Title:
Expression of the PTTG1 oncogene is associated with aggressive clear cell renal cell carcinoma

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Abstract

The pituitary-tumor transforming gene PTTG1 is a recently discovered oncogene implicated in malignant progression both endocrine and non-endocrine malignancies. Clear cell RCC (ccRCC) is cytogenetically characterized by chromosome 3p deletions that harbor the ccRCC-related VHL, PBRM1, BAP1 and SETD2 tumor suppressor genes, along with chromosome 5q amplifications where the significance has been unclear. PTTG1 localizes to the chromosome 5q region where amplifications occur in ccRCC. In this study, we report a functional role for PTTG1 in ccRCC tumorigenesis. PTTG1 was amplified in ccRCC, overexpressed in tumor tissue, and associated with high grade tumor cells and poor patient prognosis. In preclinical models, PTTG1 ablation reduced tumorigenesis and invasion. An analysis of gene expression affected by PTTG1 indicated an association with invasive and metastatic disease. PTTG1-dependent expression of the RhoGEF proto-oncogene ECT2 was observed in a number of ccRCC cell lines. Moreover, ECT2 expression correlated with PTTG1 expression and poor clinical features. Together, our findings reveal features of PTTG1 that are consistent with its identification of an oncogene amplified on chromosome 5q in ccRCC, where it may offer a novel therapeutic target of pathological significance in this disease.
Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer and accounts for approximately 39,000 new cases and 13,000 deaths per year within the United States (1). Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell carcinoma and accounts for approximately 75-80% of cases (2). ccRCC is characterized by a recurrent set of chromosomal abnormalities that includes deletions of chromosomes 3p, 9q, and 14q and amplifications of chromosome 5q and 7 (3). Deletion of chromosome 3p removes one allele of the von Hippel-Lindau (VHL) tumor suppressor gene, while somatic mutation typically inactivates the remaining allele. Biallelic loss of VHL is the most frequently reported genetic abnormality in ccRCC, occurring in the majority of cases (4, 5) and leading to stabilization of the HIF proteins and subsequent transcription of HIF target genes (6-8). Amplification of chromosome 5q is the second most common cytogenetic change associated with ccRCC, yet the significance of this abnormality is not understood (3, 9-11). Multiple cytogenetic studies have concluded that genetic alterations of chromosome 5q, particularly 5q33.2-35, are associated with the progression of ccRCC (10, 12-15). Despite the frequency of this aberration, few candidate oncogenes within this region of copy number gain have been implicated in ccRCC (16).

Pituitary tumor transforming gene (PTTG1) is a recently discovered oncogene that resides on 5q33.3 and has been implicated in the development and progression of many malignancies (17). Upregulation of PTTG1 has been correlated with aggressive disease and poor prognosis in hepatocellular carcinoma, prostate cancer, esophageal cancer and glioma, among others (18-22). PTTG1 overexpression has also been associated with the invasiveness of thyroid and colorectal cancers (23, 24). The oncogenic potential of PTTG1 was initially demonstrated by its ability to transform NIH3T3 fibroblasts upon overexpression. This was later substantiated in HEK293 cells in which overexpression of PTTG1 resulted in increased cell proliferation, anchorage-independent growth in soft agar, and tumor formation in nude mice (25). While its potent transforming ability is now well established both in vitro and in vivo, its precise oncogenic mechanism remains unclear as PTTG1 appears to be a multi-functional protein with several possible means of promoting tumorigenesis.
As a securin protein, PTTG1 regulates sister chromatid separation during mitosis and overexpression can interfere with this process, leading to aneuploidy (26). Other studies have suggested an important role for PTTG1 in influencing the tumor microenvironment, in part by increasing the expression and secretion of the important pro-angiogenic factors (25, 27-29). PTTG1 involvement in cell cycle progression and proliferation, p53-mediated apoptosis, and DNA repair has also been reported (27, 30-32). The key to PTTG1’s oncogenic effects likely lies in its role as a transcriptional activator. In 2000, Pei et al showed that the activation of the MAPK pathway led to phosphorylation of the PTTG1 transactivation domain, initiating PTTG1’s nuclear translocation and transcriptional activity (33), and direct downstream targets identified thus far include basic fibroblast growth factor (b-FGF) and c-MYC (27, 34). Mutation of the double PXXP motif required for transactivation has been shown to abrogate the transforming effects of PTTG1 expression (17, 35, 36).

