A DNA Methylation Prognostic Signature of Glioblastoma: Identification of NPTX2-PTEN-NF-κB Nexus

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Abstract
Glioblastoma (GBM) is the most common, malignant adult primary tumor with dismal patient survival, yet the molecular determinants of patient survival are poorly characterized. Global methylation of GBM samples (our cohort; n = 44) using high-resolution methylation microarrays was carried out. Cox regression analysis identified a 9-gene methylation signature that predicted survival in GBM patients. A risk-score derived from methylation signature predicted survival in univariate analysis in our and The Cancer Genome Atlas (TCGA) cohort. Multivariate analysis identified methylation risk score as an independent survival predictor in TCGA cohort. Methylation risk score stratified the patients into low-risk and high-risk groups with significant survival difference. Network analysis revealed an activated NF-κB pathway association with high-risk group. NF-κB inhibition reversed glioma chemoresistance, and RNA interference studies identified interleukin-6 and intercellular adhesion molecule-1 as key NF-κB targets in imparting chemoresistance. Promoter hypermethylation of neuronal pentraxin II (NPTX2), a risky methylated gene, was confirmed by bisulfite sequencing in GBMs. GBMs and glioma cell lines had low levels of NPTX2 transcripts, which could be reversed upon methylation inhibitor treatment. NPTX2 overexpression induced apoptosis, inhibited proliferation and anchorage-independent growth, and rendered glioma cells chemosensitive. Furthermore, NPTX2 repressed NF-κB activity by inhibiting AKT through a p53-PTEN-dependent pathway, thus explaining the hypermethylation and downregulation of NPTX2 in NF-κB-activated high-risk GBMs. Taken together, a 9-gene methylation signature was identified as an independent GBM prognosticator and could be used for GBM risk stratification. Prosurvival NF-κB pathway activation characterized high-risk patients with poor prognosis, indicating it to be a therapeutic target. Cancer Res; 73(22); 6563–73. ©2013 AACR.

Integration of Systems and Technologies

Introduction
The grade IV glioma (glioblastoma; GBM) is the most common, malignant primary brain tumor in adults. The prognosis remains poor with median survival ranging from 12 to 15 months in spite of improvements in treatment protocol (1). Genetic heterogeneity in GBM has been proposed to explain the limitations in the effectiveness of current therapies, which necessitates the need for prognostic gene signature (2). Many genetic and epigenetic alterations as well as expression of some genes have been correlated with poor or better prognosis (3–7). In addition, molecular biomarkers like methyl guanine methyl transferase (MGMT) promoter methylation, isocitrate dehydrogenase 1 (IDH1) mutation status, and Glioma-CpG Island Methylator Phenotype (G-CIMP) have been identified as GBM prognostic indicators (1, 8, 9).

Expression profiling studies have identified mRNA and microRNA signatures for classification and prognosis in GBM (10–14). However, none of the gene signatures have been translated into clinics, suggesting the need for more robust prognostic gene signature panels. Here, we have identified and validated a 9-gene methylation signature for GBM prognostication. Multivariate analysis with all known prognostic markers and signatures for GBM identified our methylation signature to be an independent GBM prognostic indicator. Furthermore, an activated NF-κB pathway was found to be associated with poor prognosis. We also show that neuronal pentraxin II (NPTX2), a component of methylation signature with risky methylation, inhibits cell growth by antagonizing NF-κB pathway through p53-PTEN activation, thus connecting the methylation signature to NF-κB pathway.

Materials and Methods

Plasmids and reporter constructs
pCMV-Entry/NPTX2 was obtained from Origene. CAPI3K (pCDNA3-CD2p110myc) was described earlier (15, 16). CAAKT (pCDNA MYR HA AKT1) was from Addgene (Plasmid 9008). PG13-Luc was described earlier (17). CAIKK, NF-κB-Luc,
PTEN-luc, shATM, and shATR plasmid constructs were obtained from Profs. I. Verma, K.N. Balaji, Dr. R.C.M. Simmen, Dr. Y. Shiloh, and Dr. T. De Lange, respectively.

