**Molecular Cancer Research**

**Tpl2 Kinase Impacts Tumor Growth and Metastasis of Clear Cell Renal Cell Carcinoma**

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**Abstract**

Due to the innate high metastatic ability of renal cell carcinoma (RCC), many patients with RCC experience local or systemic relapses after surgical resection. A deeper understanding of the molecular pathogenesis underlying advanced RCC is essential for novel innovative therapeutics. Tumor progression locus 2 (Tpl2), upregulated in various tumor types, has been reported to be associated with oncogenesis and metastatic progression via activation of the MAPK signaling pathway. Herein, the relevance of Tpl2 in tumor growth and metastasis of RCC is explored. Inspection of The Cancer Genome Atlas (TCGA) indicated that Tpl2 overexpression was significantly related to the presence of metastases and poor outcome in clear cell RCC (ccRCC), which is the most aggressive subtype of RCC. Moreover, expression of Tpl2 and CXCR4 showed a positive correlation in ccRCC patients. Depletion of Tpl2 by RNAi or activity by a Tpl2 kinase inhibitor in human ccRCC cells remarkably suppressed MAPK pathways and impaired in vitro cell proliferation, clonogenicity, anokinesis, resistance, migration, and invasion capabilities. Similarly, orthotopic xenograft growth and lung metastasis were significantly inhibited by Tpl2 silencing. Furthermore, Tpl2 knockdown reduced CXCL12-directed chemotaxis and chemoinvasion accompanied with impaired downstream signaling, indicating potential involvement of Tpl2 in CXCR4-mediated metastasis. Taken together, these data indicate that Tpl2 kinase is associated with and contributes to disease progression of ccRCC.

**Implications:** Tpl2 kinase activity has prognostic and therapeutic targeting potential in aggressive clear cell renal cell carcinoma. *Mol Cancer Res; 11(11); 1375–86. ©2013 AACR*

**Introduction**

Renal cell carcinoma (RCC) is the most common kidney tumor, whose incidence and mortality increases continuously over the past 30 years (1, 2). Although surgical intervention is effective for the majority of patients with a localized disease, approximately 30% of patients are diagnosed with metastatic diseases initially (3). In addition, a similar portion of patients with RCC who had radical surgery experiences relapses and systemic metastases (4). Despite improved understanding of dysregulated molecular pathways in RCC, there has been no significant improvement in the therapeutic strategies against advanced RCC with metastatic lesions; the 5-year survival rate of metastatic patients with RCC is about 10% (5, 6).

RCC represents a spectrum of heterogeneous histologic and genetic subtypes (7). Among pathologic subtypes, clear cell RCC (ccRCC) accounts for approximately 80% of all RCC and shows worse clinical outcomes compared with other subtypes including papillary, chromophobe, and collecting duct cell RCC; more than 90% of metastatic RCCs are ccRCCs (8, 9). Molecularly, 33% to 57% of ccRCCs harbor Von Hippel–Lindau (VHL) gene mutation, however, the clinical implications of this mutation are still controversial (10). Therefore, the identification of prognostic markers and novel therapeutic targets for ccRCC is of crucial importance for highly responsive therapeutic options for RCC.

Disregulation of mitogen-activated protein kinase (MAPK) kinases (MKK) signaling enhances tumorigenesis and tumor metastasis (11). Tumor Progression Locus 2 (Tpl2), which is known as MAP3 kinase 8 (MAP3K8), plays essential roles in tumor necrosis factor, interleukin-1 (IL-1), CD40, Toll-like receptor and G protein–coupled receptor-mediated MAPK and c-jun-NH2-kinase (JNK) pathways (12). Tpl2 has been reported to be overexpressed in various human tumors and to promote cell transformation, proliferation, migration, and invasion by the activation...
of extracellular signal–regulated kinase (ERK), Rac1, and focal adhesion kinase (FAK; refs. 12–18). Tpl2 maybe a novel target for ccRCC as its mRNA levels are significantly elevated in ccRCC compared with normal kidneys (12), and elevated MKK or ERK activity has been detected in human RCC cases (19, 20). However, little information exists about prognostic relevance and functional significance of Tpl2 in oncogenesis and metastatic conversion of ccRCC. Herein, we report that elevated Tpl2 expression in ccRCC is associated with the metastasis and poor prognosis and provide the first in vitro and in vivo evidence for its roles in the tumorigenesis and metastasis of ccRCC using short hairpin RNAs (shRNA)–targeting Tpl2 (shTpl2) and a specific Tpl2 kinase inhibitor (KI).