While overexpression of PTTG1 has now been observed in many cancer types, there is little information regarding the role of the PTTG1 oncogene in kidney cancer. Here, we show that PTTG1 overexpression in ccRCC is associated with patient survival and assess its putative role as a promoter of disease progression. In addition, we show that this 5q oncogene is genetically amplified in ccRCC and contributes to the oncogenic transformation of renal cancer cells, as well as, the progression of ccRCC from localized to invasive disease. We also demonstrate that PTTG1 regulates the expression of the Rho-GEF ECT2, another novel proto-oncogene that exhibits significant overexpression in aggressive ccRCC.

Materials and Methods

DNA copy number analysis

Tissue was collected from Spectrum Health Hospital of Grand Rapids, MI and the Cooperative Human Tissue Network (CHTN) under the VARI Institutional Review Board. All tissue was snap-frozen in liquid nitrogen immediately after nephrectomy and stored at -80°C. Genomic DNA was extracted using a Jetquick Maxiprep kit (Genomed, St. Louis, MO, USA) per the manufacturer’s instructions. The SNP mapping assay was performed according to Affymetrix’s protocol. SNP intensity data was analyzed...
using Affymetrix GeneChip Genotyping analysis software (GTYPE) version 4.0 and examined largely with the exception that a divide-and-conquer algorithm was used to segment the copy number data (See Supplemental Methods) (37). The SNP intensity data has been deposited at the Gene Expression Omnibus (GEO 25399).

**PTTG1 and ECT2 expression analysis**

Expression data was generated from the Affymetrix HG-U133 Plus 2.0 platform as previously described (15). A Cox Proportional Hazards model using cancer-specific survival (CSS) and the expression level of each examined gene as a continuous predictor. The significance values (p-values) from the Cox models are reported in the figures and the text. To visualize the relationship between gene expression levels and CSS, Kaplan-Meier survival analysis was performed. The gene expression values were turned into dichotomous variables (high and low) using an optimal cut procedure (see Supplemental Methods). To identify differentially expressed genes following PTTG1 knockdown, expression profiles from treated and control samples were generated from the Affymetrix HG-U133 Plus 2.0 using RMA analysis and data was subsequently unlogged. Genes with expression values under 20 were considered to be not expressed (NE) in the indicated cell line. Fold-change of each gene was defined to be the ratio of expression intensity of that gene in the treated sample over that in the control sample, if the ratio was positive; otherwise, the fold change was defined to be the negative of inverse value of the ratio. The gene expression data has been deposited at the Gene Expression Omnibus (GEO 25493).

**Fluorescent In-situ Hybridization**

FISH probes were prepared from purified BAC clones CTD-2155B8 (5q33.3) (Invitrogen, Carlsbad, CA, USA) and RP11-254G13 (5q11.2) (BACPAC Resource Center, bACPAC.chori.org). The BAC clone DNA was labeled with SpectrumGreen or SpectrumOrange (Abbott Molecular Inc., Des Plaines, IL, USA), by nick translation. Tumor touch preparations were prepared by imprinting thawed
tumors onto glass slides. Images were analyzed using FISHView EXPO v6.0 software (ASI) and 100 to 200 interphase nuclei were scored for each sample.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded tissues were microtome cut into 3 micron sections, deparaffinized and rehydrated. Samples were boiled in citrate buffer, cooled, and subsequently incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity. The sections were blocked with goat serum and incubated with human anti-PTTG1 antibody (Invitrogen) or anti-ECT2 antibody (Sigma Aldrich, St. Louis, MO, USA) overnight followed by incubation with biotinylated anti-rabbit IgG for 1 h at room temperature. Subsequently, the slides were stained with an avidin–biotin complex to visualize antigen–antibody complex, counterstained with hematoxylin, dehydrated and mounted. Substitution of tris buffered saline for primary antibody was used as a negative control. Sections were imaged and sent to pathologist (Dr. James Resau) for semi-quantitative analysis. Stain intensity, either nuclear or cytoplasmic, was scored on a scale of 0 (low) to 4 (high). Statistical significance of variable stain intensity between low grade ccRCC (Fuhrman grades 1 and 2) and high grade ccRCC (Fuhrman grades 3 and 4) was assessed by Wilcoxon Rank-Sum Test.

