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Imaging, Diagnosis, Prognosis

Novel Glioblastoma Markers with Diagnostic and Prognostic Value Identified through Transcriptome Analysis

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Abstract

Purpose: Current methods of classification of astrocytoma based on histopathologic methods are often subjective and less accurate. Although patients with glioblastoma have grave prognosis, significant variability in patient outcome is observed. Therefore, the aim of this study was to identify glioblastoma diagnostic and prognostic markers through microarray analysis.

Experimental Design: We carried out transcriptome analysis of 25 diffusely infiltrating astrocytoma samples [WHO grade II—diffuse astrocytoma, grade III—anaplastic astrocytoma, and grade IV—glioblastoma (GBM)] using cDNA microarrays containing 18,981 genes. Several of the markers identified were also validated by real-time reverse transcription quantitative PCR and immunohistochemical analysis on an independent set of tumor samples ($n = 100$). Survival analysis was carried out for two markers on another independent set of retrospective cases ($n = 51$).

Results: We identified several differentially regulated grade-specific genes. Independent validation by real-time reverse transcription quantitative PCR analysis found *g*rowth *a*rrest and *D*NA-*d*amage–inducible α (GADD45α) and follistatin-like 1 (FSTL1) to be up-regulated in most GBMs (both primary and secondary), whereas superoxide dismutase 2 and adipocyte enhancer binding protein 1 were up-regulated in the majority of primary GBM. Further, identification of the grade-specific expression of GADD45α and FSTL1 by immunohistochemical staining reinforced our findings. Analysis of retrospective GBM cases with known survival data revealed that cytoplasmic overexpression of GADD45α conferred better survival while the coexpression of FSTL1 with p53 was associated with poor survival.

Conclusions: Our study reveals that GADD45α and FSTLI are GBM-specific whereas superoxide dismutase 2 and adipocyte enhancer binding protein 1 are primary GBM-specific diagnostic markers. Whereas GADD45α overexpression confers a favorable prognosis, FSTL1 overexpression is a hallmark of poor prognosis in GBM patients.

Diffuse-infiltrating astrocytomas include the following entities: (*a*) diffuse astrocytoma (DA; WHO grade II), (*b*) anaplastic astrocytoma (AA; WHO grade III), and (*c*) glioblastoma (GBM; WHO grade IV). AA and GBMs constitute malignant astrocytomas and are the most common intrinsic central nervous system neoplasms (1). The prognosis of patients with these tumors is dismal, and GBM, which is the most malignant phenotypic variant, has a mean survival of 10 to 12 months (2). Currently available treatment options are multimodal, which include surgery, radiotherapy, and chemotherapy. However, these have been shown to improve survival only marginally in GBM patients.

The development of GBM has been described to occur through at least two genetic pathways resulting in the formation of primary and secondary GBMs (3). Primary GBM represents the most frequently presenting variant occurring *de novo* without an evidence of a less malignant precursor.

Patients under this category are commonly of older age and have a high rate of *epidermal growth factor receptor* (*EGFR*) gene amplification, p16 INK4A deletion, mutations in *PTEN* gene, and MDM2 amplification. In contrast, secondary GBM occurs after a preceding diagnosis of lower-grade astrocytomas. Mutations in the *p53* gene, RB alterations, and PDGFR amplification and overexpression are thought to be more common in the development of secondary GBM (3, 4). In spite of these genetic differences, it remains uncertain whether these subtypes differ significantly with respect to prognosis (5).

Microarray expression profiling of glioma allows simultaneous analysis of thousands of genes and is likely to identify molecular markers associated with tumor grade, progression, and patient survival (6, 7). In a previous study, we reported differential gene expression between low-grade astrocytoma and GBM, and proposed a role for notch pathway in progressive astrocytoma development (8). The purpose of this study was to identify genes that are differentially regulated during the development of malignant astrocytomas, in particular, GBM. Through cDNA microarray experiments, we have identified several distinct gene categories of transcripts overexpressed in different grades of astrocytoma. In addition, we have validated the identified genes that characterize GBM, in particular primary GBM. The prognostic value of some of the markers is also studied.

Materials and Methods

Tumor samples. Tumor samples were collected from patients who were operated on 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 131 samples of different grades of astrocytomas and normal brain samples were used in this study. For microarray hybridization, a set of 25 samples of diffusely infiltrating astrocytomas comprising 4 DAs, 5 AAs, 16 GBMs (6 secondary and 10 primary), and 5 normal controls were used. For subsequent real-time reverse transcription-PCR validation of selected genes, we used an independent set of 91 samples of diffusely infiltrating astrocytomas comprising 5 DAs, 31 AAs, 55 GBMs (20 secondary and 35 primary), and normal brain tissue from another set of nine controls. 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. These were used for histopathologic grading of astrocytoma and immunohistochemistry.

RNA isolation, cDNA labeling, and microarray hybridization. Total RNA was extracted from the frozen tissue by using the TRI reagent (Sigma). The RNA samples were quantified by measuring the absorbance using a spectrophotometer and visualized on a MOPS-formaldehyde gel for quality assurance. cDNA synthesis and labeling of total RNA were done using the Micromax direct labeling kit (Perkin-Elmer Life Sciences, Inc.). RNA derived from normal brain tissue was labeled with cyanine 3 dUTP, whereas that of tumor was labeled with cyanine 5 dUTP. Twenty micrograms of total RNA were used for each labeling reaction. The quality of cDNA labeling was monitored by separating small amounts of cyanine 3– and cyanine 5–labeled cDNA in an agarose gel made on a microscopic slide and scanning the gel using the laser scanner LSIV (Genomic Solutions). Cyanine 3– and cyanine 5–labeled cDNAs were added to 100 μL of 1× hybridization buffer (Ultrahyb, Sigma), incubated at 75°C for 5 min before adding to the 19 k arrays (University Health Network). Hybridization was carried out in a GeneTAC Hyb Station (Genomic Solutions) at 65°C for 4 h, 60°C for 4 h, and 55°C for 10 h. The slides were washed using medium

stringency, high stringency, and postwash buffers (Genomic Solutions) for five times each; dried; and scanned using the Scanarray Express (Perkin-Elmer).

