Human Pituitary Tumor Transforming Gene (hPTTG) Inhibits Human Lung Cancer A549 Cell Growth through Activation of p21\textsuperscript{WAF1/CIP1}

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**Abstract.** Pituitary tumor transforming gene (PTTG) is a proto-oncogene cloned from rat GH4 cells. This gene was able to induce cell transformation \textit{in vitro} and is also associated with p53-dependent and -independent apoptosis. In this study, we cloned human PTTG (hPTTG) from a pituitary tumor and then stably transfected the hPTTG into HeLa and A549 cells. An overexpression of hPTTG significantly inhibited cell growth, which was determined by the adherent cell growth properties, colony formation in soft agar and [\textsuperscript{3}H] thymidine incorporation, respectively, in HeLa and A549 cells. The inhibitory effect on cell growth was associated with the activation of p21\textsuperscript{WAF1/CIP1} in A549 cells, but not in HeLa cells. The hPTTG overexpression increased both the p21\textsuperscript{WAF1/CIP1} mRNA and protein expression levels as determined by both Northern and Western blot analysis, respectively, in A549 cells. The increased expression of p21\textsuperscript{WAF1/CIP1} mRNA was regulated at the transcription level and was independent on p53 expression because the luciferase activity increased after the co-transfection of hPTTG and p21\textsuperscript{WAF1/CIP1} promoter fragments with and without a p53 binding sequence. The subcellular distribution of hPTTG was dependent on cell type, and was predominantly in the nucleus in HeLa, Cos-7 and DU145 cells, but showed a diffuse distribution in both the nucleus and cytoplasm in A549, DLD-1 and NIH3T3 cells. These results indicate that an overexpression of hPTTG inhibits the cell growth due to different mechanisms, which are p21\textsuperscript{WAF1/CIP1}-dependent and -independent.

\textit{Key words}: Pituitary tumor transforming gene (PTTG), A549 cell, p21/WAF1/CIP1

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A new powerful transforming gene, called pituitary tumor transforming gene (PTTG), has recently been reported to be isolated from rat GH4 cells. This gene was able to induce cell transformation \textit{in vitro}, and tumor formation in nude mice [1]. Human PTTG (hPTTG) was cloned by three independent groups two years ago [2–4]. Among the three publications, the cDNA sequences of the coding region were the same in two of them, but the sequences of the 5'-uncoding region are quite different. hPTTG contains an open reading frame for 202 amino acids and demonstrates 85% identity with rPTTG protein. hPTTG has been determined to be located on chromosome 5q33, a locus associated with recurrent lung cancer and myelogenous leukemias, using fluorescent \textit{in situ} hybridization
The wide distribution of PTTG in normal tissues, including the testis, thymus, placenta, small intestine and colon indicates that this gene is not pituitary tumor specific [2, 3]. In fact, hPTTG is highly expressed in some malignant tumor cells, especially in hematopoietic neoplasms [2, 3].

The mechanism of PTTG to induce cell transformation is still not completely understood. PTTG induces mRNA and protein expression of basic fibroblast growth factor (bFGF), a major activating factor for angiogenesis, in stably transfected NIH3T3 cells [2]. A concordant expression of PTTG and bFGF was demonstrated in both human pituitary tumor and in estrogen-induced rat lactotroph tumors [5]. Some evidence also indicates that PTTG plays a potential role in the regulation of transcription [3, 6, 7]. Chien and Pei demonstrated that the co-transfection of PTTG and PTTG binding factor expression vectors induces a transcriptional activation of the bFGF promoter [6]. Pei also found that PTTG is phosphorylated by MAP kinase and is linked to a MAPK cascade by direct interaction with MEK1 through a putative SH3-domain binding site [8]. More recently, it was demonstrated that the induction of PTTG resulted in increased cell proliferation through the activation of c-Myc. PTTG binds to c-Myc promoter near the transcription initiation site and activates c-Myc transcription [7]. PTTG mRNA and protein expressions are cell cycle-dependent, which is low at the G1/S border and peaked at G2/M phase [10]. Yu et al. recently showed that an overexpression of PTTG by a transient transfection of PTTG-EGFP into cells induced apoptosis in tumor cell lines [11]. These apoptotic effects are p53-dependent in MCF-7 cells and p53-independent in MG-63 cells [11]. PTTG mRNA and protein expressions are cell cycle-dependent, which is low at the G1/S border and peaked at G2/M phase [10].