**Cell Culture**

Caki-1, ACHN, A-498, HK2, SW-156, and 786-O RCC cell lines were obtained from the American Type Culture Collection. SN12C cells were kindly provided by Dr. George Vande Woude (Van Andel Research Institute). UO-31, TK-10 and RXF393 cells were obtained from the National Cancer Institute. HK2 cell lines were obtained in November 2011 and characterized by short tandem repeat analysis by ATCC. All other cell lines were obtained prior to the new requirements for publication and, therefore, have not been authenticated. Cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), in a humidified incubator containing 5% CO₂ at 37°C.
HUVEC were obtained from Clonetics and maintained in Clonetics EBM-2 medium supplemented with EGM-2 SingleQuots or EGM-MV SingleQuots (Lonza; Basel, Switzerland).

**Immunoblotting**

Total protein was extracted from cell lines using HNTG lysis buffer supplemented with protease-inhibitor cocktail (Roche, Indianapolis, IN, USA). Briefly, cells were scraped and incubated on ice in lysis buffer for 30 minutes and subsequently centrifuged at 20,000 rpm for 20 minutes at 4°C to isolate soluble proteins. This fraction was heat-denatured and used for SDS-PAGE and subsequent transfer to a PVDF membrane. Target proteins were identified using rabbit polyclonal anti-PTTG1 (Invitrogen), mouse monoclonal anti-B-Actin (Abcam; Cambridge, MA) or rabbit polyclonal anti-ECT2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies and appropriate secondary reagents.

**Quantitative Real-Time PCR**

RNA was isolated from cells using Trizol Reagent (Invitrogen) using a standard guanidinium thiocyanate-phenol-chloroform extraction method. Taq-Man probes specific for PTTG1, ECT2, and B-Actin were obtained from Applied Biosystems (Carlsbad, CA, USA). RNA was reverse transcribed and amplified for quantitative real-time-PCR using the qScript™One-Step SYBR® Green qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD, USA) according to protocol. Analysis was performed using the comparative-Ct method normalizing target mRNA levels to B-Actin mRNA. A control human RNA sample (Applied Biosystems) was amplified and used as a calibrator for this analysis.

**RNA Interference**

PTTG1 was transiently silenced *in-vitro* using two separate siRNA’s (Invitrogen), and ECT2 was silenced using a combination of 3 siRNA’s (Invitrogen). A non-targeting siRNA (Invitrogen) was used as a negative control. SiRNA was transfected into ccRCC cell lines with lipofectamine-2000 (Invitrogen) using standard lipid transfection methods. Cells were allowed to recover from lipid reagent
permeabilization following transfection by incubation in Dulbecco’s modified eagle medium (DMEM) 10% FBS overnight before usage. Stable knockdown of PTTG1 was achieved using the pLKO.1 lentivirus based shRNA delivery system (38, 39). Plasmids carrying shRNA sequence against PTTG1 (clones TRCN0000015104, TRCN0000015105, TRCN0000015106, TRCN0000015103, TRCN0000015107) were purchased from Openbiosystem™ (Thermo Scientific, Waltham, MA, USA), while a negative control pLKO.1 vector carrying a non-targeting shRNA sequence was purchased from Addgene™ (Cambridge, MA, USA). The generation of lentivirus particles was achieved by co-transfecting pLKO.1clone with second generation packaging vectors (developed by Didier Trono and co-workers), psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259) into HEK293FT cells (Invitrogen). Virus packaging, transduction and selection of stably transduced cells were performed according to a manufacturer recommended protocol (Addgene).

**Plasmid Construction**

PTTG1 from a human cDNA pool (Clontech, CA) was amplified by PCR using forward primer (5’-CTCGGATCCACCATGGCTACTCTGATCTATG-3’) and reverse primer (5’-AGAATTCCAAATATCTATGTCACAGCAAACAGG-3’). The resulting PTTG1 PCR product was inserted into pcDNA6/myc-His B vector (Invitrogen, CA) at the Bam HI and Eco RI restriction sites.