Materials and Methods

The Cancer Genome Atlas dataset for RCC

RNA sequencing data, VHNI mutation status, and clinicopathologic data for 468 ccRCCs and 73 papillary RCCs (papRCC) were downloaded from The Cancer Genome Atlas (TCGA) data portal (http://cancergenome.nih.gov/) and the cBioPortal for Cancer Genomics (http://www.cbioportal.org/).

RCC cell line expressing shTpl2

Human ccRCC cell lines (caki-1, ACHN, A498, 786-O, and 769-P) were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 (for caki-1, 786-O, and 769-P) or minimum essential medium (MEM; for ACHN and A498) media (Gibco) supplemented with 10% FBS (In vitrino) in a humidified incubator containing 5% CO2 at 37°C. VHNI gene mutation status of each ccRCC cell line was referred from previous report (21). Caki-1 cells that stably express either nontargeting short hairpin RNA (shRNA; caki-1-shCon) or shTpl2 (caki-1-shTpl2-1 (Pf. Jeong at Medical College of Wisconsin, Milwaukee, WI) and caki-1-shTpl2-2 (Applied Biological Materials Inc.) were established by lentiviral transduction and puromycin selection (2 µg/mL; Gibco).

Western blot analysis and quantitative reverse transcription PCR

Cells were lysed in the radioimmunoprecipitation assay buffer (RIPA) lysis buffer [15 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8.0)] supplemented with 1 × phosphatase inhibitors (PhosStop; Roche Diagnostics) and a 1 × protease inhibitors cocktail (Complete Mini; Roche Diagnostics). After centrifugation at 10,000×g for 5 minutes, the supernatant was harvested. Amount of protein was analyzed using the BCA Protein Assay Kit (Thermo). Protein was separated on SDS-PAGE for 2 hours at 100V. The proteins on the gel were transferred onto polyvinylidene difluoride membranes (Whatman) for 1 hour at 100V and then probed with antibody. The antibodies targeting total Tpl2 were obtained from Santa Cruz Biotechnology and Life Technologies. Antibodies to detect total MAP–ERK kinase (MEK), phosphorylated MEK (p-MEK), total ERK1/2, phosphorylated ERK1/2 (p-ERK), total JNK, phosphor
dated JNK (p-JNK), total protein kinase B (Akt), and phosphorylated Akt (p-Akt) were from Cell Signaling Biotechnology. Internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Santa Cruz Biotechnology. For quantitative reverse transcription PCR (qRT-PCR), mRNA was extracted using a RNeasy Kit (Qiagen) and then used for subsequent reverse transcription using a SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Resulting cDNA was used for quantitative PCR (qPCR) using SYBR Green PCR Master Mix and gene-specific primers; human Tpl2, forward = 5’-CAAGTGAAGGAGCGAGAGTTT-3’, reverse = 5’-GCAAGCAATCCTCCCCAGTTC-3’; human GAPDH, forward = 5’-CATCATGCTCTGGCCTCT-3’, reverse = 5’-GGTGCTAAGCAGTTGTTG-3’.

Cell proliferation assay

Human ccRCC cells were seeded to 96-well plates at a density of 1 × 103 cells/mL in 100 µL 10% FBS/RPMI or MEM. After 1, 24, 48, or 72 hours, cell viability was determined with an EZ-Cytox Cell Viability Assay Kit (Daeil Lab.) according to the manufacturer’s instruction. For pharmacologic Tpl2 inhibition, the Tpl2 KI, 4-(3-chloro-4fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-1,7-naphthrylidine was purchased from Calbiochem (22). The naphthyridine-3-carbonitriles, which are Tpl2 inhibitors, are reported to be quite specific for Tpl2 with IC50 of 12 nmol/L to 50 nmol/L. This affinity is at least 2,000- to 10,000-fold more than those for other protein kinases such as Raf-1, MEK, p38, CAMKII, and so on (23). Alternatively, RCC cells were treated with different concentrations of TKI (dimethyl sulfoxide, DMSO) only, 1, 2.5, 5, or 10 µmol/L for 1, 24, 48, and 72 hours, and cell proliferation inhibition was analyzed using the same assay.

