Overexpression of Peptidyl-Prolyl Isomerase-Like 1 Is Associated with the Growth of Colon Cancer Cells

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Abstract

Purpose and Experimental Design: To discover novel therapeutic targets for colon cancers, we previously surveyed expression patterns among 23,000 genes in colon cancer tissues using a cDNA microarray. Among the genes that were up-regulated in the tumors, we selected for this study peptidyl-prolyl isomerase-like 1 (PPIL1), a cyclophilin-related protein.

Results: Western blot analysis and immunohistochemical staining using PPIL1-specific antibody showed that PPIL1 protein was frequently overexpressed in colon cancer cells compared with noncancerous epithelial cells of the colon mucosa. Colony formation assay showed a growth-promoting effect of wild-type PPIL1 on NIH3T3 and HEK293 cells. Consistently, transfection of short-interfering RNA specific to PPIL1 into SNUC4 and SNUC5 cells effectively reduced expression of the gene and retarded growth of the colon cancer cells. We further identified two PPIL1-interacting proteins, SNW1/SKIP (SKI-binding protein) and stathmin. SNW1/SKIP is involved in the regulation of transcription and mRNA splicing, whereas stathmin is involved in stabilization of microtubules. Therefore, elevated expression of PPIL1 may play an important role in proliferation of cancer cells through the control of SNW1/SKIP and/or stathmin.

Conclusion: The findings reported here may offer new insight into colonic carcinogenesis and contribute to the development of new molecular strategies for treatment of human colorectal tumors.

Colon cancer is one of the most common causes of cancer death throughout the world. Molecular investigations have provided evidence that multiple genetic alterations are involved in colorectal tumorigenesis. Early in those studies, loss of heterozygosity was observed frequently at loci on chromosomes 1p, 2p, 3p, 5q, 8p, 11q, 17p, 18q, and 22q (1, 2). Later, alterations in genes encoding K-ras, adenomatous polyposis coli protein (APC), β-catenin (CTNNB1), p53 (TP53), and/or conductin (AXIN2) were shown to be involved in colonic carcinogenesis (3–7). In spite of intensive study at the molecular level, however, the roles of a large number of genes that show altered expression in colorectal cancers remain unclear.

Peptidyl-prolyl isomerase-like 1 (PPIL1) was first identified as a cyclophilin-related gene encoding a protein that shares 46.0% identity in amino acid sequence with human cyclophilin A (8). It is expressed in heart, skeletal muscle, and liver (8). However, its biological function remains unclear. Cyclophilins and FK506-binding proteins are two families of peptidyl-prolyl isomerases that are extensively characterized. Cyclophilins form complexes with cyclosporin A, whereas FK506-binding proteins bind the immunosuppressant drug FK506. A third family of peptidyl-prolyl isomerases includes parvulin and Pin1. These proteins do not bind immunosuppressants but are involved in cell cycle progression and cell survival (9, 10). Pin1 is a phosphorylation-dependent peptidyl-prolyl isomerase that catalyzes the cis-trans isomerization of phosphoserine-proline and phosphothreonine-proline bonds in multiple proteins such as CDC25, RNA polymerase II, cyclin D1, p53, and Tau (11, 12). It interacts with phosphorylated-p53 when the latter is activated in response to DNA damage, altering the stability of p53 through isomerization of a motif involving a serine/threonine residue followed by a proline (Ser/Thr-Pro; refs. 10, 13). All of these peptidyl-prolyl isomerase families carry out cis-trans isomerization of the peptide bond on the NH2-terminal side of Pro residues, forming a tight bend in the polypeptide backbone that substantially changes the conformation of the protein. Through this isomerization and consequent conformational change, peptidyl-prolyl isomerases alter the functions of their target proteins (14).

To discover target molecules for development of novel diagnostic strategies and/or therapeutic drugs, we previously carried out a genome-wide analysis of gene expression in...
human colon cancer tissues by means of cDNA microarray consisting of 23,040 genes (15). In this report, we show that PPI1, a gene that is frequently up-regulated in colon cancers, promotes growth of cancer cells. In addition, we document interactions between PPI1 and two known proteins, SNW1/SKIP and stathmin, in vitro and in vivo. These data may not only lead to a more profound understanding of colorectal carcinogenesis but also provide clues for the development of novel therapeutic strategies.

