Molecular Cancer

Research

PTTG/securin activates expression of p53 and modulates its function
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Published: 08 July 2004
Received: 26 May 2004
Accepted: 08 July 2004

Molecular Cancer 2004, 3:18 doi:10.1186/1476-4598-3-18

This article is available from: http://www.molecular-cancer.com/content/3/1/18

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Abstract

Background: Pituitary tumor transforming gene (PTTG) is a novel oncogene that is expressed abundantly in most tumors. Overexpression of PTTG induces cellular transformation and promotes tumor formation in nude mice. PTTG has been implicated in various cellular processes including sister chromatid separation during cell division as well as induction of apoptosis through p53-dependent and p53-independent mechanisms. The relationship between PTTG and p53 remains unclear, however.

Results: Here we report the effects of overexpression of PTTG on the expression and function of p53. Our results indicate that overexpression of PTTG regulates the expression of the p53 gene at both the transcriptional and translational levels and that this ability of PTTG to activate the expression of p53 gene is dependent upon the p53 status of the cell. Deletion analysis of the p53 gene promoter revealed that only a small region of the p53 gene promoter is required for its activation by PTTG and further indicated that the activation of p53 gene by PTTG is an indirect effect that is mediated through the regulation of the expression of c-myc, which then interacts with the p53 gene promoter. Our results also indicate that overexpression of PTTG stimulates expression of the Bax gene, one of the known downstream targets of p53, and induces apoptosis in a human embryonic kidney cell line (HEK293). This stimulation of bax expression by PTTG is indirect and is mediated through modulation of p53 gene expression.

Conclusions: Overexpression of PTTG activates the expression of p53 and modulates its function, with this action of PTTG being mediated through the regulation of c-myc expression. PTTG also upregulates the activity of the bax promoter and increases the expression of bax through modulation of p53 expression.

Background

The pituitary tumor transforming gene (PTTG), a securin, was cloned initially from a rat pituitary tumor [1]. Subsequently, we and others cloned the human PTTG gene [2,3] and characterized its function. PTTG protein is expressed at higher than normal levels in several tumors, including those of the pituitary [4], thyroid [5], colon [6], ovary [7], testis [7] and breast [8], as well as in hematopoietic neo-

plasms [9]. In some tumors, including those of the thyroid [10], pituitary [11], esophagus [12] and colorectum [6], high levels of expression of PTTG correlate with tumor invasiveness and, more recently, PTTG has been identified as a key “signature gene”, with high expression predicting metastasis in multiple tumor types [13]. Overexpression of PTTG enhances cell proliferation, induces cellular transformation, and promotes tumor formation in nude
mice [2,3]. The involvement of PTTG in several cellular functions, such as mitosis [14,15], cell cycle progression [16], DNA repair [17] and secretion and expression of basic fibroblast growth factor (bFGF) [18] and vascular endothelial growth factor (VEGF) [19] has been reported.

Structural homology suggested that PTTG may be a mammalian securin and this has been confirmed by its involvement in regulating sister chromatid separation during mitosis [16]. The involvement of PTTG in cell signaling via the MAP kinase cascade [20] and its interaction with the c-myc promoter suggest its involvement in cellular transformation [21].

There is now considerable evidence supporting the concept that PTTG can regulate apoptosis in both a p53-dependent and a p53-independent manner [22]. In their studies, Yu et al. [22] have shown that overexpression of PTTG results in the induction of apoptosis of cells that are p53 deficient (osteosarcoma MG-63 cells), as well as cells that express functional p53 (breast tumor MCF-7 cells), with overexpression of both PTTG and p53 resulting in an increase in apoptosis of MCF-7 cells but not MG-63 cells. However, overexpression of E6, which targets p53 for degradation, eliminated p53 in MCF-7 cells but did not inhibit apoptosis on overexpression of PTTG. Analysis of the molecular basis for the interaction between PTTG and p53 by these investigators [22] showed that overexpression of PTTG in MCF-7 cells resulted in an increase in translocation of p53 protein to the nucleus. Subsequently, Bernal et al. [23] using phage-display screening identified an interaction of the p53 and PTTG proteins in vitro and in vivo. These investigators found that this interaction of p53 with PTTG inhibited the transcriptional activity of p53 and its ability to induce cell death in MCF-7 cells. These investigators did not find activation of p53 expression on overexpression of PTTG in the lung tumor cell line, H1299. In these cells, overexpression of both PTTG and p53 resulted in down regulation of p53-induced apoptosis together with down regulation of the p53-induced expression of downstream signaling genes, including Bax, SFN, and CDKN1A suggesting that, in these cells, PTTG inhibits the function of p53 rather than its expression.