Microarray image and data analysis. Image analysis was carried out with the GeneTAC Analyzer software, version 3.3 (Genomic Solutions), and filtering and assembling of data were done using MS Excel and MS Access. To begin with, the image was visually inspected and spots of questionable quality were flagged and eliminated from further consideration. In the next step, spots having a signal-to-noise ratio <1.5 in both channels and total intensity values below a threshold value were excluded. We first computed arithmetic mean and SD for background subtracted total intensities of all negative control spots (3× SSC) on the slide and then computed threshold value as arithmetic mean plus 2 SDs. If the coefficient of variation of expression ratios of duplicate spots of a given gene is >20%, then the gene was excluded from further analysis. Within-array normalization (Lowess-tip wise) and across-array normalization were done using MIDAS software (9). The cyanine 5/cyanine 3 normalized expression ratio was determined for each spot and the values from the duplicate spots within each array were averaged and log 2 transformed. All the subsequent analyses were done using log 2–transformed data. We have analyzed four grade 2, five grade 3, six secondary GBM, and ten primary GBM samples by microarray hybridization. The genes having values in at least 50% of the samples in each group were considered for further analysis. To find the significantly differentially regulated genes between normal brain and astrocytoma, and between the groups of astrocytoma, data were analyzed by Significance Analysis of Microarrays (SAM; ref. 10). SAM one-class option with estimated false discovery rate (median) <5% and fold-change cutoff of 3 was used to identify significantly differentially regulated genes between normal and tumor. SAM two-class option with estimated false discovery rate (median) <5% and fold-change cutoff of 1.5 was used to identify significantly differentially regulated genes between different groups of glioma. SAM-identified genes were subjected to hierarchical cluster analysis using TMEV software to see the substructure of the data (9).

Real-time quantitative reverse transcription-PCR. The relative quantification of the expression levels of selected genes was carried out using a two-step strategy: In the first step, cDNA was generated from RNA derived from different tissue samples using a cDNA archive kit (ABI PRISM); subsequently, real-time quantitative PCR was carried out in an ABI PRISM 7900 (Applied Biosystems) sequence detection system with the cDNA as template using gene-specific primer sets and a Dynamo kit containing SYBR green dye (Finnzyme). All measurements were made in triplicate. The genes *GARS* (glycyl-tRNA synthetase), *AGPAT1* (1-acylglycerol-3-phosphate *O*-acyltransferase 1), *ATP5G1* [ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)], and *RPL35A* (ribosomal protein L35a) were used as internal controls because 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. ΔC_T method was used for the calculation of ratios. An increase or decrease in gene expression by 4-fold (log 2 ratio = 2) or more over its mean expression in reference samples was chosen as threshold to calculate the percentage of regulated samples. Statistical significance was tested by Mann-Whitney test using GraphPad PRISM software. Sequences of reverse transcription-PCR primers and conditions used will be provided on request.

Histopathology and immunohistochemistry. Histologic sections of normal brain and tumor tissues were examined by light microscopy using H&E preparation. Tumor sections of diffusely infiltrating astrocytomas were graded using the WHO grading scheme (11). Paraffin sections (4 μm) from the tumor tissue and control samples were collected on silane-coated slides for immunohistochemistry. Newly recognized markers, namely *g*rowth *a*rrest and *D*NA-*d*amage–inducible α (GADD45α) and follistatin-like 1 (FSTL1), were validated. The primary antibodies were GADD45α (rabbit polyclonal, 1:50 dilution) and FSTL1 (rabbit polyclonal, 1:100 dilution). GADD45α antibody (clone

C-20) was obtained from Santa Cruz Biotechnology. Rabbit polyclonal antibody against purified GST-FSTL1 protein was made using standard immunization protocol. Microwave antigen retrieval was done at 400 W for 18 min in 10 mmol/L citrate buffer (pH 6.0). The antibodies used mainly for the purpose of subclassifying GBM cases were p53 (monoclonal: DO-7, Biogenix, diluted to 1:200) and EGFR (monoclonal: E-30, Biogenix, diluted to 1:50). For p53, antigen retrieval was done by heat treatment of the deparaffinized sections in a microwave oven

for 25 to 35 min at 700 W in citrate buffer (10 mmol/L, pH 6.0). For EGFR staining, the sections were pretreated with Tris-EDTA (pH 9.0) at 600 W for 30 min. All 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 min. The sections were incubated with the primary antibody overnight at 4°C. This was followed by incubation with supersensitive nonbiotin horseradish peroxidase detection
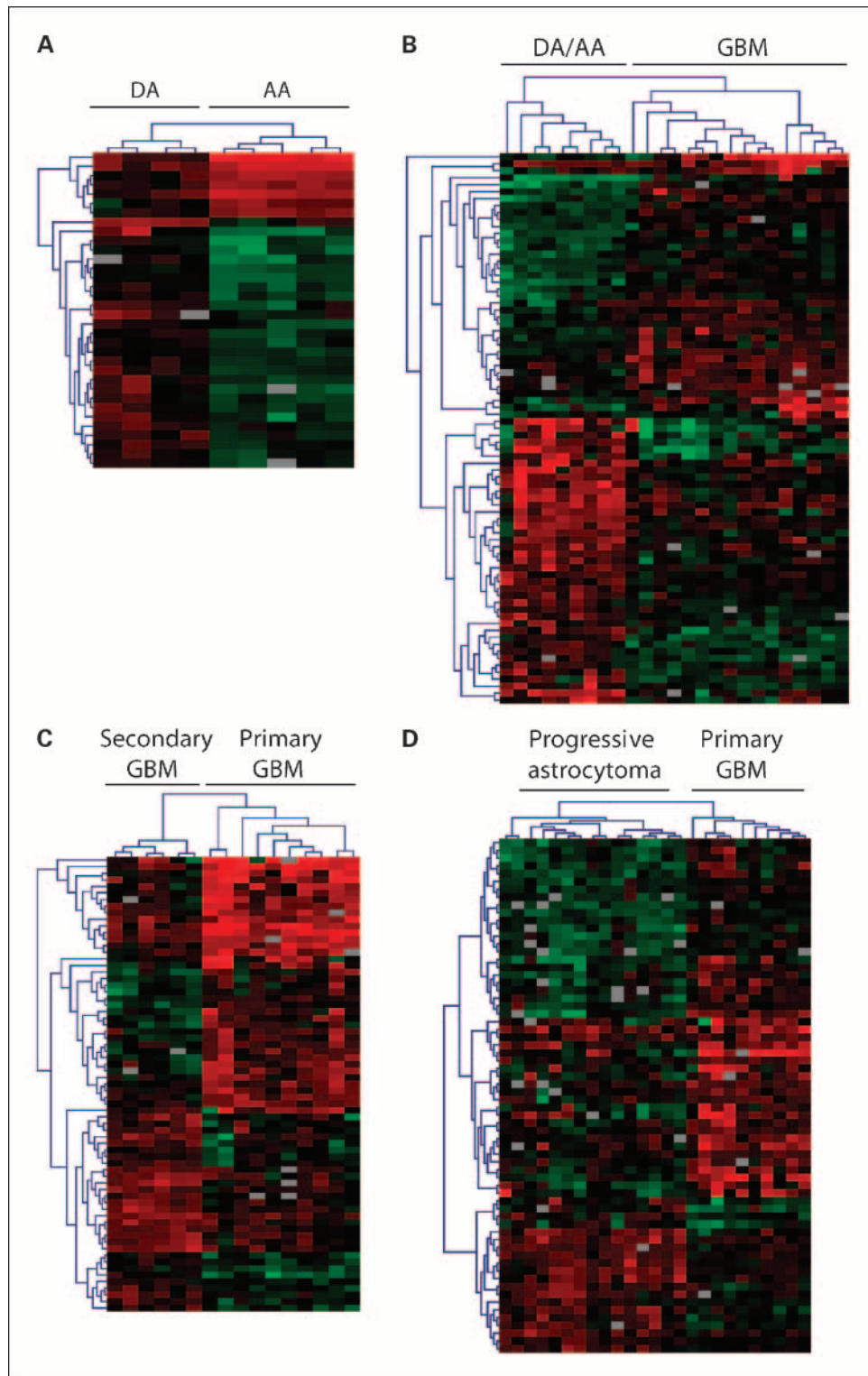
Fig. 1. Two-way hierarchical clustering of SAM-identified grade-specific genes. Normalized, log 2 – transformed expression ratios of SAM-identified differentially regulated genes were subjected to two-way hierarchical clustering using TMEV software to see if gene sets can cluster samples of different grades separately. A dual-color code was used, with red and green indicating up- and down-regulation, respectively, in the particular glioma sample compared with normal brain tissue. *A,* differentially regulated genes between DA and AA. Twenty-six genes were up-regulated in DAs as against AAs and 7 genes were up-regulated in AA as against DA. *B,* differentially regulated genes between DA/AA and GBM. Forty-one genes were up-regulated in DAs and AAs as against GBMs, and 37 genes were up-regulated in GBMs as against DAs and AAs. *C,* differentially regulated genes between secondary GBM and primary GBM. Thirty-nine genes were up-regulated in primary GBM in comparison with secondary GBM and 30 genes were up-regulated in secondary GBM in comparison with primary GBM. *D,* differentially regulated genes between progressive astrocytoma and primary GBM. Twenty genes were up-regulated in progressive astrocytoma (DA, AA, and secondary GBM) as against primary GBM and 45 genes were up-regulated in primary GBMs as against progressive astrocytoma.

