The NPTX2-PTEN-NFkB nexus is an essential component of a prognostic DNA methylation signature of glioblastoma

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Abbreviations: GBM –Glioblastoma, NFkB – Nuclear factor kappa light polypeptide gene enhancer in B cells; NPTX2 - neuronal pentraxin II, PTEN - Phosphatase and Tensin Homolog, PI3 kinase - Phosphatidylinositol 3-kinase

The authors disclose no potential conflicts of interest
Abstract

Glioblastoma is the most common, malignant adult primary tumor with dismal patient survival, yet the molecular determinants of patient survival are poorly characterized. Global methylation profile 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 glioblastoma patients. A risk-score derived from methylation signature, predicted survival in univariate analysis in our and 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 NFkB pathway association with high-risk group. NFkB inhibition reversed glioma chemoresistance and RNA interference studies identified IL6 and ICAM1 as key NFkB targets in imparting chemoresistance. Promoter hypermethylation of 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. Further, NPTX2 repressed NFkB activity by inhibiting AKT through a p53-PTEN dependent pathway, thus explaining the hypermethylation and downregulation of NPTX2 in NFkB 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. Pro-survival NFkB pathway activation characterized high-risk patients with poor prognosis indicating it to be a therapeutic target.
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 12-15 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). Additionally, 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. Further, an activated NFkB pathway was found to be associated with poor prognosis. We also show that NPTX2, a component of methylation signature with risky methylation, inhibits cell growth by antagonizing NFkB pathway through p53-PTEN activation thus connecting the methylation signature to NFkB pathway.
Materials and methods

Plasmids and reporter constructs

pCMV-Entry/NPTX2 was obtained from Origene. CAPI3K (pCDNA3-CD2p110myc) was described before (15, 16). CAAKT (pCDNA MYR HA AKT1) was from Addgene (Plasmid 9008). PG13-Luc was described before (17). CAIKK, NFKb-Luc, PTEN-luc, shATM and shATR plasmid constructs were obtained from Profs. Inder Verma, KN. Balaji, Dr. R.C.M. Simmen, Dr. Yoshi Shiloh and Dr. Titia De Lange respectively.

Cell lines and reagents

Temozolomide, Adriamycin, 5-aza-2'-deoxycytidine, MTT and EscortIII transfection reagent were purchased from Sigma, USA. 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. Abhijit Guha, University of Toronto, Toronto, Canada and grown in DMEM medium. SVG cells were obtained from Dr. Pankaj 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 while 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 (SSSIHMS) and National Institute of Mental Health and Neurosciences (NIMHANS), 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 histopathological 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 TCGA data set (Agilent expression data, 17814 genes) for gene expression differences between low-risk and high-risk groups by performing t-test analysis with FDR correction. We identified 3127 genes which were significantly differentially regulated between the high-and low-risk groups (1983 genes upregulated and 1144 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 (GO terms) associated with each network. From the list of 3127 differentially regulated genes, we chose a subset of 61 genes, with a log2 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 eight significant networks (Table ST3).

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

To construct the NFkB gene/protein interaction network, NFkB target genes were downloaded (18). A total of 150 NFkB 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 publically 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 average of the same gene in normal brain and landscape was constructed.

**Patient samples and 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**

The details are given in the supplementary information.

**Statistical analysis and Independent Validation of risk score**

The details are given in the supplementary information.
Results

Identification and validation of a nine 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 as a schematic diagram (Figure 1). We identified 9 probes whose methylation status correlated significantly with patients' survival (Table 1 and Supplementary information). A methylation risk score calculated for each patient by combining the effect of each of the 9 genes using a risk score formula (Supplementary information) divided the patients into high- and low-risk with significant survival difference (median survival:12 months versus 23 months) (Fig. 2A and Supplementary Table ST1). The strength of methylation risk score in predicting patient survival was further confirmed by ROC curve analysis and subset analysis (Supplementary Fig. SF1A and Supplementary information). The methylation risk score predicted survival in GBM data set derived from The Cancer Genome Atlas (TCGA) cohort as well (Fig. 2B and Supplementary Table ST1). The methylation risk score also divided patients significantly into three groups in both data sets with the identification of a third group with very high risk as indicated by very short median survival (Supplementary Fig. SF1B, C and Supplementary information).