Our previous analysis of the PTTG promoter revealed tumor-specific activation of the promoter in various cell lines and indicated that Sp1 and NF-Y binding sites within the PTTG promoter are involved in its activation [24]. Zhou et al. [25] recently confirmed that Sp1 and NF-Y are involved in the expression of PTTG and that these binding sites play an important role in the regulation of the expression of PTTG by p53. We therefore undertook an analysis of the mechanisms by which PTTG regulates p53 expression and function. Here, we report that PTTG regulates p53 expression and function by modulating the activity of the p53 promoter indirectly through regulation of the expression of c-myc, which binds directly to the p53 promoter sequence.

Results

PTTG upregulates expression of p53 in HEK293 and MCF-7 cells
To determine the relationship between PTTG and p53, we first determined the effect of overexpression of PTTG on p53 expression. For this purpose, we used three different cell lines. Two of these HEK293, which is a human embryonic kidney cell line, and MCF-7, which is a human breast cancer cell line, express wild-type p53, and one, PC3, which is a human prostate cancer cell line expresses a mutant form of p53. As shown in Figure 1A, western blot analysis of the lysates prepared from HEK293 and MCF-7 cells that had been transiently transfected with pcDNA3.1-PTTG revealed a significant increase in expression of both PTTG and p53 proteins compared to cells transfected with vector only. Consistent with the data reported by Rokhlin et al. [26], we did not detect p53 protein on Western blotting of lysates prepared from PC-3 cells that had been transfected with pcDNA3.1-PTTG or pcDNA3.1 vector only. To confirm that overexpression of PTTG activates expression of p53, we performed double immunohistochemical analysis of HEK293 cells transfected with pcDNA3.1-PTTG. As shown in Figure 1B, the cells that exhibited staining of the PTTG protein and showed high levels of expression of PTTG also exhibited intense staining of the p53 protein. Staining of PTTG and p53 was either absent or very weak in HEK293 cells that had been transfected with pcDNA3.1 vector alone. These results suggested that PTTG upregulates the expression of p53.

The up-regulation of expression of p53 by PTTG could be attributed to an alteration in the stability of the mRNA, an alteration in the efficiency of translation, or altered gene transcription. To determine whether the change in expression of p53 is due to an alteration in the transcription of the p53 gene, we analyzed the p53 gene promoter activity and its regulation by PTTG. As shown in Fig. 2, transient transfection of cells with pcDNA3.1-PTTG resulted in a 3-fold higher level of p53 promoter activity in MCF7 cells, a 2.3-fold higher level in PC3 cells, and a 1.6-fold higher level in HEK293 cells as compared to the levels of promoter activity in the same cell lines on transfection with pcDNA3.1 vector only. Taken together, these results clearly demonstrate that up-regulation of p53 expression is, at least in part, due to up-regulation of transcription of the p53 gene by the PTTG protein.

Up-regulation of p53 promoter activity by PTTG is mediated by regulation of expression of the c-myc gene
To map the region of the p53 promoter sequence that is required for activation by PTTG, we performed 5’ and 3’-
deletion analysis of the p53 gene promoter sequence and identified a small segment of 84 base pairs (from nucleotide -172 to -89) located near the transcription start site of p53 gene that is responsible for activation (Fig. 3). This region has been demonstrated previously to be important for p53 promoter activity [27]. DNA footprinting and gel shift assays using the 84 bp p53 promoter sequence and purified PTTG recombinant protein did not indicate direct binding of the PTTG protein to the p53 promoter sequence (data not shown), suggesting that the PTTG protein does not bind the p53 promoter sequence directly.