**Materials and Methods**

**Cell lines and tissue specimens.** Human colon cancer cell lines SN1C4 and SN1C5 were obtained from the Korean cell line bank. HEK293 (human embryonic kidney), COS-7 (monkey kidney), and NIH3T3 (mouse fibroblasts) cells were purchased from the American Type Culture Collection (Rockville, MD). All tumor tissues and corresponding nontumorous mucosae were excised with informed consent from 79 patients who underwent colectomy or endoscopic polypectomy at the Department of Surgery, Tokyo Hitachi Hospital.

**Real-time PCR (TaqMan) assay.** Extraction of RNA, preparation of cDNA, and real-time PCR were done as described previously (16). The β-actin gene (ACTB) served as a quantitative control. The sequences of primers and probes are as follows: PPI1 forward primer, 5'-TGACCCCAAGACGAGGAGTTC-3', and reverse, 5'-GGTCATCTTCAACACTTTGCTC-3'; probe, 5'-FAM-GGTGTCATCTT-TGCCTG-3' (reverse) for stathmin, and 5'-TCGAAATCCGAGCATGAGCTCACCAGC-3' (forward) and 5'-TGGTCCTGCAGTTCTCCTCTCTGTCATGC-3' (reverse) for SNW1/SKIP. The PCR products were cloned into pCMV-HA or pcDNA3.1/Myc-His (Invitrogen), respectively. COS-7 cells transfected with pFLAG-PPI1 (expressing FLAG-tagged PPI1), pCMVHA-STMN1 (expressing HA-tagged stathmin), pcDNA-SNW1/SKIP-myc (expressing myc-tagged SNW1/SKIP), or a combination of the plasmids were washed with PBS and lysed in TNE buffer. Immunoprecipitation and immunoblotting were carried out using anti-HA (3F10; Roche, Mannheim, Germany), anti-myc (9E10: Santa Cruz Biotechnologies, Santa Cruz, CA), anti-FLAG (Sigma), horseradish peroxidase–conjugated goat anti-rat IgG (Santa Cruz Biotechnologies, Santa Cruz, CA), and donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences, Bucks, United Kingdom). Immunoblot analysis with anti-PPIL1 polyclonal antibody showed that its expression was enhanced >2-fold in 10 of 14 additional colon cancer tissues compared with the corresponding noncancerous mucosa (Fig. 1A). To prepare plasmids expressing S16A, S25A, S38A, or S63A forms of mutant stathmin, we used a QuikChange Site-Directed Mutagenesis kit (Stratagene) and amplified each form using pCMV-HA-STMN1 as a template and different sets of primers according to the recommendations of the manufacturer.

**Immunoprecipitation assay.** The entire coding region of the human stathmin gene (STMN1) and SNW1/SKIP was amplified by reverse transcription-PCR using primers as follows: 5'-ATTAGTCTTCCACCATGTCCTCACTCTATGCTCCATC-3' (forward) and 5'-AATTGTACCTTCCACCATGTCCTCACTCTATGCTCCATC-3' (reverse) for stathmin, and 5'-GGTCCTGCAGTTCTCCTCTCTGTCATGC-3' (reverse) for SNW1/SKIP. The PCR products were cloned into pCMV-HA or pcDNA3.1/Myc-His (Invitrogen), respectively. COST7 cells transfected with pFLAG-PPI1 (expressing FLAG-tagged PPI1), pCMVHA-STMN1 (expressing HA-tagged stathmin), pcDNA-SNW1/SKIP-myc (expressing myc-tagged SNW1/SKIP), or a combination of the plasmids were washed with PBS and lysed in TNE buffer. Immunoprecipitation and immunoblotting were carried out using anti-HA (3F10; Roche, Mannheim, Germany), anti-myc (9E10: Santa Cruz Biotechnologies, Santa Cruz, CA), anti-FLAG (Sigma), horseradish peroxidase–conjugated goat anti-rat IgG (Santa Cruz Biotechnologies, Santa Cruz, CA), and donkey anti-rabbit IgG conjugated to horseradish peroxidase–conjugated sheep anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, United Kingdom) antibodies as described earlier (19). To prepare plasmids expressing S16A, S25A, S38A, or S63A forms of mutant stathmin, we used a QuikChange Site-Directed Mutagenesis kit (Stratagene) and amplified each form using pCMV-HA-STMN1 as a template and different sets of primers according to the recommendations of the manufacturer.