system (QD440-XAK, Biogenex). 3,3'-Diaminobenzidine (Sigma) was used as chromogenic substrate.

Brain tumor samples previously characterized for overexpression of p53 and EGFR were used as positive controls. p53 and EGFR immunoreactivity was considered positive when >20% of tumor cells stained positively (nuclear and membrane cytoplasmic labeling, respectively). GBMs were classified as primary and secondary, taking into consideration the clinical profile of patients, expression of EGFR, and p53 (5). The mean age of patients with primary GBM was 50.6 y and the mean duration of symptoms was 2.7 mo. Uniform staining for EGFR was evident in all cases and five revealed additional p53 expression. Among secondary GBMs, the mean age of the patients was 33.8 y and the mean duration of symptoms was 5.3 mo. p53 immunoreactivity was uniformly evident in all cases and two revealed additional EGFR overexpression.

The staining pattern was diffuse cytoplasmic with both GADD45α and FSTL1 antibodies. A staining intensity of 3+ in the tumor cell cytoplasm and a cutoff value of 20% tumor cell positivity were considered to label the tumor as positive. GBM tumor samples showing significantly high expression of mRNA levels by reverse transcription-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 on archival samples of GBM to assess the survival value of GADD45 and FSTLI. To analyze the survival value of GADD45α and FSTL1, we subjected a different set of 51 retrospective GBM cases, where follow-up was available, for expression of these two markers along with p53 and EGFR by immunohistochemical analysis. Case records of GBM patients operated on in the year 2002 were retrospectively analyzed and follow-up data were 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 postoperative follow-up were selected and shown to be representative of the total 96 cases of GBM operated on in that year by independent samples *t* test.

The mean age of the patients in the retrospective group analyzed was 45.3 y (18-80 y) and their mean Karnofsky performance status at presentation was 60.9 (30-90). Of these 51 patients, 35 (68.6%) underwent near-total/gross-total resection, and a subtotal resection was achieved in the rest. All the patients were referred for adjuvant therapy. The median duration of follow-up was 8 mo (1-53 mo). 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 GADD45α, FSTL1, p53, and EGFR. A cutoff of 20% for GADD45α and 40% for FSTL1 was considered for statistical analysis.

Statistical analysis. Statistical analysis was done using SPSS 10.0 software. For univariate analysis, Kaplan-Meier survival curves with log-rank statistical correlations were used. Multivariate analysis was done using the Cox proportional hazard model (Enter method), with age, operative extent of resection, Karnofsky performance status at presentation, radiotherapy, p53 expression, and EGFR overexpression as the constant clinical covariants, and adding the immunohistochemical marker as appropriate.

Results

Transcriptome analysis of astrocytomas. We analyzed the expression profile of 18,981 human genes using 19 k cDNA microarrays for 25 samples of diffusely infiltrating astrocytoma comprising 4 DA (grade II), 5 AA (grade III), and 16 GBM (grade IV; 6 secondary and 10 primary). Of these, only 14,929 genes for which data were available for >50% of the samples within each grade were considered for subsequent analysis. The data obtained from image analysis were filtered, normalized, and log 2 transformed before being used for further studies.

To identify the significantly differentially regulated genes between normal and astrocytoma samples as well as between different grades of astrocytoma, data were subjected to SAM using the one-class and two-class options as required. To obtain a visual appreciation of the sets of differentially regulated genes between different groups and to verify whether identified gene sets can differentiate samples into different groups, the data obtained from SAM were subjected to an unsupervised, two-way, average-linkage hierarchical cluster analysis with Euclidean distance as similarity metric using the TMEV software.