Sequence analysis of the 84 bp activation region of the p53 promoter revealed binding sites for the nuclear factors, NFκB and c-myc/max heterodimers. The importance of these transcription factors in the regulation of p53 promoter activity has been demonstrated previously [28]. Pei [22] had demonstrated binding of PTTG to the c-myc pro-
moter sequence near its transcription start site, and stimulation of c-myc transcription by PTTG. As PTTG does not exhibit direct binding to the p53 promoter sequence, we hypothesized that PTTG may modulate p53 promoter activity indirectly through its interaction with c-myc/max. To test our hypothesis, we investigated the role of c-myc in the regulation of p53 promoter activity. We first confirmed the effect of overexpression of PTTG on c-myc expression in HEK293 cells (data not shown). We then determined the effects of PTTG on binding of c-myc to the p53 promoter sequence by creating a mutation within the c-myc-binding sequence, CATGTG, that is known to abrogate binding of c-myc/max to the p53 promoter sequence and result in a loss of c-myc effects [28]. As shown in Fig. 4, mutation of the c-myc/CATGTG binding sequence to CATGGA resulted in a complete loss of the effects of PTTG on p53 promoter activity in both HEK293 and PC-3 cell lines, suggesting that PTTG regulates p53 promoter activity by modulating the expression of the c-myc gene, which in turn binds to the c-myc/max sequence of the p53 promoter.

To further characterize the effect of PTTG on the interaction of c-myc/max with the p53 promoter sequence, we performed gel shift analysis using a γ-[32P]-labeled probe derived from the p53 promoter sequence (from nucleotide -172 to -89) containing either the wild-type (CATCTG) or mutated (CTTGGA) c-myc/max binding sequence. As shown in Fig. 5, gel mobility shift assays of nuclear extracts prepared from HEK293 cells transfected with pcDNA3.1-PTTG indicated an enhanced binding of the c-myc protein to the c-myc/max sequence (Fig. 5A and 5B, Lane 2) compared to that observed in extracts prepared from HEK293 cells transfected with pcDNA3.1 vector only (Fig. 5A and 5B, Lane 1). This binding of c-myc to the c-myc/max sequence was specific, since the inclusion of a 20-fold molar excess of unlabeled specific sequence in the reaction mixture resulted in almost complete abrogation of this binding (Fig. 5A, Lane 3), and pre-incubation of the nuclear extract with a c-myc-specific antibody directed against the N-terminal region of the c-myc protein resulted in a supershift (Fig. 5B, Lane 5) nor supershift of the binding complex (Fig. 5B, Lane 6) was observed. It is known that the c-myc protein is highly unstable and has only a short half-life but that it can be stabilized by the binding of its partner, max, at its C-terminal [29]. This c-myc/max heterodimer binds to the c-myc/max target sequence on the p53 promoter and mediates various effects [30]. To confirm whether c-myc binds to the c-myc/max sequence alone or in association with max, we used antibodies directed against the C-terminal of the c-myc protein in the supershift assays. Inclusion of these antibodies negated the supershift of the DNA-protein complex (Fig. 5B, Lane 4), indicating that in these circumstances the C-terminal of c-myc is not accessible and is probably associated with max.

To further validate the role of c-myc in the induction of p53 promoter activity on PTTG overexpression, we utilized a dominant-negative chimeric protein (myc DN), a myc-max fusion containing the DNA-binding domain of max fused to a transactivation-deficient c-myc [31]. It has been shown previously that when myc DN is overexpressed, it can bind to E boxes with high affinity, but that it cannot induce transcription and antagonizes c-myc function in a dominant-negative way [31]. As shown in Fig. 6, co-transfection of Myc DN with the p53-promoter construct resulted in a complete loss of activation of the p53 promoter (Fig. 6A) and the induction of its expression by PTTG (Fig. 6B). These effects of Myc DN appear to be specific, since no changes in p53 promoter activity or its expression were observed in HEK293 cells when co-transfected with Myc DN and the pcDNA3.1 vector (Fig. 6A and 6B). In addition, expression of Myc DN in HEK293 cells did not block the expression of the PTTG protein.
suggesting that PTTG protein up-regulates the transcription and expression of the p53 gene and this action of PTTG is achieved through modulation of c-myc expression and function.

