TTPAL Promotes Colorectal Tumorigenesis by Stabilizing TRIP6 to Activate Wnt/β-Catenin Signaling

Hongyan Gou1,2, Jessie Qiaoyi Liang2, Lijing Zhang2, Huarong Chen2, Yanquan Zhang2, Rui Li2, Xiaohong Wang3, Jiafu Ji3, Joanna H. Tong4, Ka-Fai To4, Joseph J.Y. Sung2, Francis K.L. Chan2, Jing-Yuan Fang1, and Jun Yu2

Abstract
Copy number alterations are crucial for the development of colorectal cancer. Our whole-genome analysis identified tocopherol alpha transfer protein-like (TTPAL) as preferentially amplified in colorectal cancer. Here we demonstrate that frequent copy number gain of TTPAL leads to gene overexpression in colorectal cancer from a Chinese cohort (n = 102), which was further validated by a The Cancer Genome Atlas (TCGA) cohort (n = 376). High expression of TTPAL was significantly associated with shortened survival in patients with colorectal cancer. TTPAL promoted cell viability and clonogenicity, accelerated cell-cycle progression, inhibited cell apoptosis, increased cell migration/invasion ability in vitro, and promoted tumorigenicity and cancer metastasis in vivo. TTPAL significantly activated Wnt signaling and increased β-catenin activation and protein expression of cyclin D1 and c-Myc. Coimmunoprecipitation followed by mass spectrometry identified thyroid receptor-interacting protein 6 (TRIP6) as a direct downstream effector of TTPAL. Depletion of TRIP6 significantly abolished the effects of TTPAL on cell proliferation and Wnt activation. Direct binding of TTPAL with TRIP6 in the cytoplasm inhibited ubiquitin-mediated degradation of TRIP6 and, subsequently, increased levels of TRIP6 displaced β-catenin from the tumor suppressor MAGI1 via competitive binding. This sequence of events allows β-catenin to enter the nucleus and promotes oncogenic Wnt/β-catenin signaling. In conclusion, TTPAL is commonly overexpressed in colorectal cancer due to copy number gain, which promotes colorectal tumorigenesis by activating Wnt/β-catenin signaling via stabilization of TRIP6. TTPAL overexpression may serve as an independent new biomarker for the prognosis of patients with colorectal cancer.

Significance: TTPAL, a gene preferentially amplified in colorectal cancer, promotes colon tumorigenesis via activation of the Wnt/β-catenin pathway.

Introduction
Colorectal cancer is the third most common cancer and the second leading cause of cancer-related death worldwide (1, 2). The pathogenic mechanisms underlying colorectal cancer development appear to be complex and heterogeneous. Copy number alterations (CNA) are common somatic changes in cancer featured with gain or loss in copies of DNA sections (3). Deletions and copy number gains contribute to alterations in the expression of tumor suppressor genes and oncogenes, respectively. The stepwise accumulation of CNAs confers growth advantage and metastatic competence on cells, thus playing a crucial role in cancer initiation and progression. Recent studies have suggested that genes with CNAs are potential biomarkers and/or therapeutic targets for colorectal cancer. Therefore, detection and mapping of copy number abnormalities provide an approach for associating aberrations with disease phenotype and for localizing critical genes (4, 5). It is important to identify and functionally characterize novel genes with CNAs that are associated with colorectal cancer (4).

Chromosome 20q amplification has been commonly found in colorectal cancer and is involved in transforming adenoma to carcinoma (5, 6). Chromosome 20q amplification is also a potential indicator of poor prognosis in patients with colorectal cancer (7–9). By whole-genome sequencing (WGS), we identified that TTPAL (tocopherol alpha transfer protein-like), located at 20q13.12, was preferentially amplified in primary colorectal tumor tissues. In keeping with our finding, analyses from The Cancer Genome Atlas (TCGA) dataset showed that copy number gain of TTPAL occurs frequently in colorectal cancer and positively correlates with its upregulated mRNA expression. Searching for the public protein datasets of Human Protein Atlas, we found that higher TTPAL protein expression was shown in colorectal cancer...
tumor tissues compared with normal colon tissues from the dataset (http://www.proteinatlas.org). Moreover, high TTPAL mRNA expression is significantly associated with poor survival in patients with multiple cancer types (https://www.proteinatlas.org/ENSG00000124120-TTPAL/pathology#top; \( P < 0.001 \)). Therefore, we hypothesize that TTPAL plays an oncogenic function in colon carcinogenesis.

The role of TTPAL in human cancer remains uninvestigated. In this study, we identified the frequent overexpression of TTPAL in colorectal cancer due to its copy number gain. Further functional studies revealed that ectopic expression of TTPAL significantly promoted colorectal cancer growth by enhancing G1–S cell-cycle progression and reducing apoptosis. The tumor-promoting effect of TTPAL was revealed to be associated with the activation of Wnt/β-catenin signaling pathway by binding to thyroid receptor-interacting protein 6 (TRIP6). The direct binding of TTPAL with TRIP6 in cytoplasm inhibited TRIP6 ubiquitin degradation. Enhanced cytoplasmic TRIP6 displaced β-catenin from the tumor suppressor MAGI1 via competitive binding, which allowed β-catenin to enter into the nucleus and subsequently activated the oncogenic Wnt/β-catenin signaling. Moreover, TTPAL overexpression was significantly associated with poor survival of patients with colorectal cancer. Thus, this study revealed a novel oncogenic mechanism and the clinical application value of TTPAL in colorectal cancer.