**Cell lines and reagents**

Temozolomide, adriamycin, 5-aza-2’-deoxycytidine, MTT, and EscortIII transfection reagent were purchased from Sigma. Glioma cell lines U138, LN18, U343, LN229, U251, U87, T98G, U373 (all human glioma derived), 293, and C6 (Rat glioma derived) were obtained from the laboratory of Dr. A. Guha, University of Toronto, Toronto, Canada, and grown in DMEM medium. SVG cells were obtained from Dr. P. Seth, National Brain Research Center, New Delhi, and grown in MEM medium. The medium was supplemented with 10% FBS, penicillin, and streptomycin. No information is available about their authentication, although many common known mutations/alterations have been verified in our laboratory.

**Patient samples and clinical data**

Tumor samples used for study were obtained from patients who were operated at Sri Sathya 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. Tissues were bisected and one half was snap-frozen in liquid nitrogen and stored at –80°C until DNA/RNA isolation. The other half was fixed in formalin and processed for paraffin sections. These sections were used for the histopathologic grading of tumor and immunohistochemical staining.

**Network analysis**

To find out the key signaling pathways differently regulated between low-risk and high-risk patients, we compared gene expression profiles of The Cancer Genome Atlas (TCGA) data set (Agilent expression data, 17,814 genes) with gene expression differences between low-risk and high-risk groups by conducting t-test analysis with FDR correction. We identified 3,127 genes that were significantly differentially regulated between the high-risk and low-risk groups (1,983 genes upregulated and 1,144 genes were downregulated in high-risk compared to low-risk). To functionally interpret the role of differentially regulated genes with respect to patient survival, we carried out biological network analysis using MetaCore from Thomson Reuters, a pathway mapping tool. This tool builds biological networks from the input gene set and lists the biological processes (Gene Ontology terms) associated with each network. From the list of 3,127 differentially regulated genes, we chose a subset of 61 genes, with a log\_2 fold change >1.5 as the input for MetaCore software. Using variant shortest path algorithm with main parameters of relative enrichments of input genes and relative saturation of networks with canonical pathways, MetaCore identified 8 significant networks (Supplementary Table S3).

**NF-κB gene/protein association network and landscape analysis of gene expression**

To construct the NF-κB gene/protein interaction network, NF-κB target genes were downloaded (18). A total of 150 NF-κB target genes were used to generate an interaction network using STRING database (19) using “experiment,” “textmining,” and 0.700 confidence level as input option. To make a protein-protein interaction network, STRING curates publicly available databases for the information on direct and indirect functional protein interactions. The data file of protein-protein interaction network generated from STRING was then used in Medusa software (20) to construct a network. This network was then analyzed by ViaComplex software (21). ViaComplex plots the gene expression activity over the Medusa network topology. ViaComplex overlays expression data with the interaction network and constructs a landscape graph by distributing microarray signal with interaction network coordinates. For each gene, the average of each group (high-risk and low-risk) was plotted over the average of the same gene in normal brain and landscape was constructed.

**Other methods and additional information**

The details for additional clinical data, genomic DNA extraction, sodium bisulfite conversion, methylation array analysis, bisulfite sequencing, RNA isolation, real-time quantitative RT-PCR analysis, 5-Aza-2′-deoxycytidine treatment, transfection, colony formation assays, stable cell line generation, proliferation assay, cytotoxicity assay, CHIP assay, and PTEN activity ELISA are given in the Supplementary information.

**Statistical analysis**

To identify the CpGs whose methylation correlates with survival, methylation data, available for 44 patients, were considered for survival analysis. The probes with missing value imputed using k nearest neighbor algorithm using Imputation package in R. The assessment of survival correlation for all 27,578 probes was performed using BRB-ArrayTools survival analysis (BRB-ArrayTools is available without charge for non-commercial applications at http://linus.nci.nih.gov/BRB-ArrayTools.html). Firstly, for each CpG probe, univariate Cox proportional hazard regression was done and parametric \( P \) value was calculated. The survival time and censoring status were randomly assigned to each patient and univariate Cox proportional hazard regression was performed to re-estimate the \( P \) value for each probe. This step was repeated 10,000 times and permutation \( P \) value was calculated. To select the probes with maximum variation in \( b \) value, a standard deviation criteria of >0.2 was applied. Based on these criteria, we identified 9 probes that strongly correlated with survival.

The methylation risk score was calculated as follows. Using these 9 probes, we devised a formula, resulting in a methylation risk score that would combine their independent survival predicting capabilities (based on Cox regression coefficients derived from Cox proportional hazards analysis). Each patient was assigned a risk score that is a linear combination of \( \beta \) value of the nine selected probes weighted by their respective Cox regression coefficients.