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. SF2A, B, C, D). Upon correlation of methylation status to patient survival, NPTX2 methylation was found to be risky
while methylation of the remaining eight genes was found to be protective (Fig. 2D, Table 1 and Supplementary Fig. SF2E). 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 nine genes which form part of methylation signature are needed for prognostication (Supplementary information).

**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 data set. While 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 significant with MGMT promoter methylation showing a trend towards significance (Table 2). Interestingly, we found that all IDH1 mutant patients (n=9) and all G-CIMP + patients (n=11) were a subset of low-risk group (n=16) (Fig. 2E).

Upon correlation with gene expression subtypes, we noticed that while the low-risk group is highly enriched with proneural GBM tumors (9/12; 75%), the high-risk group was moderately enriched for mesenchymal GBM tumors (16/30; 53%) (Fig. 2E). However, survival analysis revealed that proneural low-risk patients survived significantly longer than proneural high-risk patients or all other nonproneural GBM patients (Fig. 2C). On comparison with recently published 9-gene predictor (22) using TCGA data set, we found that both signatures were significant in predicting survival (Supplementary Table ST2). On comparison with 10-
microRNA prognostic signature (14) using TCGA data set, we found the methylation risk score remain significant while the microRNA signature showed a trend towards significance (Supplementary Table ST2). Further, multivariate analysis using another cohort of glioma grade III (N=67) also identified methylation risk-score to be an independent survival predictor (Supplementary Table ST3; Supplementary Fig. SF3) (23). Thus these results put together identify methylation risk score as an independent predictor of patient survival.

**Network analysis reveals an activated NFkB pathway in high-risk patients**

We hypothesized that the nine 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 ST4), identified eight significant networks (Supplementary Table ST5). Careful analysis revealed an enrichment of NFkB pathway members in these networks. As NFkB pathway is associated with oncogenesis, tumor progression and chemoresistance in many cancers (24), we tested whether the NFkB pathway is differentially activated between low-risk and high-risk groups. First, NFkB gene/protein interaction network was constructed using the STRING database with 150 NFkB target genes as input (Supplementary Fig. SF4A). Landscape analysis of the NFkB interaction network using GBM patient gene expression data of low-risk vs. normal and high-risk vs. normal from the TCGA cohort revealed that while NFkB 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 NFkB pathway activation as evidenced by the bright red and orange color in z-axis plots (Fig. 3B), suggesting the possibility.
that higher activation of NFkB pathway in high-risk GBM may be responsible for their poor treatment response and less survival. In good correlation, pretreatment with NFkB inhibitor made only those cell lines, where NFkB is already activated, more chemosensitive but not in other cell lines, wherein NFkB activation is very low (Fig. 3C,D and Supplementary Fig. SF4B, C and D). Furthermore, silencing of p65 subunit of NFkB rendered glioma cells more sensitive to chemotherapy (Fig. 3E).

It is interesting to note that several NFkB target genes with anti-apoptotic, pro-metastasis, pro-survival and pro-inflammatory functions were upregulated only in GBM tumors from high-risk patients (Supplementary Table ST6), 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 seven selected target genes identified IL6 and ICAM1 as the key NFkB target genes to be the mediators of chemoresistance, as their down regulation rendered glioma cells sensitive to chemotherapy consistently (Fig. 3F).