Materials and Methods

Colon cancer cell lines

The colon cancer cell lines (DLD-1, HCT116, HT29, LOVO, SW480, RKO, and SW1116) were obtained from the ATCC between 2014 and 2015 and cells’ authentication were confirmed by short tandem repeat profiling. Cells were routinely cultured and maintained in DMEM supplemented with 10% FBS and antibiotics (Gibco BRL) according to the ATCC protocols, except HCT116 cells, which were cultured in complete McCoy 5A medium (Gibco BRL). Routine Mycoplasma testing was performed by PCR. Cells were grown for no more than 25 passages in total for any experiment.

Subjects and sample collection

Paired primary colorectal tumors and adjacent nontumor tissues were collected immediately after surgical resection at the Prince of Wales Hospital (Hong Kong, China; 182 primary tumors and 18 nontumor tissues) and Peking University Hospital Cancer Institute (Beijing, China; 102 primary tumors 50 nontumor tissues). The specimens were snap-frozen in liquid nitrogen and stored at 80°C and were also fixed in 10% formalin and embedded in paraffin for routine histologic examination. Biopsies from 3 cases of normal mucosa obtained during colonoscopy were recruited as healthy controls, which during colonoscopy were recruited as healthy controls, which

PCR copy number analysis

DNA was extracted from 102 frozen colorectal cancer samples of Chinese Cohort using the AllPrep DNA/RNA/Protein Kit (Qiagen). The purified genomic DNA was amplified with a probe specific to target gene TTPAL (Mm00082375_cn) by TaqMan Copy Number Assays (Applied Biosystems). The results were analyzed by CopyCaller software v2.0 (Applied Biosystems).

Subcutaneous xenograft and experimental metastasis mouse models

HCT116 and DLD1 cells stably transfected with TTPAL expression vector or empty vector (5 × 10⁶ cells/0.1 mL PBS) were injected subcutaneously into the left and right dorsal flanks of 4- to 6-week-old female Balb/c nude mice (\( n = 8 \) group), respectively. Tumor size was measured every 2 days for 2–3 weeks using a digital caliper. Tumor volume (mm³) was estimated by measuring the longest and shortest diameters of the tumor and calculating as described previously (10). At the endpoint, tumors were harvested and weighted. The excised tissues were either fixed in 10% neutral-buffered formalin or snap frozen in liquid nitrogen. Tumor sections from paraffin-embedded blocks were used for histologic examination.

For metastasis model, HCT116 cells stably transfected with TTPAL expression vector or empty vector (2 × 10⁶ cells in 0.1 mL PBS) were injected intravenously via the tail vein (\( n = 5 \)). After 6–8 weeks, mice were sacrificed and their lungs were harvested. The lungs were sectioned and stained with hematoxylin and eosin. The number of lung metastases were counted. All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Coomassie precipitation and liquid chromatography–mass spectrometry

Coomassie precipitation (co-IP) assays were carried out as described previously (11, 12). Briefly, total protein from HCT116 cells (~5 × 10⁶/reaction) stably transfected with TTPAL (Flag-tagged) expression vector or empty vector was extracted in radioimmunoprecipitation assay (RIPA) buffer supplemented with proteinase inhibitor (Novagen). Immunoprecipitation was performed using anti-Flag M2 antibody (A2220, Sigma-Aldrich). The immune complexes were precipitated by Pure Proteome Protein A/G Mix magnetic beads (LSKMAGA02, Millipore) overnight at 4°C. Beads with extracted proteins were washed three times by 50 mmol/L ammonium bicarbonate buffer and subjected to digestion by trypsin at 37°C for 2 hours (Promega). Tryptic peptides were then extracted for liquid chromatography–mass spectrometry (LC/MS) analysis.

Co-IP and Western blot analyses

Whole lysate (150 μg protein) and co-IP precipitant by anti-Flag-tag, anti-thyroid receptor–interacting protein 6 (TRIP6) antibody (ab70747, Abcam), anti-HA-tag (ab18181, Abcam), or IgG were immunoblotted with either anti-TRIP6, anti-HA, or anti-Flag (A2220, Sigma-Aldrich) antibody to confirm the interaction between TTPAL and TRIP6 that was identified by LC/MS. The lysate (1% input, 10 μg protein) was also used as a control. The antibodies used are listed in Supplementary Table S1.

Ubiquitination assay

HCT116 and DLD1 cells stably transfected with TTPAL expression vector or empty vector were cotransfected with Ubiquitin-HA for 24 hours. Then the cells were incubated in the presence or
absence of 10 μmol/L MG132 (M-7449, Sigma-Aldrich) for 12 hours. Cells were lysed with RIPA buffer supplemented with protease inhibitors. Immunoprecipitation was performed using anti-TRIP6 or IgG, respectively. Immunoprecipitated proteins were analyzed by Western blot using anti-HA (ab18181, Abcam).

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS; standard V.16.0; IBM Corporation). The Pearson correlation coefficient was used to evaluate the correlation between TTPAL gene amplification and expression in the clinical samples. The x² test was used for comparison of patient characteristics and distributions of expression and covariates by vital status. ROC curve was used to estimate the cut-off value of the methylation percentage. Cut-off value was analyzed by survival significance analysis using the tool Cutoff Finder (http://molpath.charite.de/cutoff; ref. 13). Crude relative risks (RR) of death associated with TTPAL expression were validated in patients with colorectal cancer from TCGA study.

