A Novel Regulatory Mechanism of Pim-3 Kinase Stability and its Involvement in Pancreatic Cancer Progression

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Running Title: Stabilization of Pim-3 by TCTP

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Abstract

Translationally-controlled tumor protein (TCTP/TPT1) was identified from a yeast two-hybrid screen and shown to interact with Pim-3, a member of the proto-oncogene Pim family with serine/threonine kinase activity. TCTP was aberrantly expressed in human pancreatic cancer cells and malignant ductal epithelial cells, but not in normal pancreatic duct epithelial cells adjacent to tumor foci of human pancreatic cancer tissue. Moreover, TCTP co-localized with Pim-3 both in human pancreatic cancer cells and clinical tissues. Mapping studies revealed that the interaction between Pim-3 and TCTP occurred through the C-terminal region of Pim-3 and N-terminal region of TCTP. Although Pim-3 had no effect on TCTP expression or phosphorylation, overexpression of TCTP increased the amount of Pim-3 in a dose-dependent manner. Interestingly, RNAi-mediated ablation of TCTP expression reduced Pim-3 protein but not mRNA, through a mechanism involving the ubiquitin-proteasome degradation system. As a consequence of Pim-3 instability and subsequent degradation, tumor growth in vitro and in vivo was inhibited by arresting cell cycle progression and enhancing apoptosis. Furthermore, TCTP and Pim-3 expression were significantly correlated in pancreatic adenocarcinoma specimens, and patients with highly expressed TCTP and Pim-3 presented with a more advanced tumor stage. These observations indicate that TCTP enhances Pim-3 stability to simultaneously promote and prevent cell cycle progression and apoptosis, respectively. Hence, TCTP and Pim-3 serve a pivotal role in human pancreatic cancer with important ramifications for clinical diagnostic and therapeutic implications.

Implications: The present study provides a new idea and experimental evidence for recognizing TCTP/Pim-3 pathway as a target for therapy in human pancreatic cancer.
Introduction

Pim-3, a member of the proto-oncogene Pim family with serine/threonine kinase activity, was originally identified as a depolarization-induced gene, KID-1, in the rat pheochromocytoma cell line PC12 (1). Later, KID-1 was renamed Pim-3 because of its high sequence similarity with Pim family proteins, which belong to the group of calcium/calmodulin-regulated kinase (1). Subsequently, Deneen and colleagues demonstrated that Pim-3 gene transcription was enhanced in EWS/ETS-induced malignant transformation of NIH3T3 cells (2), suggesting the involvement of Pim-3 in tumorigenesis. In line with these observations, we demonstrated that Pim-3 expression was enhanced in malignant lesions, but not normal tissues of endoderm-derived organs such as the liver (3), pancreas (4), colon (5), and stomach (6). Hepatocellular carcinoma development was accelerated in mice expressing the Pim-3 transgene selectively in liver, when these mice were treated with a hepatocarcinogen (7). We observed that Pim-3 can inactivate Bad in human pancreas and colon carcinoma cell lines by phosphorylating Ser^{112}, but not Ser^{136}, and ultimately promoting their survival (4, 5) as observed for Pim-1 and Pim-2 (8, 9). Moreover, it has been reported that Pim-3 can promote cell cycle progression by modulating the functions of molecules that regulate cell cycle progression and augment protein synthesis through the regulation of PGC-1α, eventually contributing to carcinogenesis (10). Baines and colleagues recently reported that Pim-3 suppression can sensitize pancreatic cancer cells to gemcitabine (11). We also demonstrated that Pim-3 can promote tumor growth and angiogenesis by stimulating the VEGF pathway (12). Furthermore, Pim-3 modulates Myc activity to promote tumorigenesis (13). Thus, Pim-3 is a key player in tumorigenesis, and therefore, an ideal target for cancer therapy.

The translationally controlled tumor protein (TCTP) is a highly conserved hydrophilic protein (14) that has been identified in a wide range of eukaryotic organisms, including fungi, yeast, insects, plants, and mammals (15). TCTP is also known as IgE-dependent histamine releasing factor (HRF), fortulin, P21, P23, and TPT-1 (16-18).
This protein was named TCTP because its mRNA was found to be controlled at the translational level (19). Although TCTP is found ubiquitously in tissues and cell types, its expression levels vary depending on the tissue type, growth, stress factors, and cytotoxic signals (20-22). A series of recent reports proved that TCTP plays important roles in a number of cell physiological events in cancer, cell proliferation, apoptosis regulation, stress response, gene regulation, heat shock response, and allergic response (23). TCTP can also interact with many cellular proteins, including translation elongation factors eEF1A and eEF-B-β, tubulin, actin, myeloid cell leukemia protein-1 (MCL1), Bcl-XL, p53, and Na- and K-ATPase (24, 25). However, the roles of TCTP in tumorigenesis remain largely unknown.