Association of NFkB with high risk GBM was further validated by means of an indirect approach using REMBRANDT (25) and Phillips (26) GBM data sets which had both gene expression data and patients survival information (Supplementary information). The expression levels of the same set of genes (n=3127) that are differentially regulated between low-risk and high-risk groups in TCGA data set (Supplementary Table ST4), was able to divide these data sets into two clusters with significant survival difference and NFkB activation (Supplementary Fig. SF5A, B and SF6 A, B, C, D and E). These results overall validate the survival prediction by our methylation signature and confirm the association of activated NFkB 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 down regulated in GBM samples and glioma derived cell lines compared to normal brain samples (Fig. 4B and Supplementary SF7A) and methylation inhibitor, 5-aza-2’-deoxycytidine, treatment resulted in re-expression of NPTX2 transcripts (Fig. 4C). NPTX2 transcripts levels inversely correlated with NPTX2 promoter methylation (Supplementary Fig. SF7B). 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. SF7C and D), showed increased apoptosis, reduced proliferation, decreased anchorage-independent growth and increased chemosensitivity compared to a vector stable (Fig. 4E, F, G, H). Furthermore, 5-aza-2’-deoxycytidine pretreatment sensitized U251 glioma cells to chemotherapy in a NPTX2 dependent-manner, as simultaneous silencing of NPTX2 resulted in chemoresistance (Fig. 4I and Supplementary SF7E).

NPTX2 inhibits NFkB pathway through p53-PTEN-PI3K-AKT dependent manner

Next upon investigation of the connection between NPTX2 and NFkB pathway, we found NPTX2 overexpression inhibited expression from NFkB luciferase reporter in glioma cell lines (Fig. 5A), sequence-specific DNA-binding (Supplementary Fig. SF8A), nuclear translocation of NFkB sub unit (p65) Supplementary Fig. SF9 A, B and C) and repressed six of the seven key NFkB target genes significantly (Supplementary Fig. SF8B). We also show that
NPTX2 inhibition of NFkB could be abrogated by co-expression of constitutively active forms of PI3 kinase, AKT and IKKα (Fig. 5B). Further, 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. SF10A and D). NPTX2 overexpression also induced p53 protein levels (but not transcript levels) and its targets, p21 and PTEN protein levels (Fig. 5E and Supplementary Fig. SF8C). Aptly, NPTX2 activated expression from PTEN promoter reporter construct in U343 and U87 cells and in a concentration 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. SF10B). NPTX2 also induced PTEN transcript (Supplementary Fig. SF8D). More importantly, NPTX2 overexpression resulted in 4.8 fold increase in p53 occupancy in PTEN promoter (Fig. 5F), significant activation of PTEN phosphatase activity (3 to 3.5 fold) as seen by increased PIP2 levels (Fig. 5G) and an efficient inhibition of AKT as seen by reduced pAKT levels (Fig. 5E). NPTX2 overexpression also decreased pIkB and increased total IkB levels (Supplementary Fig. SF9D). In good correlation, NPTX2 failed to inhibit NFkB promoter in p53 mutant U251 cells (Fig. 5A), p53 silenced cells (Supplementary Fig. SF10 C) 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 anti-proliferative functions may involve inhibition of NFkB pathway through p53-dependent PTEN activation leading to inhibition of PI3K-AKT-IKKα signaling.

Both ATM and ATR have been shown to have both 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 NPTX2 activation of p53 activity was compromised significantly in ATM silenced cells (Supplementary figure SF10 E and F) but not in ATR silenced cells (data not shown).

**Discussion**

In the present study, utilizing 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 NFkB pathway and high-risk which may explain their shorter survival. Inhibition of NFkB pathway made glioma cell lines with activated NFkB pathway alone sensitive to chemotherapy. NPTX2, a gene with risky methylation, was validated for its methylation and growth inhibitory functions and was found to inhibit NFkB activity through its ability to induce PTEN in a p53-dependent manner. Thus the NPTX2-p53-PTEN pathway connected the methylation signature to NFkB 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. While low-risk group showed enrichment of patients with IDH1 mutation, proneural gene expression sub type and G-CIMP positivity (8), methylation signature alone was identified as an independent survival predictor. Further, comparisons with the 9 gene (22) and 10 microRNA
panels (14) also identified our methylation signature as an independent survival predictor. Thus it appears 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 NFkB pathway in high-risk group from TCGA data set and was further validated in REMBRANDT and Phillips data sets. While the association between NFkB pathway activation and glioblastoma aggressiveness and chemoresistance as well as NFkB as a therapeutic target is known (24, 28), our study clearly shows that NFkB pathway is activated particularly in high-risk group. Our data also show that NFkB inhibition could sensitize only those glioma cells having activated NFkB pathway to chemotherapy. Further, we found IL6 and ICAM1, which are upregulated only in high-risk tumors, as the key mediators of chemosensitivity. In good correlation, IL6 and ICAM1 were found associated with invasion, angiogenesis, tumor growth, chemoresistance and prognosis in many cancers (29-32). This raises the potential use of targeting NFkB pathway in treatment protocol specifically for those patients belonging to high-risk group.

