P14ARF Suppresses Tumor-Induced Thrombosis by Regulating the Tissue Factor Pathway

Abdessamad Zerrouqi, Beata Pyrzynska, Daniel J. Brat, and Erwin G. Van Meir

Abstract

How necrotic areas develop in tumors is incompletely understood but can impact progression. Recent findings suggest that the formation of vascular microthrombi contributes to tumor necrosis, prompting investigation of coagulation cascades. Here, we report that loss of tumor suppressor P14ARF can contribute to activating the clotting cascade in glioblastoma. P14ARF transcriptionally upregulated TFPI2, a Kunitz-type serine protease in the tissue factor pathway that inhibits the initiation of thrombosis reactions. P14ARF activation in tumor cells delayed their ability to activate plasma clotting. Mechanistically, P14ARF activated the TFPI2 promoter in a p53-independent manner that relied upon c-JUN, SP1, and JNK activity. Taken together, our results identify the critical signaling pathways activated by P14ARF to prevent vascular microthrombosis triggered by glioma cells. Stimulation of this pathway might be used as a therapeutic strategy to reduce aggressive phenotypes associated with necrotic tumors, including glioblastoma. Cancer Res; 74(5): 1–8. ©2014 AACR.

Introduction

High-grade gliomas represent the most common primary central nervous system tumors in adults, and are associated with poor survival. The serious therapeutic challenge posed by these tumors, particularly glioblastoma multiforme (WHO grade 4), is due in part to their complex biology (1). In glioblastoma, complex heterotypic interactions between tumor and stromal vascular cells lead to the formation of glomeruloid microvascular proliferations in close proximity to micronecrotic regions surrounded by pseudopalisading tumor cells (2). These structures are pathognomonic to glioblastoma and are associated with poor patient prognosis. Understanding the mechanisms underlying their formation is important as it may lead to new therapeutic approaches (3).

It is well known that a major driver of vascular proliferation in glioblastoma is the tumor cell secretion of VEGF stimulated by microenvironmental hypoxia (4–7). One aspect that has not been extensively studied, however, is the consequence of plasma leakage in the tumor following VEGF-induced vascular permeability. Glioblastoma cells have a strong ability to activate the coagulation system by expressing tissue factor (TF; ref. 8), a unique cell-associated receptor for coagulation factor VIIa, a serine protease that can initiate blood coagulation (9).

Formation of a blood clot in tumor vessels is expected to render the surrounding region hypoxic and ischemic and causes micronecrosis. In response to this microenvironmental stress, tumor cells may migrate away from the obstructed vessels, possibly creating the observed pseudopalisading cell layer surrounding a micronecrotic region containing remnants of obstructed vessels (2).

Activation of the coagulation cascade is facilitated by genetic events in the tumor. Glioblastomas show overexpression of wild type and truncated constitutively active EGFR genes (10, 11). Activation of the EGFR signaling can upregulate the expression of TF (10), its ligand factor VII and the protease-activated receptors (PAR-1 and PAR-2; ref. 12). These factors come in contact with coagulation factors that have leaked out from the fenestrated vessels, and participate at the initiation step of coagulation and consequently, clot formation. Activation of the phosphoinositide 3-kinase pathway through PTEN gene loss (13), high activity of the NF-kB (14), and the development of hypoxia within the tumor can also contribute to the coagulopathy by increasing TF expression (13). Here, we were interested to know whether additional genetic events might facilitate thrombus formation through the loss of negative regulators of coagulation.

One of the most potent factors able to prevent the initiation of coagulation reactions is tissue factor pathway inhibitor-2 (TFPI2). Human TFPI2, also known as placental protein 5 (PP5), inhibits several coagulation factors, including factor VIIa, TF, factor Xa, and thrombin (15). TFPI2 is expressed in most human tissues, but tumors arising from these tissues display either reduced or undetectable expression. Several highly aggressive tumors lose TFPI2 expression due to gene silencing associated with promoter hypermethylation, suggesting it is a tumor suppressor (16–18).