The internal validation of the risk score was carried out using ROC analysis and subset analysis. Independent validation of risk score in other data sets, multivariate analysis, and additional details are given in the Supplementary Information.
Results

Identification and validation of a 9-gene prognostic methylation signature for GBM

With an aim to find methylation signature predictive of GBM survival, Cox proportional hazards regression was carried out by comparing methylation status of 27,578 CpGs and patient’s (our cohort; n = 44) survival data. The entire workflow as to how the methylation signature was identified, tested, and validated is shown in a schematic diagram (Fig. 1). We identified 9 probes whose methylation status correlated significantly with patients’ survival (Table 1 and Supplementary Materials). A methylation risk score calculated for each patient by combining the effect of each of the 9 genes using a risk score formula (Supplementary Materials) divided the patients into high-risk and low-risk groups with significant survival difference (median survival: 12 months vs. 23 months; Fig. 2A and Supplementary Table S1). The strength of methylation risk score in predicting patient survival was further confirmed by ROC curve analysis and subset analysis (Supplementary Fig. S1A and Supplementary Materials). The methylation risk score predicted survival in GBM dataset derived from TCGA cohort as well (Fig. 2B and Supplementary Fig. S1B). GBM tumors from low-risk group tend to have more methylation of NPTX2 whereas patients tend to have more methylation of NPTX2 compared to normal. Additional analysis revealed that all 9 genes, which form part of the methylation signature, are needed for prognostication (Supplementary Materials).

Multivariate regression analysis indicates 9-gene methylation signature is an independent prognosticator

Cox multivariate analysis with age, we found methylation risk score to be an independent predictor of GBM patient survival in our patient set (P = 0.001; HR = 1.267; B = 0.237). Additional multivariate analysis with various prognostic factors was carried out using TCGA dataset. Although MGMT promoter methylation (CpG probe ID #Cg02941816), IDH1 mutation, and Glioma-CpG Island Methylator Phenotype (G-CIMP) were found to be independent predictors when compared with age, an analysis that included all markers identified methylation risk score alone to be indicated by very short median survival (Supplementary Fig. S1B and S1C and Supplementary Materials).

The risk score distribution and a comparison of risk score with patient survival status among GBMs of our and TCGA cohort are shown (Supplementary Fig. S2A-S2D). Upon correlation of methylation status to patient survival, NPTX2 methylation was found to be risky, whereas methylation of the remaining 8 genes was found to be protective (Fig. 2D, Table 1, and Supplementary Fig. S2E). GBM tumors from high-risk patients tend to have more methylation of NPTX2 whereas tumors from low-risk group tend to have more methylation of the remaining genes. Additional analysis revealed that all 9 genes, which form part of the methylation signature, are needed for prognostication (Supplementary Materials).
Table 1. Details about 9-gene methylation signature that predicts survival in GBM

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Symbol(CpG ID)</th>
<th>Name</th>
<th>P-value</th>
<th>HR Low risk</th>
<th>High risk Difference</th>
<th>Low risk</th>
<th>High risk</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NPTX2 (cg12799895)</td>
<td>Neuronal pentraxin 2</td>
<td>0.0043</td>
<td>1.91</td>
<td>6.783</td>
<td>0.15</td>
<td>0.5</td>
<td>0.35</td>
</tr>
<tr>
<td>2</td>
<td>LIMD1 (cg04037228)</td>
<td>LIM domain-containing protein 1</td>
<td>0.0018</td>
<td>2.62</td>
<td>0.073</td>
<td>0.51</td>
<td>0.3</td>
<td>0.21</td>
</tr>
<tr>
<td>3</td>
<td>MBD1 (cg13603171)</td>
<td>DBH-like monooxygenase</td>
<td>0.0037</td>
<td>2.86</td>
<td>0.057</td>
<td>0.57</td>
<td>0.3</td>
<td>0.21</td>
</tr>
<tr>
<td>4</td>
<td>IRF6 (cg23283495)</td>
<td>Interferon regulatory factor 6</td>
<td>0.0055</td>
<td>2.23</td>
<td>0.107</td>
<td>0.51</td>
<td>0.33</td>
<td>0.18</td>
</tr>
<tr>
<td>5</td>
<td>FBP1 (cg17814481)</td>
<td>Fructose-1,6-bisphosphatase 1</td>
<td>0.0067</td>
<td>2.49</td>
<td>0.083</td>
<td>0.51</td>
<td>0.3</td>
<td>0.21</td>
</tr>
<tr>
<td>6</td>
<td>FMOD (cg17978476)</td>
<td>Fibromodulin</td>
<td>0.0067</td>
<td>2.56</td>
<td>0.082</td>
<td>0.69</td>
<td>0.46</td>
<td>0.23</td>
</tr>
<tr>
<td>7</td>
<td>LAD1 (cg25947945)</td>
<td>Ladinin-1</td>
<td>0.0081</td>
<td>2.6</td>
<td>0.074</td>
<td>0.66</td>
<td>0.3</td>
<td>0.36</td>
</tr>
<tr>
<td>8</td>
<td>RBPSUH (cg21835643)</td>
<td>Recombining binding protein 5</td>
<td>0.0084</td>
<td>2.5</td>
<td>0.091</td>
<td>0.46</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>9</td>
<td>GSTM5 (cg04987894)</td>
<td>Glutathione S-transferase Mu 5</td>
<td>0.0084</td>
<td>2.32</td>
<td>0.098</td>
<td>0.61</td>
<td>0.33</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Network analysis reveals an activated NF-κB pathway in high-risk patients

