Gain of TPPP as a predictor of progression in patients with bladder cancer

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Abstract. The present study investigated the role of tubulin polymerization promoting protein (TPPP) in the regulation of bladder cancer (BC) cell proliferation and migration, in addition to the association between TPPP gene copy number amplification and clinicopathological characteristics of BC. TPPP gene amplification was measured in human BC epithelial cells and samples obtained from 52 patients with BC via fluorescence *in situ* hybridization. TPPP gain was defined as mean TPPP copy number >2.2 per nucleus (cutoff). The neutrophil-to-lymphocyte ratio (NLR) was also obtained from the preoperative data of the patients. For *in vitro* assays, BC cell lines were transfected with either TPPP small interfering RNAs or scrambled control, following which cell proliferation and migration were determined using Cell Counting Kit-8 and Transwell migration assays, respectively. The percentage of cells with TPPP copy number amplification in the four BC epithelial cell lines (MGH-U1, -U1R, -U3, -U4) examined (86.0-100.0%) was found to be higher compared with that in the normal human uroepithelial cell lines (3.0 and 9.0%). Patients were divided into one- (1.9%), two- (55.8%), three- (7.7%), four- (26.9%) and five-copy (7.7%) types. Results calculated using Fisher's exact test indicated that the gain of TPPP in patients with BC associated significantly with age ($P<0.05$), advanced histological grade ($P<0.001$), tumor stage ($P<0.05$), histological type ($P<0.001$) and NLR ($P<0.05$). In MGH-U1R and MGH-U4 cells, cell proliferation and migration were revealed to be significantly lower following TPPP knockdown compared with those in cells transfected with the scrambled control. In conclusion, findings from the present study suggest that TPPP is important for cell proliferation, cell migration and BC progression, such that TPPP copy number assessment would be advised for preoperative urine cytology for urothelial neoplasia diagnosis.

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Introduction

Bladder cancer (BC) is the predominant malignancy urinary tract and is ranked as the cancer with the 4th highest incidence and as the 8th largest estimated cause of death in men in the United States (1). BC development and progression is correlated to genetic susceptibility and environmental exposure to arsenicals, pollutants, cigarette smoke, insecticides, fungicides and pesticides (2). BC is divided into two main subtypes based on the assessment of pathophysiology, namely non-muscle-invasive (NMIBC) and muscle-invasive (MIBC) (3). In Europe, ~75% patients with NMIBC exhibit high recurrence and low progression rates, whilst patients with MIBC are associated with high risks for progression and cancer-specific mortality (4). A number of studies have previously reported that fibroblast growth factor receptor 3 mutation, p53 pathway alteration and cyclin dependent kinase inhibitor 2A promoter hypermethylation are strongly associated with BC tumor histopathological characteristics, including histological grade, stage, progression and recurrence (5-8). In clinical practice, a combination of cytological and cystoscopic examination of the urothelium is considered to be the gold standard for the detection of urothelial neoplasia (9). Additionally, cytogenetic evaluations via fluorescence *in situ* hybridization (FISH) assays have revealed that numerical chromosomal aberrations that occur during the pathogenesis of BC can be used to assess gains or losses in centromere number (10-13).

Genetic aberration serves a crucial role in tumorigenesis (14,15). Using a whole-genome fine-tiling oligonucleotide array-based comparative genomic hybridization (CGH) assay, a previous study reported that gains in centromere numbers of chromosomes 5p, 7q and 19q, in addition to losses in the centromeres of chromosomes 4q, 9p and 15q, were common among patients with upper-tract urothelial carcinoma (UTUC) in end-stage renal disease (ESRD) (16). Notably, high copy number variants were also observed in high-stage and -grade tumors of UTUC from these findings (16). In another study, Yamamoto *et al* (17) reported that the gain of chromosomal region 5p15.33, which includes the tubulin polymerization promoting protein (*TPPP*) gene, was associated with the progression of BC. Consistent with this finding, a high genomic copy number of TPPP was also detected in the early stages of non-small-cell lung cancer through a high-resolution array CGH assay (18).