A frequent genetic change that occurs in glioblastoma is the loss of the CDKN2A locus. This locus encodes the tumor suppressor P14ARF (p19Arf in mice; refs. 19–24), and its loss...
prediseases to diverse tumor types, including glioblastoma (21, 23–25). P19Arf binds to and inactivates Mdm2, a negative regulator of the p53 tumor suppressor (20), thereby mediating cell cycle arrest or inducing apoptosis (22, 23, 26). P14ARF also has additional p53-independent tumor-suppressor activities, including the ability to suppress tumor angiogenesis (24, 25, 27, 28).

Here, we hypothesized that the loss of P14ARF may facilitate the initiation of the coagulation cascade in tumors by reducing the expression of negative regulators. A link between the activation of the coagulation system and the loss of P14ARF activity in cancer is currently unknown. We showed that restoring P14ARF gene expression in tumor cells inhibits the clotting process. Mechanistically, we found that in human glioblastoma, P14ARF upregulates the expression of the TFPI2 in a p53-independent manner. This upregulation is mediated by the coordinated action of SP1 and AP-1 on the TFPI2 gene promoter following P14ARF activation, thus establishing novel downstream signaling pathways for this tumor suppressor.

Materials and Methods

Cell lines and transfections

Human glioblastoma cell lines LN229 (deleted for the P14ARF gene) and LN-Z308 (devoid of endogenous p53 protein; ref. 29) were used to generate clones with doxycycline (dox) inducible expression of hemagglutinin–tagged P14ARF cDNA. Clones LN229-L16 (30) and LNZ308-C16 (31) expressing stably the reverse tetracycline-controlled transactivator (rtTA) were engineered to express dox-inducible P14ARF (A5 and C19, respectively; ref. 28). The A5 and C19 cell lines were genetically engineered to express dox-inducible P14ARF (A5 and C19, the reverse tetracycline-controlled transactivator (rtTA) were co-transfected with a plasmid containing the reverse tetracycline-controlled transactivator (rtTA) and the p-glucuronidase (p-gus) reporter gene driven by the promoter following P14ARF activation, thus establishing novel downstream signaling pathways for this tumor suppressor.

Plasma clotting assay

Tumor cells (10^7) cultured for 48 hours in medium containing 2% serum were gently rinsed twice with cold PBS, scraped from the dish, and resuspended in 1.0 mL of PBS. For the clotting assay, 200 μL of tumor cell suspension was added to 200 μL of citrated human plasma (Precision Biologic), and then 200 μL of 25 mmol/L CaCl_2 was added to the tube to initiate the clotting process. Clotting time was counted using an automated coagulation timer (Medical Laboratory Automation, Inc.), and when the liquid formed a semisolid gel, it triggered the stop of the timer. Plasma clotting times induced by tumor cells were measured in triplicate with all reagents maintained at 37°C. Positive controls for each experiment included neoplastic (thromboplastin) clotting reagent (Diagnostica Stago) in place of tumor cell suspension (Clotting time = 16–22 seconds).

Western blot analysis

Whole-cell extracts were obtained by lysing cells in 1× Laemmli sample buffer (0.1% 2-mercaptoethanol, 0.0005% bromophenol blue, 10% glycerol, 2% SDS, and 63 mmol/L Tris-HCl, pH 6.8) or radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, pH 7.4; 150 mmol/L NaCl; 0.5 mmol/L EDTA; 0.5% sodium deoxycholate; 0.1% SDS; 1% Triton X-100, and 1× mini-Complete protease inhibitor cocktail; Roche) and protein concentrations were determined using the DC protein assay (Bio-Rad). The protein samples (50–100 μg per well) were then boiled for 5 minutes after adding dithiothreitol (DTT) to a final concentration of 50 mmol/L and resolved by SDS-PAGE on either 12.5% Tris-HCl gels or 4% to 20% gradient gels (Bio-Rad), and transferred to NitroBind, pure nitrocellulose membranes (GE Water and Process Technologies). The membranes were immunoblotted using polyclonal rabbit anti-hemagglutinin (1:2000, Covance) or goat anti-P14ARF antibodies to detect P14ARF (1:1,000, Santa Cruz Biotechnology), mouse anti-human p53 (clone DO-7; 1:1,000, DAKO), mouse anti-human p21 (Ab-11; 1:500, NeoMarkers), rabbit anti-SP1 (1:500, PEP2), goat anti–β-actin (1:1,000), mouse anti–α-tubulin (1:500; Santa Cruz Biotechnology), rabbit anti-c-JUN, anti-phospho S63 and S73 c-JUN, anti-phospho T183 and Y185 JNK1/2 (1:500; Cell Signaling Technology), anti-JNK (1:300, Zymed), rabbit anti-TFPI2 (generously provided by Dr. Walter Kissel, Krakow, Poland), and mouse anti-TFPI2 (B7; Santa Cruz Biotechnology). Immunodetection was performed using the corresponding secondary horseradish peroxidase (HRP)–conjugated antibodies. HRP activity was detected using the SuperSignal West-pico Chemiluminescence Kit (Pierce).