PTTG activates bax gene expression by inducing p53 expression

To assess whether overexpression of PTTG modulates the transactivation properties of p53, we determined the effect of PTTG on bax promoter activity, as bax is a known to be a proapototic molecule that is induced by p53. The bax gene is a pro-apoptotic member of the bcl-2 gene family [32] and its regulation by p53 is well documented [33]. Co-transfection of MCF7 and HEK293 cells with pcDNA3.1-PTTG and the bax gene promoter showed a dose-dependent stimulation of the bax promoter activity in both HEK293 and MCF-7 cell lines (Fig. 7A). Western blot analysis of lysates of HEK293 and MCF-7 cell lines transfected with pcDNA3.1-PTTG revealed enhancement of the levels of bax protein compared with the levels in cells transfected with pcDNA3.1 vector only (Fig. 7B). These results suggest that the PTTG protein up-regulates bax gene transcription and expression. It remains unknown if this effect of PTTG is direct or mediated through p53 gene regulation; however, transfection of PC-3 cells, which do not express functional p53, with pcDNA3.1-PTTG did not result in an increase in the expression of bax (Fig. 7B). Taken together, these results suggest a relationship between PTTG and p53 in the regulation of p53 downstream signaling pathways by PTTG.

Overexpression of PTTG induces apoptosis

Finally, we determined the effect of PTTG overexpression on apoptosis. Apoptotic cells were not detectable on TUNEL staining of HEK293 cells that had been transiently transfected with the pcDNA 3.1 vector alone (Fig. 8A). In contrast, apoptotic cells were apparent on transient transfection of the HEK293 cells with either PTTG or p53 cDNA (Fig. 8A). Furthermore, flow cytometric analysis of HEK293 transfected with PTTG or p53 cDNA indicated that a greater number of cells in G1 phase in both PTTG-transfected (12%) and p53-transfected (22.6%) cells than in pcDNA 3.1-transfected cells (Fig. 8B), indicating that
PTTG and p53 cDNA induce cell cycle arrest at the G1 phase.

Discussion
The present study was focused on determining the mechanism(s) by which PTTG regulates the expression of p53. For this purpose, we used three different cell lines: HEK293 and MCF7, which express wild-type p53; and PC-3, which expresses a mutant form of p53 that is degraded rapidly. Overexpression of PTTG led to activation of p53 expression in the HEK293 and MCF-7 cell lines, but not the control PC-3 cells (Fig. 1A). Immunohistochemical analysis of the HEK293 cells that had been transfected with pcDNA3.1-PTTG confirmed that overexpression of PTTG protein resulted in high levels of expression of the p53 protein (Fig. 1B). The activation of the expression of the p53 protein by PTTG in MCF-7 cells is consistent with the findings of Yu et al. [22] who reported an increase in expression of p53 and its translocation into nucleus on overexpression of PTTG in these cells. In their studies, Yu et al. [22] did not find simultaneous accumulation of p53 and mdm2 in the nucleus, indicating that PTTG may not use the ARF mechanism to induce p53 expression. It is possible that PTTG may induce p53 nuclear accumulation by inhibiting mdm2 expression through other mechanisms [34]. It also is possible that enhancement of the expression of p53 may lead to the activation of other pathways, which could alter the expression of other genes or the interaction of p53 with other proteins [35]. For example, enhanced p53 may activate bax, an antagonist of Bcl-2 [36], or enhance the synthesis of insulin-like growth factor-I (IGF-I) receptor and one of its binding proteins, IGF-BP3 [37].