In the present study, we cloned a human cDNA homologue of rat PTTG from human pituitary tumors. This PTTG cDNA demonstrates 100% identity with one of three published sequences in both the coding region and 5'-uncoding region. The entire coding region of this gene was subcloned into a pIRE

 expression vector and then was transfected into HeLa and A549 cells. An overexpression of hPTTG was thus found to significantly inhibit the cell growth in both HeLa and A549 cells. The inhibitory effect on cell growth was associated with the activation of p21WAF1/CIP1 in A549 cells, but was not in HeLa cells. The subcellular distribution of hPTTG is dependent on cell type, and is predominantly nuclear in HeLa, Cos-7 and DU145 cells, and shows a diffuse distribution in both nucleus and cytoplasm in A549, DLD-1 and NIH3T3 cells.

**Materials and Methods**

*Cloning of human PTTG cDNA*

For the isolation of hPTTG cDNA, we first designed the sense/antisense primers, 5'-TCCAGGATGGCTATCTGATC-3'/5'-AGGATCATGAGAGGCACTCC-3', based on the sequence of rat PTTG cDNA [1]. PCR was performed in 30 μl of reaction mixture containing 3 μl of 10x buffer (Promega), 1.8 μl of 25 mM MgCl2, 1.8 μl of 2.5 mM deoxy-NTPs, 2 μl each of 10 pmol/μl primers, 2 μl of template cDNA synthesized from total RNAs of human pituitary tumors and 2.0 U Taq DNA polymerase, at 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec, at 53°C for 30 sec, and at 72°C for 2 min. A single band of the predicted size was visualized by 1% agarose gel electrophoresis with ethidium bromide staining, excised from gel and then subcloned into the pGEM-T-easy vector (Promega). A 453 bp of hPTTG was determined by a sequencing analysis. This fragment showed 79% identity with rPTTG cDNA and 100% identity with published hPTTG. For the isolation of the full human cDNA, 5'- and 3'-rapid amplification of cDNA ends (RACE) were performed using a Marathon cDNA amplification kit (Clontech). The first-strand and then second strand cDNA was synthesized using 2 μl of poly(A) RNA prepared from human pituitary tumors according to the manufacturer's instructions and ligated to Marathon cDNA adapter.
Both the antisense (5'-ATCATCTGAGGCAGGAAC AGAGC-3') and sense (5'-GCCTTACCTAAAGCTA CTAGAAAGG-3') primers for 5'-RACE PCR and 3'-RACE PCR were designed based on the sequence of the 453 bp fragment. The PCR products of 5'-RACE and 3'-RACE were subcloned into a pGEM-T-easy vector and then were sequenced to verify their structures.

Northern blot analysis

Tumor cell lines including THP-1, Raji (purchased from Health Science Research Resources Bank, HSRBB, Osaka, Japan), AR230 (donated by Dr. K. Muta, Kyushu University, Fukuoka, Japan), DLD-1, HeLa, A549, H1299, HepG2 (obtained from the American Type Culture Collection, ATCC) and CEM cells (kindly provided by Dr. B. Thompson, The University of Texas Medical Branch, Galveston, TX) were cultured in an appropriate medium, respectively. Total RNA was prepared by the acid guanidium thiocyanate-phenol chloroform using Isogen (Wako Pure Chemical Co., Osaka, Japan). Twenty micrograms of total RNA were electrophoresed through a 1% agarose gel containing formaldehyde and transferred to Hybond N+ membrane (Amersham). The membrane was hybridized with 32P-labeled hPTTG cDNA probe in 5 ml hybridization buffer containing 50% deionized formamide, 0.5x SSPE, 0.5x Denhardt’s solution, 0.5% sodium dodecyl sulfate (SDS), and 1 mg salmon sperm DNA (Sigma). After hybridization overnight at 50°C, the membrane was washed according to the manufacturer’s instructions and exposed to BioMax-MS film (Eastman Kodak) for 2 days at –70°C.