SAM analysis identifies grade-specific genes. Examination of 14,929 gene data sets derived from 25 astrocytoma samples through SAM analysis found 385 to be up-regulated and 911 genes to be down-regulated in all groups of astrocytoma in comparison with normal brain sample, thus confirming the fact that astrocytoma development and progression are associated with altered gene expression profile. DAs (grade II) are diffusely infiltrating low-grade astrocytoma that progress over to malignant grade III anaplastic astrocytoma and grade IV GBM over a period of 5 to 10 years (4, 5, 12). To better understand the gene expression changes during progression from DA (grade II) to AA (grade III), we compared the data obtained from four DA and five AA samples through SAM. We found 26 genes to be up-regulated in DA as against AA and 7 genes up-regulated in AA as against DA (Fig. 1A; Supplementary Table S1). AAs (grade III) are less malignant than GBMs, with AA patients having a mean survival of 2 to 3 years in comparison with GBM patients whose mean survival is <1 year (3, 5, 13, 14). To identify the genes that are differentially regulated between GBM and less malignant grades (DA and AA), we subjected the data obtained from 9 of the later group (4 DA and 5 AA) and 16 GBM (10 primary and 6 secondary) through SAM analysis. We found 41 genes up-regulated in DA and AA as against GBM and 37 genes up-regulated in GBM as against DA and AA (Fig. 1B; Supplementary Table S2). The primary and secondary GBM arise through different genetic pathways, suggesting the existence of differences in the expression profile between these two classes. Accordingly, to identify genes that are differentially regulated between primary and secondary GBM, we subjected the data obtained from 10 primary GBM and 6 secondary GBM samples through SAM analysis. We found 39 genes up-regulated in primary GBM in comparison with secondary GBM and 30 genes up-regulated in secondary GBM in comparison with primary GBM (Fig. 1C; Supplementary Table S3). Because secondary GBM progresses from lower grades (i.e., DA and AA), it is likely that these tumors share an expression profile with DA and AA as against primary GBM. Thus, to obtain the expression profile specific to progressive astrocytoma (DA, AA, and secondary GBM) as against primary GBM (*de novo* GBM), we compared the data obtained from 15 samples belonging to progressive astrocytoma (4 DA, 5 AA, and 6 secondary GBM) and 10 samples belonging to primary GBM through SAM analysis. We found 20 genes up-regulated in progressive astrocytoma as against primary GBM and 45 genes up-regulated in primary GBM as against progressive astrocytoma (Fig. 1D; Supplementary Table S4).

GADD45α and FSTL1 are GBM-specific markers. Genes that are specifically up-regulated in GBM samples (see above) included many novel genes in addition to genes that are reported by others. We have validated some of the relevant

genes by real-time quantitative reverse transcription-PCR. In concurrence with previously published reports, additional validation by real-time quantitative reverse transcription-PCR confirmed the GBM-specific expression of insulin-like growth factor binding protein 2 and collagen type-IVα2 (data not shown).

A novel gene up-regulated in primary GBM is GADD45α (DDIT1). We found >4-fold up-regulation of GADD45α transcripts in majority of primary GBM (62.80%; 22 of 35 with a median log 2 ratio of 2.30) and secondary GBM samples (50.00%; 10 of 20 with a median log 2 ratio of 2.35) as against 22.50% (7 of 31 with a median log 2 ratio of 0.20) among AA and 20.00% (1 of 5 with a median log 2 ratio of 0.80) among DA samples (P = 0.0106; Fig. 2A). We also analyzed the expression pattern of GADD45α by immunohistochemical staining (Fig. 3). Among primary and secondary GBM samples tested, 58.0% (7 of 12) and 50.0% (3 of 6) of them, respectively, showed strong positive cytoplasmic staining for GADD45α. The percentage positivity for GADD45α staining in AA (grade 3) and DA (grade 2) was found to be 14.0% (1 of 7) and 0.0% (0 of 3), respectively. The average percent positive tumor cells was found to be higher among primary GBM samples (30.0%), followed by 25.0% and 20.0% among secondary GBM and AA, respectively. Analysis of five normal brain samples showed glial cells to be negative for GADD45α staining.

Another interesting gene up-regulated in majority of GBM cases was *FSTL1*, which encodes a protein with similarity to follistatin, an activin-binding protein. The levels of FSTL1 transcripts were found to be increased >4-fold in majority of primary GBM (80.00%; 28 of 35 with a median log 2 ratio of 2.80) and secondary GBM samples (55.00%; 11 of 20 with a median log 2 ratio of 2.0) as against 12.90% (4 of 31 with a median log 2 ratio of 0.8) and 0.0% (0 of 5 with a median log 2 ratio of 0.8) among AA and DA samples, respectively, with P value reaching significance (P = 0.0042; Fig. 2B). Immunohistochemical analysis revealed that 85.0% (18 of 21) and 86.0% (9 of 15) of primary and secondary GBM, respectively, are positive for FSTL1 protein staining (Fig. 4). As expected, reduced positivity was found among AA (30.0%; 3 of 10) and DA (0.0%; 0 of 10). Staining of normal brain revealed glial cells to be negative for FSTL1 staining. The average percent positive tumor cells was found to be higher among primary GBM and secondary GBM (31% and 24%, respectively) as against only 13.0% among AA cases, suggesting that FSTL1 is expressed in very high levels among GBMs.

Identification of primary GBM-specific genes. Genes that were up-regulated in primary GBM identified through SAM analysis included many novel genes. In addition, some of the genes that have been reported previously by others were found to be validated as primary GBM-specific genes. These include chitinase 3-like 1 and Ras homologue gene family member C (data not
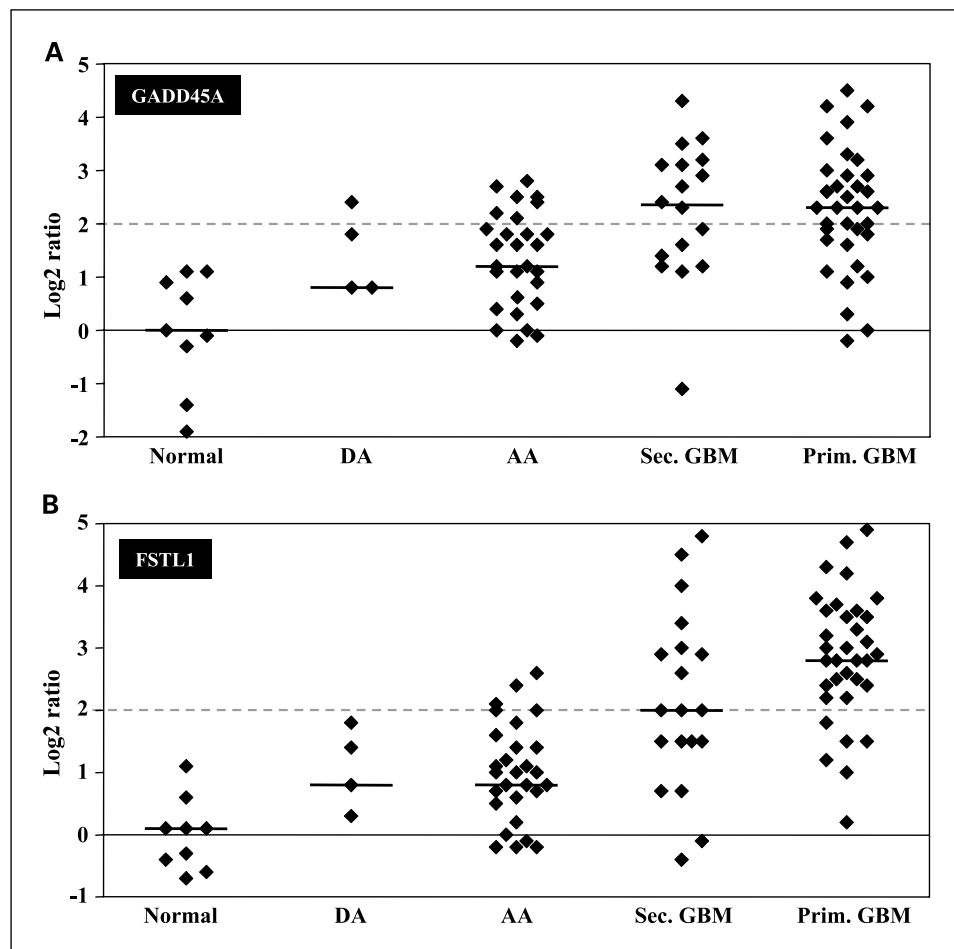
Fig. 2. Scatter plots of up-regulated genes in GBMs. Log 2–transformed gene expression ratios obtained from real-time quantitative PCR analysis are plotted for GADD45α (*A*) and FSTL1 (*B*). Each dot represents data derived from one sample. For each sample, fold change in gene expression is calculated over its mean expression in normal brain samples. Dotted line represents the fold change threshold (four-fold log 2 ratio = 2) used to calculate the percentage of regulated samples.