Considering the critical role of Pim-3 in tumor development and progression, defining regulatory mechanisms of Pim-3 signaling networks is important. In this study, in order to identify potential novel regulators of Pim-3, we performed yeast two-hybrid screening using human HeLa matchmaker cDNA library. We observed that TCTP specifically interacts with Pim-3 and enhances Pim-3 protein stability by blocking the ubiquitin–proteasome-mediated degradation of Pim-3 protein, thereby promoting tumor growth in vitro and in vivo.
Materials and Methods

Cell culture and antibodies

Human pancreatic carcinoma cell lines, PCI35 and PCI55, were gifts from Prof. Mukaido Naofumi of Kanazawa University (Kanazawa, Japan). Human pancreatic carcinoma cell lines SW1990, MiaPaca-2, PANC-1, and BxPC-3 and the human embryonic kidney (HEK) cell line 293T were purchased from the American Type Culture Collection (ATCC). Among them, SW1990, MiaPaca-2 and PANC-1 cells were cultured in RPMI 1640 supplemented with 10% FBS (Biowest, Inc., Loire Valley, France). BxPC-3 and 293T cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified 5% CO2 incubator. The authenticity of all the cell lines was confirmed by determining DNA profiling of Short Tandem Repeat (STR) while mycoplasma contamination was excluded with the help of Amelogenin (Beijing Microread Genetics Co., Ltd). The antibodies used in this study are described in Supplementary Materials & Methods.

Yeast two-hybrid screening

Human Pim-3 cDNA fragment (121-326aa) was cloned into pGBKT7 vector and used to screen a pACT2-human HeLa matchmaker cDNA library in a yeast two-hybrid system (Clontech, Mountain View, CA), because we did not detect Pim-3 protein in Hela cells (4). β-galactosidase activities were measured using o-nitrophenyl-galactoside as a substrate. Clones activating the β-galactosidase reporter gene were sequenced and analyzed.

Generation of expression vectors

An expression plasmid for TCTP tagged with the His epitope at the COOH- terminus was constructed to detect TCTP protein by the anti-His antibody. A TCTP cDNA fragment, which has an XhoI restriction site in place of the stop codon, was generated by PCR using TCTP cDNA cloned in the pACT2 plasmid as the template and two primers
The amplified fragments were digested with HindIII and XhoI (Takara, Dalian, China) and inserted into HindIII and XhoI sites of the pcDNA4 vector (Invitrogen). The Pim-3 expression vector (pcDNA4-Pim-3) and the Pim-3-shRNA vector (pSilencer-Pim-3-shRNA) were constructed as previously described (4).

Three deletion mutants of TCTP and two deletion mutants of Pim-3 were isolated by PCR using a combination of primers (Supplementary Table S1) for His-tagged constructs. PCR products spanning each fragment were cloned into the EcoRI and XhoI sites of the pcDNA4 vector, and named ΔTCTP-1 (Met1-Gly69), ΔTCTP-2 (Val70-Ala119), ΔTCTP-3 (Glu120-Cys172), ΔPim-3A (met1-pro126), and ΔPim-3B (leu121-leu326), respectively. All mutated nucleotides were confirmed by sequencing.

Co-immunoprecipitation and immunoblotting

HEK293T cells were co-transfected with the Pim-3 expression and pcDNA4-TCTP-His vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Forty-eight hours after transfection, the cells were collected and solubilized with 1 ml of NP-40 lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, pH 7.4, with complete protein inhibitor cocktail (Roche)]. Pre-cleared cell lysates were incubated with 2 μg rabbit anti-Pim-3 antibody or mouse anti-His antibody overnight at 4°C and precipitated with 20 μl protein G-Sepharose 4 Fast flow (GE Healthcare Bio-sciences AB) for 2 to 4 h at 4°C. The beads were washed three times with cell lysis buffer. Materials bound to the beads were eluted with SDS-PAGE loading buffer containing 1% β-mercaptoethanol, boiled for 5 min, and separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Immunoblotting was performed as previously described (4).

Immunofluorescence analysis

PCI55 cells were cultured for 48 h, washed and fixed with 4% paraformaldehyde/PBS, and hybridized with a combination of goat polyclonal anti-Pim-3 antibodies (1:100, Santa Cruz) and rabbit monoclonal anti-TCTP antibodies (1:100, Epitomics). Next, the
cells were sequentially incubated with Alexa Fluor 594 donkey anti-goat IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG antibodies. The signals were visualized using immunofluorescence confocal microscopy (Leica, Switzerland). Immunofluorescence analysis was performed similarly on human pancreatic cancer tissues.

Real-time quantitative (q)RT-PCR

Total RNA was extracted using the Trizol LS reagent (Invitrogen). mRNA was reverse-transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR was performed using the Applied Biosystems HT7900 PCR system with 2× QuantiFast SYBR Green PCR Master Mix (Qiagen), 0.2 μM primers (Supplementary Table S2), and <100 ng cDNA in a 25-μl reaction mixture. Relative expression of target genes was analyzed by the ΔΔCt method. Results are expressed as means ± SD.