Subcellular distribution of hPTTG

The pCMX-GFP-hPTTG expression vector was prepared in order to determine subcellular distribution of hPTTG. First, Xho I and Bam HI sites were added to the 5’ and 3’ ends of hPTTG cDNA, respectively, through PCR using pGEM-hPTTG as a template. The hPTTG cDNA was produced by Xho I-Bam HI digestion and inserted at the Sal I-Bam HI sites of pCMX-GFP which was kindly donated by Dr. H. Ogawa (Nara Institute of Science and Technology, Nara, Japan) [14]. The validity of this construct was confirmed by enzymatic digestion and sequencing. In pCMX-GFP-hPTTG, GFP was fused at the amino-terminal of a hPTTG which lacked the first 2 amino-acid residues. The pCMX-GFP-hPTTG was transfected into HeLa, A549, Cos-7, DU145, DLD-1 and NIH3T3 cells (obtained from ATCC) by SuperFect transfection reagent (Qiagen, GmbH, Germany) according to the manufacturer’s instructions. After overnight culturing at 37°C, the cells were observed using confocal fluorescence laser scanning microscopy attached to computer.

Construction of expression vector and stable transfection into HeLa and A549 cells

The entire coding region of hPTTG was isolated from pGEM-T-hPTTG vector by digestion and inserted into pIRES-neo vector (Clontech) using the EcoRI site. The construction was confirmed by enzymatic digestion, and was sequenced to verify the correct reading frame. HeLa and A549 cells were cultured in DMEM supplemented with 10% FBS and antibiotics. The pIRES-neo-hPTTG was transfected into the cells by SuperFect transfection reagent. Two days after transfection, the cells were treated with G418 (Gibco, Life Technologies) at 1 mg/ml for 3 weeks and then were continuously cultured with 0.8 mg/ml G418. For growth experiments, cells transfected with hPTTG or empty vector were plated into 24-well plate at 1 × 10^4 cells/well in DMEM medium supplemented with 10% FBS and the medium was changed every 2 days. After washing, the cells were trypsinized and then counted by Trypan blue exclusion using a hemocytometer every 24h for 6 days. The cell growth in soft agar was based on the methods described by Cowley et al. [15]. Six centimeter tissue dishes were coated with 5 ml soft agar (20% 2xDMEM, 10% FBS, 50% DMEM, 20% 2.5% agar, melted and combined at 45°C). Cells suspended in medium were then combined with 4 ml of agar mixture, and 1.5 ml of this mixture were added to each dish. Cells were plated in triplicate at a density of 10^5 cells/ml and incubated for 28 days. After washing, the cells were trypsinized and counted by trypan blue exclusion using a hemocytometer every 24h for 6 days. The cell growth in soft agar was based on the methods described by Cowley et al. [15]. Six centimeter tissue dishes were coated with 5 ml soft agar (20% 2xDMEM, 10% FBS, 50% DMEM, 20% 2.5% agar, melted and combined at 45°C). Cells suspended in medium were then combined with 4 ml of agar mixture, and 1.5 ml of this mixture were added to each dish. The cell were plated in triplicate at a density of 10^4 cells/ml and incubated for 28 days. The cells were fed with 1 ml of medium, serum, and 0.3% agar mix on a weekly basis.

[3H] Thymidine incorporation

For the DNA synthesis measurement, [3H] thymidine incorporation was performed using the method described by Cover et al. [16]. Briefly, HeLa and A549 cells transfected with pIRESneo empty vector or
hPTTG were plated onto 24-well culture dishes. After cell attachment, the medium was changed and the cells were pulsed for 3 h with 3 μCi of [3H] thymidine, and then washed three times with ice-cold 10% trichloroacetic acid, and then lysed with 300 μl of 0.3 N NaOH. Lysate (200 μl) were transferred into vials containing 5 ml of scintillation fluid, and radioactivity was quantitated by scintillation counting. Triplicates were averaged and expressed as cpm per well.