**Immunofluorescence**

Cells were fixed and permeabilized using a 1:1 Methanol/acetone solution at -20 C for 3 minutes and subsequently blocked with 10% donkey serum in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) for 1 hour. Cells were then incubated with rabbit polyclonal anti-PTTG1 antibody (Invitrogen) or anti B-Actin antibody (Abcam) diluted 1:100 in PBS with 1% BSA for 2 hours at room temperature. Cells were then incubated with FITC-conjugated and TRITC-conjugated secondary antibodies (Cell Signaling; Danvers, MA, USA), counterstained with DAPI, and imaged.
Colony formation assay

Cells with or without PTTG1 were tested for their anchorage-independent growth potential. Parental lines 786-O and SN12C and cell lines transduced with shPTTG1 or control vector were counted and 1 x 10^3 cells were placed in 2x DMEM, 20% FBS, mixed with equal volume 0.7% agarose, and plated on top of a base layer of 0.8% agar and DMEM, 10% FBS in a single well of a 6-well plate. For each cell line, 6 replicates were plated. Cells were incubated at 37 C and fed DMEM with 20% FBS twice weekly for 3 weeks until colonies became visible. The plates were methanol-fixed and stained in crystal violet for imaging.

Subcutaneous xenograft

Cells with and without PTTG1 knockdown were tested for their tumorigenic ability in nude mice. Two different parental lines, 786-O and SN12C, and their derivatives shPTTG1-786-O, con shPTTG1-SN12C, or control-vector transduced cells were cultured and 3x10^6 resuspended in 0.2 ml DMEM with 10% FBS were injected subcutaneously into the right flank of each animal. Tumor volume was caliper measured every other day and animal weight was measured weekly until tumor burden necessitated sacrifice.

Proliferation assays

Cells were seeded into 96-well plates at an approximate concentration of 500 cells per well, 12 wells per treatment. The cells were allowed to adhere overnight before cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-(4-sulfophenyl)-2H-tetrazolium assay according to the manufacturer's instruction (MTS; Promega, WI, USA). This was repeated every 24 hours for four days. The results are presented as fold change from initial measurement (t=0).

Invasion assays
Cells transfected with anti-PTTG1 siRNA or a non-targeting control siRNA were serum starved for 24 hours in culture. Subsequently, $5 \times 10^4$ ($1 \times 10^5$ for Caki-1 cells) were resuspended in 200 ul serum-free-media and placed onto BioCoat Growth Factor Reduced Matrigel invasion chambers (BD Biosciences; Pharmingen, CA, USA), which were then placed into a 24-well plate containing 800 ul DMEM plus 10% FBS as a chemoattractant. Cells were allowed to invade through the chambers for 16 hours before the chambers were swabbed and invading cells were methanol fixed and stained with crystal violet for imaging and counting. Two replicates per treatment were used and the experiment was performed in duplicate.

**ECT2 Rescue Experiment**

HK-2 cells were transfected with empty vector pcDNA6 or PTTG1 construct. The stable transfected cells were established and maintained in 20µg/mL blasticidin (Sigma, CA). Then the stable cell lines were transduced with lentivirus expressing short hairpin RNAs (shRNA) targeting ECT2 or a control construct (Santa Cruz, CA) prior subjected to cell proliferation assay and colony formation assay.

**Results**

*5q amplification results in frequent PTTG1 copy number gain*

Single nucleotide polymorphism (SNP) arrays were used to identify regions of copy number change associated with the pathogenesis of ccRCC (37). Consistent with previous reports, we observed deletion of chromosome 3p to be the most frequent cytogenetic abnormality in ccRCC. Deletion of some portion of chromosome 3p was seen in 39 of 43 (91%) cases tested by SNP 100K microarray (data not shown), a frequency consistent with previous reports (12, 37). The second most common chromosomal aberration was amplification of the long arm of chromosome 5 (Figure 1a). We observed copy number gain of this region in 26 of 43 (60%) cases and identified 5q33.2-35 as a refined region of copy number gain, amplified in all cases showing 5q amplification. Similar results regarding the minimal region of amplification have been obtained by two using similar SNP profiling studies (16, 40). Notably, we
observed a high degree of variability in amplicon size. Some cases showed gain of all of chromosome 5, whereas other events were restricted to the terminal end of chromosome 5q. Polyploidy of chromosome 5 was observed in 9 of 43 tumors. Smaller amplification events, typically involving approximately half of 5q, were more common than whole chromosome gain. The smallest amplicon detected by SNP microarray showed copy number gain from 5q33-5qter, spanning just 3 cytobands. In all cases with 5q gain, PTTG1 is located within the minimal region of amplification (Figure 1a). We noted that the oncogenes, EGR1 and CSF1R, are located in the region of PTTG1 amplification and are overexpressed in ccRCC. However, expression of these genes is not associated with overall survival (Supplemental Table 1 and data not shown).