Knockdown of TCTP expression

The selected short interfering RNA target sequence in TCTP (5′-AAGGTACCGAAAGCACAGT-3′ corresponded to 179–197 residues) and nonspecific control short interfering RNA duplexes (5′-UUCUCCGAACGUGUCACGUTT-3′) were designed and synthesized by GenePharma (Shanghai Co., Ltd.). For transient knockdown of TCTP expression, PCI55 cells were transiently transfected with the resultant siRNA using Lipofectamine 2000. The small hairpin RNA (shRNA)-encoding oligonucleotides for TCTP and scramble were prepared by Sangon (Shanghai). The annealed shRNA were inserted into the AgeI and EcoRI sites of the lentiviral plasmid pLKO.1-TRC cloning vector (Addgene, Cambridge, USA). For stable knockdown of TCTP, HEK293T cells were plated in 75-cm² culture flasks and transfected with 10 μg TCTP shRNA or scramble shRNA lentiviral vectors. The medium was changed the next day and viral supernatant was harvested 48 h later. All viral containing medium was collected, passed through 0.45-μm syringe filters. PCI55, Miapaca-2, and SW1990 cells were incubated with the lentivirus supernatant for 24 h and selected with puromycin (4 μg/ml).
Cell cycle, cell apoptosis, and cell viability

Flow cytometric analysis was conducted to examine cell cycle and apoptosis with the help of propidium iodine (Invitrogen) and human Annexin V-FITC Kit (Invitrogen), respectively, according to the manufacturer’s protocol. Cell viability was determined at 0, 24, 48, 72, 96, 120 h using the Cell Counting Kit-8 reagent (Dojindo) according to the manufacturer’s protocol. All observations were reproduced at least three times in independent experiments.

Mouse xenografts

Female Balb/c nude mice (6–8 weeks of age, weighing 18–20 g, and specific pathogen free) were obtained from Shanghai SLAC Laboratory Animals (Shanghai, China). Before the experiment, mice were divided into four groups (Mipaca-2-scramble shRNA, Miapaca-2-TCTP shRNA, SW1990-scramble shRNA, and SW1990-TCTP shRNA). Each cancer cell line (5 × 10^6/site) was injected subcutaneously into the right flank of a nude mouse. After establishment of the nude mice xenograft model, tumor sizes were measured every 3 to 4 days using micrometer calipers. Tumor volumes were calculated using the following formula: Volume = \( \frac{1}{2} \times a \times b^2 \), where \( a \) and \( b \) represent the larger and smaller tumor diameters, respectively. Tumor growth was followed for 28 days from the first injection. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals of Fudan University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Fudan University (Permit Number, SYXK(Hu)2009-0082).

Patient and tissue samples

The study included 148 patients who underwent surgery at Fudan University Shanghai Cancer Center from 2004 to 2012 after histological verification of pancreatic ductal adenocarcinoma. None of these patients received preoperative chemotherapy or radiotherapy. The patients provided written consent for the use of tumor tissue for clinical research, and the Fudan University Shanghai Cancer Center Ethical Committee
approved the research protocol. Detailed description of patients and tissue samples is provided in Supplementary Materials & Methods.

**Immunohistochemical analysis of human pancreatic cancer tissues**

Following deparaffinization and quenching of endogenous peroxidase, sections were incubated with 1% bovine serum albumin (BSA) in PBS. Subsequently, the slides were treated with rabbit anti-TCTP (1:500) and goat anti-Pim-3 (1:25) antibodies followed by incubation with donkey anti-goat IgG and goat anti-rabbit IgG antibodies, respectively. Pim-3 and TCTP immunoreactivities were visualized using the GT vision DAB kit (GeneTech). The slides were counterstained with ChemMate Hematoxylin (DakoCytomation) and mounted and observed under a microscope (Olympus). The proportion of Pim-3- or TCTP-positive cells in human pancreatic carcinoma tissues were evaluated by 2 independent pathologists without a prior knowledge on the clinical information. The scoring of TCTP and Pim-3 was performed as described in Supplementary Materials & Methods.

**Statistical analysis**

The $\chi^2$ test or Fisher’s exact probability test was used to compare clinicopathological features of the 148 patients with TCTP and Pim-3 expression. Correlation between TCTP and Pim-3 expression was evaluated using Spearman correlation analysis. Statistical analysis was performed with SPSS statistical software (IBM SPSS Statistics 20). Data were reported as the means ± SD when appropriate and p < 0.05 was considered statistically significant.
Results

Interaction between Pim-3 and TCTP

We initially screened a cDNA library to identify the gene products that can modulate Pim-3 activity as well as serve as its substrate. Using the yeast two-hybrid system with Pim-3 as bait, six candidate genes were identified (data not shown). Among them, we characterized human TCTP protein (NM_003295.2) at first. To validate the specificity of the interaction between Pim-3 and TCTP, His-tagged TCTP protein was co-expressed with Pim-3 in HEK293T cells. Immunoprecipitation with anti-Pim-3 antibodies but not control IgG, co-precipitated His-tagged TCTP (Fig. 1A, compare lanes 1 and 2). Moreover, immunoprecipitation with anti-His antibodies but not control IgG also specifically co-precipitated Pim-3 protein (Fig. 1A, lane 3 and 4). Next, we examined the interaction between Pim-3 and TCTP in PCI55 cells at endogenous levels. Immunoprecipitation with anti-TCTP antibodies, but not control IgG, co-precipitated endogenous Pim-3 (Fig. 1B, compare lanes 2 and 4). In a reciprocal co-IP experiment, the TCTP protein was found to be present in the immune complexes pulled down by the anti-Pim-3 antibodies but not by the control IgG (Fig. 1B, compare lanes 3 and 4). Furthermore, immunofluorescence analysis showed that TCTP and Pim-3 were predominantly localized in the cytoplasm and co-localized in human pancreatic cancer cell lines (Fig. 1C). These observations indicate a physical interaction between Pim-3 and TCTP, and prompted us to examine TCTP expression in human pancreatic cancer cell lines. We detected TCTP mRNA (data not shown) and protein expression in all six human pancreatic cancer cell lines that we examined (Fig. 1D and 1E). Moreover, TCTP expression correlated with Pim-3 expression in these cell lines (Spearman correlation coefficient, 1.0; p < 0.01) (Fig. 1F). To map the region necessary for the interaction between Pim-3 and TCTP, we constructed truncation vectors of TCTP and Pim-3. As shown in Fig. 1G and 1H, Pim-3 was co-precipitated with TCTP-1 deletion mutants but not with the other deletion mutant (TCTP-2 and TCTP-3). Moreover, the Pim-3 mutant lacking the C-terminal domain was not co-precipitated by TCTP, whereas the Pim-3 mutant lacking N-terminal domain was (Fig. 1I and 1J). Taken together, these
results indicate that the interaction between Pim-3 and TCTP is immediately mediated through the C-terminal region of Pim-3 and N-terminal region of TCTP (Fig. 1K).

