

Associations Between *TFPI-2* Methylation and Poor Prognosis in Glioblastomas

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Summary. *Background and Objective.* The epigenetic silencing of tumor suppressor genes plays an important role in gliomagenesis. Recently, tissue factor pathway inhibitor 2 (*TFPI-2*) has been suggested as a tumor suppressor gene involved in tumorigenesis and metastasis in some cancers. However, to date, little is known about the methylation status of *TFPI-2* gene in glioblastoma tissues. In this study, we aimed to investigate the methylation status of *TFPI-2* promoter and its associations with patient prognosis in glioblastoma.

Material and Methods. The methylation status of *TFPI-2* was investigated by methylation-specific polymerase chain reaction in 99 glioblastoma patients. The associations between patients' clinical variables and overall survival time were assessed.

Results. *TFPI-2* was aberrantly methylated in 22.2% (22/99) of glioblastoma tumors, but was not methylated in normal brain samples. The survival of patients with glioblastoma differed significantly between the methylated and unmethylated *TFPI-2* groups ($P=0.047$). The 2-year survival among patients carrying methylated *TFPI-2* tumors was significantly lower compared with that of patients with unmethylated *TFPI-2* (27% versus 4.7%, $P=0.037$).

Conclusions. The present work demonstrated that the epigenetic inactivation of *TFPI-2* by promoter hypermethylation was a frequent and tumor-specific event in glioblastoma, and *TFPI-2* promoter methylation might be considered as a prognostic marker in glioblastoma.

Introduction

Glioblastoma is one of the greatest challenges in the management of cancer patients worldwide, despite notable recent achievements in oncology. Even with aggressive surgical resections, along with recent advances in radiotherapy and chemotherapy, the prognosis for glioblastoma patients remains dismal: the median survival after diagnosis is about 14 months. The established good prognostic factors are limited, but include young age, high Karnofsky Performance Status (KPS) score, high Mini-Mental State Examination score, O6-methylguanine methyltransferase promoter methylation, and the resection of more than 98% of the tumor (1). Therefore, new markers are being investigated, which may help better diagnose and predict the course of glioblastoma. Glioblastoma is caused by an accumulation of genetic and epigenetic alterations. Gene silencing by the hypermethylation of CpG islands in the promoter regions is a common epigenetic abnormality in cancer and may lead to a loss of function of tumor suppressor gene. Identifying the aberrantly methylated genes may provide better understanding of the pathogenesis of glioblastoma and help for the development of novel tumor markers and therapeutic targets.

Recently, it has been shown that tissue factor pathway inhibitor 2 (*TFPI-2*) is highly expressed in low-grade gliomas, but minimally expressed or undetectable in glioblastomas, and that the enforced expression of this gene reduces the invasive properties of brain tumor cells (2). The knockdown of *TFPI-2* was associated significantly with increased glioma cell proliferation, migration, and invasion (3). Promoter region analysis revealed a high GC-rich content with 3 transcription initiation sites (4). Besides gene locus deletion and aberrant splicing, the mechanism responsible for *TFPI-2* down-regulation in tumor cells has been mainly attributed to promoter hypermethylation (5). These findings let us hypothesize that this gene could be a target for epigenetic silencing in glioblastoma.

This study aimed at evaluating the methylation status of *TFPI-2* in glioblastoma tumor tissues to support our hypothesis that *TFPI-2* is epigenetically inactivated in glioblastomas by promoter hypermethylation and plays a role as a tumor suppressor gene in gliomagenesis.

Material and Methods

Glioblastoma Tumor and Normal Brain Tissues. In total, 99 glioblastoma tumor specimens were collected in the Clinic of Neurosurgery, Hospital of Lithuanian University of Health Sciences (Kaunas, Lithuania), from 2003 to 2009. Each patient gave

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his/her written informed consent. The database was closed in November 2010. Diagnoses were established by experienced pathologists according to the World Health Organization (WHO) classification. One sample of human brain DNA (Zymo Research, USA) was included as a control. Glioblastoma samples were stored in liquid nitrogen until DNA extraction.

The following clinical data were recorded for each patient: age at the time of operation, gender, tumor multifocality, duration of the last follow-up, and patient's status. Survival time was recorded for all the cases, and the overall survival was estimated from surgery to death or the last contact with live patients.