Additional analysis of methylation signature and survival identified neuronal pentraxin II (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 down regulate cyclinD1 leading to cell cycle arrest and apoptosis (33). Our study demonstrates that NPTX2 is methylation silenced in GBMs, could be re-expressed 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 has 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 NFκB activity suggesting that its functions may involve activating or inhibiting signaling pathways which are modulators of NFκB pathway. p53 is known to activate PTEN leading to inhibition of PI3K-AKT pathway (35, 36). In our study, we demonstrate that NPTX2 activates PTEN at the level of transcription in ap53-dependent manner leading to PI3 kinase inhibition. Further, we also show that NPTX2, through its ability to activate PTEN, inhibits AKT, which has been shown to be a key upstream activator of NFκB 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 NFκB may involve p53-PTEN activation leading to PI3K-AKT-IKKα inhibition. Further, it explains why NPTX2 is methylation silenced in high-risk group wherein NFκB pathway is activated.

Thus our study uncovers a novel pathway (through NPTX2) of a complex interaction network between differential methylation of nine genes and alteration of global gene regulation leading to modulation of signaling, in particular NFκB 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 and high-risk groups which would facilitate individualized therapeutic modality. Mechanistic investigation identified activated NFκB signaling as the potential cause of poor prognosis in high-risk patients suggesting NFκB as a therapeutic target in particular for patients who do not respond to current treatment protocols.
Acknowledgements

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References


Table 1: Details about 9 gene methylation signature that predicts survival in GBM

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<th>s. no.</th>
<th>Symbol (CpG id)</th>
<th>Name</th>
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<th>Hazard ratio</th>
<th>Our patient data set (Median β value)</th>
<th>TCGA data set (Median β value)</th>
<th>Difference</th>
<th>Low risk</th>
<th>High Risk</th>
<th>Difference</th>
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**Table 2:** Multivariate Cox regression analysis of methylation risk score and other Prognostic markers using TCGA cohort.

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<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></td>
<td>1.14</td>
<td>0.131</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IDH1</td>
<td>65</td>
<td>0.476</td>
<td>-0.472</td>
<td>0.316</td>
</tr>
<tr>
<td>Methylation risk score</td>
<td></td>
<td>1.026</td>
<td>0.029</td>
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</tr>
<tr>
<td>G-CIMP</td>
<td>205</td>
<td>0.400</td>
<td>-0.916</td>
<td>0.215</td>
</tr>
<tr>
<td>Methylation risk score</td>
<td></td>
<td>1.030</td>
<td>0.030</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>III. Multivariate analysis of all the markers in TCGA data set</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>65*</td>
<td>NA</td>
<td>NA</td>
<td>0.275</td>
</tr>
<tr>
<td>MGMT</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>0.064</td>
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<tr>
<td>IDH1</td>
<td></td>
<td>NA</td>
<td>NA</td>
<td>0.322</td>
</tr>
<tr>
<td>G-CIMP</td>
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<td>NA</td>
<td>0.416</td>
</tr>
<tr>
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<td></td>
<td>1.250</td>
<td>0.223</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>III. Multivariate analysis of all the markers in TCGA data set (Except IDH1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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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<td>-1.112</td>
<td>0.016</td>
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<tr>
<td>G-CIMP</td>
<td></td>
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<tr>
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<td></td>
<td>1.100</td>
<td>0.096</td>
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*Multivariate analysis that included all markers was carried out in two 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 with 65 patients while the analysis involving MGMT, G-CIMP, age and methylation risk score (without IDH1) was carried out with 205 patients.
**Figure legends**

**Figure 1:** Schematic diagram of the entire workflow as to how the 9-gene signature was identified, tested and validated is shown.