**Statistical analysis.** Statistical significance was determined by Sheffe's F test, using a commercially available software, Statview 5.0 (SAS Institute, Cary, NC). A difference of P < 0.05 was considered statistically significant.

**Results**

**Elevated PPI1 expression in colorectal cancer cells.** Among the genes that were up-regulated in the colon cancer tissues investigated on our 23,040 gene microarray, expression of PPI1 gene was elevated in all informative cases (data not shown). Subsequent real-time PCR (TaqMan) assay showed that its expression was enhanced >2-fold in 10 of 14 additional colon cancer tissues compared with the corresponding noncancerous mucosa (Fig. 1A). To examine expression of PPI1 protein in colon cancer tissues, we did immunoblot analysis with anti-PPI1 polyclonal antibody using an additional five pairs of colon cancer tissues and the corresponding noncancerous mucosa. In agreement with the elevated PPI1 transcript, PPI1 protein was augmented in all five cancer tissues compared with the noncancerous
mucosa (Fig. 1B). Immunohistochemical staining with the anti-PPIL1 antibody revealed that nuclei and/or cytoplasms of tumor cells were more strongly stained in 26 of 29 colon cancer tissues than their corresponding noncancerous epithelial cells (Fig. 2A-C). In addition, lamina propria cells showed weak staining, which might correspond to low levels of PPIL1 expression in noncancerous tissues of the colon. Notably, cancerous cells at an early stage of tumor localized within mucosal and submucosal layer were also positively stained. On the other hand, no epithelial cells in normal crypts were stained with the antibody (Fig. 2A and B, arrowheads). We further examined its expression in 18 adenomas of colon, which were all negative for PPIL1 (Fig. 2D). These data corroborated elevated PPIL1 expression in colon cancer cells and suggest that PPIL1 may be involved in malignant transformation of colon tumor.

**Effect of exogenous PPIL1 on growth of mammalian cells.** To disclose a potential biological function of PPIL1 in colorectal tumorigenesis, we constructed plasmids expressing wild-type PPIL1 (pcDNA-PPIL1-myc) and mutant PPIL1 (pcDNA-mtPPIL1-myc) and carried out colony formation assays with NIH3T3 and HEK293 cells. Transfection of pcDNA-PPIL1-myc produced a significantly larger number of colonies than did the negative control (pcDNA-LacZ-myc) or mutant plasmids (pcDNA-mtPPIL1-myc; data not shown for HEK293; Fig. 3A and B). This result was confirmed by three independent experiments, suggesting that elevated expression of PPIL1 might confer growth-promoting effects on cancer cells.

**Effect of PPIL1 siRNAs on growth of colon cancer cells.** To investigate the role of PPIL1 in colorectal tumorigenesis, we constructed plasmids expressing siRNAs specific to PPIL1 (psiH1BX-PPIL1), and examined their effects on expression of the gene in colon cancer cells and on subsequent growth of those cells. Among the three plasmids expressing PPIL1-siRNAs, both psiH1BX-siB and psiH1BX-siC markedly suppressed constitutional expression of PPIL1 protein in SNUC5 as well as in SNUC4 colon cancer cells at 48 hours after transfection,
whereas mild effect was observed with constructs psiH1BX-siA (Fig. 3C). In accord with this suppression of PPIL1, transfection of SNUC5 or SNUC4 cells with psiH1BX-siB or psiH1BX-siC suppressed their growth at day 10 of transfection; although the number of colonies was unchanged, the number of viable cells was significantly fewer compared with the control, psiH1BX-EGFP (Fig. 3D). In addition, mild growth suppressive effect was detected with psiH1BX-siA, indicating a positive correlation between gene knockdown effect and growth suppressive effect of the siRNA (Fig. 3D). We further analyzed sub-G1, G0-G1, S, and G2-M population in cell cycle by flow cytometry at day 10. Although sub-G1 population was unchanged, the population of S phase was significantly decreased in cells transfected with psiH1BX-siB (7.3%) compared with those with psiH1BX-EGFP (13.9%; Fig. 3E).