Fig. 3. Immunohistochemical validation of GADD45α overexpression in astrocytoma. Results of staining for GADD45α were as follows: sections from normal brain, negative for staining (*A*); DA, negative for staining (*B*); AA, negative for staining (*C*); secondary GBM, positive for staining (*D*); and primary GBM, positive for staining (*E*).
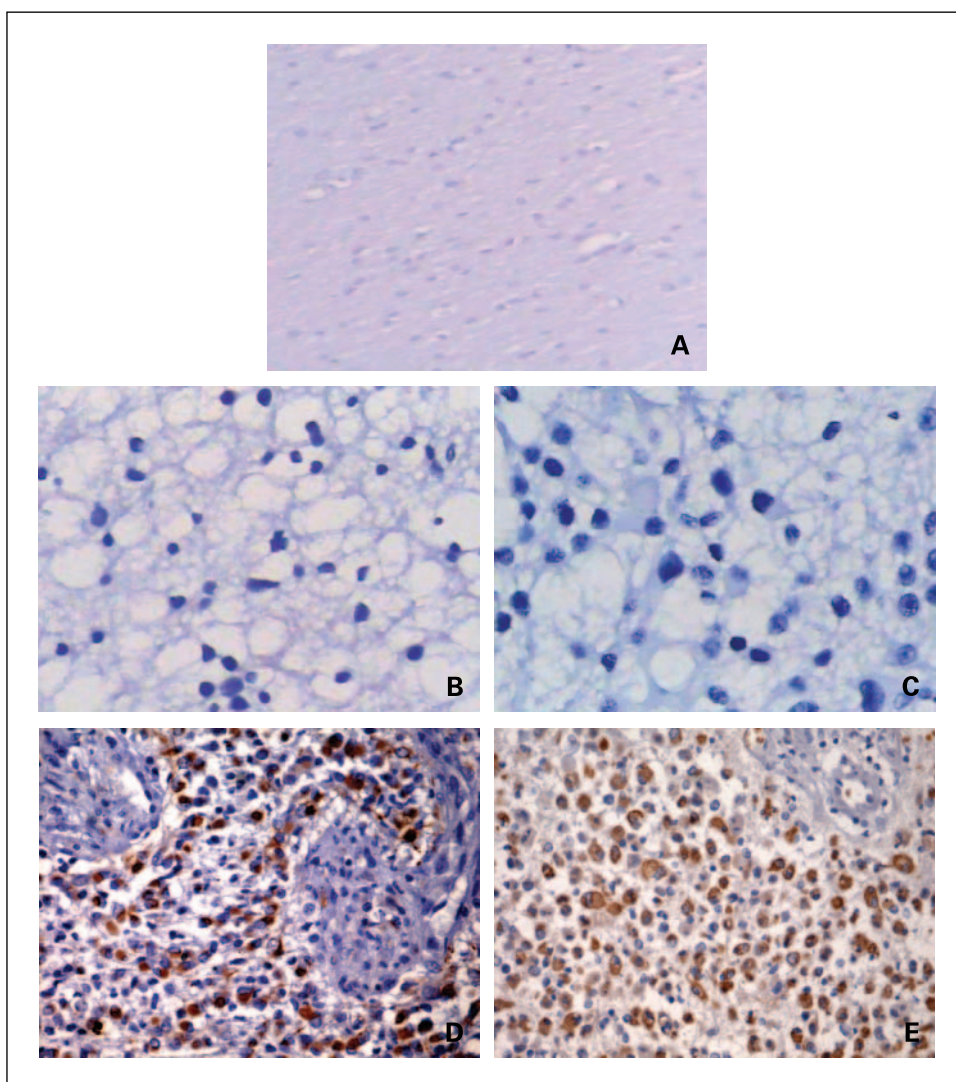
shown). For some of the novel genes, we have carried out real-time quantitative PCR and immunohistochemical validation wherever antibody was available on an independent set of tumor samples.

Manganese superoxide dismutase (SOD2), located in the mitochondria, was up-regulated >4-fold in majority of primary GBM (71.4%; 25 of 35 with a median log 2 ratio of 2.70) in comparison with secondary GBM (20.00%; 4 of 20 with a median log 2 ratio of 0.15), AA (16.10%; 5 of 31 with a median log 2 ratio of 0.20), and DA (0.0%; 0 of 5 with a median log 2 ratio of 1.20) with a *P* value of 0.001 (Fig. 5A). Adipocyte enhancer binding protein 1 (AEBP1) was up-regulated >4-fold in the majority of primary GBM (57.0%; 20 of 35 with a median log 2 ratio of 2.4) as against secondary GBM (40.0%; 8 of 20 with a median log 2 ratio of 1.00), AA (12.9%; 4 of 31 with a median log 2 ratio of -1), and DA (20.0%; 1 of 5 with a median log 2 ratio of 0.8), with a *P* value of 0.0269 (Fig. 5B).