Pim-3 has no effect on TCTP expression or phosphorylation

We previously observed that Pim-3 can prevent cell apoptosis and eventually lead to progression of human pancreatic cancer (4). Hence, we postulated that functional interaction of Pim-3 and TCTP can promote human pancreatic carcinogenesis. When HEK293T cells with endogenous TCTP protein were transiently transfected with the Pim-3 expression vector, TCTP protein expression did not change (Fig. 2A). Moreover, TCTP protein levels were marginally affected even when the amounts of Pim-3 (Fig. 2B) or incubation periods were increased (data not shown). Furthermore, when MiaPaca-2 cells with a low level of Pim-3 protein, were transiently transfected with Pim-3, TCTP expression was not enhanced (Fig. 2C). When PC155 cells with a high level of Pim-3 protein was depleted of endogenous Pim-3, TCTP expression did not change discernibly (Fig. 2D). It was previously reported that TCTP can be phosphorylated mainly at Ser^46, but not at Thr^65 (26). Pim-3 overexpression and ablation of Pim-3 expression increased and decreased the amount of phospho-Ser^112 Bad, respectively, but with few effects on the levels of phospho-TCTP^Ser^46 or total amount of Bad in human pancreatic cancer cells (Fig. 2E and 2F). These observations indicate that Pim-3 has minimal effect on TCTP expression or phosphorylation states.

TCTP enhances the protein stability of Pim-3 by blocking ubiquitin–proteasome degradation of Pim-3

As TCTP can stabilize Mcl-1 protein (15), we assumed that TCTP may enhance Pim-3 protein levels by stabilizing Pim-3. To address this possibility, we transiently co-transfected HEK293T cells with a fixed amount of Pim-3 and increasing quantities of the His-tagged TCTP expression vectors. Pim-3 protein levels increased as the amounts of the transfected His-tagged TCTP vectors were increased (Fig. 3A). Moreover, ablation of endogenous TCTP protein significantly decreased the amount of Pim-3 protein but not
Akt, indicating the specificity of the TCTP-mediated effect on Pim-3 levels (Fig. 3B). The elevation of these protein levels could be either due to enhanced mRNA expression, protein synthesis, or inhibition of protein degradation. We found that TCTP siRNA treatment did not affect Pim-3 mRNA levels (Fig. 3C). On the contrary, transient transfection of the TCTP expression vector in HEK293T cells delayed the degradation of Pim-3 protein (Fig. 3D). Conversely, ablation of TCTP with siRNA enhanced the degradation of Pim-3 protein, compared to scrambled siRNA in PCI55 cells (Fig. 3E). Moreover, TCTP siRNA-mediated enhancement in Pim-3 protein degradation was abrogated by a proteasomal inhibitor, MG132 (Fig. 3F). Furthermore, TCTP siRNA treatment promoted Pim-3 ubiquitination in PCI55 cells (Fig. 3G). Taken together, these results indicate that TCTP can increase Pim-3 protein stability by blocking the ubiquitin–proteasome-mediated degradation of Pim-3 in human pancreatic cancer cells.

**Ablation of TCTP protein prevents Pim-3-mediated tumor growth by arresting cell cycle progression and enhancing apoptosis**