The mechanisms by which PTTG up-regulates the expression of p53 may include enhancement of the stability of mRNA, an improvement in translation efficiency, or enhanced transcription of the gene. Our studies suggest that the enhanced expression of the p53 gene is due, at least in part, to an increase in p53 promoter activity. It has

![Figure 4](image)

**Figure 4**
Activation of the p53 promoter by PTTG is mediated through the c-myc/max sequence. HEK293 (open bars) and PC3 (solid bars) were co-transfected with the pcDNA3.1-PTTG and p53/-172/-89 (wild-type myc/max binding sequence) or the p53/-172/-89-Δc-myc (mutated myc/max binding sequence) promoter construct. Transfections were performed in duplicate and the results are expressed as mean ± S.E.M of four independent experiments (*, p < 0.05 by Student's t-test).
been shown that the PTTG possesses transactivating [38] and DNA binding properties [21]. We therefore expected that PTTG might regulate p53 promoter activity by virtue of its direct binding to the p53 gene promoter sequence; however, our DNA foot printing and gel shift assays showed no direct binding of PTTG protein to the p53 promoter sequence, suggesting that PTTG modulates p53 promoter activity through an indirect mechanism. Subsequent 5' and 3' deletion analysis of the p53 gene promoter identified the sequence between nucleotides -172 to -89, which contains a c-myc/max binding sequence, as being responsive to PTTG activation. In previous studies, Pei [21] demonstrated binding of PTTG protein to the c-myc gene promoter sequence near its transcription start site and its activation by PTTG. Therefore, it is possible that the enhancement of expression of the p53 gene by PTTG is a result of enhancement of the expression of c-myc. The resulting c-myc would then interact with its partner, max, to form a heterodimer that binds to the p53 promoter and regulates its transcription. Binding of the c-myc protein to this sequence was confirmed by gel mobility shift and super shift assays, which revealed specific bind-

Figure 6
c-myc is essential for activation of p53 expression by PTTG. A: Co-transfection of HEK293 cells with the c-myc dominant-negative (Myc DN) construct, p53 gene promoter and PTTG cDNA. Transfections were performed in duplicate and results are expressed as mean of two independent experiments. B: Western blot analysis of HEK293 cells transfected with either pcDNA 3.1, the c-myc dominant-negative (Myc DN) and/or PTTG cDNA. Expression was detected using specific antibodies. β-Actin antibody was used as a control to determine the variation in protein concentration and loading.
ing of the c-myc protein to this sequence. Site-directed mutagenesis of the c-myc/max sequence resulted in a complete loss of binding of the c-myc protein and p53 promoter activation by PTTG. The importance of c-myc in regulating expression of the p53 gene by PTTG is further supported by the results generated using a c-myc dominant-negative construct that abolished the induction of p53 gene expression by PTTG. These results demonstrate clearly that PTTG up-regulates the expression and transcription of the p53 gene by modulating the expression of the c-myc gene and its binding to the c-myc/max sequence on the p53 promoter.

PTTG is an oncogene and has been shown to induce cellular transformation in vitro and promote tumor formation in nude mice [2]. In an attempt to define the mechanism by which PTTG contributes to tumorigenesis, Bernal et al. [23] used phage display screening and determined that the p53 protein interacts with the PTTG protein both in vitro and in vivo leading to inhibition of the transcriptional activity of p53 and its ability to induce cell death. In contrast to our results, these investigators did not find that PTTG altered the expression of p53. In their studies, these investigators showed a very low or marginal increase in the levels of PTTG protein on transfection with PTTG cDNA. Therefore, the discrepancy between Bernal’s findings and ours may be attributable to the requirement for a threshold amount of PTTG protein for induction of p53 promoter activity. Zhou et al. [25] showed suppression of p53 expression in cells treated with the DNA-damaging drugs doxorubicin and bleomycin. This drug-induced suppression of p53 was shown to be dependent on the presence of functional p53. In their studies, these investigators [25] showed direct suppression of PTTG expression by p53 through its interaction with the NF-Y transcription factor binding sequence of the PTTG promoter, suggesting that the PTTG gene is a target of p53 and may play a role in the p53-mediated cellular response to DNA damage.