**Figure 2:** Risk stratification of GBM based on methylation risk score. **A, 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 nine genes from methylation signature (our patient set; n=44). **E)** Heat map of methylation status (β value) of nine genes from methylation signature (TCGA set; n=65) for whom G-CIMP status, glioma gene expression sub types, IDH1 mutation status and methylation β values were available. In **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. In **E**, above the heat map, each sample is additionally color coded as described in the key.

**Figure 3:** NFkB pathway association with high-risk GBM. Landscape analysis of NFkB network (**Fig. SF4A**) using NFkB target gene expression in low-risk compared to normal brain (**A**) and high-risk compared to normal brain (**B**). **C** and **D** Human glioma cell lines were treated with BAY 11-7082 (4µM) for 12 hours and then treated with indicated drugs for 48 hours followed by viability measurement. The proportion of viable cells from untreated cells/Bay treated cells was considered as 100% (NS - P>0.05). **E** U343 and U138 cells were transfected with siRNAs against NFkB (p65) and after 36 hours, treated with indicated drugs. The proportion of live cells was quantified after 48hrs. The proportion of viable cells from siControl was considered as 100%. **F** U343 and U138 cells were transfected with siRNAs against indicated genes and after
36 hours, treated with Temozolomide (125, 250, 500, and 1000μM) and Adriamycin (0.1, 0.2, 0.4, and 0.8μg/mL). After 48 hours of the drug treatment, proportion of live cells was quantified. IC₅₀ values of control siRNA was considered as 100% and the ratio of control siRNA IC₅₀ to that of indicated siRNA are shown as percent IC₅₀.

**Figure 4:** Validation of methylation status and growth inhibitory function of NPTX2 gene. **A)** Bisulfite sequence analysis of the NPTX2 promoter. Percentage methylation was established as total percentage of methylated cytosines from 7–10 randomly sequenced colonies. **B)** NPTX2 transcript levels obtained from RT-qPCR (present study group) or microarray data (TCGA and REMBRANDT) are plotted. **C)** Total RNA was isolated from glioma cell lines after treatment with 5aza2dC and NPTX2 transcript level was quantified by RT-qPCR. For each sample, fold change in gene expression is calculated over its mean expression in untreated sample. **D)** G418-resistant colonies were selected after transfection of glioma cell lines with Vector or pCMV-NPTX2. Mean colony counts are displayed as percentage of vector control. **E)** The active caspase 3 levels was measured using FITC-DEVD-FMK in U343/Vector and U343/NPTX2 cells and shown as mean intensity. **F)** Cell proliferation was measured as number of cells for U343/Vector and U343/NPTX2 clones and plotted. The difference in proliferation at different time points was found to be significant (p value of <0.01). **G)** U343/Vector and U343/NPTX2 clones were subjected to soft agar colony formation and the number of colonies were counted and shown keeping U343/Vector as 100%. The difference was found to be significant (p <0.028). **H)** U343/Vector and U343/NPTX2 clones were treated with varying concentrations of indicated drugs (Adriamycin: 0.1, 0.2, 0.4, and 0.8 μg/mL; Taxol: 2, 4, 8, and 16μM; Temozolomide: 125, 250, 500, and 1000μM). At 48 hours post drug addition, the proportion of live cells was quantified by MTT assay. The proportion of viable cells from untreated cells was
considered as 100%. Percent IC$_{50}$ is shown. I) U251 cells were transfect ed with either control siRNA or NPTX2siRNA. At 48 hours post-transfection, the cells were either untreated or treated with 5aza2dC (5 µM). After 24 hours of 5aza2dC addition, the cells were treated with various amounts of Adriamycin-0.1, 0.2, 0.4, and 0.8µg/mL or Temozolomide-125, 250, 500, and 1000µM. After 48 hours of the drug treatment, proportion of live cells was quantified by MTT assay. The absorbance of control cells was considered as 100%. The proportion of viable cells for indicated concentrations are shown.