TPPP, also known as p25, was first identified as a brain-specific protein that is primarily expressed in oligodendrocytes and the neuropil (19). TPPP has been previously reported to induce the formation of aberrant microtubule assemblies and was considered to be a novel marker for Parkinson's disease and α-synucleinopathy (20). TPPP is a microtubule-associated protein (MAP) in vertebrates that polymerizes tubulin into normal or aberrant microtubules, depending on its concentration and phosphorylation state (21-23). It also serves as a scaffolding protein to deliver tubulin heterodimers to nascent protofilaments or block the formation of mitotic spindles (24,25). Amplification of TPPP may confer a growth advantage to urothelial cells resulting from abnormalities in tubulin assembly and spindle formation (17). By contrast, the expression of TPPP mRNA was reported to be significantly lower in hepatitis C virus-positive hepatocellular carcinoma tissues compared with that in adjacent non-cancerous tissues from patients with secondary metastatic liver malignancies, where low TPPP mRNA expression indicated a poor prognosis (26).

The present study investigated whether TPPP may represent a potential target for the evaluation of BC clinicopathology, serve as an index of risk assessment in patients with BC and explored the possible *in vitro* role of TPPP in cell proliferation, cell migration and invasion in BC using TPPP RNA interference.

Materials and methods

Tissue specimens. The frozen tissue samples were obtained from 52 patients with urothelial BC (sex, 44 men and 8 women; average age, 65.24±13.35 years; age range, 37-84 years) who received BC diagnoses at Chang Gung Memorial Hospital, Taiwan, between April 2004 and April 2015. The tissue specimens were frozen at -80˚C until use. Patients received transurethral resection of bladder tumor (TUR-BT) and were staged with either CT scan or MRI imaging study. All patients were diagnosed with urothelial carcinoma. All tissue samples were retrieved from Biobank and Tissue Bank Core Lab at Chang Gung Memorial Hospital. Written informed consent was obtained from all participants before surgical samples were deposited to Biobank and Tissue Bank Core Lab of Chang Gung Memorial Hospital. The study was approved by the Human Subject Research Ethics Committee/Institutional Review Board at Chang Gung Memorial Hospital at Linkou (approval no. IRB No.103-1999C; Taoyuan, Taiwan). Each case of urothelial BC was evaluated by examining the data in the medical records, including tumor size, pathological type, histological staging and grading, from the Department of Pathology at Chang Gung Memorial Hospital at Linkou. The pathological staging and grading were assigned according to the American Joint Committee on Cancer Cancer Staging Manual (8th edition) Clinical Practice Guidelines in Bladder Cancer (2017) (27). Patients who were diagnosed with urothelial BC and first received TUR-BT were included in this study. Patients who had known infections or other concurrent severe and/or uncontrolled medical diseases in this study were excluded. Pregnant or breastfeeding women were also excluded.

Cell culture. Four human BC epithelial cell lines, namely MGH-U1, MGH-U1R, MGH-U3, and MGH-U4, were provided by Dr Chi-Wei Lin (Massachusetts General Hospital, Boston, MA, USA). The characteristics of these four cell lines were reported in detail in a previous study (28). Briefly, MGH-U1 is the subline of T24 (29), which was established from a grade 3, stage B bladder tumor. MGH-U1R is a doxorubicin-resistant cell line that was derived from the MGH-U1 (30). The MGH-U3 cell line was established from a grade 1, stage A bladder tumor whereas the MGH-U4 cell line was established from a stage 0 bladder tumor with atypia (31). STR analysis was performed to demonstrate that the MGH-U1 was a subline of T24 (data not shown). The cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.). SV-HUC-1 cells, a normal human uroepithelial cell line and MC-SV-HUC T-2, a tumorigenic human urothelial cell line, were purchased from the American Type Culture Collection and cultured in Ham's F12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 7% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin and 2 mM L-glutamine. All cell lines were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

