Small interfering RNA against PTTG: A novel therapy for ovarian cancer

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Abstract. Ovarian epithelial cancer is a significant cause of death among women, accounting for 5% of all female cancer-related fatalities. A lack of reliable detection methods and resistance to chemotherapy agents are considerable obstacles in the treatment of this cancer. Recently, high-level expression of the pituitary tumor transforming gene (PTTG) was found in a wide range of tumors, including ovarian cancers. Elevated PTTG levels were found to induce cellular transformation in vitro and tumor formation in nude mice. Therefore, we hypothesize a correlation exists between the levels of PTTG expression and tumorigenesis, and that down-regulation of PTTG levels will result in the suppression of tumor growth. We used small interfering RNA (siRNA) to silence PTTG expression in human A2780 ovarian carcinoma cells and assessed the effect of PTTG silencing in tumor formation in vitro and in vivo. The siRNA directed against PTTG reduced its expression at both the mRNA and protein levels. A fifty percent reduction in cell proliferation was achieved in cells constitutively expressing PTTG siRNA compared to vector or control-siRNA transfected cells. Furthermore, colony formation in soft agar was reduced by 70% in PTTG siRNA stable cell lines. Using nude mice, we showed that animals injected with A2780 cells constitutively expressing PTTG-siRNA decreased the incidence of tumor development and tumor growth. Taken together, these results strongly suggest that PTTG may serve as an important molecular target for the discovery of new anticancer agents and treatment strategies.

Introduction

Ovarian cancer continues to be a significant cause of death among women in the western world. With an increasing number of cases not diagnosed due to a lack of appropriate screening methods or detected at late stages, rising incidence affected approximately 20,000 individuals in 2006. The etiology of ovarian cancer is not clearly understood; however, it is believed that multiple genetic lesions are required to initiate ovarian epithelial cancer. Currently, chemotherapy is among the standard management approaches (1), yet resistance to chemotherapeutic agents is a treatment obstacle for many patients (2). Therefore, a novel therapeutic approach is needed.

Initially cloned from the rat pituitary tumor (3), pituitary tumor transforming gene (PTTG), also known as securin, is an oncogene that is highly expressed in many tumors including pituitary (4,5), lung (6), colon, and ovarian tumors (7-10). On the other hand, PTTG expression in normal tissue is very low except for the testis (8). Sharing the same function as the yeast pds1 (11), securin shares no sequence homology to any other protein identified to date. Based on work from our laboratory and others, securin has been suggested to be a multifunctional protein. Some of its functions include inhibition of sister chromatids separation (11), DNA repair (12), trans-activation of other genes (13), and secretion and expression of angiogenic and metastatic factors (14-16). Studies with NIH3T3 (3,8) and HEK-293 cells (16) demonstrated the ability of PTTG to promote cellular transformation in vitro and tumor formation in vivo. Furthermore, PTTG overexpression induces aneuploidy (17) genomic instability (18), and invasiveness (19).

By generating PTTG null mice (PTTG -/-), the physiological function of PTTG in cellular transformation was demonstrated. PTTG -/- mice developed splenic and testicular hypoplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division, however, these mice were viable and fertile (20). Furthermore, crossing PTTG -/- with Rb heterozygous mice (Rb +/-) resulted in a 30% pituitary tumors compared to 87% in PTTG +/- / Rb (+/+) mice (21). Previous studies with antisense oligonucleotides targeted against PTTG in ovarian tumor cell line (SK-OV3) and cervical tumor cell line (HelaS3) (22,23), were effective at reducing cell proliferation. Recent work with siRNA directed against PTTG in hepatoima cells was successful at reducing tumorigenic phenotypes of these cells in p53-dependent manner (24). Collectively, these data suggest an important role of PTTG in tumorigenesis.