Survival value of GADD45α and FSTL1. To analyze the survival value of GADD45α and FSTL1, we subjected a different set of 51 retrospective GBM cases where follow-up was available for expression of these markers. In GBM patients, although 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 (15–17). Therefore, for the purpose of multivariate analysis, we immunostained the sections to study the expression of p53 and EGFR to analyze the significance of their coexpression with GADD45 and FSTL1 with respect to patient survival.

In univariate analysis, the median survival of the group positive for GADD45α was 13 months compared with 7 months in the group that was negative (data not shown). However, this did not reach statistical significance ($P = 0.96$). Further, in multivariate analysis, this seemingly favorable effect of GADD45α positivity was retained, with a trend toward statistical significance ($P = 0.051$; odds ratio, -3.711; Fig. 6A). These results indicate that cytoplasmic overexpression of GADD45α in GBMs probably confers a survival advantage to these patients.

With respect to correlation of expression of FSTL1 with survival, it independently did not correlate with survival. However, its coexpression with p53 was associated with a poorer survival. By univariate analysis, the median survival of the group

that coexpressed p53 and FSTL1 was 8 months, compared with 13 months in the group that did not coexpress the two markers ($P = 0.04$; Fig. 6B). Similarly, by multivariate analysis, whereas FSTL1 expression by itself did not correlate with survival, its coexpression with p53 again was associated with poorer survival (Fig. 6C). These data put together suggest that expression of GADD45α and FSTL1 have prognostic value.

Discussion

Diffusely infiltrating astrocytomas are heterogeneous neoplasms and are currently being diagnosed and graded by well-defined histopathologic criteria (4). GBMs constitute the most malignant form of this group of astrocytoma. Despite the fact that several genetic alterations have been described in GBMs, they have not been very useful in prognostication or for therapeutic stratification (18). Recently, microarray-based expression profiling studies have revealed that molecular subclassification of malignant astrocytoma, particularly GBMs, could be of prognostic value (6). Therefore, identification of these molecular subclasses of GBMs could greatly facilitate

prognostication and our ability to develop effective treatment protocols.

We have used cDNA microarray–based expression profiling of 25 diffuse-infiltrating astrocytoma samples belonging to various grades to identify genes expressed in a grade-specific manner. We have identified several genes, whose expression is characteristic to particular grades of astrocytoma. Further, by using real-time quantitative PCR, we were able to confirm specific expression of many genes in GBM, particularly primary GBM. Similar to reported information in the literature, we were able to show GBM-specific expression of insulin-like growth factor binding protein 2 and collagen type-IVα2, and primary GBM-specific expression of chitinase 3-like 1 (CHI3L1/YKL40) and RhoC. More importantly, we were able to identify four novel markers: GADD45α and FSTL1 as GBM-specific markers, and SOD2 and AEBP1 as primary GBM-specific markers. The grade-specific expression of GAD45a and FSTL1 was also shown by immunohistochemical staining. In addition, survival analysis revealed that GADD45α and FSTL1 are prognostic markers as well.

We found GADD45α to be up-regulated in majority of GBM cases as against DA and AA. GADD45α is a member of a group of genes whose transcript levels are increased following stressful
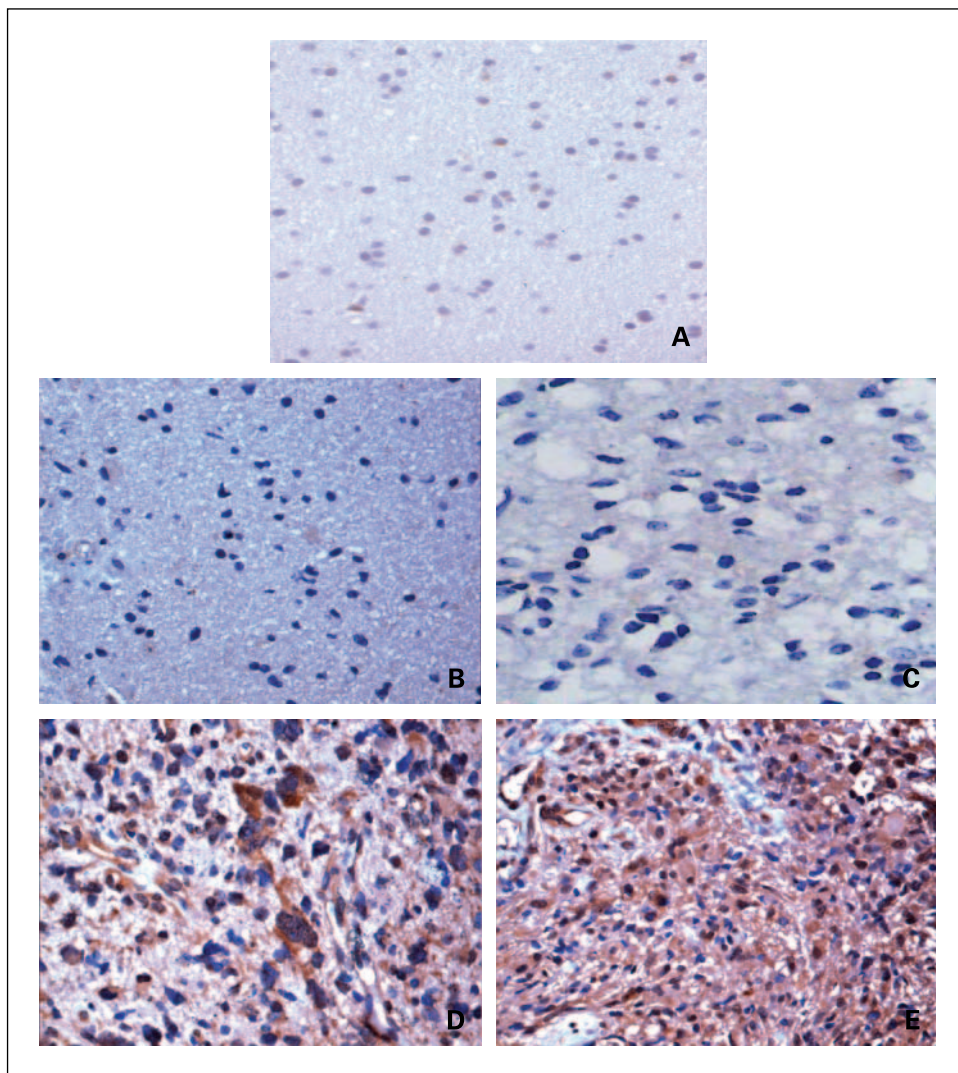
Fig. 4. Immunohistochemical validation of FSTL1 overexpression in astrocytoma. Results of staining for FSTL1 were as follows: sections from normal brain, negative for staining (*A*); DA, negative for staining (*B*); AA, negative for staining (*C*); secondary GBM, positive for staining (*D*); and primary GBM, positive for staining (*E*).