Table 1. Univariate and multivariate Cox regression analyses of potential poor prognostic factors in colorectal cancer patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Chinese cohort - mRNA level</th>
<th></th>
<th></th>
<th>Chinese cohort - protein level</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>RR (95% CI)</td>
<td>P</td>
<td>Multivariate</td>
<td>RR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>1.021 (0.997–1.045)</td>
<td>0.091</td>
<td>1.020 (0.996–1.045)</td>
<td>0.110</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>0.696</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td></td>
<td>1.132 (0.608–2.107)</td>
<td>1.128 (0.606–2.101)</td>
<td>1.128 (0.606–2.101)</td>
<td>1.112 (0.596–2.107)</td>
<td>1.128 (0.606–2.101)</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>1</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td>0.964 (0.36–1.736)</td>
<td>0.904</td>
<td>1.216 (0.602–2.457)</td>
<td>0.586</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td>1</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
<tr>
<td>Rectum</td>
<td></td>
<td>1</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td>Moderate or high</td>
<td>1.789 (0.961–3.330)</td>
<td>0.067</td>
<td>High</td>
<td>2.202 (0.909–4.447)</td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td>1</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
<tr>
<td>TNM</td>
<td></td>
<td>0.142</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
<tr>
<td>Early</td>
<td></td>
<td>0.227 (0.137–0.374)</td>
<td>&lt;0.0001</td>
<td>0.202 (0.122–0.355)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Late</td>
<td></td>
<td>1</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
<tr>
<td>TTPAL expression</td>
<td></td>
<td>High</td>
<td>1.954 (1.258–3.036)</td>
<td>2.441 (1.544–3.858)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td></td>
<td>1</td>
<td>1</td>
<td>No comparison</td>
<td>0.893</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 1. Overexpression of TTPAL is due to copy number gain in colorectal cancer and predicts poor prognosis of patients with colorectal cancer. A, Copy number of TTPAL is preferentially amplified in colon and rectum cancers as compared with other cancer types. Data were from TCGA studies, with sample sizes indicated under each cancer type. COAD, colon adenocarcinoma; READ, rectum adenocarcinoma; BRCA, breast invasive carcinoma; HNSC, head and neck squamous cell carcinoma; LUSC, lung squamous cell carcinoma; KIRC, kidney renal clear cell carcinoma; BLCA, bladder urothelial carcinoma; LUAD, lung adenocarcinoma; UCEC, uterine corpus endometrioid carcinoma; OV, ovarian serous cystadenocarcinoma; GBM, glioblastoma multiforme; STAD, stomach adenocarcinoma. B, TTPAL copy number was positively correlated with its mRNA expression in Chinese and TCGA cohorts by the Pearson correlation coefficient analysis.

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS; standard V.16.0; IBM Corporation). The Pearson correlation coefficient was used to evaluate the correlation between TTPAL gene amplification and expression in the clinical samples. The x² test was used for comparison of patient characteristics and distributions of expression and covariates by vital status. ROC curve was used to estimate the cut-off value of the methylation percentage. Cut-off value was analyzed by survival significance analysis using the tool Cutoff Finder (http://molpath.charite.de/cutoff; ref. 13). Crude relative risks (RR) of death associated with TTPAL expression were validated in patients with colorectal cancer from TCGA study.
TTPAL expression were estimated by univariate Cox proportional hazards regression model first. Multivariate Cox model was then constructed to estimate the adjusted RR for TTPAL expression. Overall survival in relation to expression was evaluated by the Kaplan–Meier survival curve and the log-rank test. Mann–Whitney U test or Student t test was performed to compare the variables of two groups. mRNA expression z-scores (RNA Seq V2 RSEM) from 382 colorectal cancer samples were downloaded from TCGA via eBioportal. Nonparametric Spearman correlations in expression levels between TTPAL and Wnt target genes were then computed. The difference in cell viability and tumor growth rate between two groups of nude mice was determined by repeated-measures ANOVA. P values <0.05 were taken as statistical significance.

Results
TTPAL is frequently overexpressed in colorectal cancer due to copy number gain
By WGS analysis to compare genetic alterations in primary colorectal cancer tissues with corresponding peripheral blood samples, we identified TTPAL to be preferentially amplified in colorectal cancer tumor tissues by WGS (Supplementary Fig. S1A). TTPAL was also frequently amplified in colon and rectum cancers from TCGA cohort but less amplified in other cancers (Fig. 1A), demonstrating that copy number gain of TTPAL may be specifically associated with colorectal tumorigenesis. We further quantitated the copy number and mRNA expression of TTPAL in colorectal cancer samples by TaqMan copy number assay and qRT-PCR, respectively. In 102 patients with colorectal cancer, 48% (49/102) of primary colorectal cancer tissues exhibited TTPAL copy number gain, which was positively correlated with its mRNA expression (R² = 0.3537, P < 0.0001; Fig. 1B). This finding was further validated in colorectal cancer samples from TCGA cohort, with copy number gain detected in 70.2% of samples (≥1.2 fold, 436/621) and mRNA expression also positively correlated with copy number (n = 376, R² = 0.5632, P < 0.0001; Fig. 1B). These results demonstrate that TTPAL is commonly overexpressed in colorectal cancer due to copy number gain.

TTPAL expression is upregulated in colon cancer cells and primary colorectal cancer tumors
We then examined TTPAL expression in colon cancer cells and primary colorectal cancer tissues. TTPAL expression at both mRNA and protein levels was detected in all 7 colon cancer cell lines (DLD1, LOVO, RKO, HT29, HCT116, SW480, and SW1116), but not in normal colon tissues (Fig. 1C). TTPAL mRNA expression was significantly upregulated in primary colorectal cancer tumors as compared with their adjacent normal tissues as determined by qRT-PCR (Fig. 1D). Specifically, the upregulation of TTPAL mRNA in colorectal cancer was validated in three independent cohorts of paired tumor and adjacent normal samples including Beijing Chinese cohort (n = 50; P < 0.0001), Hong Kong Chinese cohort (n = 18; P < 0.0001), and the TCGA cohort (n = 23; P < 0.0001; Fig. 1D). Significant upregulation was also observed in colorectal cancer (n = 286) compared with unpaired adjacent normal tissues (n = 39) from TCGA study (P < 0.0001; Fig. 1D). Western blot and IHC staining further confirmed the upregulation of TTPAL at the protein level in colorectal cancers as compared with adjacent normal tissues (Fig. 1E and F).