Northern blot analysis

To generate probes specific for each human gene of interest, the reverse transcription PCR amplification was performed. The sequences of the PCR forward (Fwd) and reverse (Rev) primers were as follows: P14ARF-Fw: 5′-aaa cta tgg atg gtc ggc agg ttc ttc tgt g3′, P14ARF-Rev: 5′-act tgg atc cca tca tgg acc tgt tct tct a3′; TFPI2 Fw: 5′-cga agg caa cgc caa caa ttt cta 3′, TFPI2 Rev: 5′-tct cgg att cta tgg caa aag cga 3′, c-JUN Fw:5′-agc caa acc tca gca act tca acc 3′, c-JUN Rev:5′-ttc cag tct tgg cgt tag cat cag cgc 3′, CDKN1A/P21-Fw: 5′-gca ggg tgt tgg gtt gaa aag 3′, CDKN1A/P21 Rev: 5′-ctc gta aag caa cag gta g3′. The annealing temperatures used in PCR reactions were either 60°C or 62°C. Northern blot analyses were generated as previously described (28, 33).

Luciferase reporter assay of TFPI2 promoter activity

Transcription factor binding sites in the TFPI2 gene promoter were identified using Proscan version 7.1 (http://www-bimas.cit.nih.gov/molbio/proscan/). A5 cells were transfected with plG3-luciferase reporter plasmids driven by the 222 bp promoter of the TFPI2 gene; either wild type (p222-luc), or
Subsequently, the cells were grown in medium with 2% serum. C2A5 cells were grown in 150-mm dishes at 3°C using the manufacturer’s protocol with minor adjustments.

Chromatin immunoprecipitation assays

Cell lysate.

The chromatin immunoprecipitation (ChIP) assays were performed with a commercial kit (Upstate Biotechnology) containing point mutations in the AP-1 and SP1 binding sites (p222-mtAP1luc, p222-mtSP1luc, and p222-mtAP-SP1luc; ref. 34) using Fugene for 24 hours. Serum was then added to 2% cells does not affect the expression of TF. An extract of U87MG glioma cells cultured in 1% oxygen was used as positive control (Pos.Ctrl.) for TF expression.

Figure 1. P14ARF inhibits plasma clotting by upregulating the TFPI2 expression. A, left, Western blot analysis showing the tight regulation of P14ARF induction with dox in LN229-A5 human glioma cells. The induction of P14ARF increases p53 levels, which in turn increases the expression of the p53 downstream target, p21. The treatment of LN229-L16 cells (P14ARF null) with dox has no effect on p53 or p21, indicating the absence of nonspecific effects of dox. Right, Western blot analysis showing the reduction in TFPI2 expression upon P14ARF silencing in transformed human astrocytes. B, induction of P14ARF in A5 cells increases the clotting time of pooled normal human plasma, which can be prevented by the silencing of the TFPI2 expression. The treatment of L16 with dox has no effect on plasma coagulation time. Neoplastine was used as a positive control of plasma clotting. The unpaired two-tailed Student t-test was used to assess the statistical differences between experimental groups, “P > 0.05; “P > 0.01; n = 3. C, induction of P14ARF with dox in A5 cells does not affect the expression of TF. An extract of U87MG glioma cells cultured in 1% oxygen was used as positive control (Pos.Ctrl.) for TF expression. D, Northern blot analysis showing that the induction of P14ARF expression enhances the transcription of TFPI2 in A5 cells and that the silencing of TFPI2 with specific siRNA decreases its mRNA levels. α-Tubulin, β-actin, and GAPDH were used as loading controls.