In the present study, we reduced PTTG expression levels using PTTG specific small interfering RNA (siRNA) to...
investigate the consequences of overexpression of PTTG for tumor cell proliferation and growth. The use of siRNA has been a powerful tool used by many researchers to decrease expression or silence target genes. Once introduced into the cell, short 19-21 bp siRNA binds to complementary specific mRNA strand. These short, double-stranded RNA mediate the degradation of complementary mRNA through the recruitment of RNA silencing complex (RISC) (25). Studies in plants and C. elegans showed that siRNA can affect gene expression by inducing promoter methylation or by inhibiting protein synthesis (25). Our data suggest that reducing PTTG in ovarian cancer cells A2780 leads to the inhibition of tumor cell proliferation and a lower incidence of tumor development in nude mice.

Materials and methods

**Cell lines and cell culture.** Human epithelial ovarian carcinoma cell line (A2780) was obtained from Dr Denise Connolly (Fox Chase Cancer Center, Philadelphia, PA). These cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Atlanta, GA) and maintained in a humidified atmosphere at 37°C and 5% CO₂. Colon cancer cell lines HCT116 (wild-type) and PTTG knockout (PTTG -/-) were obtained from Dr Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and grown according to instructions from the supplier. The cells lines were cultured on a routine basis every 3-4 days.

**Generation of plasmid constructs.** To generate constructs constitutively expressing PTTG-siRNA or control-siRNA, we cloned PTTG-siRNA or control siRNA into pSUPER.retro.puro (OligoEngine, Seattle, WA) expression plasmid vector. Sequences used were for PTTG sense (5'-GAT CCC CTG GGA GAT CTC AAG TTT CAT CAA AGA GAC ATG AAA CTT GAG ATC TCC CAT TTT TA-3') and antisense (5'-AGG TAC CAA AAA AAT GTA TTG GCC TGT ATT AGT CTC TGG TAC AGG CCA ATA CAT TTT TTA-3') and antisense (5'-GAT CCC GTA TTG GCC TGT ATT AGT CTC CTA AGG CCA ATA CAT TTT TA-3') and antisense (5'-AGG TAC CAA AAA AAT GTA TTG GCC TGT ATT AGT CTC TGG TAC AGG CCA ATA CAT TTT TTA-3') and antisense (5'-GAT CCC CTG GGA GAT CTC AAG TTT CAT CAA AGA GAC ATG AAA CTT GAG ATC TCC CAT TTT TA-3') and antisense (5'-AGG TAC CAA AAA AAT GTA TTG GCC TGT ATT AGT CTC TGG TAC AGG CCA ATA CAT TTT TTA-3'). The oligonucleotides were obtained from Invitrogen. The sequences were flanked by BglIII and HindIII restriction enzyme sequences for cloning purpose. Equal amounts of sense and antisense primers were mixed and annealed in a 50-μl reaction by heating at 90°C for 4 min followed by 70°C for 10 min, 60°C for 30 min, 37°C for 20 min, and 25°C for 10 min. The pSUPER.retro.puro expression vector was digested with BglIII and HindIII and purified on agarose gel. The annealed oligos were purified on agarose gel and directionally cloned into pSUPER.retro.puro plasmid vector, which directs the synthesis from the H6 promoter. All plasmid constructs were verified by nucleotide sequence analysis.

**Generation of cell lines that express siRNA constructs.** To generate stable clones constitutively expressing siRNA, A2780 cells were plated in 6-well plates 24 h prior to transfection. Cells were transfected with vector alone, control-siRNA, or PTTG-siRNA cloned into pSUPER.retro.puro plasmid (see above) using transfectin transfection reagent (Bio-Rad, Hercules, CA) as described previously (8). Forty-eight hours after transfection, cells were trypsinized and plated in a 100-mm dish. Cells were treated with puromycin (Sigma-Aldrich Co., St. Louis, MO) 8 μg/ml for 2 weeks, cell lines were selected. Expression of each cell line was determined by Western blot analysis.

