06	2.70	14.28	3.01	15.64	8.40	0.043	Significant
Normals Vs DAs	3.49	0.68	2.06	2.70	7.63	1.21	2.98	6.75	0.018	Significant
AAs Vs GBMs	14.28	3.01	15.64	8.40	27.54	4.68	31.43	14.50	0.066	Approaching significance
DAs Vs GBMs	7.63	1.21	2.981	6.75	27.54	4.68	31.43	14.50	0.066	Approaching significance
DAs Vs AAs	7.63	1.21	2.981	6.75	14.28	3.01	15.64	8.40	0.692	Not significant

[a]Standard error of mean; [b]Standard deviation; [c]$p < 0.05$ is considered significant.

in neutrophils.[16] Visfatin has been found to be elevated in serum of patients with type 2 diabetes mellitus in human and animal models.[12] An increase in Visfatin plasma concentration has been demonstrated in during obesity development and in patients with type 2 diabetes.[12] The role of PBEF1 in the biology of human solid tumors is unclear. Given the relative specificity of increased expression in GBMs relative to anaplastic astrocytomas, it is possible that PBEF1 protein is an integral component of GBM development and/or may contribute to GBM-specific histological characteristics, which distinguish them from lower grades of astrocytoma. It is also possible that elevated levels of PBEF1 in GBMs may deregulate signaling pathways resulting in activation of pro-survival pathways and chemoresistance. Indeed, it has been shown that NAmPRTase can inhibit apoptosis under experimental conditions in neutrophils and impart chemoresistance in cancer cells.[16,17] PBEF1 overexpression has also been shown to predict poor response to doxorubicin-based primary chemotherapy in breast cancer patients.[18] Further, NAD^+, the final product of salvage pathway catalyzed by NAmPRTase, is an essential coenzyme that is also used as a substrate in several biochemical reactions, such as those catalyzed by poly (ADP-ribose) polymerase (PARP1), sirtuins and ADP-ribosyl cyclase.[19] In particular, NAmPRTase, the rate-limiting component in the NAD^+ biosynthesis pathway, regulates the function of the Sir2 ortholog, Sirt1, in mammalian cells.[11] A potent inhibitor of NAmPRTase, FK866, can reduce cellular NAD + levels and induce apoptosis in tumors.[20]

Thus the overexpression of PBEF1/NAmPRTase in GBM patients may result in poor survival and open room for developing therapeutic small molecules targeting this enzyme. Indeed the survival analysis in our study suggests a prognostic value for PBEF1 expression. Taken together, we have identified PBEF1 as a potential grade specific astrocytoma serum marker of prognostic importance. More importantly, this marker could serve as a potential therapeutic target for malignant astrocytoma.