Overexpression of TTPAL is an independent predictor of poor survival in patients with colorectal cancer
We further evaluated the clinicopathologic and prognostic significance of TTPAL expression in patients with colorectal cancer. We evaluated TTPAL mRNA expression in primary colorectal cancer tissues from 102 Chinese patients. By classifying samples into low and high expression groups using cut-off finder, TTPAL overexpression was detected in 64.7% (66/102) of primary colorectal cancers. No correlation was found between TTPAL expression and clinicopathologic features such as age, gender, location, differentiation, and Tumor–Node–Metastasis (TNM) stage (Supplementary Table S2). However, TTPAL overexpression was associated with an increased risk of cancer-related death by univariate Cox regression (RR = 2.202, 95% CI: 1.090–4.447, P = 0.028; Table 1). In particular, after adjustment for potential confounding factors, TTPAL overexpression was found to be an independent risk factor for shorter median overall survival in colorectal cancer by multivariate Cox regression analysis (RR = 2.146, 95% CI: 1.061–4.340, P = 0.034; Table 1). Kaplan–Meier survival curves showed that patients with colorectal cancer with TTPAL overexpression had significantly poorer overall survival based on the log-rank test (P < 0.05; Fig. 1G).

TTPAL protein expression was further evaluated in primary colorectal cancer tissues from another cohort of Chinese patients (n = 182) by IHC using tissue microarrays. No correlation was found between TTPAL protein expression and clinicopathologic features such as age, gender, location, differentiation, and TNM stage (Supplementary Table S2). Further univariate and multivariate Cox regression analyses showed that TTPAL protein expression was also an independent predictor of poor survival in patients with colorectal cancer (multivariate RR = 2.441; 95% CI: 1.544–3.858, P < 0.0001) besides TNM stage (Table 1). As shown in the Kaplan–Meier survival curves, colorectal cancer patients with high TTPAL protein expression had significantly shorter survival than those with low expression (P = 0.002, log-rank test; Fig. 1G). Moreover, the prognostic significance of TTPAL expression was well validated in TCGA cohort (Fig. 1H). These findings collectively indicated that TTPAL may serve as an oncogenic function in vitro. A, Overexpression of TTPAL in HCT116 and DLD1 cells, confirmed by RT-PCR and Western blot analysis, significantly increased cell viability and colony formation ability. B, Knockdown of TTPAL by siTTPAL in SW480 and SWIT16, confirmed by RT-PCR and Western blot analysis, significantly inhibited cell viability and colony formation ability. C, Overexpression of TTPAL significantly increased cells in S-phase, while knockdown of TTPAL showed the opposite effect. D, Western blot analysis showed TTPAL enhanced protein levels of cyclin-D1, CDK4, and PCNA, while knockdown of TTPAL had the opposite effect. E, Overexpression of TTPAL inhibited cell apoptosis by flow cytometry after Annexin V/7-AAD dual staining, while knockdown of TTPAL promoted cell apoptosis. F, Western blot analysis showed TTPAL expression reduced activation of caspases-8, -9, -7, -3, and PARP, while knockdown of TTPAL exhibited the opposite effect. G, Overexpression of TTPAL promoted cell migration as shown by wound-healing assays. H, Overexpression of TTPAL significantly increased cell invasion ability as shown by Matrigel invasion assay. I, Knockdown of TTPAL significantly inhibited migration ability. J, Knockdown of TTPAL significantly decreased cell invasion ability. K, TTPAL increased expression of mesenchymal marker (vimentin) and decreased expression of epithelial markers (E-cadherin and claudin-7), while knockdown of TTPAL showed the opposite effects.
TTPAL expression promotes colon cancer cell growth

The frequent overexpression of TTPAL in colorectal cancer prompted us to investigate its potential oncogenic role in colorectal cancer. To this end, we performed in vitro gain- and loss-of-function assays on TTPAL. Ectopic expression of TTPAL in HCT116 and DLD1 cells (Fig. 2A) significantly increased cell viability and clonogenicity (Fig. 2A). On the other hand, TTPAL knockdown by RNA interference in SW480 and SW1116 cells markedly inhibited cell viability and clonogenicity (Fig. 2B). These results indicated that TTPAL plays an oncogenic role in colon cancer.

TTPAL promotes cell-cycle progression and inhibits cell apoptosis

We analyzed the cytokinetic effect of TTPAL. Overexpression of TTPAL led to a significant decrease in G1 phase cells and a concomitantly increase in S-phase cells in both HCT116 and DLD1 cells by BrdU staining (all P < 0.01; Fig. 2C). The promoting effect of TTPAL on cell-cycle progression was confirmed by the increased protein expression of two master G1–S checkpoint regulators (cyclin D1 and CDK4) and the proliferation marker proliferating cell nuclear antigen (PCNA; Fig. 2D). These results prompted us to investigate its potential oncogenic role in colorectal cancer.

Consistently, overexpression of TTPAL significantly upregulated the protein levels of cyclin D1, c-Myc, β-catenin, and active β-catenin, while TTPAL knockdown diminished expression of these key Wnt signaling genes (Fig. 2B). Moreover, Wnt signaling inhibitor IWP-2 significantly inhibited cell growth in DLD1 and HCT116 cells stably transfected with TTPAL as compared with DMSO treatment. IWP-2 also eliminated the growth-promoting effect exerted by TTPAL overexpression (P > 0.05 for IWP-2-treated TTPAL-overexpressed cells vs. DMSO-treated vector-transfected cells, Fig. 2C). TTPAL knockdown showed weaker effect on proliferation in RKO cells, which do not harbor mutation in APC or CTNNB1 gene (Supplementary Fig. S2B). These results demonstrate that the oncogenic role of TTPAL is largely mediated by activation of Wnt/β-catenin signaling.