Cell-cycle analysis

Cells were fixed by 70% ethanol at 4°C for 30 minutes and then stained in PBS with RNase (100 µg/mL) and propidium iodide (40 µg/mL; BD Biosciences) at 37°C for 30 minutes. Cell cycle was analyzed using a FACSCalibur (BD Biosciences).

Focus forming assay

Caki-1 control cells (shCon) or Tpl2 stable knockdown cells (shTpl2-1 and shTpl2-2; 300 cells/well) were seeded in 6-well plates and maintained in 10% FBS/RPMI for 3 weeks. In another experiment, caki-1 cells were cultured with Tpl2 KI (DMSO only, 2.5 or 5 µmol/L) for 3 weeks. The number of viable colonies per well was counted after staining with 0.2% crystal violet.

Anoikis evaluation

Cells undergoing death by anoikis were identified and quantified using the Cytoselect 24-well Anoikis Assay Kit (Cell Biolabs) according to the manufacturer’s instructions.

In vitro Transwell migration and invasion assay

After 24 hours serum starvation, 1 × 104 cells suspended in 300 µL serum-free RPMI or MEM media were loaded

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Tpl2 Is a Novel Prognostic Marker and Therapeutic Target in ccRCC

Figure 1. Upregulation ofTpl2 in human primary ccRCCs is correlated with metastasis and worse clinical outcome. A, the differential mRNA expression ofTpl2 in different stages of ccRCCs and papRCCs derived from TCGA RNA sequencing dataset. , P < 0.05. Column — mean, error bar — SEM. B, comparison ofTpl2 mRNA expression of ccRCCs with VHL gene mutation status. Column — mean, error bar — SEM. C The differential mRNA expression of Tpl2 in different stages of ccRCCs with wild-type VHL or mutated VHL gene . , P < 0.05. Column — mean, error bar — SEM. D, Kaplan–Meier graphs representing the probability of cumulative OS in patients with ccRCC stratified according to Tpl2 level in their primary tumors. High, moderate, and low expression of Tpl2 were designated as more than mean + SD, mean – SD approximately to mean + SD and less than mean – SD, respectively. The log-rank test was used to analyze statistical significance. E, survival analysis was stratified according to the VHL mutation status.

into a top insert of 24-well microchemotaxis chambers (Costar). Next, 10% FBS/RPMI or serum-free RPMI supplemented with 200 ng/mL recombinant human chemokine (C–X–C motif) ligand 12 (CXCL12; Prospec) was added to the bottom chamber as a chemoattractant. Cells were allowed to migrate through an 8-μm pore polycarbonate filter or invade through the filter precoated with 100 μg of Matrigel (BD Bioscience) for 24 hours. The upper side of the membrane was scraped with a cotton swab to remove cells that had attached but not migrated or invaded and the migrating or invading cells attached to the lower membrane surface were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin (H&E). The degree of migration/invasion was assessed in each well by counting the number of cells in 10 randomized fields at ×100 magnification. The degree of migration and invasion was adjusted by the cell viability assay to correct for cell proliferation effects of 10% FBS or 200 ng/mL CXCL12 treatment (corrected migration or invasion index = counted migrating or invading cell number/percentage of viable cells; refs. 24, 25).

Spheroid counting and invasion assay in three-dimensional system

The three-dimensional (3D) culture was carried out, as described previously (26). Briefly, caki-1-shCon, caki-1-shTpl2-1, or caki-1-shTpl2-2 cells (2 × 10³ cells/well) were
seeded in lipid-based low-cell adhesion 96-well plates (Lipidure) in 10% FBS/RPMI on day 0. On day 1, 3, 6, and 9, morphology of spheroid was observed; spheroids were dissociated into single cells by Accumax buffer (Millipore), and then cell numbers were counted. For spheroid cell counting assay, on day 3, culture media was changed by 2% Matrigel (BD Bioscience) containing 10% FBS/RPMI media. The number of secondary spheroid was counted on day 9.