DNA Isolation and Bisulfate Modification. Tumor DNA was extracted from 25–40 mg of frozen tissue using the ZR Genomic DNA™ Tissue MiniPrep (Zymo Research, USA) according to the manufacturer's protocol. The methylation status of *TFPI-2* gene promoter was determined by the bisulfite treatment of DNA. DNA (400 ng) was used for bisulfite modification. DNA modification was performed using EZ DNA methylation kit (Zymo Research, USA), and all the procedures were done according to the manufacturer's protocol. Bisulfite-treated DNA was eluted in 40 μ L of distilled water and stored at -80°C until methylation-specific polymerase chain reaction (MS-PCR).

Methylation-Specific Polymerase Chain Reaction. The methylation status of the *TFPI-2* promoter region was determined by MS-PCR. The primers distinguishing unmethylated (U) and methylated (M) alleles were taken from the article (6) and were as follows: methylated forward 5'-TTCGTTTCGTATAAAGCGGGTATTC-3' and reverse 5'-CCGTCAAAAAACAACAAAATCG-3' (166 bp); unmethylated forward 5'-TTTGTTTTGTATAAAGTGGGTATTTGG-3' and reverse 5'-CATCAAAAAACAACAAAATCAAC-3' (165 bp). The amplicon of these MSP primers contains 18 CpG loci. Each PCR reaction contained 20 ng of sodium bisulfite-modified DNA. The MSP reaction was performed in a total volume of 20 μ L, using 10 μ L of Maxima® Hot Start PCR Master Mix (Thermo fisher Scientific, USA) with Hot Start Taq DNA polymerase and 10 pmol of each primer (Metabion International AG, Germany). The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, 38 cycles at 94°C for 30 seconds, at 61°C for 1 minute and at 72°C for 1 minute, and final step at 72°C for 5 minutes. For each set of methylation-specific PCR reactions, human blood lymphocyte DNA treated with bisulfite served as an unmethylated DNA control, and the Bisulfite-Converted Universal Methylated Human DNA Standard (Zymo Research, USA) was used as a positive

methylation control. A water blank control was also included. The PCR products were separated on 2% agarose gels with ethidium bromide and visualized under UV illumination. PCR analyses were repeated twice.

Statistical Analysis. The SPSS Statistics 19 (SPSS Inc., Chicago, IL) software package was used for statistical analysis. Associations between gene methylation data and clinical features of glioblastoma patients were analyzed by the chi-square test. To estimate survival functions, the Kaplan-Meier method was employed. To compare survival between groups, the log-rank test was used. A *P* value of <0.05 was considered significant.

Results

Characteristics of Glioblastoma Patients. The median patients' age at diagnosis was 61.0 years (range, 26–88). The male-to-female ratio was 1:1.3. The median age of men ($n=42$) and women ($n=57$) was 58.0 years (range, 34–84) and 61.0 years (range, 26–88), respectively. The mean survival in the glioblastoma sample ($n=99$) was 14.6 months (SD, 16.04; range, 0.20–88.4), while the median survival time was 8.9 months (95% CI, 6.9–10.8). Most glioblastoma patients (64%) survived less than 12 months after operation.

Hypermethylation of *TFPI-2* Promoter in Glioblastoma Tumors. The methylation status of the *TFPI-2* promoter in glioblastoma tumor samples was detected by the MS-PCR assay. The methylation status of the *TFPI-2* promoter was evaluated in 99 glioblastoma tumors. Hypermethylation of the promoter was detected in 22.2% (22/99) of the glioblastoma tumors, but not in normal brain tissues. The analysis of methylation status in the samples is shown in Fig. 1. The bands with both primer sets were detected in some, but not all, glioblastoma samples most likely because of the existence of nonmalignant cells in a fraction of the samples or that only one allele of the gene was methylated. Among 22 *TFPI-2*-methylated cases, 6 patients (27%) were younger than 60 years and 16 individuals (73%) were older than 60 years. Moreover, there was a tendency toward more frequent *TFPI-2* methylation in the patients older than 60 years, which was of borderline significance ($P=0.057$) (Table). Next, the associations between *TFPI-2* promoter methylation and 2-year survival were analyzed. The 2-year survival was higher in patients carrying the unmethylated *TFPI-2* gene as compared with those with the methylated *TFPI-2* gene. For this analysis, the patients were divided into two groups: the patients who survived 2 years or more (group 1) and the patients who survived less than 2 years (group 2). The group 1 comprised 21 patients, and the *TFPI-2* promoter was methylated only in one patient (4.7%). In the group 2,

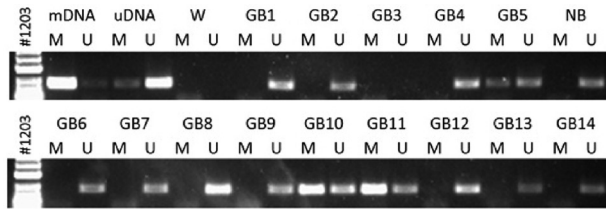


Fig. 1. Analysis of methylation status of the *TFPI-2* CpG island promoter in glioblastoma by the methylation-specific polymerase chain reaction assay

Molecular weight markers are shown on the left. mDNA, methylated DNA control; uDNA, unmethylated DNA control; W, water control; GB1-GB14, glioblastoma tumor samples; NB normal brain sample. The presence of visible PCR products in those lanes marked "U" indicates the presence of unmethylated allele genes, and "M" indicates the presence of methylated genes.