We hypothesized that the 9 genes, which form part of the methylation signature, might modify the global gene expression consequently, altering some key signaling pathways differently between low-risk and high-risk patients, thus explaining the difference in their survival. Biological network analysis using differentially regulated genes between low-risk and high-risk groups derived from TCGA (Supplementary Table S4) identified 8 significant networks (Supplementary Table S5). Careful analysis revealed an enrichment of NF-κB pathway members in these networks. As NF-κB pathway is associated with oncogenesis, tumor progression, and chemoresistance in many cancers (24), we tested whether the NF-κB pathway is differentially activated between low-risk and high-risk groups. First, NF-κB gene/protein interaction network was constructed using the STRING database with 150 NF-κB target genes as input (Supplementary Fig. S4A). Landscape analysis of the NF-κB interaction network using GBM patient gene expression data of low-risk versus normal and high-risk versus normal from the TCGA cohort revealed that NF-κB interaction network is very minimally upregulated in low-risk (as seen by very little red and orange color in z-axis plot; Fig. 3A). However, the GBM tumors from the high-risk group showed much higher level of NF-κB pathway activation as evidenced by the bright red and orange color in z-axis plots (Fig. 3B), suggesting the possibility that higher activation of NF-κB pathway in high-risk GBM may be responsible for their poor treatment response and less survival. In good correlation, pretreatment with NF-κB inhibitor made only those cell lines, wherein NF-κB is already activated, more chemosensitive but not in other cell lines, wherein NF-κB activation is very low (Fig. 3C and D and Supplementary Fig. S4B–S4D). Furthermore, silencing of p65 subunit of NF-κB rendered glioma cells more sensitive to chemotherapy (Fig. 3E).
GBM Prognostic DNA Methylation and NPTX2-NF-κB Link

It is interesting to note that several NF-κB target genes with antiapoptotic, prometastasis, prosurvival, and proinflammatory functions were upregulated only in GBM tumors from high-risk patients (Supplementary Table S6), but were either downregulated or not changed in tumors from low-risk patients, when compared to normal, thus suggesting the potential roles of these set of genes in imparting chemoresistance. Silencing each of the 7 selected target genes identified interleukin (IL)-6 and intercellular adhesion molecule (ICAM)-1 as the key NF-κB target genes to be the mediators of chemoresistance, as their downregulation rendered glioma cells sensitive to chemotherapy consistently (Fig. 3F).

Association of NF-κB with high-risk GBM was further validated by means of an indirect approach using REMBRANDT (25) and Phillips (26) GBM datasets, which had both gene expression data and patients survival information (Supplementary Materials). The expression levels of the same set of genes (n = 3,127) that are differentially regulated between low-risk and high-risk groups in TCGA dataset (Supplementary Table S4) was able to divide these datasets into 2 clusters with significant survival difference and NF-κB activation (Supplementary Figs. S5A and S5B and S6A–S6E). These results overall validate the survival prediction by our methylation signature and confirm the association of activated NF-κB pathway with poorer prognosis in high-risk GBMs.