Western blot analysis

A Western analysis of p53, p21WAF1/CIP1 and β-actin was performed as described previously [17] using specific antibodies purchased from Santa Cruz Biotechnology (p21WAF1/CIP1 and β-actin) and PharMingen (p53), respectively. The cells transformed by hPTTG were cultured in medium with 0.8 mg/ml G418. The cells were then washed with PBS and lysed with SDS sample buffer (62.5 mM Tris- HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT and 0.2 mM/L PMSF supplemented with 0.2 trypsin inhibitory units/ml aprotinin, 0.7 μg/ml pepstatin, and 1 μg/ml leupeptin). The samples were sonicated for a few seconds to shear the DNA and reduce the viscosity. Twenty micrograms of protein were mixed with an equal volume of 2 x electrophoresis buffer and then boiled for 3 min. The samples were electrophoresed on a 10% SDS-polyacrylamide gel and proteins were transferred to a Hybond-P, PVDF membrane (Amersham Life Science). Blots were rinsed in Tris-buffered saline-0.1% Tween-20 (TBS-T) and blocked with 4% BSA overnight at 4°C. The blots were incubated with mouse anti-human p53 (1 : 1,000), goat anti-human p21WAF1/CIP1 (1 : 1,000), and goat anti-human β-actin (1 : 200) antibodies, respectively, for 1 h at room temperature. After washing with TBS-T 3 times, the blots were probed with horseradish peroxidase-labelled anti-mouse IgG (1 : 2,000) or anti-goat IgG (1 : 500), respectively, in blocking buffer. Proteins were detected using a chemiluminescence luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the bands were visualized by autoradiography.

Plasmid construction and luciferase analysis

p21WAF1/CIP1 promoter fragments were generated from A549 genomic DNA by PCR. The sequences of the sense and antisense primers to amplify a 2296 bp fragment of p21WAF1/CIP1 promoter containing p53 binding sequence were 5'-GCTTGGGCAGCAGGCT GTGG-3' and 5'-GCGCGGCCCTGATATACA-3', respectively [18]. A specific sense primer (5'-CTGGCATAGAAGGGCTGTGG-3') was designed and combined with the antisense primer described above to amplify a 2227 bp p21WAF1/CIP1 promoter fragment without p53 binding sequence. The PCR fragments were subcloned into the pGEM-T-easy vector, sequenced and then inserted into Hind III site of the pGL3 basic vector. Transfection was performed using SuperFect reagents (Qiagen, GmbH, Germany) according to the manufacturer’s instructions. Briefly, A549 cells (1 × 10^6) were seeded in a 6-well plate 12 h prior to transfection, and they were transfected with total 0.9 μg of DNA constructs. One hundred nanograms of Renilla luciferase control reporter pRL-SV40 (Promega Co. Madison, WI), as an internal standard, were added per well to assess the transfection efficiency. The transfected cells were incubated at 37°C for 3 h and then were cultured in 10% FBS-containing medium overnight. On the day after transfection, the cells were lysed and harvested to perform a luciferase analysis using a Dual-luciferase reporter assay system purchased from Promega according to the technical manual.

Statistical analysis

For the analysis of [3H] thymidine incorporation, protein expression level and luciferase activity, the results were expressed as the mean ± SD from four independent experiments. Statistical analysis was performed using the Statview software. All data were evaluated for unpaired variables to compare two or more groups. P<0.05 was considered to be statistically significant.

Results

Full cDNA cloning and tumor cell expression

A 719-bp cDNA was isolated using 5'- and 3'-RACE from human pituitary tumors. This hPTTG has an open reading frame of 609 bp encoding a 202 amino acid protein (GenBank accession number AF095287), and has 100% identity with one of three published hPTTG sequences [2] in coding region and in 5'-
uncoding region, except for two bp differences in the 3’-uncoding region.

A Northern blot analysis of hPTTG revealed about 0.75 kb mRNA signal (Fig. 1). The strongest expression in THP-1 and Raji cells, and middle to weak expression in CEM, HeLa, AR230, DLD-1, H1299, HepG2 and A549 cells were detected. The transcript size of hPTTG seemed to be relatively small in the CEM-C7, Raji and HepG2 cells.