Table 1. Associations Between *TFPI-2* Promoter Methylation and Clinicopathological Parameters of Glioblastoma: Age, Sex, Multifocality, and 2-Year Survival

Variable	n	<i>TFPI-2</i> Promoter Methylation		P
		Methylated n=22	Unmethylated n=77	
Age				
<60 years	45	6	39	0.057
≥60 years	54	16	38	
Gender				
Male	31	11	31	0.468
Female	57	11	46	
Multifocal				
Yes	7	1	6	0.999
No	92	21	71	
Survival				
<24 months	78	21	57	0.037
≥24 months	21	1	20	

the methylation of the *TFPI-2* gene promoter was identified in 21 of the 78 cases (27%). A significant difference was observed comparing the group 1 and the group 2 ($P=0.037$). Further analysis showed that there were no significant associations between the methylation of the *TFPI-2* gene promoter and gender ($P=0.468$) or tumor multifocality ($P>0.99$) (Table 1).

These results suggest that the methylation of CpG islands in the *TFPI-2* gene may be an important prognostic factor for glioblastoma. To test this hypothesis, all the clinical data for a prognostic value separately for age, multifocality, and overall survival using the Kaplan-Meier curves were analyzed (P values were generated using the log-rank test) (Fig. 2). The patients younger than 60 years demonstrated better survival when compared with their older counterparts ($P<0.0001$) (Fig. 2A). The survival of patients with multifocal glioblastoma was worse comparing with that of the patients with non-multifocal glioblastoma ($P=0.007$) (Fig. 2B). The patients with the methylated *TFPI-2* gene had a sig-

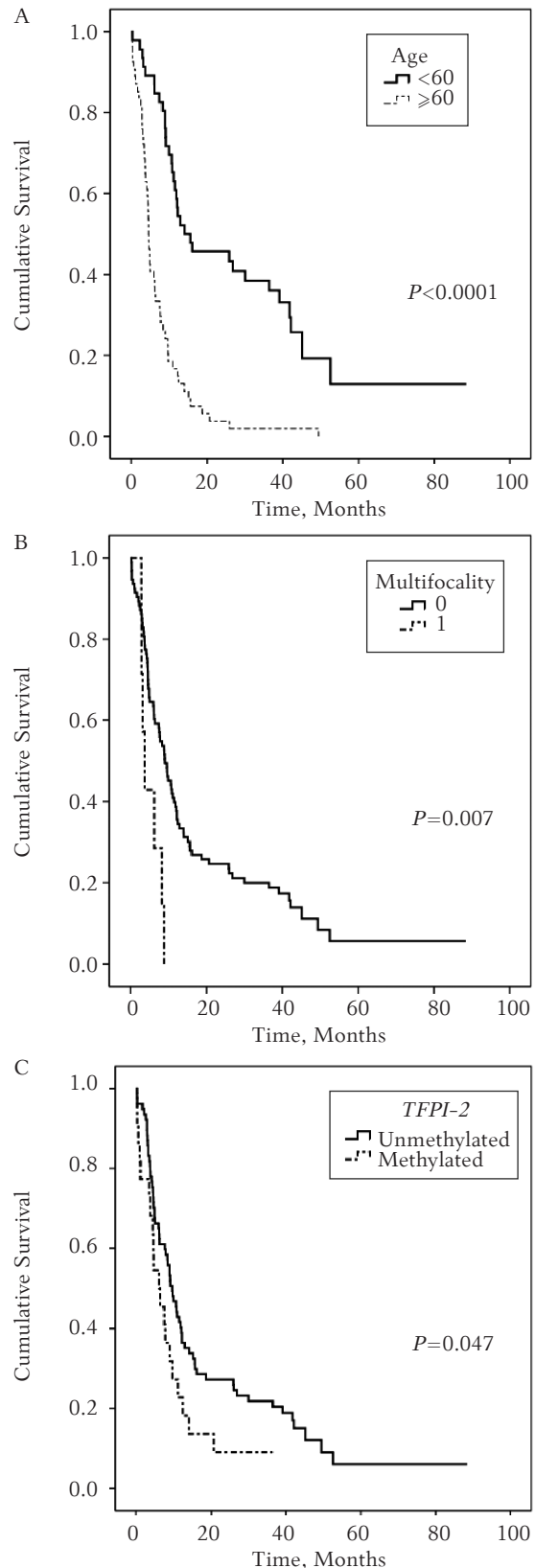


Fig. 2. Overall survival rate in glioblastoma patients regarding clinical and gene variables revealed by univariate analysis, which would significantly affect the survival time (log-rank test, $P<0.05$)