containing point mutations in the AP-1 and SP1 binding sites (p222-mtAP1luc, p222-mtSP1luc, and p222-mtAP-SP1luc; ref. 34) using Fugene for 24 hours. Serum was then added to 2% cells. Twenty-four hours after transfection, cells were left untreated or treated with 2 μg/mL of dox for 36 hours. Cells were lysed and the relative luciferase activities were measured using a Luciferase Reporter Assay System (Promega) and a luminometer (Sirius model, Berthold Detection Systems) and incubated overnight (4°C) with 1 μg/mL of Acetyl–Histone H3 antibody (Cell Signaling), 1.5 μg/mL of antibodies against c-JUN (N20), SP-1 (PEP2), or with nonspecific rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology, Inc.). Formaldehyde-fixed DNA–protein complexes were pulled down with protein A–conjugated agarose beads and extracted with 1% SDS and 0.1 mol/L NaHCO₃. The protein–DNA crosslinking was reversed at 65°C for 5 hours, and the released proteins were eliminated through digestion with protease K (40 μg/mL, 1 hour at 45°C). The coimmunoprecipitated genomic DNA was adding formaldehyde directly to the culture medium to a final concentration of 1%. The cells were washed with cold PBS, lysed for 10 min with 1% SDS, 10 mmol/L Tris-HCl, pH 8.0, and sonicated four times for 10 seconds each on ice (Sonic dismembrator Model 100; Fisher Scientific), and then cell debris were removed by centrifugation. Aliquots were taken to control for DNA input. The remaining lysate was diluted 10 times in 0.01% SDS, 1% Triton X-100, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0, and 150 mmol/L NaCl, phosphatase/protease inhibitors, precleared with agarose beads/salmon sperm DNA and incubated overnight (4°C) with 1 μg/mL of Acetyl–Histone H3 antibody (Cell Signaling), 1.5 μg/mL of antibodies against c-JUN (N20), SP-1 (PEP2), or with nonspecific rabbit immunoglobulin G (IgG; Santa Cruz Biotechnology, Inc.). Formaldehyde-fixed DNA–protein complexes were pulled down with protein A–conjugated agarose beads and extracted with 1% SDS and 0.1 mol/L NaHCO₃. The protein–DNA crosslinking was reversed at 65°C for 5 hours, and the released proteins were eliminated through digestion with protease K (40 μg/mL, 1 hour at 45°C). The coimmunoprecipitated genomic DNA was

Chromatin immunoprecipitation assays

The chromatin immunoprecipitation (ChIP) assays were performed with a commercial kit (Upstate Biotechnology) using the manufacturer’s protocol with minor adjustments. A5 cells were grown in 150-mm dishes at 3 × 10⁶ cells per plate in medium with 2% serum ± dox (2 μg/mL) for 48 hours. Subsequently, the cells were fixed for 10 minutes at 37°C by
purified using mini columns (Qiagen) and eluted with 10 mmol/L Tris-HCl, pH 8.0. The primers used for PCR to amplify the TFPI2 promoter encompassing the c-JUN- and SP1-binding sites were TFPI2 Fwd (-268 to -244) 5'-AGGAAAGTTCGGAGGCACTGTT-3' and TFPI2 Rev (-42 to -24) 5'-TGGGGCAAGGGTGTCGGAGAAA-3'. The expected size of the PCR product is 244 bp. c-JUN Fwd 5'-GGT CGG GAG GCA TCT TAAT-3' and c-JUN Rev, 5'TTC AGT CTA GGT TAG TTT GGG-3' primers were used to amplify the region –221 to –16 of the c-JUN promoter (205 bp).