**Western blot analysis.** Cells were cultured in complete growth media to 80-90% confluence. Cells were washed and collected in cold PBS and lysed with lysis buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA). Protein concentration for each sample was assayed by Bradford Method (Bio-Rad Laboratories). Forty micrograms of protein from each sample was resolved on 15% SDS polyacrylamide gel and transferred to nitrocellulose membranes (GE Healthcare Biosciences, Piscataway, NJ). Membranes were blocked overnight with 5% non-fat milk/Tween (20 mM Tris/HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.6) (TBST). Membranes were incubated with puromycin antiserum diluted at (1:1,500) (9) for 1 h at room temperature followed by 3 washes with TBST and then incubated with HRP-conjugated goat anti-rabbit secondary antibody diluted at 1:5,000. The immune complexes were detected by ECL reagents (GE Healthcare Biosciences). The blots were stripped and reprobed with β-actin antibody (Sigma-Aldrich Co.) to examine equal loading.

**Cell proliferation assay.** To determine the effect of PTTG down-regulation on cell proliferation, A2780 cells transfected with vector, control siRNA, or PTTG siRNA plasmid were seeded (2,500 cells/well) in 96-well plate in triplicate in complete culture growth media containing 8 μg/ml puromycin and incubated overnight at 37°C. The next day, medium was replaced with growth medium containing 5% FBS. Cell proliferation was performed daily using a non-radioactive assay system from Promega (Madison, WI). Briefly, after every 24 h, 20 μl of MTS reagent (CellTiter 96 AQueous) was added to each well and incubated at 37°C for 1 h. Absorbance was recorded at 490 nm (8). Experiments were repeated 3 times.

**Colony formation assay.** To test the effect of PTTG silencing on anchorage independent growth of A2780 cells, cells transfected with vector, control siRNA, or PTTG siRNA plasmid in log phase were trypsinized and counted. Ten thousand cells were mixed with 1.0 ml of 0.33% agar in complete medium/dish and plated in 35-mm tissue culture dishes layered with 2.0 ml of 0.50% agar. Cells were treated with fresh growth medium every 4-5 days for 2 weeks until the colonies were formed. Colonies were counted and photographed. Experiments were repeated twice.

**Tumor development in nude mice.** To assess in vivo activity of PTTG silencing, A2780 cells transfected with control plasmid, control siRNA, or PTTG siRNA were trypsinized, washed twice with PBS, and resuspended in RPMI media without serum and antibiotic. Cells (2x10⁶/site) were injected subcutaneously into both flanks of 4 to 6-week-old nu/nu
mice (NCI). Five mice were used in each group. After 12 days of injection, tumor volumes were recorded every fourth day for 2 weeks (6). For control experiments, mice were injected with colon cancer cell line HCT116 and PTTG knockout HCT116/PTTG (-/-) cell lines. Tumor volumes (mm$^3$) were calculated according to the formula: tumor volume (mm$^3$) = (long dimension) x (short dimension)$^2$/2. After 4 weeks of injection of cells, animals were sacrificed; tumors were collected, measured, and weighed. One part of each tumor tissue was fixed in buffered formalin and another part was frozen in liquid nitrogen and stored at -80˚C for future use. In vivo studies were carried out in accordance with the University of Louisville Institutional Animal Care and Use Committee guidelines.