As PTTG1 is a known oncogene that maps within the region of chromosome 5q amplification, we examined the copy number status and expression levels of this gene in ccRCC in greater detail. Examination of the copy number status of PTTG1 locus using the Broad Institute’s Genomic Identification of Significant Targets in Cancer (GISTIC; http://www.broadinstitute.org/igv/GISTIC) module shows copy number gain at the PTTG1 locus in 60% of cases tested (data not shown), the same frequency of amplification observed in our SNP array study. To further investigate copy number gain at the PTTG1 locus at 5q33.3, interphase fluorescent in-situ hybridization (FISH) was performed on ccRCC tumor tissue touch-prep samples (Supplemental Table 2, Figure 1d). FISH analysis showed between two and nine copies of the PTTG1 gene in ccRCC samples, confirming frequent amplification at this locus. More than two copies of the PTTG1 oncogene were observed in 9 of 11 tumors tested by FISH. Some tumors showed large-scale amplification, as detected by increased copy number of a control probe located at 5q11.2 immediately adjacent to the centromere. In contrast, other tumors showed increased copy number of only the PTTG1 probe at 5q33.3, indicative of smaller amplification events (Figure 1d).

*PTTG1 is overexpressed in ccRCC at both the mRNA and protein levels*

Next, we examined the expression of PTTG1 in ccRCC. We observed a significant increase in PTTG1 mRNA levels in carcinoma tissue specimens relative to non-diseased kidney as measured by gene
expression microarrays (Figure 1b and c and Supplementary Figure S1). Elevated PTTG1 mRNA expression is associated with high grade (Fuhrman grade 3 and 4) versus low grade (Fuhrman grade 1 and 2; P = 0.04), high tumor stage (P = 0.002), and decreased patient post-operative survival (P = 0.008). Expression of PTTG1 does not follow a bi-model expression pattern in which a subset of samples contains extremely high levels of PTTG1 and is associated with decreased survival rates. Rather, increased expression of PTTG1 is associated with a proportional decrease in survival probability (Supplemental Figure 1) suggesting PTTG1 plays a role in the progression of this disease and the acquisition of an aggressive phenotype.

PTTG1 overexpression was confirmed by immunohistochemical staining of ccRCC tumors and adjacent normal kidney (Figure 2). Elevated PTTG1 protein levels were seen in ccRCC sections relative to normal kidney with the strongest staining observed in high grade tumors. There was a significant difference (P < 0.05) in staining intensity between low grade tumors (Fuhrman grades 1 and 2) and high grade tumors (Fuhrman grades 3 and 4). These findings are consistent with recent reports of PTTG1 gene and protein expression patterns that have identified PTTG1 in a gene signature of ccRCC pulmonary metastases and demonstrated that PTTG1 protein expression is associated with high nuclear grade, tumor stage, and tumor size (41, 42). Furthermore, we observed predominately nuclear staining with some high grade cases exhibiting both strong nuclear and cytoplasmic staining, consistent with a previous report of both PTTG1 nuclear positivity and cytoplasmic mislocalization in malignant esophageal squamous cell carcinoma (ESCC) tissues (22).

**PTTG1 influences ccRCC tumorigenesis both in vitro and in vivo**

In order to study the importance of the PTTG1 oncogene in vitro and in vivo, we examined the level of PTTG1 protein expression in several ccRCC and other kidney-derived cell lines (Figure 3a). Compared with tumor derived cell lines, non-transformed human umbilical vascular endothelial cells (HUVEC) and HK2 immortalized renal proximal tubular epithelial cells showed low expression of PTTG1. In contrast, 7 of 9 (78%) ccRCC cell lines examined have easily detectable levels of PTTG1.
Notably, one of the two cell lines that did not show high levels of PTTG1 (SW-156) is not tumorigenic in vivo (data not shown). Two PTTG1-positive cell lines, SN12C and 786-O, were selected for further study. SN12C contains a wild-type allele of VHL (VHL wild-type) while 786-O contains a mutated allele of VHL (VHL null).