Interaction of PPIL1 with SNW1/SKIP. Because Dictyostelium discoideum cyclophilin Cyp2 or Schizosaccharomyces pombe cyclophilin CypE binds to SnwA or Snw1p, respectively, we examined whether PPIL1 may associate in vivo with SNW1/SKIP, the human counterpart of SnwA and Snw1p. We transfected COS7 cells with pFLAG-PPIL1, in combination with or without pcDNA-SNW1/SKIP-myc plasmids that express myc-tagged SNW1/SKIP protein. Immunoprecipitation with anti-FLAG antibody using extract from cotransfected cells revealed coimmunoprecipitated myc-tagged SNW1/SKIP protein (Fig. 4A, top, lane 8). On the other hand, immunoprecipitation with anti-myc antibody using the extract detected coimmunoprecipitated FLAG-tagged PPIL1 protein (Fig. 4A, bottom, lane 8). These data suggested that PPIL1 associates with human SNW1/SKIP.

Interaction of PPIL1 with stathmin in vitro and in vivo. To explore further the function of PPIL1, we searched for additional PPIL1-interacting proteins using a bacterial two-hybrid screening system. We screened 1.2 × 10^6 clones from a fetal brain library transfected with pBT-PPIL1 as bait, and identified 98 positive colonies. Among the 98 positive clones, one containing stathmin produced more colonies than did cells without transfected pBT-PPIL1 (data not shown). We transfected COS-7 cells with plasmids expressing HA-tagged stathmin (pCMVHA-STMN1) and carried out Western blotting with cell extracts and anti-HA antibody. This experiment produced two bands corresponding to an 18 kDa protein of the expected size.
size as well as a 20 kDa molecule, suggesting the existence of modified form(s) of stathmin (Fig. 4B, lane 1). In line with this notion, we observed 18 kDa forms of stathmin alone, when the extract was treated with λ phosphatase (data not shown). As stathmin seems to have four putative Ser/Thr phosphorylation sites (Ser16, Ser25, Ser38, and Ser63; ref. 19), we prepared four plasmids, each expressing a S16A, S25A, S38A, or S63A form of stathmin. When each construct was transfected into COS-7 cells, the S16A, S25A, or S63A mutant expressed both (18 and 20 kDa) forms of stathmin (Fig. 4B, lanes 2, 3, and 5). However, the S38A mutant expressed the 18 kDa form alone (Fig. 4B, lane 4), suggesting that the heavier protein (20 kDa) was generated as a result of phosphorylation at the Ser38 site.

To confirm an association between PPIL1 and stathmin in normal mammalian cells, we transfected COS-7 cells with the plasmid expressing FLAG-tagged PPIL1 (pFLAG-PPIL1), with or without inclusion of pCMVHA-STMN1, and carried out immunoprecipitation assays. Western blotting of immunoprecipitants with anti-HA antibody after hybridization with anti-FLAG antibody corroborated in vivo binding between PPIL1 and stathmin (Fig. 4C, lane 3). On Western blots, the band corresponding to the 20 kDa form of stathmin was more intense than the band corresponding to the 18 kDa form. Because the amount of both forms were nearly equal in cells transfected with pCMVHA-STMN1 (Fig. 4C, lane 2), PPIL1 may have a higher affinity to the 20 kDa protein, a phosphorylated form of stathmin. Additional immunoprecipitation assay with anti-HA antibody using extract from cells cotransfected with pFLAG-PPIL1 and S38A (pCMVHA-S38A-STMN1) stathmin showed that PPIL1 was also capable of interacting with the unphosphorylated form of stathmin (Fig. 4C, lane 5). PPIL1 also associated with S16A, S25A, and S63A mutants (data not shown).