FISH assay. TPPP gene amplification was detected using dual-color FISH analysis as reported previously (32). For touch imprint cytology smears, a single 1.0x1.0x0.3 cm fresh tumor sample was cut and the surface touched against uncharged slides to cover at least 60% of the slide surface (33). Fresh slides were aged at room temperature for at least 24 h. BC cells or touch imprint cytology smears from the bladder tumor samples were then fixed with fixative solution (methanol: Acetic acid=3:1 v/v) at room temperature for 30 min. The fixed samples were preserved at -20˚C for subsequent examination.

For dual-color probe preparation, information in publicly available databases (https://asia.ensembl.org/index.html), and the BAC clone for the short arm of chromosome 5 from the Roswell Park Cancer Institute (RPCI) library RP11 (clone RP11-837K1, chr 5:640447-820395 (180 kb); Invitrogen; Thermo Fisher Scientific, Inc.), which contained the TPPP gene, was selected in this study. In preparing DNA probe by

nick-translation, the BACs were isolated using Presto™ Mini Plasmid Kit (cat. no. PDH300; Geneaid Biotech Ltd.) and labeled with a BIO-PROBE Nick translation DNA labeling system (cat. no. ENZ-42710; Enzo Life Sciences Inc.), dNTPs (10x A4 dNTP mix; Roche Diagnostics GmBH) and Green-dUTP fluorescent dye (cat. no. 02N32-050; Abbott Molecular Inc.) at 15˚C for 45 min. After nick translation, the DNase I was inactivated by heating at 75˚C for 15 min. The probes were then precipitated with 0.3 M sodium acetate and 70% ethanol at -20˚C for 40 min. The probes were dissolved in the hybridization solution [50% formamide, 2x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7), 10% dextran sulfate]. To perform DNA-FISH, fixed samples were treated with denaturation buffer (70% formamide, 2x SSC, pH 7) at 58˚C for 1 min, before samples were washed in PBS and dehydrated through an ethanol gradient (70, 85 and 100% for 2 min each). Samples were heated at 75˚C for 5 min to separate DNA strands before 12.5 ng/μl of probes in hybridization buffer were applied and incubated at 37˚C overnight. After hybridization, the samples were washed in wash buffer I (0.4x SSC, 0.3% Tween-20; 75˚C) and wash buffer II (2x SSC, 0.1% Tween-20; room temperature) for 2 min each three times and with 1 μg/ml DAPI at room temperature for 30 min (Sigma-Aldrich; Merck KGaA) for nuclear staining. In addition, the accuracy and specificity of the probe were confirmed through hybridization onto commercially available CGH Metaphase Target Slides (cat. no. 06J96-001; Abbott Pharmaceutical Co. Ltd.). The hybridization conditions were the same as for the samples. The results indicated that the probe was annealed with DNA sequences at 5p15.33 (data not shown). The fluorescent images were recorded at x630 magnification using a fluorescence microscope (Leica DM2500; Leica Microsystems GmbH) and analyzed using FISHView EXPO version 5.5 software (Applied Spectral Imaging, Ltd.). According to the definition of Yamamoto *et al* (17), the criterion for TPPP gain was defined as a mean copy number of TPPP per nucleus of >2.2 (cutoff).

Neutrophil-to-lymphocyte ratio determination. Before TUR-BT, lab experiments including hemogram and biochemistry exam were performed as part of routine clinical examination. Neutrophil count, lymphocyte count, red cell distribution width and mean platelet volume were obtained for each patient. The neutrophil-to-lymphocyte ratio (NLR) was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. The optimum cutoff value for the NLR was determined to be ≥2.43 after receiver operating characteristic analysis, following a previously described method (34).