**Figure 7**
Overexpression of PTTG activates the expression of bax by upregulating p53 expression. **A:** MCF7 cells (open bars) and HEK293 cells (solid bars) were co-transfected with the bax promoter and increasing amounts of the pCDNA3.1-PTTG expression vector. Transfections were performed in duplicate and the results are expressed as the mean ± S.E.M of four independent experiments (*, p < 0.05 by Student’s t-test). Results are represented as the fold increase compared to control. **B:** Western blot analysis of HEK293, MCF7 and PC3 cells transfected with PTTG for the expression of bax after 48 hours of transfection. β-Actin antibody was used as a control to assess any variation in protein concentration and loading.
Our results show that overexpression of PTTG activates p53 expression, and that this action of PTTG is achieved through the regulation of the expression of c-myc, which in turn regulates the expression of the p53 gene, by its direct binding to the c-myc/max sequence of the p53 promoter. The importance of c-myc in the induction of the expression of p53 by PTTG is further revealed by our studies using a c-myc dominant-negative construct (Fig 6A and 6B). These results are consistent with those of Kirch et al. [28] and Levine [39] who also documented the importance of c-myc for p53 expression and activation. Furthermore, c-myc has been reported to promote apoptosis by destabilizing mitochondrial integrity in cooperation with proapoptotic members of the BCL-2 family including bax [40] and it is considered as an important transcription factor [41]. Thus, its role in the induction of p53 expression seems to be of physiologic importance in the control of genomic stability in cells.

Figure 8
PTTG overexpression induces apoptosis. A: HEK293 cells were transfected with pcDNA 3.1 vector (1), PTTG cDNA (2), or p53 cDNA (3) for 48 hours before the TUNEL assay. B: Transfected HEK293 cells also were analyzed for apoptosis by flow cytometry. Plots show an accumulation of G1 cells upon transfection with PTTG and both an induction of sub-G1 and loss of G2 cells upon p53 transfection. Data are representative of two independent experiments, performed in triplicate.
To assess the significance of the activation of p53 by PTTG with respect to the function of p53 in apoptosis and activation of downstream signaling genes, we analyzed apoptosis using TUNEL staining as well as the activation of the bax promoter. The bax gene encodes a pro-apoptotic member of BCL-2 gene family [32] and its regulation by p53 is well documented [33]. Our results indicate that overexpression of PTTG induces bax promoter activity (Fig. 7A). PTTG overexpression also induces apoptosis in HEK293 cells (Fig. 8). These results are in agreement with the earlier reports of PTTG overexpression inducing apoptosis by p53-dependent and independent mechanisms [22]. It would seem that when both of these apoptotic pathways fail, PTTG can support the survival of aneuploid cells, thereby supporting tumor growth.

**Conclusions**

Our studies reveal that the PTTG protein can up-regulate expression of p53 in cells dependent on their p53 status. This stimulatory effect of PTTG is indirect and is mediated through c-myc. PTTG also up-regulates the activity of the bax promoter and increases the expression of bax through modulation of p53 expression.

**Methods**

**Construction of reporter plasmids**

We cloned the human p53 gene promoter from nucleotides -531 to -3 (p53/-531/-3) into the promoterless and enhancerless basic pGL3 vector (Promega, Madison, Wisconsin). Briefly, the p53 gene promoter sequence (from nucleotide -531 to -3) was amplified using human genomic DNA (Promega, Madison, Wisconsin) as a template and selected sense/antisense primers (sense; 5’-TACTGAGCTCGGGACAGACCTACG-3’; antisense; 5’-TACTGAGCTCGGGACAGACCTACG-3’) in PCR. The 5’ and 3’ deleted constructs of the p53 promoter were generated by amplifying the desired sequence using p53/-531/-3 cDNA as a template and specific primers (Table 1) followed by their subcloning into pGL3 vector. The deleted constructs were designated as p53/-233/-3, p53/-172/-3, p53/-108/-3 and p53/-172/-89. The primers were designed to contain sequences for SacI and XhoI restriction enzymes (underlined) for cloning purpose. We generated a pcDNA3.1-PTTG plasmid by subcloning the full-length PTTG cDNA amplified from the human testis into pCDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA) as described previously [2]. We generated pcDNA3.1-p53 plasmid by subcloning the full length human p53 cDNA into pCDNA3.1. c-myc dominant-negative expression construct pBabe-cyc/max-64 was a generous gift from Dr. Hartmut Land, University of Rochester.