To establish a functional role for PTTG1 in ccRCC, the endogenously expressed oncogene was targeted using RNA interference in SN12C and 786-O cells. Stable knockdowns were established in these cell lines by lentiviral transduction of shRNA sequences targeting PTTG1. The knockdown efficiency over multiple passages was verified by immunoblotting (Figure 3b). PTTG1 knockdown cells were then tested for their proliferative and colony-formation abilities relative to both the parental line and a vector-transduced control cell line. There was only a marginal effect on the proliferative ability of 786-O cells and no significant effect on SN12C cells in vitro in standard monolayer liquid culture as measured by MTS assays (Figure 3c). Cells were cultured in soft agar to assess the colonigenic potential of non-adherent cells. Colony formation in the knockdown cells was diminished consistent with a role for the PTTG1 oncogene in ccRCC tumor initiation and early growth (Figure 3d,e). The importance of PTTG1 in ccRCC tumorigenesis was further demonstrated by the decrease in tumor growth observed in nude mice upon targeting the oncogene in ccRCC cell lines. When subcutaneously xenografted into nude mice, both targeted lines showed significantly reduced tumorigenicity relative to parental and control cells (Figure 4). Mean tumor volume was reduced by approximately 50% in both the 786-O-shPTTG1 and SN12C-shPTTG1 lines.

**Identification of PTTG1 associated genes**

To identify potential proteins that are influenced by PTTG1, we performed gene expression analysis on mock transfected cell lines and cell lines in which PTTG1 expression was diminished by siRNA transfection. The efficiency of siRNA-mediated PTTG1 knockdown was analyzed by both immunoblotting and immunofluorescence, which revealed almost complete ablation of PTTG1 (Supplementary Figure S2). Subsequent gene expression microarray analysis indicated that expression of
several genes was affected by PTTG1 downregulation, including the Rho GTPase-activating proto-oncogene ECT2 (Supplemental Table 3). ECT2 is a guanine exchange factor (GEF) for the Rho family of GTPase’s (Rho, Rac, CDC42), and is capable of activating these proteins. Activation of Rho and subsequent Rho-dependent actin polymerization is critical for regulating actin dynamics in both cellular locomotion and cellular division (cytokinesis). PTTG1 suppression led to an approximately 2-fold decrease in ECT2 signal as measured by gene expression microarray and we confirmed this finding at both the mRNA and protein levels by quantitative RT-PCR and immunoblotting. siRNA inhibition of PTTG1 resulted in an approximate 4-fold decrease in ECT2 transcript in 786-O and Caki-1 ccRCC cells as measured by qRT-PCR (Figure 5a). This PTTG1-mediated regulation of ECT2 expression was seen at the protein level in four cell lines tested (786-O, Caki-1, ACHN, and SN12C; Figure 5b and c). This relationship between PTTG1 and ECT2 is also supported by expression levels in ccRCC tumors. Similar to PTTG1, expression of ECT2 is associated with high Fuhrman grade and poor survival (Figure 5d and e). In addition, there is a strong association between PTTG1 and ECT2 expression (Figure 5f and g). This statistically significant (P < 0.001) clinical correlation strengthens the direct relationship observed between the two genes at both transcript and protein levels in vitro.

PTTG1-mediated upregulation of the ECT2 proto-oncogene and MMP1 metalloproteinase, two genes involved in cellular motility and invasion, suggested that PTTG1 may play a role in promoting the cellular locomotion/invasion phenotype as opposed to a proliferative phenotype (Figure 3c). To examine the role PTTG1 function has on this aspect of ccRCC cell behavior, we compared the invasive ability of several ccRCC cell lines before and after PTTG1 knockdown. Matrigel-coated Boyden chamber invasion assays demonstrated that siRNA-mediated depletion of PTTG1 could reduce the invasive potential of all four cell lines tested when compared to transfection with a non-targeting siRNA (P < 0.05)(Figure 6).