Knockdown of TPPP expression. ON-TARGETplus SMARTpool siRNA of TPPP was synthesized by Dharmacon (cat. no. L-019695-01-0010; GE Healthcare Dharmacon, Inc.), where the following four siRNA sequences were designed: 5'-CCACCGGAAUCACCCGAUA-3', 5'-GGUUGGUGCCCACGAGUUA-3', 5'-CAAAGUGUCUCGCGGAUCA-3' and 5'-GACAAGCAGUCAUCGGAAU-3'. The siRNA scramble control was synthesized by Biotools Co., Ltd with the siRNA sequence 5'-UUCUCCGAACGUGUCACGUTT-3'. MGH-U1, MGH-U1R and MGH-U4 cells lines were transfected with either 30 pmol TPPP siRNAs or scramble control in medium without antibiotics using the Lipofectamine® RNAiMAX reagent (Thermo Fisher Scientific, Inc.) combined, according to the manufacturer's protocol. After transfection, the cells were used to the further experiments, such as cell proliferation, *in vitro* cell migration and western blotting in the indicated time point.

Protein extraction and western blotting analysis. The TPPP siRNA (0, 24, 48, 72, 96 h) treated BC cells (MGH-U1, -U1R, -U4) were lysed in ice-cold modified RIPA (mRIPA) lysis buffer (50 mM Tris-Cl, pH 7.8; 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and 0.5% NP-40; 0.1 ml per 10^6 cells, respectively) on ice for 20 min and 4x sample buffer (cat. no. 1610747; Bio-Rad Laboratories, Inc.) at 95˚C for 10 min. For western blotting, 20 μl of cell lysates were separated by 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF) followed by blocking in Tris-buffered saline with 0.05% Tween-20 (TBST) containing 5% non-fat milk for 1 h, immunoblotting in primary antibody for 2 h and secondary antibody for 1 h at room temperature. Blots were visualized using a Immobilon™ Western Chemiluminescent HRP Substrate (cat. no. WBKLS0500; Merck Millipore) and analyzed by UVP ChemStudio Plus with software VisionWorks LS 8.22 (Analytic Jena AG). The primary antibodies used were anti-TPPP (1:2,000; cat. no. ab92305; Abcam) and anti-α-tubulin (1:10,000; cat. no. MS-581; Thermo Fisher Scientific, Inc.). The secondary antibodies were horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:5,000; cat. no. AP132P; Chemicon International, Inc.) and HRP conjugated goat anti-mouse IgG (1:5,000; cat. no. 31430; Thermo Fisher Scientific, Inc.).

Cell viability measurement. BC cell viability was measured using a Cell Counting Kit-8 (CCK-8) cell viability assay (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Briefly, the cells were seeded into 96-well plates at a density of $5x10^3$ cells/well. After seeding for 24 h, the cells were transfected with TPPP siRNAs for 0, 24, 48, 72, and 96 h and incubated at 37˚C in a humidified atmosphere with 5% CO_2. Subsequently, the cells were incubated with CCK-8 solution (10 μl/well) for 1 h at 37˚C before the absorbance value at 450 nm was measured in each well using a microplate reader.

In vitro migration assay. BC cell (MGH-U1, -U1R, -U4) migration was assessed using Transwell assays (24-wells; pore size, 8-μm; Corning, Inc.). After knockdown of TPPP expression for 48 h, the trypsinized cells were first suspended in RPMI 1640 medium containing 1% FBS at a density of $1x10^5$ cells before being added to the upper chamber of the well. Subsequently, RPMI 1640 medium with 10% FBS was added to the lower chamber. After incubation for 6 h at 37˚C, cells that migrated to the lower surface of Transwell were stained with Giemsa stain solution (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, imaged under a light microscope (magnification, x200) and counted from nine randomly selected fields per well.