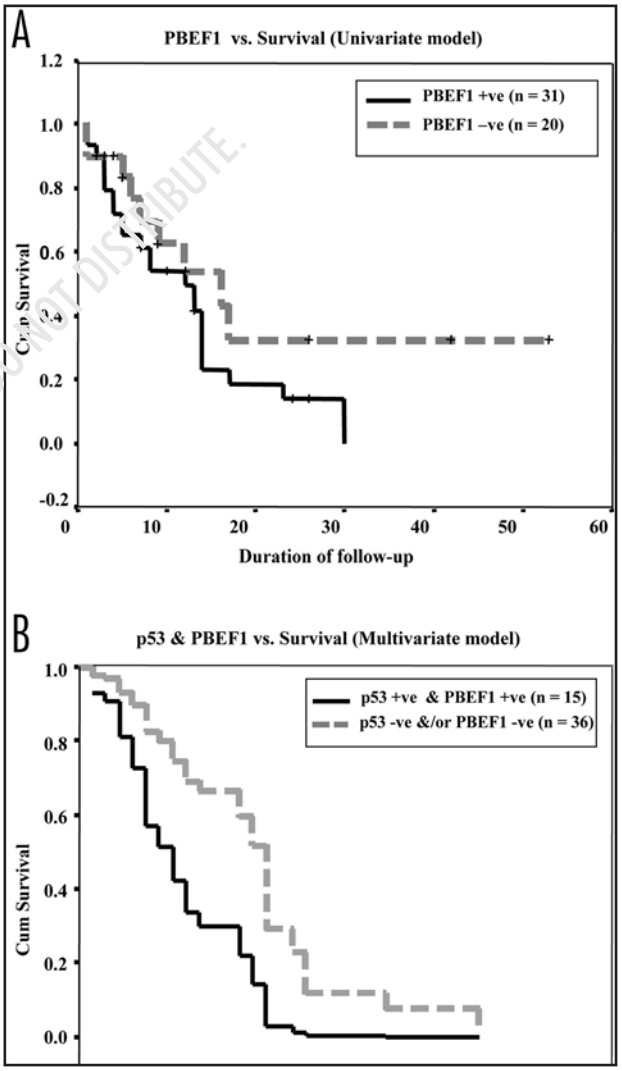

Figure 4. PBEF1 expression and survival of patients with GBM. Kaplan-Meier survival estimates for 51 GBM patients are calculated for p53, EGFR1 and PBEF1 staining. (A) Survival curves for the groups positive and negative for PBEF1 in univariate analysis. The cases which were positive for PBEF1 (black line) had a poorer survival than the cases which were negative (grey line) (p = 0.16), (B) Survival curves for the groups positive and negative for co-expression of p53 and PBEF1, in multivariate analysis. The group positive for both the markers (black line) had a poor survival as compared to the group negative for both or either of them (grey dotted line).

Materials and Methods

Tumor samples. Tumor samples were collected from patients, who were operated at Manipal Hospital, Sri Satya Sai Institute of Higher Medical Sciences and National Institute of Mental Health and Neurosciences, Bangalore, India. Normal brain tissue samples (anterior temporal lobe) obtained during surgery for intractable epilepsy were used as control samples. A total of 100 samples which

included different grades of astrocytomas [(five DAs (Gr. II), thirty one AAs (Gr. III) and fifty five GBMs (Gr IV)] and nine normal brain samples were used in this study. Tissues were bisected and one half was snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation. The other half was fixed in formalin and processed for paraffin sections and these were used to identify the histopathological analysis and immunohistochemistry.

Serum collection. Blood samples were collected from patients diagnosed to have diffuse astrocytoma of varying grades prior to surgery. Control blood samples were collected from patients who were operated for intractable epilepsy (where normal brain tissue served as control for RT-qPCR and immunohistochemistry). The blood samples were allowed to clot at room temperature for no more than 72 hrs, and were then centrifuged at 4°C for 5 min at 1000 rpm. The serum (upper phase) was separated and stored at -20°C until use.

RNA isolation and RT-qPCR. Total RNA was extracted from the frozen tissue by TRI® Reagent (Sigma, USA). The RNA samples were quantified by measuring the absorbance using a spectrophotometer and visualized on a MOPS-Formaldehyde gel for quality assurance. The relative quantitation of expression levels of selected genes was carried out using two step strategy: in the first step, cDNA was generated from RNA derived from different tissue samples using cDNA Archive kit (ABI PRISM); subsequently real-time quantitative PCR was carried out in ABI PRISM 7900 (Applied Biosystems) sequence detection system with the cDNA as template using gene specific primer sets and Dynamo kit containing SYBR green dye (Finnzyme, Finland). All measurements were made in triplicate. The genes RPL35A (ribosomal protein L35a), AGPAT1 (1-acylglycerol-3-phosphate O-acyltransferase 1), ATP5G1 (ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)) and GARS (glycyl-tRNA synthetase) were used as internal controls as their expression levels were found to be unaltered in the array experiments. Normal brain tissue samples from nine different epilepsy patients were used as reference. Delta delta CT method was used for the calculation of expression ratios. An increase or decrease in gene expression by 3 fold (log2 ratio = 1.585) or more over its mean expression in reference samples was considered significant. Statistical significance was tested by Mann-Whitney test using GraphPad PRISM® software. Sequences of RT-PCR primers and conditions used will be provided on request.