Due to the reduction of ECT2 expression following PTTG1 knockdown we also investigated the effects of directly targeting the ECT2 oncogene in ccRCC cells. Moreover, ECT2 knockdown in HK-2 cells rescued the effect of overexpression of PTTG1 on both cell proliferation and colony formation (Figure 7). However, in contrast to PTTG1, siRNA inhibition of ECT2 expression resulted in a dose-
dependent growth inhibition of ccRCC cells which promptly acquire a spindle-shaped morphology (Supplementary Figure S1). Flow-cytometric analysis revealed that cells lacking ECT2 accumulate in G2/M phase, consistent with ECT2’s role in cytokinesis. Subsequent immunofluorescent staining showed frequent multinucleation resulting from a failure to complete cytokinesis (Supplementary Figure S3), as previously described in glioma cells (43).

**Discussion**

While copy number gain of chromosome 5q constitutes the second most frequent chromosomal aberration in ccRCC, the significance of this abnormality in the development and progression of the disease has not been explained to date. We show that PTTG1, a recently described oncogene, is located in this region of amplification. PTTG1 is both overexpressed in poor prognosis renal tumors and shown to play an important functional role in vitro. Following PTTG1 knockdown, the effect on ccRCC cell proliferation is modest; however, ccRCC cells exhibit renewed anchorage dependence. As growth in soft agar is a hallmark of neoplastic transformation, it appears that efficient targeting of the endogenously overexpressed PTTG1 oncogene can cause ccRCC cells to revert to a nontransformed phenotype. When these cells are injected into mice, they show a significant reduction in tumorigenesis. The tumors that eventually develop in these mice show restored PTTG1 expression, indicative of selection for cells expressing PTTG1, underscoring the importance of this oncogene in ccRCC pathogenesis (data not shown).

PTTG1 overexpression in human tumor samples is correlated with high Fuhrman grade and high tumor stage, indicative of more aggressive tumors and more invasive tumors respectively. This clinical observation was corroborated by cellular studies in vitro, which showed PTTG1-dependent overexpression of two important activators of invasion, the MMP1 metalloproteinase and the ECT2 Rho-GTPase GEF, a proto-oncogene discovered based upon its transforming abilities. Furthermore, suppression of PTTG1 significantly reduces the invasive phenotype of these cells in vitro. Due to its
overexpression in high grade, high stage, and poor prognosis tumors, as well as, clinical correlation to PTTG1 expression, we examined the relationship between PTTG1 and ECT2 expression.

At the protein level, ECT2 expression is strongly influenced by PTTG1 expression in ccRCC cells in vitro, and these genes are coordinately overexpressed in a subset of high stage, poor prognosis ccRCC tumors clinically. It is interesting to note that Ito et.al identified a number of other Rho family members and Rho-interacting proteins as probable PTTG1 downstream targets in ESCC (22). However, whether PTTG1 lies upstream, downstream, or part of a complex with ECT2 remains to be examined in greater detail. The association between ECT2 and PTTG1 implies that PTTG1’s promotion of the invasive/anchorage independent growth contribute more to the tumorigenic phenotype than an effect on cellular proliferation. Hirata et al. implicated ECT2 in esophageal carcinoma invasion, suggesting that the PTTG1 and ECT2 oncogenes may be coordinately activated in ESCC much like we have observed in ccRCC (44). Either through direct DNA-binding activity or through interactions with other transcription factors such as p53 and Sp1.

In the ongoing search for molecular points of therapeutic intervention for various malignancies, transcriptional activators such as PTTG1 have generally not been successful as drug targets, though this has been a point of contention (45). In addition to the targeted inhibition of kinases, it has previously been shown that GEF’s, such as ECT2, represent viable targets for the development of novel anti-invasion cancer therapeutics (46). The development of resistance to monotherapy and incomplete response rates has necessitated the identification of additional targets for anticancer drug development for use in either personalized therapeutic regimens or combination therapies. Herein, we have identified PTTG1 as a potential candidate oncogene in ccRCC, contributing to both tumorigenesis and the invasive phenotype of ccRCC cells, and furthermore, identified a downstream effector of this oncogene in the Rho-GEF ECT2.