**NPTX2 is methylated in GBM and has a growth inhibitory function**

NPTX2 gene, a component of methylation signature, was particularly interesting as its methylation is risky. Bisulfite sequencing validated the hypermethylation in GBMs and glioma cell lines as against normal brain samples (Fig. 4A). NPTX2 transcripts were found to be downregulated in GBM samples and glioma-derived cell lines compared to normal brain samples.

Figure 2. Risk stratification of GBM based on methylation risk score. A and B, Kaplan–Meier graphs of (25th percentile used as cut-off) our patient cohort and TCGA cohort, respectively. C, Kaplan–Meier survival curve among proneural low-risk (green), proneural high-risk (red), and all nonproneural (blue) GBM tumors. D, heat map of methylation status (β value) of 9 genes from methylation signature (our patient set; n = 44). E, heat map of methylation status (β value) of 9 genes from methylation signature (TCGA set; n = 65) for whom G-CIMP status, glioma gene expression subtypes, IDH1 mutation status, and methylation β values were available. D and E, the rows represent genes and columns represent patients. A color code with yellow and blue indicating high and low methylation, respectively, was used. The red line divides patients into low-risk and high-risk groups. E, above the heat map, each sample is additionally color-coded as described in the KEY.

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and methylation inhibitor, 5-aza-2'-deoxycytidine, treatment resulted in reexpression of NPTX2 transcripts (Fig. 4C). NPTX2 transcripts levels inversely correlated with NPTX2 promoter methylation (Supplementary Fig. S7B). Further investigation revealed that exogenous overexpression of NPTX2 suppressed colony formation (Fig. 4D). NPTX2 stable clone of U343 glioma cell line, which overexpresses NPTX2 transcripts and protein (Supplementary Fig. S7C and S7D), showed increased apoptosis, reduced proliferation, decreased anchorage-independent growth, and increased chemosensitivity compared to a vector stable (Fig. 4E–H).

Furthermore, 5-aza-2'-deoxycytidine pretreatment sensitized U251 glioma cells to chemotherapy in an NPTX2-dependent manner, as simultaneous silencing of NPTX2 resulted in chemoresistance (Fig. 4I and Supplementary S7E).

NPTX2 inhibits NF-κB pathway through p53-PTEN-PI3K-AKT–dependent manner

Next upon investigation of the connection between NPTX2 and NF-κB pathway, we found NPTX2 overexpression inhibited expression from NF-κB luciferase reporter in glioma cell lines (Fig. 5A), sequence-specific DNA-binding (Supplementary Fig. S8A), nuclear translocation of NF-κB subunit (p65; Supplementary Fig. S9A–S9C), and repressed 6 of the 7 key NF-κB target genes significantly (Supplementary Fig. S8B). We also show that NPTX2 inhibition of NF-κB could be abrogated by coexpression of constitutively active forms of PI3 kinase, AKT, and IKKa (Fig. 5B). Furthermore, NPTX2 overexpression activated PG13-Luc, a p53-dependent reporter, efficiently in U343 and U87 cells, in a concentration-dependent manner in 293 cells as well as in a p53-dependent manner as it failed to activate in p53 mutant U251 cells (Fig. 5C) and p53 silenced cells (Supplementary Fig. S10A and S10D). NPTX2 overexpression also induced p53 protein levels (but not transcript levels) and its targets, p21 and PTEN protein levels (Fig. 5E and Supplementary Fig. S8C). Aptly, NPTX2-activated expression from PTEN promoter reporter construct in U343 and U87 cells, as well as in a p53-dependent manner in 293 cells as well as in a p53-dependent manner as it failed to activate PTEN reporter in p53 mutant U251 cells (Fig. 5D) and p53 silenced cells (Supplementary Fig. S10B). NPTX2 also induced PTEN transcript (Supplementary Fig. S8D). More importantly, NPTX2 overexpression resulted in

Table 2. Multivariate Cox regression analysis of methylation risk score and other prognostic markers using TCGA cohort