Statistical analysis. For cell proliferation assay, One-way ANOVA (PRSIM software, version 3.0; Graph pad, San Diego, CA) was performed and the difference between groups was determined by Tukey-Kramer multiple comparison test. For tumor formation in nude mice, the difference between controls. A high level of PTTG expression was observed in 7 out of 10 tumor tissues compared to normal tissues (Fig. 1). Consistent with our results, Cho-Rok et al (24), using an adenovirus system expressing PTTG siRNA to down-regulate PTTG expression, recently showed a significant reduction in tumor growth in vitro and in vivo for hepatoma cells. Taken together, these results suggest that down-regulation of PTTG expression can reduce tumor development and growth. However, use of siRNA is short-lived and the use of adenovirus to deliver siRNA to diminish PTTG expression has limitations. It can infect normal tissues and is accompanied by acute toxicity when used at a high dosage. To overcome this, we generated stable clones constitutively expressing control-siRNA or PTTG-siRNA when transfected with the vector containing PTTG siRNA or control siRNA. We cloned PTTG-siRNA or control-siRNA into pSUPER.retro.puro vector as described in Materials and methods. Positive clones were identified by restriction of the plasmid DNA with EcoRI and HindIII restriction digestion and tumorigenesis, and that down-regulation of PTTG expression in tumors will lead to suppression of tumor growth and development. In our recent studies, we showed that down-regulation of PTTG in lung tumor cells (H1299) by PTTG siRNA resulted in the inhibition of colony formation and tumorigenesis, and that down-regulation of PTTG in lung tumor cells (H1299) by PTTG siRNA resulted in the inhibition of colony formation and tumor development and growth in nude mice (6).

Results and Discussion

Transfection of ovarian tumor A2780 cells with PTTG siRNA down-regulates the expression of PTTG. In our previous studies using Northern blotting, reverse transcriptase/polymerase chain reaction (RT/PCR), and immunohistochemical analysis, we showed high levels of expression of PTTG mRNA and protein in ovarian tumors and tumor derived cell lines (8-10). In the present study, we used an ovarian cancer profiling array (Clontech, Palo Alto, CA) that included normalized cDNA from 10 different ovarian tumors and 10 corresponding normal ovarian tissues, along with negative and positive controls. A high level of PTTG expression was observed in 7 out of 10 tumor tissues compared to normal tissues (Fig. 1). These results confirm our previous results for the high levels of expression of PTTG in most of the ovarian tumors and tumor-derived cell lines. Cloning and sequencing of PTTG from various ovarian tumors showed sequence identity with the human testis cDNA (10), suggesting that the oncogenic function of PTTG is not due to its mutation but rather to its overexpression. Based on these results, we hypothesized that a correlation exists between the level of expression of PTTG and tumorigenesis, and that down-regulation of PTTG expression in tumors will lead to suppression of tumor growth and development. In our recent studies, we showed that down-regulation of PTTG in lung tumor cells (H1299) by PTTG siRNA resulted in the inhibition of colony formation in vitro and tumor development and growth in nude mice (6).
endonucleases. The A2780 cells were transfected with vector, control-siRNA, or PTTG-siRNA construct. Forty-eight hours after transfection, cells were treated with 8 μg of puromycin for 2 weeks and several cell lines were selected and expanded. Using Western blot analysis, we selected two cell lines showing different levels of reduction of PTTG expression; one that showed partial reduction of PTTG expression (designated as PTTG-siRNA Clone I) and another that showed complete reduction of PTTG expression (designated as PTTG-siRNA Clone II) (Fig. 2). We also selected two cell lines each from vector transfected and control siRNA transfected cells.

**Effect of down-regulation of PTTG on cell proliferation.** In our previous studies, we showed that overexpression of PTTG in the mouse fibroblast cell line (NIH 3T3) and human embryonic kidney cell line (HEK 293) resulted in an increase in cell proliferation, cellular transformation in vitro, and tumor development in nude mice (8,16). To investigate whether down-regulation of PTTG expression contributes to cell proliferation, we compared cell proliferation of A2780 cells stably transfected with PTTG siRNA with control siRNA and vector transfected cells. Cells were seeded in quadruplicate and allowed to attach overnight, were washed in serum-free medium, and then incubated with medium containing 5% FBS. Cell proliferation was determined using a non-radioactive assay (MTS assay) from Promega as described in Materials and methods. As shown in Fig. 3, PTTG-siRNA Clone I did not show a significant change in cell proliferation compared to control siRNA or vector transfected cells. In contrast PTTG-siRNA Clone II showed a significant reduction in cell proliferation compared to control siRNA or vector transfected cells (Fig. 3). Previous studies from our lab and
others showed that down-regulation of PTTG expression by transient transfection of tumor cells with antisense oligonucleotide (22,23), PTTG siRNA (6), or PTTG siRNA adenovirus (24) reduced cell proliferation of ovarian cervical cancer cells (HelaS3), ovarian cancer cells (SK-OV3), lung cancer cells (H1299), and hepatoma cells (SH-J1), respectively. Consistent with these results, Melmed and colleagues showed reduced cell proliferation of pancreatic B cells (26) in PTTG knockout animals, suggesting the importance of PTTG in cell proliferation.