Statistical analysis

Statistical analysis was performed using an unpaired Student t test (two-tailed). P < 0.05 was considered significant.

Results

To determine whether P14ARF regulates tumor-induced clot formation, we used two cell systems: one with inducible P14ARF overexpression and another with silencing of endogenous P14ARF. P14ARF-null human glioma cells (LN229-A5) conditionally express P14ARF under a tetr-on system (Fig. 1A, left; ref. 28). LN229-L16 parental cells (rtTA positive, but not expressing P14ARF) were used as control for the nonspecific effects of dox. The induction of P14ARF by dox resulted in p53 stabilization and downstream increase of its target p21 in A5 cells. To modulate physiologic endogenous P14ARF, we used RNA interference in E6/E7-Ras-hTert transformed human astrocytes (Fig. 1A, right), which led to an increase in HDM2 stability as expected (35).

To test whether P14ARF could modulate thrombus formation by glioma cells, we used a plasma coagulation assay. Thromboplastin (neoplastine) was used as positive control. Doxycycline induction of P14ARF in A5 cells delayed plasma clotting, whereas it had no effect on parental L16 cells used as controls (Fig. 1B). We first examined whether this effect on coagulation might reflect a decrease in TF expression, but found barely detectable levels in A5 cells, which were unaffected by P14ARF (Fig. 1C). Because TFPI2 is a negative regulator of plasma clotting at its initiation step, we then investigated whether it was induced by P14ARF. Northern blot analysis shows that the induction of P14ARF in A5 cells induced TFPI2 gene transcription (Fig. 1D). Conversely, the specific silencing of TFPI2 in A5 cells (Fig. 1D) was able to accelerate clot formation, thus reversing P14ARF-mediated inhibition (Fig. 1B). The silencing of P14ARF in E6/E7-Ras-hTert astrocytes led to a significant decrease of TFPI2 expression (Fig. 1A, right panel), with a concomitant increase of plasma clotting time (Fig. 1B). These data provide evidence for the role of P14ARF in the inhibition of plasma coagulation, and this control is mediated through the transcriptional activation of the anticoagulant factor TFPI2.

To start deciphering the signaling pathway connecting P14ARF to TFPI2, we first examined whether it was dependent on p53 as P14ARF induction activates the p53 pathway in A5 cells (Fig. 1A). As expected, siRNA-mediated silencing of p53 in A5 cells caused a significant reduction of its downstream target CDKN1A/p21 transcription. Yet, it did not inhibit the activation of TFPI2 transcription by P14ARF; in fact, it even magnified its activation (Fig. 2A and B). The dispensability of p53 in P14ARF-mediated induction of TFPI2 expression (Fig. 2C) and anticoagulation activity (Fig. 2D) was further shown in TFPI2-null LNZ308-C19 human glioma cells, which stably express dox-inducible P14ARF. Dox had no effect on the parental LNZ308-C16 cells (rtTA positive, but not expressing P14ARF), which were used as controls for nonspecific effects of dox. Taken together, these results confirm that the regulation of TFPI2 by P14ARF is P53 independent and suggest the existence of another cellular pathway coupling P14ARF to TFPI2.

The promoter region of the human TFPI2 gene contains several putative binding sites for the transcription factor AP-1 upstream of the transcription initiation site (36, 37). Therefore, we examined whether P14ARF modulates c-JUN levels or its activity. Northern blot analysis showed an increase in c-JUN expression upon induction of P14ARF expression in A5 and C19 cells (Fig. 2A and C). The role of c-JUN in the upregulation of TFPI2 by P14ARF was confirmed by silencing c-JUN in A5 cells (Fig. 3A). We then examined whether the increase in c-JUN also resulted in an increased expression of its activated
phosphorylated form. A robust upregulation of phospho-c-JUN was observed (Fig. 3B). We next examined whether P14ARF could directly affect the activity of JNK, the upstream kinase that activates AP-1 by phosphorylating c-JUN (38). The pharmacologic inhibition of JNK decreased the levels of both total and phosphorylated c-JUN and strongly antagonized P14ARF-mediated upregulation of TFPI2 mRNA and protein expression (Fig. 3B and C). Together, these results suggest that P14ARF can upregulate TFPI2 expression through the activation of JNK phosphorylation. The activated JNK can then phosphorylate its substrate c-Jun and lead to an autoamplifying c-JUN expression loop that results in AP-1 activation (38).