**Figure 5:** NPTX2 inhibits NFkB pathway through a p53-PTEN dependent pathway. A) NPTX2 vector or control vector (2 µg) was transfected along with NFkB luciferase reporter (1 µg) and after 36 hours, luciferase activity was measured and plotted. B) NPTX2 vector or control vector (2 µg) was transfected along with NFkB luciferase reporter (1 µg) and constitutively active form of either PI3K, AKT or IKK (1 µg) and after 36 hours, luciferase activity was measured and plotted. C) NPTX2 vector or control vector (2 µg) was transfected along with pG13 luciferase reporter (1 µg) and after 36 hours, luciferase activity was measured and plotted. For 293 cells, 0.5µg, 1µg and 2µg of NPTX2 vector or control vector was used. D) NPTX2 vector or control vector (2 µg) was transfected along with PTEN promoter luciferase reporter (1 µg) and after 36 hours, luciferase activity was measured and plotted. For 293 cells, 0.5µg, 1µg and 2µg of NPTX2 expression vector or control vector was used. For all experiments above, the luciferase activity of control vector was considered as 100%. E) Cell extracts from 293 cells transfected with either vector or NPTX2 overexpression construct (10 µg) or U343/vector and U343/NPTX2 stables were subjected to western blot analysis for NPTX2 (Myc tag), p53, p21, PTEN, pAKT, AKT and Actin proteins. F) The chromatin isolated from 293 cells transfected with vector or NPTX2 construct (20 µg) was used for immunoprecipitation with control or p53 antibody and used for
PCR specific p53-binding region of PTEN. G) Cell extracts from 293 or U343 cells transfected with vector or NPTX2 construct (20 μg) used for immunoprecipitation using control or PTEN antibody. The immunoprecipitates were used to perform PTEN activity ELISA. For all experiments, at - test was carried out to test the significance of the observed differences between two conditions and a \( p < 0.05 \) is represented with an asterisk, \( p < 0.01 \) is represented as double asterisks and \( p<0.001 \) is represented as triple asterisks.
Figure 2

A. Low risk (n = 12; median survival = 23 months) vs. High risk (n = 32; median survival = 12 months) with p = 0.001

B. Low risk (n = 37; median survival = 23 months) vs. High risk (n = 168; median survival = 15 months) with p = 0.0007

C. Low risk proneural (n = 9; median survival = 53 months) vs. High risk proneural (n = 5; median survival = 10 months) with p = 0.028

D. Heat map - NPTX2, LIMD1, MOXD1, IRF6, FBP1, FMOD, LAD1, RBPSUHL, GSTM5

D. 65 TCGA GBM samples - G-CIMP status (n=65), IDH1 status, Glomia subtypes, Gene expression subtypes (n=42), IDH1 mutation status (n=65)

E. Color code for heat map:
- G-CIMP status (n=65)
- Cluster1-G-CIMP + (n = 11)
- Cluster3-G-CIMP - (n = 36)
- Cluster2-G-CIMP - (n = 18)
- IDH1 status
- IDH1 WT
- IDH1 Mu
Figure 3

A. Graph showing percent viability for different treatments.

B. Graph showing percent viability for different treatments.

C. Graph showing percent viability for different treatments.

D. Graph showing percent viability for different treatments.

E. Graph showing percent viability for different treatments.

F. Graph showing percent viability for different treatments.

Shukla et al., 2013; Figure 3
The NPTX2-PTEN-NFκB nexus is an essential component of a prognostic DNA methylation signature of glioblastoma

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