Ablation of TCTP can destabilize Pim-3 protein in human pancreatic cancer cells while reduction of Pim-3 expression can arrest cell cycle progression and promote apoptosis (4). Hence, we investigated whether ablation of endogenous TCTP would affect the cell cycle and apoptosis in human pancreatic cancer cells. TCTP shRNA, but not scrambled shRNA treatment markedly diminished Pim-3 proteins in PCI55, MiaPaca-2, and SW1990 cell lines (Fig. 4A) and significantly retarded cell proliferation compared to scramble shRNA (Fig. 4B). TCTP shRNA treatment increased the ratio of G0/G1 phase cell population with reduced S and G2/M phase population, compared with scrambled shRNA treatment (Fig. 4C). Moreover, the stable transfection of TCTP shRNA resulted in a markedly higher ratio of apoptotic cells as evidenced by enhanced phosphatidylserine externalization (Fig. 4D–4F). Furthermore, in comparison to a subcutaneous injection with control shRNA-transfected cells, subcutaneous injection with TCTP shRNA-transfected cells resulted in a lower tumor formation frequency and a smaller tumor mass in nude mice 4 weeks after injection, (43% for MiaPaca-2-TCTP
shRNA cells and 67% for SW1990-TCTP shRNA cells, respectively; 100% for both of MiaPaca-2-scramble shRNA and SW1990-scramble shRNA cells) (Fig. 4G and 4H). Consistent with these observations, ablation of TCTP decreased Pim-3 expression and the amounts of phosphorylated p21, the molecules that participate in cell progression at G0/G1 phase at the tumor sites in the injected mice (27) (Fig. 4I). Cyclin B1 and Cdc25C, molecules that participate in cell progression at G2/M phase, were also decreased (Fig. 4I). Likewise, ablation of TCTP decreased Pim-3 expression, and eventually diminished the amounts of phosphor-Ser112-Bad and Bcl-XL at the tumor sites in the injected mice, without any effects on the amounts of Bad (Fig. 4I) and some other proteins related with cell cycle (data not show). Collectively, these observations indicate that TCTP can stabilize Pim-3 and promote cell cycle progression and cell viability, eventually promoting human pancreatic carcinogenesis.

Clinical relevance of TCTP and Pim-3 expression in pancreatic ductal adenocarcinoma (PDAC)

Finally, to investigate the clinical relevance of TCTP and Pim-3 expression, we performed immunohistochemistry on 148 resected PDAC tissues. TCTP and Pim-3 staining patterns were cytoplasmic, and both TCTP and Pim-3 expression were positive in more than 90% of PDAC tissues. Immunofluorescence analysis showed that TCTP and Pim-3 localized to the cytoplasm and co-localized in human pancreatic cancer tissues (Fig. 5A). Moreover, TCTP protein was abundantly detected in malignant ductal epithelium cells and some pancreatic acinar cells, but not in normal pancreatic duct epithelial cell adjacent to tumor foci (Fig. 5B). Positive reactions were not observed when control IgG was used as the primary antibody instead of the anti-TCTP antibody (data not shown), indicating the specificity of the reaction. Furthermore, the expression patterns of TCTP and Pim-3 exhibited perfect concordance in serial sections of human pancreatic cancer tissues (Fig. 5C). TCTP expression significantly correlated with Pim-3 expression in 148 human pancreatic ductal adenocarcinoma specimens (Spearman correlation coefficient, 0.518; p < 0.01) (Fig. 5D). Furthermore, higher TCTP and Pim-3
expression in PDAC was significantly associated with an advanced tumor stage (p = 0.001 and p = 0.008; Table 1). Finally, higher TCTP expression also correlated with nodal metastasis (p = 0.024), but neither TCTP nor Pim-3 expression did show any significant correlation with age, gender, tumor location, tumor size, and tumor differentiation (Table 1). These observations suggest that enhanced TCTP and Pim-3 expression may be involved in the malignant progression of human pancreatic cancer.
Discussion

We previously observed that a proto-oncogene with serine/threonine kinase activity, Pim-3, is aberrantly expressed in various malignant lesions, but not normal tissues of endoderm-derived organs such as the liver, pancreas, colon, and stomach (3-5, 28). Moreover, it can contribute to tumorigenesis by inhibiting the apoptosis of tumor cells and promoting their cell cycle progression. Pim kinase family consists of three members, Pim-1, Pim-2, and Pim-3, which exhibit marked sequence similarity, especially in their kinase domains. As Pim kinases do not possess a regulatory domain and are constitutively active when they are expressed (29), the activity of Pim kinases is largely regulated at transcriptional, translation, and post-translational levels (30). Furthermore, a very short half-life of their mRNA and protein (31) suggests the importance of regulation of Pim kinase protein levels.

Pim-1 and Pim-3 have been shown to bind to the serine/threonine protein phosphatase 2A (PP2A), resulting in their dephosphorylation, ubiquitination, and proteasomal degradation (32, 33). Moreover, heat shock 70-kDa protein 1A/B (HsP70) binds ubiquitylated Pim-1 and promotes its proteasomal degradation. On the contrary, heat shock protein 90β (HsP90) can stabilize Pim-1 protein by binding to it, and the inhibition of HsP90 induced rapid degradation of Pim-1 (34, 35). However, the molecular mechanisms of Pim-3 expression in carcinogenesis still remain largely unknown. To define the detailed regulatory machinery, we conducted yeast two-hybrid screening to identify Pim-3-interacting proteins and demonstrated for the first time that TCTP directly interacts with Pim-3. TCTP is a multi-functional protein and can interact with many cellular proteins. TCTP binds to p53 to promote its proteasomal degradation (24, 25), whereas TCTP interacts with Mcl-1 to enhance its stability (15). Indeed, Pim-3 protein stability is enhanced by its interaction with TCTP, which can block the ubiquitin-proteasome-mediated degradation of Pim-3.