**Cell culture and transfection**

We purchased HEK293, PC-3 and MCF-7 cell lines from American Type Culture Collection (ATCC). All cells were cultured under conditions recommended by the supplier. For transient transfections we seeded the cells in six-well tissue culture plates 24 hours prior to transfection. Cells were transfected with appropriate plasmid DNA (1 µg/well) using Fugene-6 as a transfection reagent (Boehringer Mannheim) as described previously [2]. We used pRenila-Luc (100 ng) (Promega, Madison, Wisconsin) as an internal control. After 48 hours of transfection, the cells were harvested and assayed for luciferase and renilla luciferase activities using dual-luciferase reporter system (Promega) and quantified using a Zylux Femtometer FB12 luminometer.

**Western blot analysis**

The cells were harvested in lysis buffer (50 mM Tris-HCl, 8.3; 100 mM NaCl; 0.1% Triton X-100; 1 mM PMSE; 1 µg/ml leupeptin; 1 µg/ml pepstatin and 1 µg/ml aprotinin) and denatured in loading SDS sample buffer by heating at 95°C for 3 minutes. Fifty µg of proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to Hybond nitrocellulose membrane (Amer-
sham Biosciences). The membranes were blocked in 5% non-fat milk for 1 hour at room temperature, followed by incubation in primary antibody for 1 hour. The antibodies used were anti-PTTG (1:1,500 dilution) [42], anti-p53 (1:2,000 dilution, Zymed Laboratories, San Francisco, California), anti-bax (1:500 dilution, Sigma, St. Louis, Missouri), and actin (1:5,000 dilution, Sigma). Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 was used to dilute the antibodies and wash the membranes. Proteins were visualized using the ECL system (Amersham Biosciences) according to the supplier’s instructions.

**Site-directed mutagenesis**

Site-directed mutagenesis of the putative c-myc/max binding sequence of the human p53 gene promoter carrying a 3-nucleotides change in p53/-172/-89 construct was carried out using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) using a sense 5'-TCCCCCTCCCTGGACTCAAGCAGCGCTA-3' ; antisense 5'-TACGCCTCGTCTGAGTCCAAGGGAGGGGATCCCCTCCCTTGGACTCAAGACTGGCGCTA-3' in PCR. Changing the sequence CATCTG in the wild-type sequence to CTTGGA created the mutation in the c-myc/max binding sequence. The construct was sequenced to confirm the mutation and was designated p53/-172/-89-Δc-myc.

**Preparation of nuclear extracts and DNA footprinting analysis**

Nuclear extracts from HEK293 cells transfected with pcDNA3.1 or pcDNA3.1-PTTG were prepared according to Panek et al. [43]. Briefly the cells were collected in ice-cold phosphate-buffered saline (PBS) and pelleted at 3,700 rpm for 10 minutes. The packed cell volume (PCV) of the cells was measured and then the cells were resuspended (3 volumes of PCV) in hypotonic buffer (1 M HEPES, pH 7.9, 1 M MgCl2 and 1 M KCl) and incubated for 10 minutes on ice. The resuspended cells were then transferred to a glass homogenizer and homogenized. The lysis of the cells was confirmed by Trypan blue dye exclusion. After homogenization, the nuclei were sedimented by centrifugation at 4,600 rpm for 15 minutes and then resuspended (3 volumes of the sedimented nuclei) in low-salt buffer (1 M HEPES, pH 7.9, 0.15 M MgCl2, 0.5 M EDTA, 20 mM KCl and 25% glycerol) and mixed gently, followed by addition of high-salt buffer (1 M HEPES, pH 7.9, 0.15 M MgCl2, 0.5 M EDTA, 1 M KCl and 25% glycerol). The high-salt buffer was added drop wise to the mixture with gentle mixing for 30 minutes. The mixture was centrifuged at 5,000 rpm for 15 minutes. The supernatant containing the nuclear extract was dialyzed against 100 volumes of dialysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT) for 1 hour. The mixture was centrifuged for 20 minutes at 5,000 rpm to pellet any precipitated proteins. and the supernatant containing the nuclear extract was analyzed to determine the protein content and stored at -70°C.