Acknowledgments

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References


Figure Legends

Figure 1. The PTTG1 gene locus is amplified in ccRCC. (a) A heatmap reflecting chromosome 5 copy number in ccRCC (n=43). White color indicates equal copy number as compared to normal tissue; the red and blue colors are reflective of copy number gain and loss, respectively, compared with normal tissue. The intensity of the color indicates significance of copy number increase or decrease as described in the Materials and methods. The location of the PTTG1 FISH probe and the control FISH probe used in Figure 1d and e are also shown. (b) Expression levels of PTTG1 as determined by gene expression microarray. Non-diseased samples (N) and ccRCC tumors as shown. The tumor samples are grouped by grade (1-4). Increased PTTG1 expression is associated with high grade (Fuhrman grade 3 and 4) versus low grade (Fuhrman grade 1 and 2; P < 0.05). Significance was determined using a Wilcoxon Rank Sum Test. (c) Kaplan-Meier curve of PTTG1 expression and cancer-specific survival in ccRCC samples partitioned into high and low groups as described in the Materials and methods. (d) Representative FISH images of samples described in Supplemental Table 1. False color images of DAPI counterstaining (blue), a PTTG1 locus specific probe (red) and a chromosome 5 centromeric probe (green) are shown.

Figure 2. PTTG1 protein expression is associated with high grade ccRCC. PTTG1 protein levels were examined in ccRCC and normal kidney tissue sections by immunohistochemistry. Representative (a) non-diseased kidney, (b) low grade ccRCC, and (c) high grade ccRCC tumors are shown. (d) Semi-quantitative scoring of the image intensity of non-diseased (Normal Kidney), low grade (Fuhrman grades 1 and 2), and high grade (Fuhrman grades 3 and 4) ccRCC. Asterisks (*) indicate significantly increased scores (P < 0.05) as determined using a Wilcoxon Rank Sum Test.

Figure 3. Knockdown of PTTG1 prevents colony formation in soft agar. PTTG1 protein levels were determined by Western blotting in (a) the indicated cell lines and (b) cell lines stably transduced to express control or shRNA sequences targeting PTTG1. (c) Cell number was measured using an MTS
assay in 786-O cells and SN12C cell transfected with control or PTTG1-targeting siRNA. (d) Representative images and (e) quantification 786-O and SN12C cells and cells transfected with control or PTTG1-targeting shRNAs after culture in soft agar for 3 weeks (** P < 0.01).

**Figure 4. Knockdown of PTTG1 reduces the growth of tumor cell xenographs.** Tumor volume of 786-0 and SN12C cells transfected with control or PTTG1-targeting shRNAs.

**Figure 5. PTTG1 expression influences ECT2 expression.** Gene expression microarray analysis was performed on control and PTTG1 targeting siRNAs in 786-O and Caki-1 cells. (a) ECT2 expression in 786-0 and Caki-1 cells and cells transfected with control or PTTG1-targeting siRNAs. (b) ECT2 proteins levels as determined by Western blotting in 786-O cells and cells transfected with control or various PTTG1-targeting siRNAs. (c) PTTG1 and ECT2 expression in other indicated ccRCC cell lines following transfection with either control or PTTG1-targeting siRNA. (d) Expression levels of ECT2 in human ccRCC tumor samples. Non-diseased specimens (N) and ccRCC tumors are shown with the tumors grouped by grade (1-4). The number of samples in each group is shown. Increased ECT2 expression is associated with elevated nuclear grade (P < 0.01). (e) Kaplan-Meier plot of PTTG1 expression and cancer-specific survival in ccRCC samples partitioned into high and low groups as described in the Materials and methods. (f, g) Correlation between PTTG1 and ECT2 expression levels in clinical ccRCC samples.

**Figure 6. PTTG1 knockdown reduces the invasive ability of ccRCC cell lines.** Representative images of crystal violet-stained parental 786-O cells (a) and cells transfected with control (b) or PTTG1-targeting (c) siRNAs following chemotactic invasion through Matrigel. (d) Quantification of invasive cells in indicated cell lines transfected with control or PTTG1 targeting siRNAs. (** P < 0.001, * P < 0.05).
Figure 7. **ECT2 knockdown prevents PTTG1-mediated growth.** HK-2 cells that stably over-expressed PTTG1 were treated with ECT2-targeted shRNA (a) PTTG1 and ECT2 protein levels were determined by Western blotting in the indicated cell (b) Cell number was measured using an MTS assay (**P < 0.01). (c) Colonies formation of indicated modified HK-2 cell lines after culture in soft agar for 5 weeks. (d) Quantification of colonies number for the indicated cell lines (**P < 0.01).
Figure 7
Expression of the PTTG1 oncogene is associated with aggressive clear cell renal cell carcinoma

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