Comparisons of TCTP sequences in 24 eukaryotes (36) revealed the presence of two highly conserved regions in TCTP protein; one region from 45 to 55 residues and another region from 129 to 147 residues. Several proteins can interact with TCTP by binding to
either region (Supplementary Table S3) (26, 36-40). We proved that the N-terminal portion of TCTP (residues 1–69) and the C-terminal portion of Pim-3 were required for their physical interaction. Although the crystal structure of Pim-3 has not yet been determined, Pim-3 seems to exhibit a similar structure to Pim-1, due to its extraordinarily high amino acid sequence similarity with Pim-3. The Pim-1 kinase structure adopts a two-lobe kinase fold with a deep and intervening cleft (29). The N-terminal and C-terminal lobes are composed of β-sheets and α-helices, respectively, while the two domains are connected through the hinge region (residues 121-126). TCTP could interact with the C-terminal but not the N-terminal portion-possessing Pim-3 mutant, indicating that the α-helices in the C-terminal portion are mainly involved in binding to TCTP.

Pim kinases can phosphorylate p27Kip1 and regulate its expression at both transcriptional and post-translational levels, to promote tumorigenesis (41). Moreover, Pim-2 overexpression in HCT116 cells leads to enhanced phosphorylation of p21 to increase its stability (42). Furthermore, Pim-3 can augment protein synthesis (13) and regulate transcriptional activity of Myc (31). However, overexpression or ablation of Pim-3 failed to induce any changes in TCTP protein expression or phospho-TCTPser46 levels. Thus, Pim-3 has few effects on TCTP expression or phosphorylation levels, even though Pim-3 can bind to TCTP.

TCTP was overexpressed in many human cancer tissues including liver (43), colorectal (44), prostate (45), breast (46), and lung cancer (47). Likewise, TCTP was detected in pancreatic cancer cell lines, and malignant duct epithelial cells and some normal acinar cells but not normal pancreatic duct epithelial cells adjacent to tumor foci. Moreover, TCTP expression positively correlated with Pim-3 in pancreatic cancer cell lines and tissues. Furthermore, patients with high TCTP and Pim-3 expression often had advanced tumor stage. Thus, TCTP may promote pancreatic cancer by preserving Pim-3 expression.

All Pim kinase members can bind to and phosphorylate CDK inhibitor, p27, at its threonine residues and induce the binding of p27 to 14-3-3 protein, resulting in its nuclear export and proteasome-dependent degradation (41). Moreover, Pim-1 can
promote cell cycle progression by phosphorylating and modulating the functions of molecules involved in cell cycle progression such as Cdc25A, cyclinD1-associated kinases (48), Cdc25C-associated kinase 1 (C-TAK1)(49), and G1-specific inhibitor p21 (Waf) (50). Given the high sequence identity between Pim-1 and Pim-3, it is likely that Pim-3 can also modulate these molecules like Pim-1. Indeed, cell cycle progression is accelerated in hepatocytes of transgenic mice, which express human Pim-3 cDNA selectively in hepatocytes (7). Moreover, a small-molecule Pim-3 kinase inhibitor markedly retarded in vitro growth of human pancreatic cancer cell lines by inducing G2/M arrest (51), suggesting a potential role for Pim-3 in cell cycle progression. Consistently, ablation of TCTP decreased the amounts of Pim-3, Cdc25C, cyclin B1, and phospho-p21T145, but not the total amounts of p21. Furthermore, TCTP ablation arrested cell cycle progression at the G1 phase in human pancreatic cancer cells, similar to when the cells were depleted of Pim-3.

Diraison and colleagues recently reported that increased TCTP expression reduced the sensitivity of pancreatic β cells to apoptosis (52). We previously observed that Pim-3 shRNA treatment in vitro enhanced the apoptosis of various types of cancer cells (3-5). Likewise, TCTP shRNA destabilized and induced the degradation of Pim-3, and promoted the apoptosis of human pancreatic cancer cells. Moreover, TCTP shRNA-treated human pancreatic cancer cells exhibited a weaker tumorigenic ability than scrambled shRNA-treated cells, when they were injected into nude mice. Furthermore, we detected a much lower level of Pim-1 and Pim-2 mRNA than Pim-3 mRNA in human pancreatic cancer cells that we examined (Supplementary Fig. S1). Thus, it is likely that TCTP can regulate the cell cycle and cell apoptosis mainly by Pim-3-mediated in human pancreatic cancer cells.

Several independent groups developed small-molecule inhibitors against Pim kinases including flavonol quercetargetin, imidazole[1,2-b]pyridazines, bezylindene-thiazolidine-2,4-dione, 3,5-disubstituted indole derivatives, pyrazolo[3,4-g]quinoxaline derivatives, 1,6-dihydropyrazolo[4,3-c]carbazoles and 3,6-dihydropyrazolo[3,4-c]carbazoles derivatives (53), and pyrrolo[2,3-a]carbazole and
pyrrolo[2,3-g]indazoles derivatives (54, 55). Among them, 1,6-dihydropyrazolo[4,3-c]carbazoles, 3,6-dihydropyrazolo[3,4-c]carbazoles and pyrrolo[2,3-g]indazoles could be used as an interesting molecular tool to study Pim-3 biological functions (53). Consistently, we also demonstrated that stemonamide synthetic intermediates derivative can inhibit Pim-3 as well as Pim-1 and Pim-2 activities and can reduce tumor growth in vivo xenograft models using a human pancreatic cancer cell line without causing major adverse effects (56, 57). TCTP but not Pim-3 expression was well correlated with nodal metastasis in human pancreatic cancer patients. Thus, given the fact that TCTP can bind and modulate several molecules, TCTP can contribute to pancreatic cancer development and progression besides its effects on Pim-3. If so, the targeting of TCTP may supplement Pim-3 inhibitors for the treatment of various types of cancer, which exhibit enhanced Pim-3 and TCTP expression.