The 84 base pair (-172 to -89) fragment of human p53 promoter was amplified using [γ-32P] end-labeled sense or antisense primer (see Table 1 for primer sequences) and the p53/-172/-89 construct as a template in PCR as described above. This [32P]-labeled DNA fragment was purified on a 2% agarose gel. DNA footprinting analysis was carried out in a 25 µL reaction volume containing 200 mM HEPES, pH 7.9, [32P]-labeled DNA fragment (40,000 cpm) and 50 ng/µL purified PTTG recombinant protein [42]. The binding reaction was allowed to proceed for 10 minutes on ice. The reaction was terminated by addition of 50 µL of stop solution A (10 mM MgCl2, 5 mM CaCl2 and 6.5 ng/µL of yeast transfer RNA) followed by digestion of DNA with 0.2 unit of DNase I (Promega Biotech, WI) for 1 minute at room temperature. The DNA digestion reaction was terminated by addition of stop solution B (20 mM EDTA, pH 8.0, 1.0% SDS and 0.2 M NaCl). DNA was extracted with phenol/chloroform, and separated by electrophoresis through 8% acrylamide/7 M urea gels. The gel was dried and subjected to autoradiography.

**Electrophoretic mobility shift assay (EMSA)**

The [32P]-labeled DNA probe (84 bp) containing either the wild-type or mutated c-myc/max binding sequence was purified from agarose gel and used in gel shift assays. Briefly, 4 µg of nuclear extract was incubated with the γ-[32P] labeled probe (25,000 cpm) in EMSA buffer (50 mM Tris-HCl, pH 7.5; 5 mM MgCl2; 250 mM NaCl; 2.5 mM EDTA; 25 mM DTT and 20% glycerol) and 250 ng/ml poly (dl-dC) in a 25 µl reaction volume for 30 minutes at room temperature. For super shift assays, the nuclear extract was incubated with PTTG antiserum (1:1,500 diluted), N-terminal anti-c-myc rabbit polyclonal antibody (0.5 µg, Santa Cruz Biotechnology, Santa Cruz, CA) or C-terminal anti-c-myc monoclonal antibody (0.5 µg, Zymed Laboratories, San Francisco, CA) for 30 minutes at room temperature prior to the addition of labeled probe. DNA-protein complexes were separated on 4% polyacrylamide gels and analyzed by autoradiography.

**Double immunostaining of cells for PTTG and p53 proteins**

HEK293 cells were grown on polylysine-coated chamber slides (Nunc International Corp., Naperville, IL), for 24 hours and then transfected with pcDNA3.1 or pcDNA3.1-PTTG cDNA as described above. After 48 hours of transfection, the cells were fixed with 4% freshly prepared paraformaldehyde for 8 minutes and then treated with 0.1% Nonidet P-40 for 5 minutes. Cells were treated with 5% normal goat serum for 60 minutes to block nonspecific binding followed by incubation with preimmune serum
Apoptosis assay and flow cytometric analysis

Apoptosis analysis of the cells was carried out using TUNEL assay kit (Roche, Indianapolis, IN). HEK293 cells were transiently transfected with pcDNA 3.1-PTTG or pcDNA 1.50 plasmid as described above. After 48 hours of transfection, the cells were subjected to TUNEL assay following the supplier’s instructions. Briefly, the cells were washed with PBS, air-dried and fixed in 4% paraformaldehyde pH 7.4 for 1 hour at room temperature followed by incubation in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 minutes on ice. The cells were then washed twice with PBS and air-dried. Fifty μl of TUNEL reaction mixture was added to each sample and incubated in the dark in a humidified chamber for 60 minutes at 37°C. The cells were washed three times with PBS and examined under Olympus X-70 fluorescence microscope. For flow cytometric analysis cells were transfected with pcDNA3.1-PTTG or pcDNA3.1-p53 cDNA for 24 hours and analyzed as described by Zhou et al. [25].

List of abbreviations

PTTG, pituitary tumor transforming gene; Myc DN, c-myc dominant-negative expression construct; EMSA, electrophoretic mobility shift assay.

Authors’ contributions

TH carried out most of the experimental work; SSK carried out the immunohistochemistry experiments and helped in evaluation of the data and manuscript preparation. Both authors read and approved the final version of the manuscript.

Acknowledgment

This work was supported by grants from NIH/NCI 82511 and Kentucky Lung Cancer Program.

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