Fig. 5. Scatter plots of up-regulated genes in pGBMs. Log 2–transformed gene expression ratios obtained from real-time quantitative PCR analysis are plotted for SOD2 (*A*) and AEBP1 (*B*). Each dot represents data derived from one sample. For each sample, fold change in gene expression is calculated over its mean expression in normal brain samples. Dotted line represents the fold change threshold (four-fold log 2 ratio = 2) used to calculate the percentage of regulated samples.
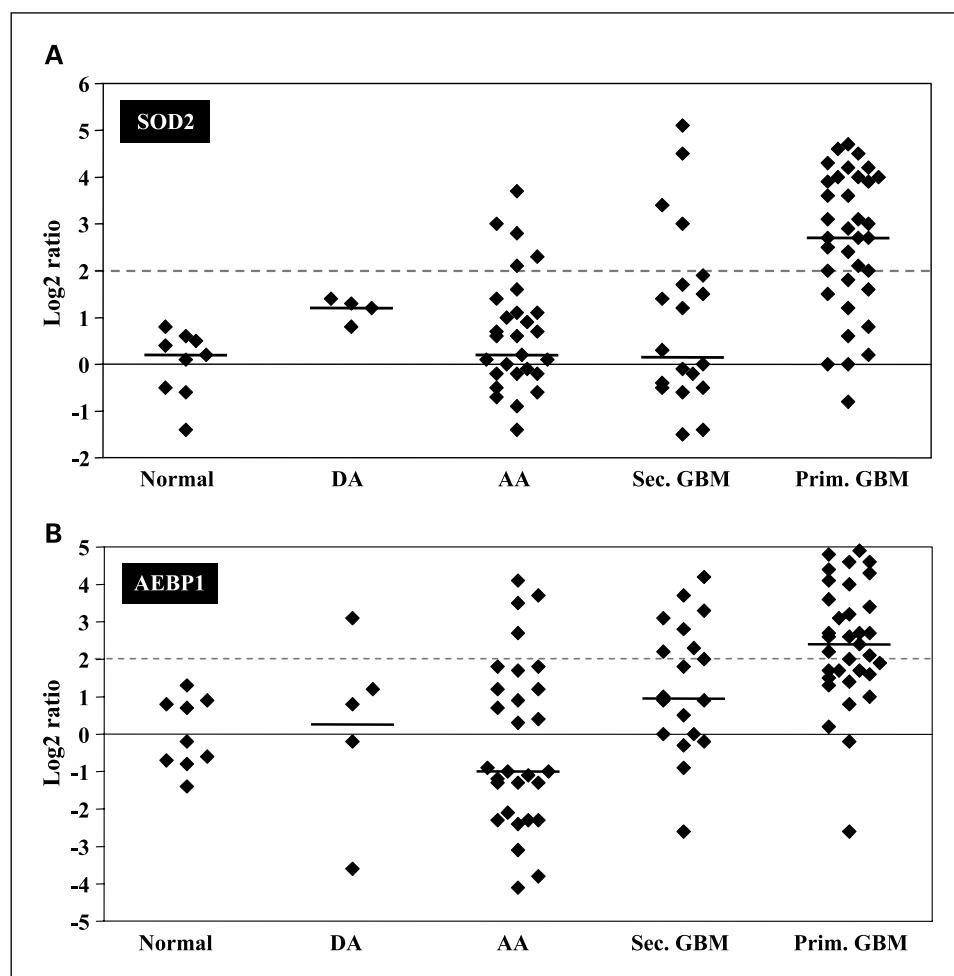
growth arrest conditions and treatment with DNA-damaging agents (19). Under stress conditions like DNA damage, GADD45α protein is known to induce growth arrest and apoptosis by activating p38/c-Jun-NH$_2$-kinase pathway via MTK1/MEKK4 kinase (20). Further, it was found that 13.6% of invasive ductal carcinomas of the pancreas had mutation in GADD45α, and the expression of Gadd45α, combined with that of p53, significantly affected the survival of patients with resectable invasive ductal carcinomas of the pancreas (21). Given this information, it was surprising to see the elevated RNA and protein levels of GADD45α in majority of primary GBMs compared with normal brain and lower grades of astrocytoma. However, immunohistochemical analysis revealed that GADD45α was primarily located in cytoplasm. GADD45α being a nuclear protein, cytoplasmic sequestration might reflect a functionally inactive protein. Thus, one would consider that cytoplasmic localization of GDD45α in GBM might lead to functionally inactive protein and poor patient survival. In contrast to this notion, cytoplasmic overexpression seems to provide survival advantage to the GBM patients as per our results. One way to explain this paradox is as follows: GADD45α overexpression in human fibroblast is shown to cause cell cycle arrest (22). However, there are conflicting reports that GADD45 proteins induce as well as inhibit apoptosis under varying conditions (23). Interestingly, it was found that GADD45 proteins bind to MTK1 and activate its

kinase activity. Further, it was found that expression of GADD45 genes induces p38/c-Jun-NH$_2$-kinase activation and apoptosis, which can be partially suppressed by coexpression of a dominant negative MTK1 mutant (20). There is also a report suggesting that GADD45 proteins interact with p38 kinase directly during stress-induced signaling and is believed to play an ancillary function to GADD45 interactions with MTK1 (24). This set of data clearly furnish evidence for a function of GADD45 proteins in cytoplasm as well. It is proposed that the outcome of GADD45-mediated activation of p38 and c-Jun-NH$_2$-kinase, resulting in cell cycle arrest, DNA repair and cell survival, or apoptotic cell death, is determined by the nature of the stress stimulus, its magnitude, and the levels of GADD45 proteins in cytoplasm to bring out effective interactions. Although our results indicate that increased expression of GADD45α levels correlate with GBM, a subgroup of patients having higher levels of GADD45α protein in tumors had good prognosis. Perhaps the very high cytoplasmic levels of GADD45α might reflect an efficient activation of p38/c-Jun-NH$_2$-kinase pathway leading to apoptosis of the tumor cells, thus providing survival advantage to these patients.