Subcellular distribution of hPTTG

To determine the subcellular distribution of hPTTG, HeLa, A549, Cos-7, DU145, DLD-1 and NIH3T3 cells were transfected with pCMX-GFP-hPTTG to visualize the green fluorescence. After 24 h transfection, the cells were scanned using confocal laser scanning microscopy (Leica TCS-SP System, Leica Microsystems, Heidelberg, Germany) using a filter for FITC. The cells were imaged for green fluorescence by excitation at the 488 nm line with an argon laser, and the emission was viewed through a 496 to 505 nm band pass filter. HeLa, Cos-7 and DU145 cells transfected with pCMX-GFP-hPTTG showed the localization of the GFP-hPTTG fused protein predominantly in nucleus, while the fluorescence in A549, DLD-1 and NIH3T3 cells transfected with hPTTG demonstrated a diffuse distribution throughout the cytoplasm and nucleus (Fig. 2). These results indicate that the subcellular distribution of hPTTG is dependent on the cell type and may also explain the different results reported by others [3, 10, 13].

Overexpression of hPTTG suppresses the cell growth

To determine whether the overexpression of hPTTG suppresses the cell growth, the entire coding region of hPTTG was cloned into pIRES expression vector under the control of the cytomegalovirus (CMV) promoter and the resulting plasmid was then transfected into HeLa and A549 cells. The empty vector was used as a negative control. The cells were then treated with 1 mg/ml G418 for 3 weeks. All of the HeLa and A549 parental cells were killed by G418 within this period. An overexpression of hPTTG in HeLa and A549 cells
was confirmed by Northern blot analysis (Fig. 3). In response to overexpression of hPTTG in both HeLa and A549 cells, we observed profound alterations in cell growth. As shown in Fig. 4, both HeLa and A549 cell growth was markedly inhibited after hPTTG transfection. On the 6th day of observation, the number of HeLa cells transfected with hPTTG was about 40% of the cells transfected with the empty vector. In contrast, the hPTTG transfected A549 cells were nearly completely arrested. An overexpression of hPTTG caused cells to exhibit an increased size and flattened morphology as well as enlarged nuclei (Fig. 5), especially in the A459 cells. The control cells transfected with empty vector grew small and rounded in a manner similar to that of parental cells. Aneuploidy, which was uncommon in control cells, was also frequently observed in hPTTG transfected HeLa and A549 cells.

The inhibitory effect of hPTTG on cell growth was also determined by soft agar colony formation. The cells transfected with hPTTG or empty vector were plated at low confluence (15,000 cells per 60 mm dish) and grown for 4 weeks. As shown in Fig. 6, cells transfected with empty vector formed large colonies on soft agar. In contrast, the cells transfected with hPTTG grew slowly and formed small colonies, and even A459 cells became big in size, which was consistent with the adherent cell growth properties.

An analysis of [3H] thymidine incorporation revealed that hPTTG caused a significant decrease in DNA synthesis to about 50% and 20% of the control cells transfected with empty vector in HeLa and A549 cell, respectively (Fig. 7).

**hPTTG activates p21^{WAF1/CIP1} in A549 cells**

Previous studies indicated that PTTG overexpression caused p53-dependent apoptosis in MCF-7 cells and p53-independent cell cycle arrest and subsequent apoptosis in MG-63 osteosarcoma cells [11]. We investigated the expression levels of p53 and p21^{WAF1/CIP1} in both HeLa and A549 cells transfected with hPTTG. Total RNA and protein were extracted as described in the Materials and Methods, and mRNA and protein expression levels of p53 and p21^{WAF1/CIP1} were determined by both a Northern blot and Western blot analysis, respectively. p53 mRNA levels showed no changes in the hPTTG transfected HeLa and A549 cells compared with those in the control cells. In contrast, an overexpression of hPTTG induced a significant increase of p21^{WAF1/CIP1} mRNA in A549 cells (Fig. 8). Even the p53 protein level in A549 cells transfected with hPTTG slightly increased to over 1.2-fold that of the control cells, but the difference was not statistically significant (p>0.05) (Fig. 9). In line with the increase of p21^{WAF1/CIP1} mRNA expression level, an overexpression of hPTTG induced a significant increase of p21^{WAF1/CIP1} protein in A549 cells transfected with hPTTG (p<0.01) (Fig. 9). Neither
p53 or p21\(^{\text{WAF1/CIP1}}\) mRNAs and proteins changed due to an overexpression of hPTTG in HeLa cells (Figs. 8 and 9). These results indicated that hPTTG-induced suppression of cell growth in A549 and HeLa cells is due to different mechanisms.