A, age (>60 vs. ≤60 years); B, multifocality (nonmultifocal vs. multifocal); C, methylation status of *TFPI-2* gene (unmethylated vs. methylated).

nificantly poorer prognosis for overall survival than the patients with unmethylated *TFPI-2* ($P=0.047$) (Fig. 2C).

Discussion

The capability of glioblastoma tumor cells to infiltrate the surrounding brain parenchyma critically limits the effectiveness of current treatment (7). Tumor invasion is a complex, multistep process, and the mechanisms resulting in degradation of the extracellular matrix (ECM) and tumor cell migration and invasion have not been completely elucidated yet (5). The matrix degradation can be promoted by the imbalance between proteolytic enzymes (proteases) and their inhibitors (8, 9). Additional studies have shown that *TFPI-2* inhibits tumor-related angiogenesis, and some members of the ECM, therefore, implicate tumor invasion and progression (10). It has been previously shown that *TFPI-2*, a broad-range proteinase inhibitor, is highly expressed in low-grade gliomas, but minimally expressed or undetectable in glioblastomas, and that the enforced expression of this gene reduces the invasive properties of brain tumor cells (2).

The aberrant methylation of the *TFPI-2* promoter CpG islands in human cancers and cancer cell lines has been widely documented to be responsible for the diminished expression of mRNA encoding *TFPI-2* and decreased or inhibited the synthesis of *TFPI-2* protein during cancer progression (11).

The transcriptional silencing of *TFPI-2* by hypermethylation in the promoter region has been recently demonstrated in many types of human cancers: the *TFPI-2* promoter was methylated in 88.6% (62/70) of nasopharyngeal carcinoma primary tumors (5), 93% (51/55) of colorectal tumor samples (12), 27.1% (36/133) of non-small cell lung cancer (6), and 18% (7/38) of primary gastric carcinomas (13). The aberrant methylation of *TFPI-2* was also detected in 73% (102/140) of pancreatic cancer xenografts and primary pancreatic adenocarcinomas, and was more likely to be in older patients with pancreatic cancer (14).

However, the methylation status of the *TFPI-2* gene has not been investigated in glioblastoma tissues. For the first time, we have examined the methylation status of *TFPI-2* in the tumor DNA of glioblastoma patients. In this study, the hypermeth-

ylation of the *TFPI-2* promoter was detected in 22 of the 99 glioblastoma samples (22.2%), but not in the normal brain DNA, which implied that the epigenetic silencing of the *TFPI-2* pathway might be involved in gliomagenesis. Although the promoter hypermethylation of *TFPI-2* was frequently found in glioblastoma, it was not associated with patients' sex, age, or tumor multifocality in our series. Concerning patient outcome, a significant association was found between the methylation of *TFPI-2* and overall survival of patients ($n=99$, $P=0.047$, Kaplan-Meier analysis, log-rank test). In our set of glioblastoma patients, the methylation of the *TFPI-2* gene promoter was present in 4.7% of long-term survivors with glioblastoma, distinguishing this series from classical glioblastomas, which showed such a characteristic in 27% of the cases ($P=0.037$). The overall methylation rate of this gene in the total glioblastoma sample was 22.2%, a percentage which is similar as described in primary gastric carcinomas and lung cancers, but much lower than in nasopharyngeal carcinomas or colorectal tumors (5, 6, 12, 13). These findings suggest that the prognostic value of *TFPI-2* promoter hypermethylation could be tissue-specific. Therefore, such characteristics may suggest the methylation of *TFPI-2* as a molecular marker for glioblastoma. The restoration of *TFPI-2* expression in tumor tissues inhibits invasion, tumor growth, and metastasis, which creates a novel possibility in cancer patient treatment (11).

Conclusions

The present work demonstrated that the epigenetic inactivation of *TFPI-2* by promoter hypermethylation was a frequent and tumor-specific event in glioblastoma, and *TFPI-2* promoter methylation might be considered a prognostic marker in glioblastoma.

Acknowledgments

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Statement of Conflicts of Interest

The authors state no conflict of interest.

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