TTPAL directly interacts with TRIP6

To identify the direct interacting partners of TTPAL in colorectal cancer, we performed co-IP followed by LC/MS. TTPAL binding candidates were then identified by comparing the anti-TTPAL-Flag IP products of TTPAL (Flag)-overexpressed cells with those of control cells (Fig. 3D). Among the identified candidates with ≥3 mapping peptides, 7 genes have been reported with functions that may be associated with cancer development (Supplementary Table S3). Among them, TRIP6 is of interest due to its involvement in Wnt signaling pathway (14). To validate the interaction between TTPAL and TRIP6, Flag-TTPAL and HA-TRIP6 (or TRIP6) were co-expressed in HCT116 and DLD1 cells, and total proteins were immunoprecipitated with anti-Flag or anti-HA/anti-TRIP6 antibodies. Western blot results showed that TTPAL and TRIP6 could be coprecipitated by each other in both cells. Moreover, the interaction of endogenous TTPAL and TRIP6 in SW480 and SW1116 cells was confirmed by immunoprecipitation–Western blot analyses (Fig. 3E), indicating the direct interaction between TTPAL and TRIP6. We next examined the expression and intracellular distribution of TTPAL and TRIP6 by immunofluorescence. Confocal microscopy images showed that TTPAL colocalized with TRIP6 in the cytoplasm of HCT116 and DLD1 cells following transfection of TTPAL and HA-TRIP6 (Fig. 3F, 1). The localization of the two proteins was further confirmed by Western blotting of membrane, cytoplasmic, and nuclear fractions, which showed that TTPAL and TRIP6 was mainly localized in the cytoplasm of HCT116 and DLD1 cells (Fig. 3F, 2).

TTPAL promotes tumorigenicity and metastasis by regulating TRIP6 and Wnt/β-catenin signaling in vivo

In light of our in vitro findings, we tested the effect of TTPAL in vivo. Results showed that stable transfection of TTPAL expression vector in HCT116 and DLD1 cells significantly promoted the growth of tumor volume over the entire assay period and increased tumor weight at the end point in subcutaneous xenograft models (Fig. 4A). Overexpression of TTPAL was confirmed by IHC staining in DLD1 and HCT116 xenografts (Fig. 4B). Cell proliferation was significantly promoted as determined by Ki-67 staining, and apoptosis was significantly reduced as evidenced by the TUNEL staining, in both TTPAL-overexpressed xenografts as compared with controls (Fig. 4B). Moreover, the expression of protein-1 (AP-1), MAPK/ERK (SRE), Wnt/β-catenin, Akt (FHRE), and NFκB. Ectopic expression of TTPAL significantly increased the activity of Wnt signaling as demonstrated by TOPFlash/FOPFlash luciferase reporter assay, and knockdown of TTPAL showed the opposite effect on Wnt activity (Fig. 3A; Supplementary Fig. S2A). Consistently, overexpression of TTPAL significantly upregulated the protein levels of cyclin D1, c-Myc, β-catenin, and active β-catenin, while TTPAL knockdown diminished expression of these key Wnt signaling genes (Fig. 3B). Moreover, Wnt signaling inhibitor IWP-2 significantly inhibited cell growth in DLD1 and HCT116 cells stably transfected with TTPAL as compared with DMSO treatment. IWP-2 also eliminated the growth-promoting effect exerted by TTPAL overexpression (P > 0.05 for IWP-2-treated TTPAL-overexpressed cells vs. DMSO-treated vector-transfected cells, Fig. 2C). TTPAL knockdown showed weaker effect on proliferation in RKO cells, which do not harbor mutation in APC or CTNNB1 gene (Supplementary Fig. S2B). These results demonstrate that the oncogenic role of TTPAL is largely mediated by activation of Wnt/β-catenin signaling.
Figure 3.
TTPAL activates Wnt signaling pathway and interacts with TRIP6. 

A, Overexpression of TTPAL significantly increased TOPflash/TOPflash luciferase reporter (Wnt) activity in DLD1 cells, while knockdown of TTPAL significantly suppressed Wnt activity in SW480 cells. 

B, Western blot analysis results showed that TTPAL expression enhanced the expression of Wnt signaling–related markers, while knockdown of TTPAL showed the opposite effect. 

C, Wnt signaling inhibitor IWP-2 treatment abolished the promoting effects of TTPAL on cell proliferation and clonogenicity in HCT116 and DLD1 cells. 

D, Co-IP followed by LC/MS identified TRIP6 to be a TTPAL-binding protein. 

E, Co-IP followed by Western blot analyses confirmed the binding between TTPAL and TRIP6 in ectopic and endogenous. 