To further determine whether or not the increased expression of p21 mRNA was regulated at the transcription level, we made the p21\(^{\text{WAF1/CIP1}}\) promoter expression constructs with and without the p53 binding sequence, as described in Materials and Methods, and also determined the luciferase activity. As shown in Fig. 10, both the luciferase reporters driven by p21\(^{\text{WAF1/CIP1}}\) promoter with and without the p53 binding sequence displayed a strong promoter activity which was approximately 100-fold greater than the basic vector. The co-transfection of p21\(^{\text{WAF1/CIP1}}\) promoter and hPTTG expression vector induced luciferase activity ranging from 1.5- to 3.3-fold over p21\(^{\text{WAF1/CIP1}}\) promoter alone. In addition, no significant difference was seen between the p21\(^{\text{WAF1/CIP1}}\) promoters with or without the p53 binding sequence co-transfection with hPTTG (p>0.05). These results thus indicate that hPTTG regulates the p21\(^{\text{WAF1/CIP1}}\) expression at the transcription level and that this effect is independent of the presence or the absence of p53.

**Discussion**

In this study, we have isolated hPTTG cDNA from a human pituitary tumor by RACE and demonstrated that the coding region and 5' untranslated region of this cDNA showed 100% identity with one of three published sequences [2]. The transcript was found to be highly expressed in acute monocytic leukemia THP-1 cells and Burkitt lymphoma Raji cells. A relatively low expression was observed in HeLa, AR230, DLD-1 H1299, HepG2, and A549 cells. The wide distribution of hPTTG in these cancer cell lines implies that hPTTG may play an important role in cancer cell growth and proliferation. In fact, the expression levels of hPTTG in human pituitary tumors were much lower than those in tumor cells (data not shown). The transcript size was relatively smaller in the CEM-7, Raji and HepG2 cells than that in other cells. These different transcript sizes had been noticed in human tissue...
and were smaller in the testis than in pituitary tumors. This phenomenon may be due to differential splicing or use of an alternative polyadenylation site for transcription [19].

Early studies indicated the hPTTG signal to be mainly located in the cytoplasm in Jurkat cells and pituitary tumors [3, 10]. In contrast, a more recent study found it to be located in the nucleus of JEG-3 cells [13]. Our results demonstrated that the subcellular distribution of PTTG was dependent on the cell type, which was predominantly in the nucleus in HeLa, Cos-7 and DU145 cells, while showing a diffuse distribution throughout the nucleus and cytoplasm in A549, DLD-1 and NIH3T3 cells. These different results may be due to the different cells and methods used in these studies including our present one. PTTG has been demonstrated to be a securin which inhibits sister chromatid separation and also directly binds to c-Myc promoter [7, 9]. From these functions, PTTG must be located in the nucleus, while PTTG also binds to cytosolic ribosomal protein S10 and DnaJ homologue [12]. Based on these findings, the dual intracellular localization of PTTG is rational and may indicate that it demonstrates different functions in different cells.