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. of patients</th>
<th>HR</th>
<th>B (coefficient)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Univariate analysis TCGA dataset</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>205</td>
<td>1.033</td>
<td>0.033</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MGMT</td>
<td>205</td>
<td>0.226</td>
<td>−1.325</td>
<td>0.004</td>
</tr>
<tr>
<td>IDH1</td>
<td>197</td>
<td>0.441</td>
<td>−0.916</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>G-CIMP</td>
<td>205</td>
<td>0.298</td>
<td>−1.211</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methylation risk score</td>
<td>205</td>
<td>1.146</td>
<td>0.136</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>II. Multivariate analysis with TCGA dataset</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>205</td>
<td>1.027</td>
<td>0.027</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Methylation risk score</td>
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<td>1.109</td>
<td>0.103</td>
<td>0.005</td>
</tr>
<tr>
<td>MGMT</td>
<td>205</td>
<td>0.320</td>
<td>−1.138</td>
<td>0.013</td>
</tr>
<tr>
<td>Methylation risk score</td>
<td>205</td>
<td>1.14</td>
<td>0.131</td>
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<tr>
<td>IDH1</td>
<td>65</td>
<td>0.476</td>
<td>−0.472</td>
<td>0.316</td>
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<td>1.026</td>
<td>0.029</td>
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<td>Methylation risk score</td>
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<td>0.400</td>
<td>−0.916</td>
<td>0.215</td>
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<tr>
<td>III. Multivariate analysis of all the markers in TCGA dataset</td>
<td></td>
<td></td>
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<tr>
<td>Age</td>
<td>65</td>
<td>NA</td>
<td>NA</td>
<td>0.275</td>
</tr>
<tr>
<td>MGMT</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.064</td>
</tr>
<tr>
<td>IDH1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.322</td>
</tr>
<tr>
<td>G-CIMP</td>
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<td>NA</td>
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<td>Methylation risk score</td>
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<td>0.223</td>
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<td>III. Multivariate analysis of all the markers in TCGA dataset (except IDH1)</td>
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<td></td>
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<tr>
<td>Age</td>
<td>205</td>
<td>1.026</td>
<td>0.026</td>
<td>0.001</td>
</tr>
<tr>
<td>MGMT</td>
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<tr>
<td>G-CIMP</td>
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<td>0.904</td>
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<tr>
<td>Methylation risk score</td>
<td>1.100</td>
<td>0.096</td>
<td></td>
<td>0.038</td>
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</tbody>
</table>

*Multivariate analysis that included all markers was carried out in 2 ways as IDH1 mutation was available only for 65 patients among 205 patients who had CpG methylation values for all 9 probes that formed part of methylation signature. The analysis that included MGMT, IDH1, G-CIMP, age, and methylation risk score was carried out with 65 patients, whereas the analysis involving MGMT, G-CIMP, age, and methylation risk score (without IDH1) was carried out with 205 patients.*/
4.8-fold increase in p53 occupancy in PTEN promoter (Fig. 5F), significant activation of PTEN phosphatase activity (3–3.5 fold) as seen by increased PI(3)P levels (Fig. 5G), and an efficient inhibition of AKT as seen by reduced pAKT levels (Fig. 5E). NPTX2 overexpression also decreased pXb and increased total IkB levels (Supplementary Fig. S9D). In good correlation, NPTX2 failed to inhibit NF-xB promoter in p53 mutant U251 cells (Fig. 5A), p53 silenced cells (Supplementary Fig. S10C), and in PTEN null U87 cells (Fig. 5A). These results together suggest that NPTX2 is a glioma growth inhibitory gene, which can modulate chemosensitivity, and its antiproliferative functions may involve inhibition of NF-xB pathway through p53-dependent PTEN activation, leading to inhibition of PI3K-AKT-IKKα signaling.

Both ATM and ATR have been shown to have overlapping and independent functions in phosphorylation and subsequent activation of p53 during cellular genotoxic stress (27). To define the role of ATM/ATR proteins in NPTX2 activation of p53, the ability of NPTX2 to activate p53-dependent reporter activity was measured in either ATM or ATR silenced cells. Interestingly, we found that NPTX2 activation of p53 activity was compromised significantly in ATM silenced cells (Supplementary Fig. S10E and S10F) but not in ATR silenced cells (data not shown).