Orthotopic xenografts implantation and in vivo metastasis assay

All animal experiments were approved by the appropriate Institutional Review Boards of the Samsung Medical Center (Seoul, South Korea) and conducted in accord with the "National Institute of Health Guide for the Care and Use of Laboratory Animals" (NIH publication No. 85–23, revised in 1996). A total of 1 × 10⁶ caki-1 shCon or caki-1 sh-Tpl2-1 cells were injected into subcapsular area of the left kidney of female 6- to 8-week-old BALB/c-nu mice. Four weeks after the implantation, kidneys and lungs were harvested and assessed for tumor weight. Tumor weight was calculated by subtracting the weight of the right normal kidney (or lung) from that of the left tumor-bearing kidney (or lung). When right kidney (or lung) was heavier than left, the weight of the tumor was recorded as zero. For in vivo experimental metastasis assay, 2 × 10⁶ caki-1 sh-Con or caki-1 sh-Tpl2-1 cells were injected into the tail vein of female 6- to 8-week-old BALB/c-nu mice. Four weeks later, pulmonary nodules were counted both macroscopically and microscopically. Harvested organs were processed for paraffin-embedded sections, which were stained with H&E to confirm pathologically the presence of orthotopic renal or lung metastatic tumors. In addition, to examine effects of Tpl2 silencing on MAPK signaling in vivo tumors, immunohistochemistry (IHC) was conducted. Paraffin-embedded orthotopic renal xenografts sections (4 to 6 μm thick) were mounted on slides, which were used to determine Tpl2, p-ERK and p-JNK expression levels. The antibodies used were from the companies indicated in parentheses: anti-Tpl2 (Santa Cruz Biotechnology); anti-p-ERK and anti-p-JNK antibodies (Cell Signaling Technology).

Statistical analysis

All data are presented as mean ± SD or mean ± SEM for Fig. 1. For comparison of Tpl2 mRNA expression levels between different clinical stages, ANOVA was used. Overall survival (OS) curves were estimated by the Kaplan–Meier method, and the resulting curves were compared using the log-rank test. Significance of difference between the experimental groups and controls was assessed by the Student t test. Difference is significant if P value is < 0.05.

Results

Clinical implications of Tpl2 in RCC

To understand clinical relevance of Tpl2 in RCC, we investigated whether elevated expression of Tpl2 in RCC portends poor clinical outcomes. With the mRNA sequencing data including Tpl2 and CXCR4 retrieved from TCGA (http://cancergenome.nih.gov), VHL mutation status and clinicopathologic data of patients with RCC were obtained. Stage IV metastatic ccRCCs (n = 80) have significantly higher level of Tpl2 mRNA than stage I and II localized ccRCCs (n = 271; Fig. 1A; P = 0.043), whereas this expression difference was not observed in papRCCs (n = 76; Fig. 1A). Although level of Tpl2 mRNA expression of VHL wild-type ccRCCs (n = 335) was not different that of VHL mutant ccRCCs (n = 133; Fig. 1B), significant elevation of Tpl2 mRNA in metastatic ccRCC was identified only in the VHL wild-type ccRCCs (Fig. 1C). Kaplan–Meier plots of survival for patients with ccRCC showed that OS was significantly decreased with upregulation of Tpl2 (Fig. 1D; Log-rank test, P < 0.001). This correlation between upregulation of Tpl2 and worse prognosis was observed in not only VHL wild-type ccRCCs (P < 0.001) but also VHL mutant ccRCCs (P = 0.021; Fig. 1E). In contrast, papRCC patients with high level of Tpl2 mRNA showed similar clinical outcome compared with those with low or moderate level of Tpl2 mRNA (Supplementary Fig. S1). These analyses suggest that Tpl2 would play a role in the progression of ccRCC, not in papRCC.