ELISA assay. PBEF1 levels were determined for all of the serum samples, using the Visfatin EIA kit from ALPCO Diagnostics according to the manufacturer's protocol. Absorbance was read using Molecular Devices SPECTRAMax® 340PC Microplate Reader. Protein concentrations were estimated by interpolation of the non-linear regression curve plotted using four parameter logistic equation. To compare the differences between the data obtained from different groups, Mann-Whitney test was performed. A P value of less than 0.05 was considered as statistically significant difference between the compared data sets. GraphPad PRISM® Version 4.0 was used for these purposes.

Histopathology and immunohistochemistry (IHC). Histological sections of normal brain and tumor tissues were examined by light microscopy using hematoxylin and eosin (H&E) preparation. Tumor sections of diffusely infiltrating astrocytomas were graded using the WHO grading scheme.[10] Paraffin sections (4 μm) from the tumor tissue and control samples were collected on silane coated slides and IHC for PBEF1 was carried out as follows: rabbit polyclonal antibody against purified GST-PBEF1 protein was made using standard immunization protocol. Microwave antigen retrieval was done at 400 watt for 18 mins in 10 mM citrate buffer, pH 6.0. The sections were further treated with methanol and 5% hydrogen peroxide to block endogenous peroxidase followed by washes with PBS buffer (pH 7.6). Skimmed milk powder (5%) was used to block background staining for 45 mins. The sections were incubated with the primary antibody (diluted to 1:1000) at room temperature for 2 hours. This was followed by incubation with supersensitive non-biotin HRP detection system (QD440-XAK, Biogenex). "3,3'-Diaminobenzidine" (Sigma) was used as the chromogenic substrate.

The staining pattern of PBEF1 was found to be granular and diffuse cytoplasmic. A staining intensity of 3+ in the tumor cell cytoplasm with more than 30% cells showing positive staining was taken to consider the tumor to be positive. GBM tumor samples showing significantly high expression of mRNA levels by RT PCR was taken as positive control. A negative control slide in which the primary antibody was excluded was incorporated with each batch of slides.

Immunohistochemistry for PBEF1 expression in archival samples of GBM to assess the survival value of PBEF1. To analyze the survival value of PBEF1, we subjected a different set of 51 retrospective GBM cases where follow up was available, for expression of PBEF1 along with p53 and EGFR by immunohistochemical analysis. Case records of GBM patients operated in the year 2002 were retrospectively analyzed and follow up data collected. Postal questionnaires were sent to all these patients, requesting their present functional status and date of expiry, when appropriate. Of these, 51 cases of adult, supratentorial, lobar GBM patients who underwent an open surgery, and had at least one post-operative follow-up, were selected and were shown to be representative of the total 96 cases of GBM operated in that year, by independent samples T-test.

The mean age of the patients in the retrospective group analyzed was 45.3 years (Range: 18–80 yrs) and their mean Karnofsky Performance Status (KPS) at presentation was 60.9 (Range: 30–90). 35 of these 51 patients (68.6%) underwent near-total/gross-total resection, and a sub-total resection was achieved in the rest. All the patients were referred for adjuvant therapy. The median duration of follow-up was 8 months (Range: 1–53 months).

The formalin fixed, paraffin embedded blocks of the 51 cases were retrieved. Fresh sections (4 μm) were collected on silane coated slides and immunostained for PBEFI, p53 (monoclonal: DO-7, Biogenix, USA, diluted to 1:200) and EGFR (monoclonal: E-30, Biogenix, USA, diluted to 1:50). For p53 staining, antigen retrieval was performed similar to that for PBEF1, but for 25–35 mins at 700 W. For EGFR staining, the sections were pretreated with Tris-EDTA pH 9.0 at 600 W for 30 mins. For p53 and EGFR, brain tumor samples previously characterized for their overexpression were used as positive controls. p53 and EGFR immunoreactivity was considered positive when more than 20% of tumor cells stained positively (nuclear and membrane cytoplasmic labeling respectively). A cut off of 50% cell positivity for PBEF1 was considered for statistical analysis

Statistical analysis was done using SPSS 10.0 software. For univariate analysis, Kaplan-Meier survival curves with Log-Rank statistical correlations were employed. Multivariate analysis was done using Cox proportional hazard model (Enter method) using age, operative

extent of resection, KPS at presentation, radiotherapy, p53 expression and EGFR overexpression as the constant clinical co-variants and adding PBEF1 expression to these.

Acknowledgements

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