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References:


Table 1. Correlation of TCTP and Pim-3 expression with clinicopathological features in 148 PDAC specimens.

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<tr>
<th>Clinicopathological Patients Variable</th>
<th>TCTP expression</th>
<th>p</th>
<th>Pim-3 expression</th>
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*p < 0.05
Legends to Figures

Figure 1. Association of TCTP with Pim-3 in human pancreatic cancer cell lines. A. Co-immunoprecipitation of TCTP and Pim-3 in transiently transfected HEK293T cells. Cell lysates were obtained from HEK293T cells transiently transfected with pcDNA4-Pim-3 and pcDNA4-TCTP-His. The resultant lysates were subjected to immunoprecipitation and immunoblotting. B. Co-immunoprecipitation of endogenous TCTP and Pim-3 in PCI55 cells. Cell lysates were obtained from PCI55 cells. The resultant lysates were subjected to immunoprecipitation and immunoblotting. One tenth of the cell lysates from PCI55 were subjected to immunoblotting with anti-TCTP or anti-Pim-3 antibodies as positive controls. C. Co-localization of TCTP and Pim-3 in human pancreatic cancer cells. PCI55 cells were immunostained with a combination of anti-TCTP and anti-Pim-3 antibodies as described in Materials and Methods. The fluorescent images were digitally merged. Yellow coloration in overlay panels indicates co-localization of Pim-3 and TCTP. Nuclei were counterstained with DAPI. D. TCTP and Pim-3 expression in human pancreatic cancer cell lines was detected by western blotting. \( \beta \)-actin was used as an internal control. E. The expression levels of TCTP, Pim-3, and \( \beta \)-actin were quantified using NIH ImageJ software. TCTP/\( \beta \)-actin and Pim-3/\( \beta \)-actin ratios were calculated for each cell line. F. Correlation between TCTP and Pim-3 expression in human pancreatic cancer cell lines, with a linear regression line and Spearman correlation significance (Spearman correlation coefficient 1, \( p < 0.01 \)). G. Schematic representation of cDNA constructs for each TCTP deletion mutant. H. Cell lysates were obtained from HEK293T cells transiently co-transfected with Pim-3 and TCTP deletion mutants. The resultant lysates were immunoprecipitated and immunoblotted. I. Schematic representation of cDNA constructs for each Pim-3 deletion mutant. J. Cell lysates were obtained from HEK293T cells transiently co-transfected with TCTP and Pim-3 deletion mutants. The resultant lysates were immunoprecipitated and immunoblotted. K. Schematic representation of the presumed interaction between Pim-3 and TCTP proteins.
Figure 2. Pim-3 has no effect on TCTP expression or phosphorylation. A and B. Cell lysates were obtained from HEK293T cells transiently transfected with pcDNA4-Pim-3 expression vector, empty pcDNA4 vector (A), or the indicated amounts of Pim-3 expression vector (B). The resultant lysates were subjected to immunoblotting with the indicated antibodies. C and D. Cell lysates were obtained from MiaPaca-2 cells transiently transfected with pcDNA4-Pim-3 expression or empty pcDNA4 vector (C) or from PCI55 cells transiently transfected with Pim-3 shRNA or scrambled shRNA (D). The resultant lysates were subjected to immunoblotting with the indicated antibodies. E and F. Cell lysates were obtained from MiaPaca-2 cells stably overexpressing pMEI-5-Pim-3 or empty pMEI-5 vector (E) or from PCI55 cells stably transfected with Pim-3 or scramble shRNA vectors (F). The resultant lysates were subjected to immunoblotting with the indicated antibodies.

Figure 3. TCTP enhances the protein stability of Pim-3 by blocking the ubiquitin-proteasome degradation of Pim-3. A. pcDNA4-Pim-3 expression vector (0.5 μg) was co-transfected with the indicated amount of pcDNA4-TCTP-His or empty pcDNA4 vector into HEK293T cells. After 48 h, cell lysates were analyzed by western blotting with the indicated antibodies. B. The cell lysates were obtained from PCI35 or PCI55 cells transiently transfected with TCTP or scramble siRNA. The resultant lysates were subjected to immunoblotting with the indicated antibodies. C. Immunoblotting for TCTP and real-time qRT-PCR for TCTP and Pim-3 in TCTP siRNA-treated PCI55 cells (left). Cell lysates were obtained from PCI55 cells transiently transfected with TCTP or scramble siRNA. After 48 h, the cell lysates were obtained and subjected to immunoblotting. Real-time RT-PCR analysis was performed to quantify TCTP and Pim-3 mRNA levels as described in Materials and Methods. The results are expressed as mean ± SD **, p < 0.01 vs scrambled siRNA (right). D. pcDNA4-Pim-3 expression vector (0.5 μg) was co-transfected with pcDNA4-TCTP-His (+; 1.5 μg) or empty pcDNA4 (-; 1.5 μg) vector into HEK293T cells. At 24 h after transfection, the cells were treated with cycloheximide (CHX, 30μg/ml) for various time intervals. Cell lysates were then...
analyzed by immunoblotting with indicated antibodies (left). The percentage of Pim-3 protein level was determined using densitometry scanning (NIH ImageJ software) (right).