Statistical analysis. Statistical analyses were performed using the SPSS statistical software package (version 22.0; IBM Corp.). A contingency table was generated for Fisher's exact probability test. In the *in vitro* cell assays, data were

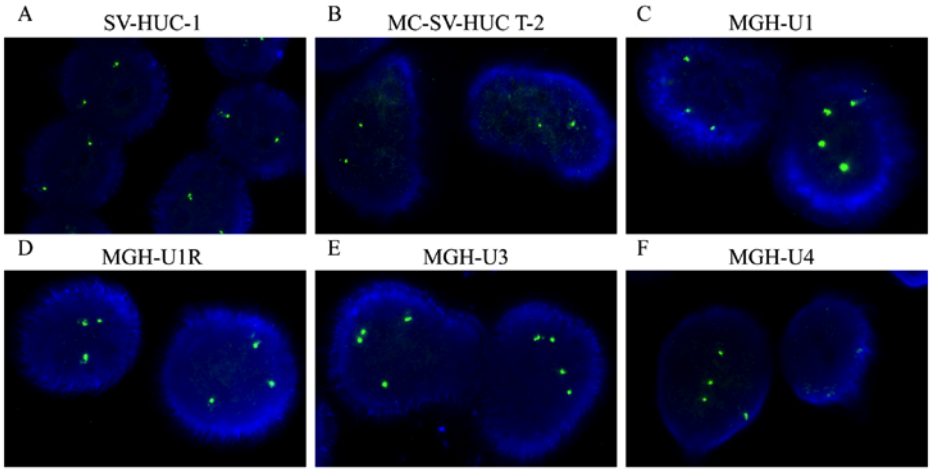

Figure 1. Comparison of TPPP copy numbers between the normal human uroepithelial and bladder cell lines, as assessed using fluorescence *in situ* hybridization. TPPP copy numbers were assessed in (A) SV-HUC-1, (B) MC-SV-HUC T-2, (C) MGH-U1, (D) MGH-U1R, (E) MGH-U3 and (F) MGH-U4 cells. Blue staining represents DAPI. Magnification, x630. TPPP, tubulin polymerization promoting protein.

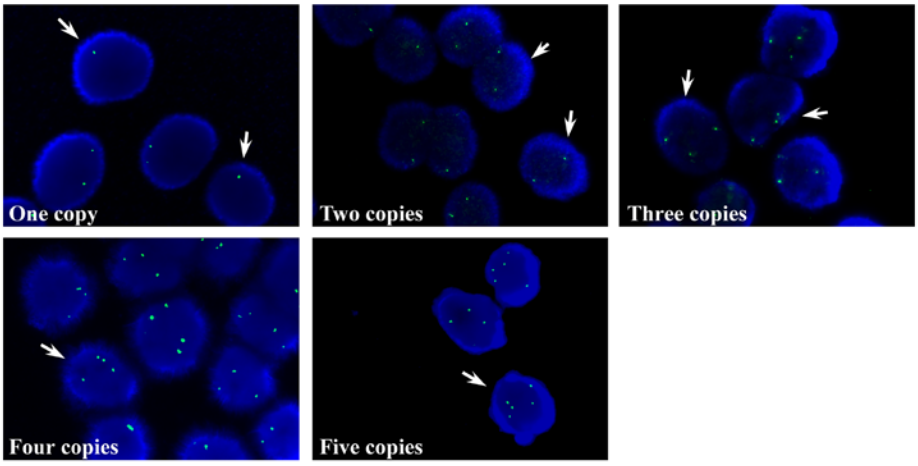

Figure 2. Representative images of cells with ≤5 copy numbers of TPPPP variants among touch imprint cytology smears obtained from patients with bladder cancer. Copy number variants of TPPP were detected by fluorescence *in situ* hybridization assay. Blue staining represents DAPI. Magnification, x630. TPPP, tubulin polymerization promoting protein.