Discussion

In this study, we revealed that PPIL1 interacts with stathmin and SNW1/SKIP. Because stathmin is a cytoplasmic protein and SNW1/SKIP localizes in the nucleus, it is unlikely that these three proteins form a complex. PPIL1 may have several target proteins in the cytoplasm as well as in the nucleus. In line with this notion, its subcellular localization was observed in both intracellular compartments. Among other prolyl isomerase proteins, Pin1, CypA, Cyp40, FKBP25, and FKBP52 have been shown to localize in the cytoplasm and nucleus (9). These proteins have several target molecules; Pin1 interacts with Tau in the cytoplasm and RNA polymerase II in the nucleus, whereas CypA associates with calcineurin in the cytoplasm and apoptosis inducing factor in the nucleus (11, 20, 21). Because PPIL1, Pin1, and CypA do not contain a defined nuclear localization signal, the target proteins may regulate their subcellular localization. These data also indicate that prolyl isomerases should regulate multiple proteins, which is consistent with the fact that Pin1 regulates a number of proteins, including CDC25, RNA polymerase II, p53, Tau, and so on (11).

Stathmin, also known as oncoprotein 18 or leukemia-associated phosphoprotein 18, was initially identified as a molecule that becomes phosphorylated in response to various extracellular signals. Stathmin controls the dynamic changes...
of microtubules by its phosphorylation. It destabilizes microtubules by stimulating microtubule catastrophes and sequestering tubulin dimers (19). Therefore, elevated expression of PPIL1 may play a role in remodeling of microtubules in proliferating and invasive cancer cells. Notably, stathmin expression is often elevated in leukemias and in carcinomas of breast, ovary, and prostate (19). Stathmin contains four Ser phosphorylation sites (Ser16, Ser25, Ser38, and Ser63; ref. 19). We detected two forms of stathmin with different migration on SDS-PAGE; one was a 20 kDa phosphorylated form and the other an 18 kDa unphosphorylated form of Ser38. Interestingly, immunoprecipitation assay revealed that most of PPIL1 associated with the phosphorylated form of stathmin, which is reminiscent of a phosphorylation-dependent association between Pin1 and p53 (10, 13). Pin1 associates with multiple Ser/Thr-Pro of target proteins, leading to their conformational change, where a WW domain in Pin1 interacts with stathmin-like domain. In addition, phosphorylation-dependent association between Pin1 and p53 (10, 13). Pin1 associates with multiple Ser/Thr-Pro of target proteins, leading to their conformational change, where a WW domain in Pin1 is responsible for the recognition. Among the four Ser residues in stathmin, Ser25 and Ser38 are followed by Pro. Therefore, we investigated association of PPIL1 with mutant forms of stathmin containing S25A, S38A, or their combination, which showed an interaction of PPIL1 with all mutant proteins (Fig. 4C; data not shown). Therefore, phosphorylation in other residues may play a role in the association. Alternatively, PPIL1 associates with both unphosphorylated and phosphorylated forms of stathmin, although PPIL1 may have higher affinity to the phosphorylated form. Future identification of the responsible region(s) in stathmin for the binding and investigation using phosphorylation-specific antibodies to stathmin may uncover the mechanisms of regulation by the interaction with PPIL1.