E. TCTP or control scramble siRNA was transfected transiently into PCI55 cells. At 24 h after transfection, the cells were treated with cycloheximide for various times as indicated. Cell lysates were then analyzed by immunoblotting with the indicated antibodies (left). The percentage of Pim-3 protein level was determined using densitometry scanning (NIH ImageJ software) (right).

F. PCI55 cells were transiently transfected with TCTP (+) or scrambled siRNA (-). At 24 h after transfection, the cells were treated with either DMSO or MG132 (1 μg/ml) for an additional 4 h. Cell lysates were obtained and subjected to the immunoblotting with the indicated antibodies.

G. PCI55 cells were transiently transfected with TCTP siRNA (+) or scrambled siRNA (-). At 24 h after the transfection, the cell lysates were prepared and immunoprecipitated with anti-Pim-3 antibodies. The immune complex was then analyzed by immunoblotting with anti-ubiquitin (UB) or Pim-3 antibodies.

Figure 4. The effects of ablation of endogenous TCTP on cell viability and cell apoptosis of human pancreatic cancer cell lines. A. TCTP or scramble shRNA was stably infected into PCI55, MiaPaca-2, or SW1990 cells using a lentivirus vector. TCTP and Pim-3 expression was detected by immunoblotting. β-actin was used as an internal control.

B. PCI55, MiaPaca-2, or SW1990 cells were stably transfected with TCTP shRNA or scramble shRNA. Three thousand of the resultant cells were plated in a 96-well microplate. After 12 h, when the cells were adhered to the microplate, was designated as time 0. The cell numbers were determined as the indicated time intervals using the Cell Counting Kit-8 reagent and the ratios were compared to time 0. Point, mean (n = 5); bars, SD.

C. PCI55, MiaPaca-2, or SW1990 cells were stably transfected with TCTP shRNA or scrambled shRNA. The proportion of cells in each cell cycle phase was determined as described in Materials and Methods.

D–F. Apoptosis of PCI55, MiaPaca-2, and SW1990 cells, which were stably transfected with TCTP or scrambled shRNA. Cells were stained with a combination of propidium iodide and Annexin V as
described in Materials and Methods. The number in each quadrant indicates the proportion of the cells present in the quadrant. G. Incidence of tumor formation in nude mice induced by injection of MiaPaca-2 or SW1990 cells, which were stably transfected with TCTP or scramble shRNA. H. Tumor sizes were measured twice a week. The mean and SD were calculated and are shown here. I. Cell lysates were obtained from PCI55, MiaPaca-2, and SW1990 cells, which were stably transfected with TCTP or scramble shRNA. The resultant lysates were subjected to immunoblotting with the indicated antibodies as described in Material and Methods.

**Figure 5. Clinicopathological analysis of TCTP and Pim-3 expression in pancreatic ductal adenocarcinoma (PDAC).** A. Co-localization of TCTP and Pim-3 in human pancreatic cancer tissue. Human pancreatic cancer tissues were immunostained with a combination of anti-TCTP and anti-Pim-3 antibodies as described in Materials and Methods. The fluorescent images were digitally merged. B. TCTP expression was detected on the section of normal pancreatic duct epithelium adjacent to tumor foci (left and middle panels) and pancreatic adenocarcinoma (right panel) by immunohistochemistry. C. TCTP and Pim-3 expression was analyzed by immunohistochemistry on serial sections of different human pancreatic cancer tissues. D. Cross-tabulation of TCTP and Pim-3 expression detected by immunohistochemistry in 148 pancreatic ductal adenocarcinoma tissues. Correlation between TCTP and Pim-3 was significant on Spearman correlation analysis (Spearman correlation coefficient 0.518, \( p < 0.01 \)).
Zhang et al. Figure 2

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Zhang et al. Figure 4

A

PCI55
MinPaca-2
SW1990

TCTP shRNA scramble shRNA scramble shRNA

\[ TCTP \]

\[ \text{Plim-3} \]

\[ \beta-\text{Actin} \]

B

PCI 55
MinPaca-2
SW1990

Cell viability ratio

Time after passage (hour)

Time after passage (day)

Time after passage (day)

C

PCI55
MinPaca-2
SW1990

Percentage of cells

D

PCI55-scramble-shRNA
PCI55-TCTP-shRNA

E

MinPaca-2-scramble-shRNA
MinPaca-2-TCTP-shRNA

F

SW1990-scramble-shRNA
SW1990-TCTP-shRNA

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H

MinPaca-2

SW1990

\[ \text{scramble-shRNA} \]

\[ \text{TCTP-shRNA} \]

\[ \beta-\text{Actin} \]
Zhang et al. Figure 5

A

TCTP  Pim-3  DAPI

Merged  Merged

TCTP/Pim-3  TCTP/Pim-3/DAPI

B

normal pancreatic duct epithelium

adjacent to tumor foci  pancreatic adenocarcinoma

C

D

TCTP expression positive correlated with Pim-3 expression

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Molecular Cancer Research

A Novel Regulatory Mechanism of Pim-3 Kinase Stability and its Involvement in Pancreatic Cancer Progression

Fei Zhang, Bin Liu, Zhen Wang, et al.

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