F, TTPAL and TRIP6 are mainly colocalized in cytoplasm as demonstrated by confocal immunofluorescence analysis (F1) and Western blot of membrane, cytoplasmic, and nuclear fractions (F2) in HCT116 and DLD1 cells cotransfected with TTPAL and TRIP6 expression vectors. Mem, membrane; Cyto, cytoplasm; Nuc, nucleus.
Figure 4.
TPAL enhances tumorigenicity and metastasis in nude mice. A, HCT116 cells stably expressing TPAL expression promoted subcutaneous tumor growth as compared with control vector transfection in both HCT116 (A1) and DLD1 (A2) cells, both in terms of tumor volume over the entire assay period and tumor weight at the end point. B, IHC staining confirmed TPAL overexpression in HCT116 and DLD1 subcutaneous xenografts, which enhanced cell proliferation (by Ki-67 staining) and inhibited cell apoptosis (by TUNEL staining). IHC staining results also showed that TPAL increased TRIP6 and β-catenin expression in xenografts. C, Western blot analysis further confirmed that TPAL expression in HCT116 and DLD1 xenografts increased the expression of TRIP6 and β-catenin. D, Overexpression of TPAL promoted experimental metastasis of HCT116 cells in vivo. Representative images of lungs and hematoxylin and eosin (H&E) staining of lung tissues from nude mice injected with TPAL- or control vector-transfected HCT116 cells. Quantitative analysis showed that TPAL expression significantly increased the number of metastatic lesions. E, Knockdown of TPAL by stably expressing shTPAL inhibited subcutaneous tumor growth as compared with shNC transfection in SW480. F, Knockdown of TPAL significantly decreased the number of metastatic lesions in SW480 cells.
Figure 5.
TTPAL exerts its oncogenic function, partially depending on TRIP6 in colorectal cancer. A, Knockdown of TRIP6 in HCT116 and DLD1 cells with stable TTPAL overexpression was confirmed by RT-PCR and Western blot analysis. B, Knockdown of TRIP6 significantly abolished the promoting effect of TTPAL on colon cancer cell growth. C, Knockdown of TRIP6 significantly abolished the promoting effect of TTPAL on clonogenicity of colon cancer cells. D, Knockdown of TRIP6 significantly decreased migration ability of colon cancer cells that was promoted by TTPAL. E, Protein expression of key factors in Wnt signaling pathway in TTPAL-overexpressed HCT116 and DLD1 cells with or without transient knockdown of TRIP6. F, Knockdown of TRIP6 significantly abolished the TOPFlash/FOPFlash luciferase activity induced by TTPAL. G–J, TRIP6 overexpression in TTPAL-inhibited SW480 cells rescued cell proliferation (G), clonogenicity (H), migration (I), and Wnt activation (J).
β-catenin and TRIP6 was dramatically increased in TTPAL-overexpressed HCT116 and DLD1 xenografts by IHC, validating the molecular basis identified in vitro (Fig. 4B). TTPAL overexpression promoted lung metastasis in nude mice (P < 0.01; Fig. 4D). Furthermore, stable knockdown of TTPAL in SW480 cells significantly inhibited tumorigenicity (Fig. 4E) and metastasis in nude mice (Fig. 4F). Collectively, these results in immunodeficient nude mice suggest that TTPAL promoted colorectal tumorigenicity and may also facilitate metastasis.

The oncogenic role of TTPAL is dependent on TRIP6

We then examined the importance of TRIP6 in TTPAL-mediated oncogenic function in colorectal cancer. TRIP6 mRNA expression was significantly increased in colorectal cancer tumor tissues as compared with adjacent normal tissues in TCGA cohort (P < 0.001, Supplementary Fig. S3A). TRIP6 mRNA was readily expressed in all 7 colon cancer cells tested, but not in normal colon tissues (Supplementary Fig. S3B). TRIP6 expression promoted cell proliferation and clonogenicity in DLD1 and HT-29 cells, whereas TRIP6 silence showed significant suppressive effect on cell proliferation and clonogenicity in SW480, SW1116, and HCT116, suggesting the oncogenic role of TRIP6 in colorectal cancer (Supplementary Fig. S4). To investigate the effect of TRIP6 on the TTPAL-mediated cell proliferation and metastasis, HCT116 and DLD1 cells stably transfected with TTPAL or control vectors were cotransfected with siRNA against TRIP6 (Fig. 5A). TRIP6 knockdown significantly abolished the promoting effect of TTPAL on cell viability (Fig. 5B), clonogenicity (Fig. 5C), and migration ability of both HCT116 and DLD1 cells (Fig. 5D). We next examined whether TTPAL activated Wnt signaling pathway through mediating TRIP6. Indeed, TRIP6 knockdown abolished the TTPAL-induced protein expression of Wnt effectors (cyclin D1, c-Myc, β-catenin, and active β-catenin) by Western blot analysis (Fig. 5E). TRIP6 knockdown also blunted the TTPAL-activated Wnt signaling as evidenced by luciferase reporter assay in HCT116 and DLD1 cells (Fig. 5F). These results suggested that the oncogenic role of TTPAL and its effect on activating Wnt signaling were dependent on TRIP6. Consistently, TRIP6 overexpression in TTPAL-inhibited SW480 cells can rescue cell proliferation (Fig. 5G), clonogenicity (Fig. 5H), migration (Fig. 5I), and Wnt activation (Fig. 5J).

TTPAL inhibits ubiquitin-mediated degradation of TRIP6 protein

To gain insights into how TRIP6 mediates the role of TTPAL, we further evaluated the effect of TTPAL on TRIP6. We observed that TRIP6 protein expression was upregulated by ectopic expression of TTPAL in HCT116 and DLD1 cells, and decreased after knockdown of the endogenous TTPAL in SW1116 cells (Fig. 6A, 1). TRIP6 protein was also upregulated by TTPAL in xenografts of nude mice (Fig. 4C and D). However, mRNA level of TRIP6 was not changed by overexpression or knockdown of TTPAL (Fig. 6A, 1). Moreover, the protein levels of TRIP6 in various colorectal cancer cell lines were positively correlated with the protein levels of TTPAL in these cell lines (Fig. 6A, 2), indicating that TTPAL may stabilize TRIP6 in colorectal cancer. We therefore assessed whether TTPAL regulated the stability of TRIP6 protein. We treated TTPAL or control vector–transfected cells with the protein synthesis inhibitor cycloheximide. As shown in Fig. 6B, 1, TRIP6 was more stable in the presence of TTPAL in HCT116 and DLD1 cells. To investigate whether TTPAL increased the stability of TRIP6 by affecting its ubiquitination/degradation, we treated cells with the proteasome inhibitor MG132 after cotransfecting ubiquitin-HA and TTPAL/control vectors in HCT116 and DLD1 cells, and then cellular lysates were immunoprecipitated with anti-TRIP6 or IgG. As speculated, TTPAL decreased TRIP6 ubiquitination and increased its protein level (Fig. 6B, 2), inferring that TTPAL binds to TRIP6 and inhibits the ubiquitin-mediated degradation of TRIP6 protein.