Because the TFPI2 gene promoter can also bind SP1 (36), and we previously showed that P14ARF can upregulate the transcriptional activity of SP1 (28), we further examined whether SP1 played a role in TFPI2 activation by P14ARF. The silencing of SP1 abrogated the induction of TFPI2 mRNA and protein by P14ARF, even in the presence of induced c-jun (Fig. 3D and E). These data suggest that SP1 and c-jun cooperate to mediate the activation of TFPI2 gene expression by P14ARF.

To further examine the respective roles of SP1 and AP-1 in P14ARF induction of TFPI2 gene expression, we performed luciferase reporter and ChIP assays (Fig. 4). P14ARF induction by dox mediated a 2.5-fold increase in the transcription of a transiently transfected TFPI2 promoter driven luciferase reporter, which was abrogated by the pharmacologic inhibition of JNK. Mutation of the AP-1- and SP1-binding sites in the TFPI2 promoter singly or in combination totally abrogated the stimulatory effect of P14ARF, suggesting that both transcription factors are necessary for the gene activation (Fig. 4A). These data also suggest that neither transcription factor is sufficient to mediate gene induction, but rather they act in a cooperative fashion, as each individual siRNA was sufficient to neutralize P14ARF-mediated reporter activation. SP1 (but not AP-1) appears to also contribute to the basic activation of the promoter since its silencing decreases the basal reporter gene activity. We then investigated whether P14ARF increases the binding activity of c-JUN and/or SP1 to the endogenous TFPI2 promoter. ChIP assays demonstrated that the induction of P14ARF increased the binding of SP1 and c-JUN on the TFPI2 promoter (Fig. 4B). The coordinated activation of the TFPI2 promoter by SP1 and c-JUN was not due to cooperation in DNA binding as c-JUN silencing did not alter SP1 binding (Fig. 4C). IgGs served as negative control and Histone H3 as positive control. These results support a model whereby P14ARF increases AP-1-binding activity by indirectly augmenting the expression levels of phosphorylated c-JUN through JNK activation. In contrast, there is increased SP1 binding to the TFPI2 promoter (Fig. 4B and C) and c-JUN (Fig. 4D) promoters without altering SP1-expression levels (Fig. 3E), in agreement with our prior findings that P14ARF enhances DNA binding and transcriptional activity of SP1 by freeing it from a negative interaction with HDM2 (28). Indeed, silencing of HDM2 further potentiated SP1 binding to the c-JUN promoter (Fig. 4D).
Finally, to determine whether SP1 and c-JUN are the downstream mediators of P14ARF’s negative regulation of coagulation, we silenced SP1, c-JUN, and TFPI2 in A5 cells. We found that all three siRNAs were able to antagonize the ability of P14ARF to delay plasma clotting (Fig. 5). These findings confirm that the anticoagulation activity of P14ARF requires TFPI2 and its upstream regulators of transcription c-JUN and SP1.

Discussion

As part of their malignant phenotype, glioblastomas display distinct pathologic features, including micronecrotic areas surrounded by pseudopalisading cells. The biologic events triggering the appearance of these structures are incompletely understood, but there is growing evidence that they are in part initiated by vascular obstruction following blood clotting, depriving cells from oxygen and nutrients (39, 40). The genetic causes behind the glioblastoma coagulopathy and the signaling events leading to the activation of the coagulation system in the tumor are poorly defined.