Discussion
In this study, using a cohort of patients of newly diagnosed GBM, treated uniformly and followed up prospectively, we...
have identified a 9-gene methylation signature using Cox proportional hazards model that can predict survival. Furthermore, the methylation signature was validated in independent cohorts. Multivariate analysis using TCGA data set with all known prognostic markers, gene signatures, and microRNA signature identified methylation signature as an independent GBM prognostic indicator. Network analysis revealed an association between activated NF-κB pathway and high-risk patients, which may explain the short survival of high-risk patients. Inhibition of NF-κB pathway made glioma cell lines with activated NF-κB pathway alone sensitive to chemotherapy. NFPTX2, a gene with risky methylation, was validated for its methylation and growth inhibitory functions and was found to inhibit NF-κB activity through its ability to induce PTEN in a p53-dependent manner. Thus, the NFPTX2-p53-PTEN pathway connected the methylation signature to NF-κB pathway.
The strength of our methylation signature is that multivariate analysis with all known prognostic markers and signatures identified it as an independent predictor of GBM survival. Although low-risk group showed enrichment of patients with IDH1 mutation, proneural gene expression subtype and G-CIMP positivity (8), methylation signature alone was identified as an independent survival predictor. Furthermore, comparisons with the 9 gene (22) and 10-microRNA panels (14) also identified our methylation signature as an independent survival predictor. Thus, it seems that our 9-gene methylation signature is novel, robust, specific, and the only independent predictor of survival.

To provide biological insight for survival prediction by methylation signature, with the use of network analysis, our study identified specific activation of NF-κB pathway in high-risk group from TCGA dataset and was further validated in REMBRANDT and Phillips datasets. Although the association between NF-κB pathway activation and GBM aggressiveness and chemoresistance as well as NF-κB as a therapeutic target is known (24, 28), our study clearly shows that NF-κB pathway inhibition could sensitize only those glioma cells having activated NF-κB pathway to chemotherapy.

Furthermore, we found IL-6 and ICAM1, which are upregulated in glioma cells having activated NF-κB and chemoresistance, and prognosis in many cancers (29–32).
This raises the potential use of targeting NF-κB pathway in treatment protocol specifically for those patients belonging to high-risk group.

Additional analysis of methylation signature and survival identified NPTX2 as a very important gene as its methylation is found to be risky with a higher methylation in high-risk groups. NPTX2 has been shown to be methylation silenced in pancreatic cancer, shown to induce BAX, possibly involving p53 activation, and shown to downregulate cyclin D1, leading to cell-cycle arrest and apoptosis (33). Our study shows that NPTX2 is methylation silenced in GBMs, could be reexpressed by methylation inhibitors and overexpression inhibits cell proliferation, anchorage-independent growth, and reexpression sensitizes glioma cells to chemotherapy. High levels of NPTX2 mRNA and protein have been reported in neuronal cells and in subpopulation of glial cells in normal brain (34). This may explain the reduced methylation and the consequent expression of NPTX2 in low-risk group, which is enriched for proneural gene expression type. Our work also shows that NPTX2 overexpression inhibits NF-κB activity, suggesting that its functions may involve activating or inhibiting signaling pathways, which are modulators of NF-κB pathway. p53 is known to activate PTEN, leading to inhibition of PI3K-AKT pathway (35, 36). In our study, we show that NPTX2 activates PTEN at the level of transcription in a p53-dependent manner, leading to PI3 kinase inhibition. Furthermore, we also show that NPTX2, through its ability to activate PTEN, inhibits AKT, which has been shown to be a key upstream activator of NF-κB pathway (37, 38). Although NPTX2 expression induces p53 protein level without a change in its transcript levels and may involve ATM, the exact mechanism at present remains to be investigated. These results suggest that NPTX2 inhibition of NF-κB may involve p53-PTEN activation, leading to PI3K-AKT-IKKα inhibition. Furthermore, it explains why NPTX2 is methylation silenced in high-risk group wherein NF-κB pathway is activated.

Thus, our study uncovers a novel pathway (through NPTX2) of a complex interaction network between differential methylation of 9 genes and alteration of global gene regulation, leading to modulation of signaling, in particular NF-κB pathway, between low- and high-risk groups. More importantly, this work identifies the 9-gene methylation signature as an independent GBM prognostic signature. It can be used to stratify the GBM patients into low-risk and high-risk groups, which would facilitate individualized therapeutic modality. Mechanistic investigation identified activated NF-κB signaling as the potential cause of poor prognosis in high-risk patients, suggesting NF-κB as a therapeutic target in particular for patients who do not respond to current treatment protocols.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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