Impaired proliferation, clonogenicity, and anoikis resistance of human RCC cells by the depletion of Tpl2

To establish a functional role for Tpl2 in ccRCC, expression of Tpl2 in various kinds of ccRCC cell lines [VHL wild-type ccRCC cells = caki-1, ACHN; VHL deficient (mutated or hypermethylated) ccRCC cells = A498, 786-O, 769-P]. Levels of Tpl2 mRNA and protein of VHL wild-type ccRCC cells were higher than those of VHL deficient ccRCC cells (Fig. 2A). As correlation between Tpl2 expression and worse clinical outcome of ccRCC was more obvious in VHL wild-type ccRCC, Caki-1 cells that show higher expression of Tpl2 than the other VHL wild-type ccRCC cell line ACHN (Fig. 2A), were selected to test functional roles of Tpl2

Figure 2. Depletion of Tpl2 activity–impaired proliferation, colony formation, and anoikis resistance ability of human ccRCC caki-1 cells. A, relative Tpl2 mRNA and protein level were analyzed by qRT-PCR (left) and immunoblot analysis (right), respectively, in five different human ccRCC cell lines. GAPDH = internal control (for qRT-PCR) and loading control (for immunoblot analysis). B, relative Tpl2 mRNA and protein level were analyzed by qRT-PCR (left) and immunoblot analysis (right), respectively, in caki-1 cells with Tpl2-silencing shRNA (shTpl2-1 or shTpl2-2) or nontargeting shRNA (shCon). GAPDH = internal control (for qRT-PCR) and loading control (for immunoblot analysis). C, IL-1β (100 ng/mL)-mediated MAPK signaling activation was analyzed by Western blot analysis in caki-1 cells with shTpl2-1 or shCon. GAPDH = loading control. D, alteration in IL-1β-induced MAPK signaling activation by the in vitro treatment of a Tpl2 KI was analyzed by Western blot analysis in caki-1 cells. GAPDH = loading control. E, suppressed proliferation of caki-1 cells by Tpl2 gene silencing and Tpl2 KI treatment. F, evaluation of colony formation in accordance with Tpl2 knockdown inhibition and Tpl2 KI treatment. G, anoikis resistance of caki-1 shCon and shTpl2-1 cells was examined. a and c = ×20 magnification; b and d = ×60 magnification. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
in ccRCC. Stable knockdown of Tpl2 and appropriate control cells were established in Caki-1 by lentiviral transduction of sh-Tpl2-1, sh-Tpl2-2, and sh-Con. The knockdown efficiency was verified by qRT-PCR and Western blot analysis (Fig. 2B).

Because of the major function of Tpl2 is to activate downstream MAPKs in response to IL-1 stimulation (27), measuring the phosphorylation levels of MAPKs including MEK, ERK, and JNK could therefore reflect the inhibition of Tpl2. Both shRNA-mediated knockdown of Tpl2 and in vitro Tpl2 KI treatment suppressed of the activation of IL-1β-induced Tpl2-mediated MAPK downstream signaling pathways in caki cells (Fig. 2C and D, respectively). These MAPK downstream signaling pathways are known to be crucial at the intersection of several oncogenic pathways that regulate cell proliferation and survival (6, 7). In vitro proliferation of caki-1 cells was significantly reduced in Tpl2 knockdown cells (both in sh-Tpl2-1 and sh-Tpl2-2) compared with control cells (Fig. 2E) and the suppressed proliferation was accompanied with a significant delayed cell-cycle progression; increase in G1 (sh-Tpl2-1 = 54.2%, sh-Con = 37.8%) and decrease in S (sh-Tpl2-1 = 35.4%, sh-Con = 46.4%) phase cell ratio (Supplementary Fig. S2). Dose-dependent inhibition of proliferation was also observed by the Tpl2 KI (Fig. 2E; P = 0.021 at 2.5 μmol/L, P = 0.0004 at 5 μmol/L and P = 0.0002 at 10 μmol/L). Moreover, the clonogenicity of caki-1 cells was significantly diminished by Tpl2 gene silencing (all P = 0.005) and pharmacologic inhibition (37% reduction at 2.5 μmol/L, P = 0.008; 83% reduction at 5 μmol/L, P = 0.002; Fig. 2F).