Our previous work has shown that glioblastoma cells are able to activate coagulation by expressing TF, a unique cell-associated receptor for coagulation factor VIIa and a key initiator of blood coagulation (13). Here, we report that the tumor suppressor p14ARF is able to delay clot formation by inducing the transcription of TFPI2 in malignant human glioma cells, and thereby inhibiting the early steps of coagulation. TFPI2, also called PP5, is expressed and secreted primarily in the extracellular matrix of a wide range of cells. TFPI2 contains three Kunitz-type inhibitor domains that mediate its anticoagulation activity through binding the factor VIIa–TF complex (41). A tumor-suppressive role for TFPI2 was initially suggested by the fact that it is abundant in normal tissues, but its expression decreases with tumor grade (42). Reduction in expression with tumor progression is in part explained by TFPI2 epigenetic silencing. The gene is
methylated in more than 20% of glioblastomas, but not in normal brain (17, 43), and in many other cancers, including carcinomas of the pancreas (44), gastric system (45), oesophagus (46), and prostate (18). This tumor-associated epigenetic silencing of TFP2 may be a common mechanism that causes an imbalance in tumors in favor of the local increase of procoagulation factors, which may trigger vascular thrombosis, hypoxia, and necrosis.

Our findings now show that the loss of the CDKN2A locus, one of the most common genetic defects in cancers, also contributes to the regulation of P14ARF expression in tumors. The regulation of this gene by P14ARF is new, and the underlying mechanisms are unknown. Given that P14ARF is known to exert some of its tumor-suppressive functions through raising the levels of p53, we considered the involvement of p53 transcriptional activity in the regulation of TFP2. However, we found that P14ARF upregulated the transcription of TFP2 independently of p53, consistent with the absence of p53-binding sites in the human TFP2 promoter. After considering candidate transcription factors with binding sites in the TFP2 promoter region, we found that the silencing of SP1 and c-JUN, a major component of the AP-1 transcription factor, significantly abrogated the p14ARF-mediated induction of TFP2. ChIP further showed that the binding of both transcription factors to the TFP2 promoter was enhanced by p14ARF, and reporter assays suggested that gene activation might require the cooperation of AP-1 and SP1, as neither was sufficient. These results are consistent with previous reports demonstrating that the activation of AP-1 and SP1 with phorbol esters can upregulate TFP2 (36, 47).

The activation of AP-1 by P14ARF is the result of a signaling cascade that starts with P14ARF, activating the phosphorylation of JNK. The latter then phosphorylates c-JUN, which leads to an autoactivating transcriptional loop of c-JUN expression and further amplifies phospho-c-JUN levels (38, 48). How exactly P14ARF might increase JNK phosphorylation remains to be determined. JNK has many upstream activators (49), and each one of them might be a potential target for P14ARF. Further studies are needed to explore this activation. Unlike for c-JUN, we did not observe a difference in the levels of SP1 protein upon P14ARF induction. We recently showed that P14ARF could activate SP1 DNA binding and transcription of the TIMP3 gene by freeing SP1 from a negative regulation by HDI2 (28). The same mechanism likely applies to P14ARF regulation of SP1 binding to the TFP2 and c-JUN promoters, the latter leading to indirect amplification of AP-1-mediated TFP2 gene activation.

In summary, we report a new tumor-suppressive activity for P14ARF as an inhibitor of tumor-induced coagulation. This activity is mediated by a JNK/c-JUN activation cascade, which leads to AP-1 activation, and transcriptional upregulation of TFP2 expression in coordination with SP-1 activation. Altogether, these findings suggest that TFP2 expression can be a barrier to tumor development and that loss of its expression through epigenetic or genetic alteration of P14ARF or its downstream effectors may contribute to the aggressiveness of glioblastomas. These findings also point to potential therapeutic implications for TFP2 as its specific Kunitz domains could be used to antagonize thrombosis in tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A. Zerrouqi, E.G. Van Meir

Development of methodology: A. Zerrouqi, D.J. Brat

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Zerrouqi, B. Pyrzynska, D.J. Brat

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Zerrouqi, B. Pyrzynska, E.G. Van Meir

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Zerrouqi

Study supervision: A. Zerrouqi, E.G. Van Meir

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