In our microarray, expression levels of stathmin were markedly elevated in the colon cancer tissues we examined. During mitosis, stathmin is inactivated by phosphorylation; this process promotes polymerization and holds assembly of mitotic spindles (22). It also has been reported that the incorporation of tubulin into microtubules is regulated by the interaction with stathmin-like domain. In addition, phosphorylation of the four serines (Ser16, Ser25, Ser38, and Ser63) occurs sequentially at the G2-M transition (19). Recent studies reported that a stathmin phosphorylation gradient is necessary for correct spindle formation in mitotic cells (23). Therefore, phosphorylation of the Ser16 and isomerization of the following Pro by PPIL1 may be essential for the accelerated growth of neoplastic cells. Given that phosphorylated stathmin induces destabilization of microtubules (19), PPIL1 may induce the dissociation of stathmin from microtubules through the conformational change of stathmin. In addition, suppression of stathmin expression by antisense oligonucleotides inhibits growth and reverses many of the phenotypes associated with malignant transformation (24). These data tempt us to hypothesize that phosphorylation of stathmin by specific kinases, such as CDK1, CDK2, mitogen-activated protein kinase, and p38 (19), in combination with elevation of PPIL1 expression, stimulates progression of the cell cycle and remodeling of the cytoskeleton in cancer cells.

We also proved that PPIL1 interacts with human SNW1/SKIP, a transcriptional coactivator. In agreement with their interaction, both PPIL1 and SNW1/SKIP were included in the spliceosome complex (25). Because SNW1/SKIP protein plays an important role in transcription regulation, pre-mRNA splicing and cell cycle regulation (26), PPIL1 may modulate genes associated with proliferation or cell cycle progression through the regulation of SNW1/SKIP. Of note, SNW1/SKIP synergized with Ski and released cells from G2 arrest induced by Rb (27). Therefore, the PPIL1-SNW1/SKIP interaction may enhance the oncogenic activity of Ski and promote cell cycle progression. Although SNW1/SKIP is reported to be a phosphoprotein (28), its phosphorylation site(s) and mechanism(s) of modification remain to be clarified. Interestingly, human SNW1/SKIP contains two prolines preceded by Ser (Ser-Pro) at codon 225 and 233 within the SKIP domain. In addition, these residues are conserved from lower eukaryotes implying the importance of these residues in their function. Because the residues are within the responsible region for the binding with Ski (29), Smad2/3 (30), Rb (27), or HPV-E7 (31), phosphorylation and subsequent conformational change may affect its interaction with these proteins. Further investigations on phosphorylation of SNW1/SKIP as well as the functional role of the interaction between PPIL1 and SNW1/SKIP may uncover not only the role of elevated PPIL1 in cancer cells but also mechanisms underlying transcription, splicing, and polyadenylation.

In this report, we documented that PPIL1 showed growth-promoting activity in normal mammalian cells, and that reduction of its expression by siRNA suppressed the growth of colon cancer cells. The development of drugs to antagonize the function of PPIL1 may thus be a rational strategy for therapy of colon cancer. Because PPIL1 expression is elevated in cervical, gastric, and pancreatic cancers, and chronic myeloid leukemia in our microarray data (data not shown), inhibitors of PPIL1 may be effective anticancer drugs for a wide range of human tumors. Although further investigation of the function of PPIL1 will be necessary, the data provided here should contribute to a more profound understanding of colonic carcinogenesis and to development of novel therapeutic strategies for colon cancer.

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References

Article on Overexpression of Peptidyl-Prolyl Isomerase Like-1 Association with Colon Cancer Cell Growth

In the article on Overexpression of Peptidyl-Prolyl Isomerase Like-1 in the January 1, 2006 issue of Clinical Cancer Research, a reference was missing in the Results section. The first sentence in the subheading, Interaction of PPIL1 with SNW1/SKIP, should read, as follows: Because *Dictyostelium discoideum* cyclophilin Cyp2 or *Schizosaccharomyces pombe* cyclophilin CypE binds to SnwA or Snw1p, respectively (Skruzny M, Ambrozkova M, Fukova I, et al: Cyclophilins of a novel subfamily interact with SNW/SKIP coregulator in *Dictyostelium discoideum* and *Schizosaccharomyces pombe*. Biochim Biophys Acta 2001:1521:146–51), we examined whether PPIL1 may associate in vivo with SNW1/SKIP, the human counterpart of SnwA and Snw1p. Also, the first author in reference 26 is P. Folk.

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