Down-regulation of PTTG expression in A2780 cells results in inhibition of colony formation in soft agar. A soft agar growth assay was used to analyze the effect of PTTG down-regulation on the anchorage-independent property of A2780 cells stably transfected with PTTG siRNA. Ten thousand cells from each cell line were plated in soft agar and allowed to grow for 12 days. A 70% reduction in the total number of colonies was detected in PTTG-siRNA Clone II (Fig. 4). These results are consistent with our results obtained with the H1299 lung tumor cell line (6), confirming that down-regulation of PTTG expression is capable of reversing the transformation ability of PTTG in vitro.
Down-regulation of expression of PTTG abrogates tumor formation in nude mice. In our previous studies, we showed that overexpression of PTTG in NIH3T3 and HEK293 cells resulted in tumor development in nude mice (8,16). Consistent with these results, Melmed and his colleagues recently showed that overexpression of PTTG in pituitary in transgenic animals using the oGSU promoter leads to enlargement of the pituitary, development of pituitary adenomas, and hyperplasia of the prostate (27). In our recent studies using ovarian epithelial cell-specific expression of PTTG with an MISIIR promoter in transgenic animals, we showed enlargement of the ovary, development of glandular hyperplasia, and cystic dilation of the endometrium (unpublished results). In contrast, knockdown of PTTG results in hypoplasia of the testis and spleen, and hyperplasia of thymus (20). Furthermore, knockdown of PTTG resulted in a reduction of pituitary tumor development from 87 to 30% in PTTG (-/-)/Rb (+/-) animals compared to Rb (+/-) animals (21), suggesting an important role of PTTG in tumorigenesis. Therefore, we sought to determine whether the tumorigenic potential of ovarian tumor cells (A2780) in vivo would be affected by down-regulation of PTTG. We undertook standard xenograft experiments in athymic nude mice. A2780 cells stably transfected with vector, control siRNA, or PTTG siRNA were transplanted to both flanks of 5 to 6 week-old mice and tumor formation was monitored over a period of 4 weeks as described previously (6). Control siRNA transfected or vector transfected cells produced large tumors in all the animals. Whereas PTTG-siRNA Clone II seems to have lost this tumorigenic potential (Fig. 5). Out of 5 animals injected with cells transfected with PTTG-siRNA Clone II, only 1 animal developed tumors at the site of injection. However, tumor volume in these animals was comparatively smaller than tumors developed in animals injected with vector transfected or control siRNA transfected cells. In contrast, animals injected with PTTG-siRNA Clone I developed large tumors similar to control-siRNA or vector transfected cells. These results suggest that complete, but not the partial reduction of PTTG expression in hepatoma cells on infection with PTTG siRNA adenovirus.

In conclusion, our results clearly demonstrate that down-regulation of PTTG in ovarian tumor cells (A2780) results in the inhibition of cell proliferation and colony formation in vitro and tumor development in vivo. Based on these results, we conclude that PTTG may serve as a potential molecular target for the development of small molecules that can either inhibit the expression of PTTG or block its tumorigenic function to treat cancer. Based on the tendency of patients to develop resistance to chemotherapeutic agents, understanding of PTTG down-regulation holds the potential to serve as a novel strategy and an alternative to existing treatments.

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References