FSTL1 or follistatin-related protein (*FRP*), a gene that encodes a protein with similarity to follistatin, an activin-binding protein, is found to be induced four-fold in majority of primary GBMs in this study. *FSTL1* contains an FS module, a follistatin-like sequence containing 10 conserved cysteine residues and is
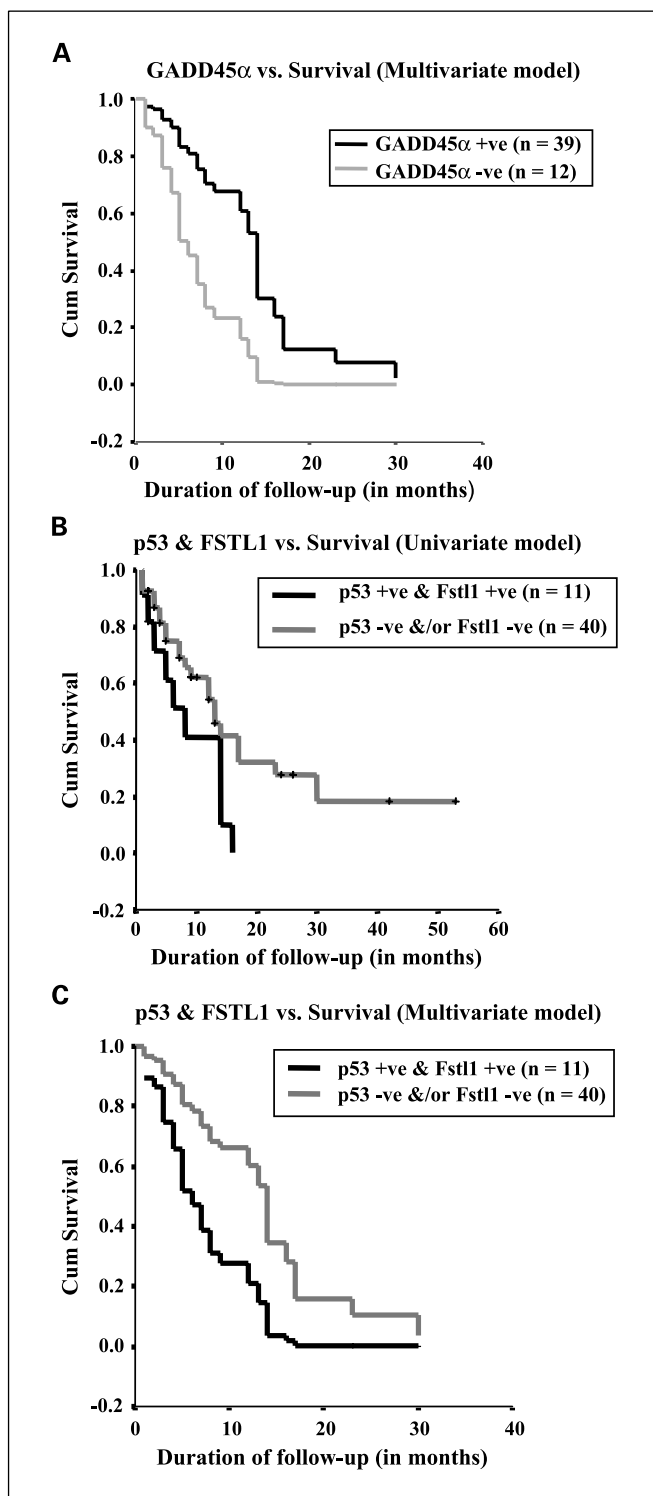
Fig. 6. Survival of patients with GBM. Kaplan-Meier survival estimates for 51 GBM samples are calculated for p53, EGFR1, GADD45α, and FSTL1 staining. *A,* survival curves for the groups positive and negative for GADD45α, in multivariate analysis. The cases that were positive for GADD45α (*black line*) had a better survival than the cases that were negative (*gray line*), with a trend toward statistical significance. *B,* survival curves for the groups positive and negative for coexpression of p53 and FSTL-1 in univariate analysis. The median survival for the group positive for both markers was 8 mo (*black line*) compared with 13 mo for the group negative for both or either markers (*gray line*). *C,* survival curves for the groups positive and negative for coexpression of p53 and FSTL-1, in multivariate analysis. The group positive for both markers (*black line*) had poor survival compared with the group negative for both or either markers (*gray line*).

thought to be an autoantigen associated with rheumatoid arthritis (25). *FSTL1* was originally identified as TSC-36 (TGFβ stimulated clone 36; ref. 26) and an estrogen-regulated gene (27). TSC-36 expression has been shown to inhibit growth of lung cancer cells (28) and proliferation of migration in vascular smooth muscle cells (29). In contrast to these observations, FSTL1 has been shown to promote metastasis in prostate cancer cells (30). FSTL3, a member of a folllistatin-like family of genes, has been recently shown to promote proliferation of breast cancer cells (31). However, the actual role of FSTL1 in human cancers, particularly astrocytomas, is largely unknown. Interestingly, in addition to GBM-specific overexpresison, coexpression of FSTL1 with p53 was found to be associated with poor survival both in univariate and multivariate analysis.

SOD2, located in the mitochondria, was up-regulated in primary GBM in comparison with secondary GBM, AA, and DA. The *SOD2* gene encodes an intramitochondrial free radical scavenging enzyme, which is the first line of defense against superoxide produced as a byproduct of oxidative phosphorylation. This enzyme catalyzes the dismutation of the superoxide anion to H_2O_2, which is converted to water by catalases and peroxidases. SOD2 was initially thought to be a tumor suppressor protein as its overexpression inhibited cellular proliferation and reduced levels have been seen in many cancer cell lines compared with their counterparts (32). However, many recent reports showed high levels of SOD2 in thyroid cancer, central nervous system tumors, and acute leukemia, and also correlated with high metastatic gastric and colorectal carcinoma (33). Further, it has been shown that increased metastatic potential of *SOD2*-overexpressing tumor cell lines may be attributed to their enhanced matrix metalloproteinase production (34). In view of the above, perhaps the higher expression of SOD2 in GBM also contributes to increased invasive potential of these tumors.

AEBP1 was up-regulated >4-fold in majority of primary GBM as against secondary GBM, DA, and AA. AEBP1, a transcriptional repressor with carboxypeptidase activity, binds to a regulatory sequence, adipocyte enhancer 1, located in the proximal promoter region of the *adipose P2* gene, which encodes the adipocyte fatty acid–binding protein (35). AEBP1 has been shown to interact with the tumor suppressor protein PTEN and inhibit its function, thus promoting cell proliferation (36). Further, AEBP1 levels were found to be higher in proliferative preadipocytes whereas its expression was abolished in terminally differentiated, nonproliferative adipocytes (37). Thus, overexpression of AEBP1 in primary GBM is likely to promote proliferation.

Thus, we have identified and validated a set of novel candidate genes whose expression at the transcript level is associated with the WHO malignancy grade of diffusely infiltrating astrocytomas. We have also identified the prognostic value of some of these markers. The knowledge about their possible roles in glioma development is still limited. Therefore, further studies are required to more precisely characterize the functional significance of these genes in glioma progression as well as their potential application for glioma grading and the assessment of prognosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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