Generally, in the absence of the anchorage, epithelial cells undergo anoikis, a form of apoptosis by disruption of the cell adhesion and cell-extracellular matrix (ECM) interactions (28). The ability of transformed cells to survive under anchorage independent (anoikis-resistance) is correlated with the metastatic potential of a tumor. To elucidate the role of Tpl2 activity and metastasis further, effects of Tpl2 silencing in caki-1 orthotopic and in vivo models were separately evaluated in an experimental lung metastasis model via tail vein injection of 1 × 10^6 caki-1-shCon (n = 9) or sh-Tpl2-1 (n = 9). Tpl2 gene silencing effectively suppressed primary tumor growth of caki-1 cells (Fig. 5A, P = 0.004). Moreover, no spontaneous lung metastases was detected in Tpl2 knockdown group, while all animals harboring caki-1-shCon showed microscopic lung metastases (Fig. 5A, arrows, P < 0.001). When the role of Tpl2 in the RCC metastasis was separately evaluated in an experimental lung metastasis model via tail vein injection of 2 × 10^5 caki-1-shCon (n = 10) or caki-1-shTpl2-1 (n = 10), Tpl2 knockdown...
showed meaningful reduction of lung metastatic foci (Fig. 5B, arrows, \( P = 0.005 \)) and the increase of lung weight by lung metastasis (data not shown). IHC analysis of orthotopic xenografts confirmed the downregulation of Tpl2 and subsequent decreased in phosphorylation of ERK and JNK in shTpl2-1 group, compared with those of control group (Fig. 5C). Consistent with the clinical implication revealed by the analysis of TCGA database, our in vivo data suggested that Tpl2 is crucial in the tumorigenesis and progression of ccRCC.
Figure 4. Inhibitory effects of Tpl2 depletion on cell proliferation, migration, and invasion in various ccRCC cell lines. A, Three different ccRCC cells with wild-type VHL (ACHN) and deficient VHL (786-O and 769-P) were treated with 10 μmol/L Tpl2 KI for 72 hours. Cell proliferation was assessed. B and D, migration and invasion was determined using Boyden Transwell chambers following treatment with 5 μmol/L Tpl2 KI in ACHN cells (B), 786-O cells (C) and 769-P cells (D). **, P < 0.01; ***, P < 0.001.
Decrease in CXCL12-induced downstream signaling activation and chemotaxis/chemoinvasion by Tpl2 silencing

Previously, it was reported that chemokine receptor type 4 (CXCR4)–toll-like receptor 2 (TLR2) complex mediated activation of MEK/ERK signaling pathway which is implicated in tumor progression and metastasis of pancreatic cancer (29). As CXCL12/CXCR4 signaling is closely related with the progression of ccRCC, correlation between Tpl2 and CXCR4 using dataset of TCGA (ccRCC, n = 335) was analyzed and, interestingly, expression of Tpl2 and CXCR4 showed significant positive correlation (Fig. 6A; Rs = 0.336, P = 0.0095). Therefore, the effects of the Tpl2 knockdown on the CXCR4-mediated downstream signaling pathways were examined.

In vitro a CXCR4 ligand, CXCL12 (200 ng/mL), stimulation activated CXCR4 (Akt, MEK, and ERK) and Tpl2 (MEK and ERK) downstream signaling components of caki-1 cells (Fig. 6B). Those activations by CXCL12 (200 ng/mL) were significantly impaired by Tpl2 gene silencing (Fig. 6B). Concomitantly, CXCR4-dependent chemotaxis and chemoinvasion were suppressed significantly in both caki-1-shTpl2-1 and caki-1-shTpl2-2 compared with caki-1-shCon (Fig. 6C, P = 0.004 and P = 0.008, respectively). These data indicate that endogenous Tpl2 could regulate CXCR4-dependent metastatic potential in ccRCC.