Both the rat and human PTTG have been demonstrated to induce cell transformation in vitro and in vivo [1, 2]. Estrogen-induced PTTG expression occurs simultaneously with early pituitary lactotroph transformation and the appearance of newly formed pituitary arterial networks [5]. The PTTG mRNA levels in invaded pituitary tumors are much higher than in tumors confined to the pituitary fossa [13]. These previous studies clearly indicate that PTTG is a novel oncogene. In this study, we demonstrated that hPTTG overexpression inhibited the cell growth in human cervical adenocarcinoma HeLa cells and lung cancer A549 cells by the transfection of hPTTG into these cells. These inhibitory effects were evidenced by a decrease in the cell number, colony size in soft agar and thymidine incorporation. PTTG overexpression led to altered cell morphological properties including an increased cell size and flattened morphology as well as enlarged nuclei, characteristic of cellular senescence. In line with a previous study [11], PTTG also induced aneuploidy in both HeLa and A549 cells in our study. The dual effects of PTTG on the inhibition of cell growth and aneuploidy thus indicate that PTTG is involved in the regulation of cell proliferation. Yu et al.
demonstrated that the PTTG mRNA or the protein expression was cell cycle dependent [10, 11]. Namely, the PTTG expression was low at the G1/S border, gradually increased during S phase, and peaked at G2/M [10].

To further determine the possible mechanism of PTTG’s inhibitory effect on cell growth, we investigated both the mRNA and protein expressions of p53 and p21\(^{\text{WAF1/CIP1}}\). p53 has been demonstrated to mediate PTTG-induced apoptosis in MCF-7 cells, because PTTG up-regulated and translocated p53 to the nucleus, and because the HPV E6 protein, a p53 inactivator, prevented PTTG-induced apoptosis [11]. Our results indicated that hPTTG overexpression inhibited the cell growth through activation of p21\(^{\text{WAF1/CIP1}}\) in A549 cells. Both the p21\(^{\text{WAF1/CIP1}}\) mRNA and protein expression levels increased in A549 cells transfected with hPTTG. The hPTTG regulated p21\(^{\text{WAF1/CIP1}}\)
mRNA at the transcription level as evidenced by a promoter analysis, in which the luciferase activity driven by p21\textsuperscript{WAF1/CIP1} promoter was up-regulated by the co-transfection of hPTTG. This effect was considered to be independent of the p53 expression for the following two reasons. First, neither the p53 mRNA nor the protein expression levels increased by hPTTG. Second, hPTTG increased the luciferase activity in cells transfected with p21\textsuperscript{WAF1/CIP1} promoter expression vectors with and without the p53 binding sequence. p21\textsuperscript{WAF1/CIP1}, since a cyclin kinase inhibitor can induce cell growth arrest by inactivating cyclin-dependent kinase (Cdks) or by inhibiting the activity of proliferating cell nuclear antigen (PCNA). This indicates that the transcription of the p21\textsuperscript{WAF1/CIP1} gene is activated by p53-dependent and -independent mechanisms. The induction of p21\textsuperscript{WAF1/CIP1} by a p53-independent pathway has been demonstrated by previous studies [20–22]. Both the serum and various growth factors, such as platelet-derived growth factor, fibroblast growth factor and epidermal growth factor, were able to induce p21\textsuperscript{WAF1/CIP1} in embryonic fibroblasts from p53 knock-out mice [23]. The transforming growth factor beta (TGFβ) induces p21\textsuperscript{WAF1/CIP1} expression by a direct up-regulation of transcription [24, 25]. An overexpression of the p21\textsuperscript{WAF1/CIP1} results in both G1/S and G2/M arrest and has also been shown to effectively suppress tumor growth [26, 27]. Our study also demonstrated that the hPTTG induced suppression of HeLa cell growth was p21\textsuperscript{WAF1/CIP1}-independent. Neither the p21\textsuperscript{WAF1/CIP1} mRNA nor the protein increased by hPTTG in HeLa cells. These results indicate that hPTTG overexpression inhibits the cell growth by multiple pathways.

In summary, we demonstrated that hPTTG overexpression inhibits the cell growth by different mechanisms, namely, the mechanism is p21\textsuperscript{WAF1/CIP1}-dependent in A549 cells but -independent in HeLa cells. Clarifying the mechanism for hPTTG’s effect on cell growth will be helpful in developing new methods of cancer treatment in the future. The next challenge is to elucidate the molecular events regarding how hPTTG activates the p21\textsuperscript{WAF1/CIP1} in A549 cells.

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