expressed as the mean ± standard deviation from three independent measurements and compared using two-tailed unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Gain of TPPP copy number in BC. According to the criteria of TPPP gain from a previous definition (17), the copy number of TPPP variants was found to be amplified in the four BC cell lines tested, namely MGH-U1 (primarily three or four copies; Fig. 1C), MGH-U1R (three copies; Fig. 1D), MGH-U3 (three copies; Fig. 1E) and MGH-U4 (four copies; Fig. 1F) cells. However, TPPP downregulation occurred in the SV-HUC-1 (two copies; Fig. 1A) and MC-SV-HUC T-2 (two copies; Fig. 1B) cell lines. Following quantification, the percentage of cells carrying ≥2.2 copies of TPPP of the total number of cells counted ranged from 86.0-100.0% for the four BC cell lines, whilst the percentages of SV-HUC-1 and MC-SV-HUC T-2 cells exhibiting TPPP amplification were found to be only 9.0 and 3.0%, respectively (Table I). Subsequently, the copy numbers of TPPP in the tissue samples obtained from patients with BC tissues were examined via touch imprint cytology smears. Consistent with the data of the BC cell lines, the TPPP copy numbers in the tissue samples exhibited either one, two and ≥ three spots (Fig. 2). The percentages of patients identified with the respective TPPP copy numbers were as follows: i) 1.9% with one copy; ii) 55.8% with two copies; iii) 7.7% with three copies; iv) 26.9% with four copies; and v) 7.7% with five copies (Table II). FISH results demonstrated that copy number amplification of TPPP in four human BC cell lines were higher than in the two normal human uroepithelial cell lines.

Association between the gain of TPPP and clinicopathological characteristics of patients with BC. Analysis of BC samples with TPPP gain, defined as the number of TPPP per nucleus >2.2, indicated that TPPP gain was associated significantly with age (≥60 years; P<0.05), advanced histological grade (P<0.001) and tumor stage (P<0.05), histological subtype (papillary vs. non-papillary urothelial carcinoma; P<0.001)

Table I. Genetic copy number data of TPPP in normal human uroepithelial and bladder cancer cell lines.

		Percentage of the alterations in TPPP copy number		
Cell lines	Cell number count	Normal (%)	One copy deletion (%)	Amplification (%)
SV-HUC-1	432	89.0	2.0	9.0
MC-SV-HUC T-2	379	96.0	1.0	3.0
MGH-U1	372	11.0	3.0	86.0
MGH-U1R	438	5.0	0.2	94.8
MGH-U3	386	0	0	100.0
MGH-U4	452	8.0	1.0	91.0

TPPP, tubulin polymerization promoting protein.

Table II. Statistics of the 52 patients with bladder with various copy numbers of tubulin polymerization promoting protein.

			Amplification (copy)		
Copy no.	Normal	One copy deletion	3	4	5
Patients, n (%)	29 (55.8%)	1 (1.9%)	4 (7.7%)	14 (26.9%)	4 (7.7%)

Table III. Association between the gain of TPPP and clinicopathological characteristics of patients with bladder cancer.

	TPPP gain[a]		
Category	-	+	P-value[b]
Sex			
Male (n=44)	27	17	0.260
Female (n=8)	3	5	
Age			
≥60 years (n=38)	18	20	<0.050
<60 years (n=14)	12	2	
Tumor grade			
High grade (n=33)	11	22	<0.001
Low grade (n=19)	19	0	
Tumor stage			
0a-I (n=31)	22	9	<0.050
II-IV (n=21)	8	13	
Tumor size[c]			
≥4 cm (n=14)	8	6	1
<4 cm (n=31)	17	14	
Histological subtype			
Papillary urothelial carcinoma (n=22)	19	3	<0.001
Non-papillary urothelial carcinoma (n=30)	11	19	
Neutrophil-to-lymphocyte ratio[c]			
≥2.43 (n=26)	11	15	<0.05
<2.43 (n=23)	17	6	

[a]For the definition of TPPP gain, the criteria was previously published by Yamamoto *et al* (17). [b]Analyzed using Fisher's exact probability test. [c]Missing data: 7 patients no tumor size records; 3 patients no neutrophil-to-lymphocyte ratio. TPPP, tubulin polymerization promoting protein.