Discussion

In the past 10 years, progresses in the understanding of RCC biology have resulted in the successful clinical development of antiangiogenic- and mTOR-targeted drugs.
However, the majority of patients with RCC still succumb to the disease, as current targeted therapies and other adjuvant chemotherapeutic regimens showed limited therapeutic effects. Moreover, useful biomarkers for advanced RCC need to be elucidated further (30). Progression and metastasis can be summarized in a series of steps starting with local invasion, followed by intravasation, survival in the circulation, extravasation, initiation of micrometastasis at a distant site, and vascularization of the new metastatic tumor (31).

While overexpression ofTpl2 has been observed in different types of human tumors (12) and a recent article reported significant upregulation of Tpl2 in metastatic prostate cancer samples (32), there is little evidence about the role of the Tpl2 oncogene in RCC. Herein, we showed that Tpl2 promotes ccRCC tumorigenesis and metastasis in vitro and in vivo by regulating diverse steps including cell-cycle progression that leads to increased proliferation, anoikis resistance, clonogenicity, migration, and invasion. Several antitumor and antimetastatic activities by Tpl2 silencing in human RCC cells would be resulted from impaired TPL2-mediated MAPK signaling activation and blocking effects on CXCR4-dependent axes including AKT and ERK pathways. These results suggest that targeting of Tpl2, which has a multifaceted role in ccRCC growth and metastatic progression could be an effective therapeutic strategy for ccRCC.
Interestingly, prognostic implication of Tpl2 upregulation was observed only in ccRCCs but not in papRCCs. In addition, those clinical meanings were stronger in VHL wild-type ccRCCs than in VHL mutant ccRCCs. RCC is heterogeneous pathologically and molecularly. The most frequent is ccRCC, which accounts for approximately 80% of all RCCs and has been reported to have most frequent somatic mutations or hypermethylation of the VHL gene. Although somatic mutation of the VHL gene is among the most frequent genetic alterations observed in ccRCCs, recent study reported that absence of VHL alteration is associated with advanced stages and diminished survival in sporadic ccRCC (33, 34). Regarding those correlations, Tpl2 is associated with most aggressive subtypes of RCC and could contribute to the aggressiveness of RCC, indicating a value as a novel prognostic factor and therapeutic target in ccRCC.

The CXCR4 receptor is involved in the metastatic process of RCC and its overexpression predicts a worse prognosis in RCC (35–38). CXCR4 and CXCL12 have multiple roles in tumor growth and metastasis, cancer cell-tumor microenvironment interactions, and angiogenesis, activating the Akt/MAPK signaling pathway (39–41). More importantly, Tpl2 knockdown trapped the CXCR4-dependent biologic axes and downregulated the Akt and ERK signaling pathways in our study. This indicates that Tpl2 silencing is a powerful therapeutic approach for controlling RCC growth and metastasis by interfering with the cross-talk involved in CXCR4-mediated downstream signaling. Furthermore, precise mechanistic studies elucidating the interrelationships between Tpl2 and CXCR4-mediated signaling are needed.

To the best of our knowledge, this is the first report showing that MAPK pathway activation and cross-talk with CXCR4/CXCL12 axis downstream signaling depend on Tpl2 contributes to both tumorigenesis and metastasis of ccRCC cells. Our data showed that Tpl2 is a pivotal member of several intracellular signaling pathways including MEK/ERK and MKK4/JNK. Activation of MAPK signaling pathways including ERK and JNK plays a crucial role in tumorigenesis, metastasis, and angiogenesis of RCC, and overexpression of MAP2K is observed in human RCC cases (42–44). The Raf/MEK/ERK signaling pathway, which is overactivated in RCC, regulates cell-cycle progression, apoptosis resistance, cellular motility, angiogenesis, and drug resistance (45). In addition, JNK is selectively activated in ccRCC specimens and, induces epithelial–mesenchymal transition, suggesting that JNK represents a novel molecular target that is selectively activated in and drives the growth of ccRCCs (46). In addition to previously reported anticancer effects of combined sorafenib and ERK inhibitor or simvastatin treatment suppressing Akt or ERK signaling pathway (47, 48), our findings can serve as the preclinical foundation for a combination targeting both the Tpl2/MEK/ERK and JNK pathways and the Ras/Raf/MEK/ERK pathway may lead to new therapeutic intervention strategies for ccRCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions


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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.J. Seol, S. Kim


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