TTPAL activates Wnt/β-catenin signaling by promoting TRIP6–MAGI1 interaction

TRIP6, containing the same PDZ-binding motif with β-catenin, can compete with β-catenin to bind to the PDZ domain of the tumor suppressor MAGI1 (Supplementary Fig. S5; ref. 14). We therefore hypothesize that TRIP6 protein increased by TTPAL binds to MAGI1 and subsequently release more β-catenin to ultimately activate Wnt/β-catenin signaling. To confirm the interaction between TRIP6 and MAGI1 in a more physiologic context, co-IP studies were carried out. HCT116 and DLD1 cells were transiently transfected with TRIP6, and cellular lysates were then immunoprecipitated with anti-TRIP6 or IgG. Western blot analysis confirmed the presence of MAGI1 in anti-TRIP6 IP products of both cells (Fig. 6C). To assess the effect of TTPAL on TRIP6–MAGI1 interaction, TTPAL or control vectors were transfected in HCT116 and DLD1 cells, and cellular lysates were immunoprecipitated with anti-TRIP6 or IgG. Western blot results showed that ectopic expression of TTPAL increased TRIP6 protein level and the binding of TRIP6–MAGI1 (Fig. 6C). By overexpressing TRIP6 and/or MAGI1 in HCT116 and DLD1 cells followed by co-IP and Western blot assays, we further showed the binding between MAGI1 and β-catenin was reduced by the presence of TRIP6 (Fig. 6D). In line with these results, both TRIP6 overexpression and MAGI1 knockdown...
significantly activated Wnt signaling, as evidenced by the increased protein levels of active β-catenin and the increased TOPFlash/FOPFlash reporter activities (all \( P < 0.01 \)) in HCT116 and DLD1 cells (Fig. 6F). TRIP6 promoted interaction between TCF4 and β-catenin (Fig. 6F). Moreover, PDZ binding–defective mutant, the deletion of the 8 C-terminal aa residues of TRIP6, was conducted (14), while mutant TRIP6 could not bind to MAGI1 (Fig. 6G). Wild-type TRIP6 induced Wnt activation as compared with mutant TRIP6 or vector transfection controls, which was indicated by the increased levels of active β-catenin and TCF4 (Fig. 6H, 1). In accordance with this, wild-type TRIP6 significantly promoted cell proliferation, clonogenicity, and migration in HCT116 and DLD1 cells (Fig. 6H, 2 and 3). Consistent with the in vitro and in vivo findings, TTPAL expression positively correlated with multiple target genes of β-catenin in primary colorectal cancer tissues from TCGA cohort, including CMYC, CCND1, ASCL2, APCDD1, AXIN2, EDN1, FGF18, MET, NAV2, and VEGFA (Fig. 6I), further supporting the activation of Wnt/β-catenin signaling by TTPAL. Collectively, our results suggest that upregulation of TTPAL increases the level of TRIP6 by inhibiting its ubiquitin-mediated degradation, and subsequently TRIP6 competitively binds with MAGI1 to release β-catenin; this sequence of events allows more β-catenin to enter the nucleus and activates oncogenic Wnt/β-catenin signaling to promote colorectal tumorigenesis (Fig. 6I).

Discussion

In this study, amplification of TTPAL was identified by WGS. We revealed that the amplification of TTPAL was positively associated with its mRNA overexpression in the 102 colorectal cancer cases in Chinese cohort (\( P < 0.001 \)) and 376 colorectal cancer cases in TCGA cohort (\( P < 0.001 \)), suggesting that increased TTPAL copy number contributes to the upregulation of TTPAL. TTPAL was located on chromosome 20q13.12, which is one of the most frequently amplified regions in colorectal cancer and in other cancer types such as breast, ovarian, and gastric cancers (7, 15, 16). Previously, we have reported amplification of SLC12A5 (9), the same location with TTPAL, contributes to cell proliferation and cell-cycle progression, inhibiting apoptosis and increasing metastatic abilities. The effects of TTPAL in promoting migration and invasion abilities in vitro and in inducing lung metastasis in nude mouse models suggested the potential role of TTPAL in facilitating colorectal cancer metastasis in vivo. Additional investigation using TTPAL transgenic and orthotopic mouse models are essential for future investigating the significance of TTPAL in colorectal cancer metastasis.

We demonstrated that TTPAL could activate the TOPFlash/ FOPFlash activity in colon cancer cells. TTPAL-induced Wnt signaling activation was also evidenced by β-catenin activation and increased protein levels of cyclin D1 and c-MyC. Furthermore, Wnt signaling inhibitor abolished the oncogenic effect of TTPAL on cell proliferation and clonogenicity. To understand the molecular basis of the role of TTPAL, co-IP of TTPAL followed by protein sequencing identified TRIP6 as an interacting partner of TTPAL. The direct interaction between TTPAL and TRIP6 was confirmed by Western blot analysis of co-IP products. TTPAL was colocalized in the cytoplasm with TRIP6 by confocal immunofluorescence assay and Western blot analysis. In addition, the expression of β-catenin and TRIP6 was dramatically increased in TTPAL-expressing xenografts tumors as compared with control xenografts. These results collectively suggest that TTPAL function via activating Wnt signaling and interacting with TRIP6.

TRIP6 was reported to promote tumor growth in several cancer types and involve in Wnt signaling (18–20). In keeping with this, we revealed that expression of TRIP6 significantly enhanced in colorectal cancer, signifying its oncogenic role in colorectal cancer. We then uncovered that TRIP6 knockdown in colon cancer cells could significantly blunt the effects of TTPAL on cell proliferation, colony formation, migration, and Wnt signaling activation. Conversely, TRIP6 expression can rescue Wnt activation and cancer-related phenotypes in TTPAL-inhibited SW480 cells, inferring that the oncogenic role of TTPAL in colorectal cancer is, at least in part, dependent on TRIP6.
To explore how TTPAL activates Wnt signaling through TRIP6, we assessed the interplay between TTPAL and TRIP6. We found that the direct binding of TTPAL and TRIP6 were existing in cytoplasm. TTPAL–TRIP6 complex may recruit lipid or proteins that inhibited activity of ubiquitin ligases, in turn, suppressed ubiquitin-mediated degradation of TRIP6. Previous studies have shown that TRIP6 contains the same PDZ-binding motif as β-catenin, and both TRIP6 and β-catenin are physiologic ligands of the PDZ domain–containing tumor suppressor MAGI1, therefore TRIP6 could bind to MAGI1 competitively with β-catenin (14, 21–25). Our results demonstrated that TTPAL expression promoted TRIP6–MAGI1 interaction, which in turn inhibited the binding between MAGI1 and β-catenin. Meanwhile, both TRIP6 overexpression and MAGI1 silence dramatically induced Wnt signaling activation. In addition, the impact of these findings was further strengthened by the observation that induction of TRIP6 promoted interaction between TCF4 and β-catenin. While PDZ-binding–defective mutant of TRIP6 was incapable of binding to MAGI1 and stimulating cancer-related phenotypes. Moreover, TTPAL expression was positively correlated with Wnt target genes in primary colorectal cancer tissues from TCGA cohort. Hence, these data imply that the direct binding of TTPAL with TRIP6 in cytoplasm inhibited ubiquitin-mediated degradation of TRIP6. Then TRIP6, which contains the same PDZ-binding motif as β-catenin, competitively bound to the PDZ domain–containing tumor suppressor MAGI1 to release β-catenin. This sequence of events allowed β-catenin to enter the nucleus and ultimately promoted the oncogenic Wnt/β-catenin signaling cascade.

However, enhanced cell proliferation was seen even in the presence of a Wnt inhibitor. Moreover, TTPAL knockdown showed weak effect on inhibiting cell growth in RKO cells, whose Wnt signaling is not hyperactivated. These results inferred the potential existence of Wnt-independent mechanism of cell proliferation induced by TTPAL in colorectal cancer. In fact, oncogenic signaling pathways including NFκB, Akt, JNK-AP-1, Hippo have been reported mediated by TRIP6 in cancer (20, 21). Our results demonstrated that TTPAL expression promotes TRIP6–MAGI1 interaction, which in turn inhibited the binding between MAGI1 and β-catenin. Hence, these data imply that the direct binding of TTPAL with TRIP6 in cytoplasm inhibited ubiquitin-mediated degradation of TRIP6. Then TRIP6, which contains the same PDZ-binding motif as β-catenin, competitively bound to the PDZ domain–containing tumor suppressor MAGI1 to release β-catenin. This sequence of events allowed β-catenin to enter the nucleus and ultimately promoted the oncogenic Wnt/β-catenin signaling cascade.

In summary, we demonstrate that TTPAL overexpression due to gene amplification is a common event in colorectal cancer. TTPAL plays a pivotal oncogenic role by activating Wnt/β-catenin signaling pathway through binding to and stabilizing TRIP6 in colorectal carcinogenesis. Therapeutic intervention of TTPAL might be a novel strategy for blunting uncontrolled growth and metastasis in Wnt/β-catenin–addicted colorectal cancer. Moreover, TTPAL may serve as an independent prognostic marker for patients with colorectal cancer.

Disclosure of Potential Conflicts of Interest

F.K.L. Chan reports receiving speakers bureau honoraria from Pfizer, Takeda, and AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: H. Gou, J.Q. Liang, J.Y. Fang, J.Y. Yu

Development of methodology: L. Zhang, Y. Zhang, J.Y. Fang, J.Y. Yu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. L., X. Wang, J.H.M. Tong, K.F.-F. To, J.Y. Yu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.Q. Liang, X. Wang, K.F.-F. To, J.Y. Yu

Writing, review, and/or revision of the manuscript: J.O. Liang, K.F.-F. To, J.Y. Sung, J.Y. Yu

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.Q. Liang, H. Chen, Y. Zhang, J.Y. Sung, J.Y. Yu

Study supervision: J.Q. Liang, J.J.Y. Sung, J.Y. Yu

Acknowledgments

This project was supported by National Key R&D Program of China (2016YFC1303200, 2017YFE0109700). Science and Technology Program Grant Shenzhen (JCYJ2018030715125271, JCYJ20170416315451462). Postdoctoral Natural Science Foundation of China (2017M622802). RGGF Hong Kong (14111121, 14163817), National Natural Science Foundation of China (NSFC; 81773000), Vice-Chancellor’s Discretionary Fund CIUHK and CIUHK direct grant; Shenzhen Municipal Science and Technology Shenzhen Virtual University Park Support Scheme to CIUHK Shenzhen Research Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 27, 2018; revised March 16, 2019; accepted April 19, 2019; published first April 24, 2019.
TTPAL Promotes Colorectal Tumorigenesis by Stabilizing TRIP6 to Activate Wnt/β-Catenin Signaling

Hongyan Gou, Jessie Qiaoyi Liang, Lijing Zhang, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-18-2986

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2019/04/24/0008-5472.CAN-18-2986.DC1

Cited articles
This article cites 27 articles, 5 of which you can access for free at:
http://cancerres.aacrjournals.org/content/79/13/3332.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/79/13/3332.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.