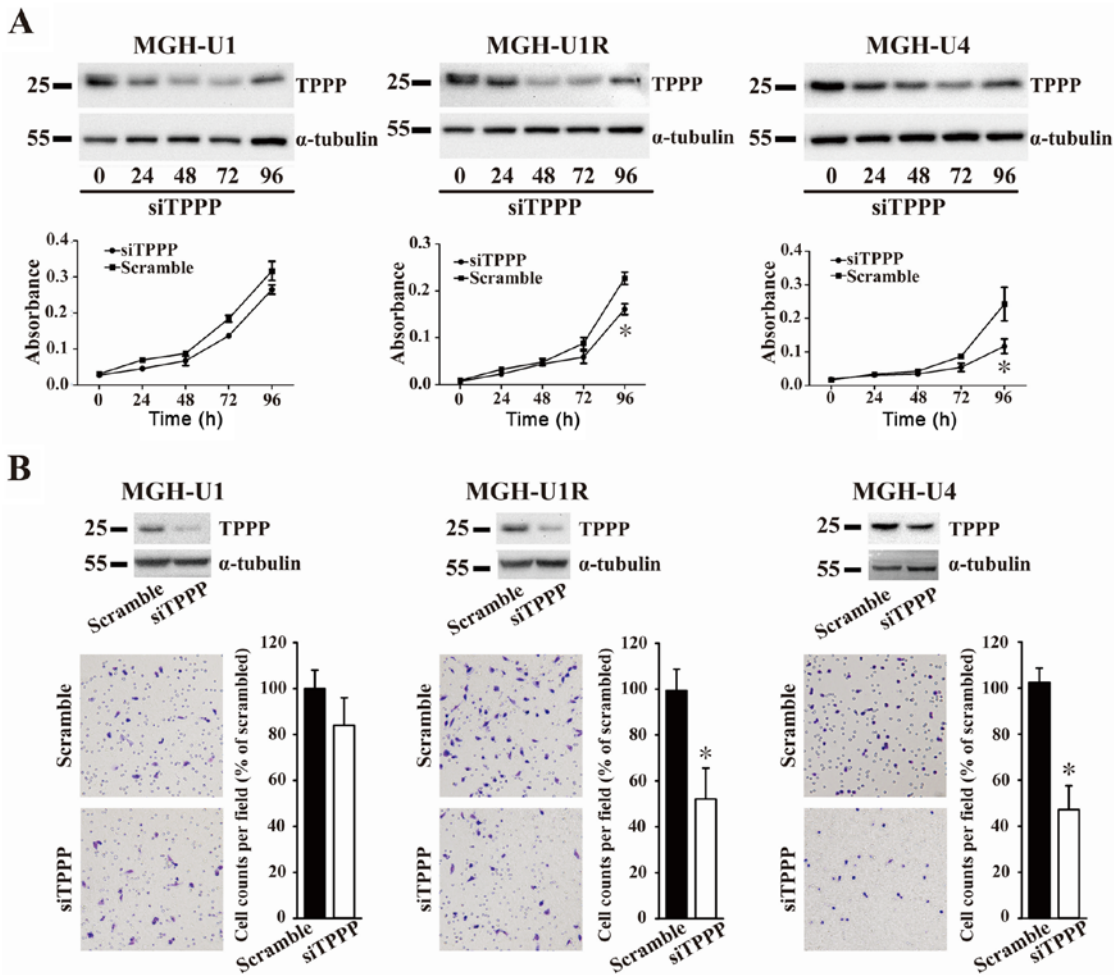

Figure 3. Knocking down TPPP expression suppresses MGH-U1R and MGH-U4 cell proliferation and migration. (A) Western blotting data of BC cells that were transfected with either the TPPP siRNA or scramble control for 0, 24, 48, 72 and 96 h. Cell Counting Kit-8 was used to measure the cell proliferation rates of the BC cells. (B) Western blotting data of BC cells that were transfected with either the TPPP siRNA or scramble control for 72 h. To measure cell migration, cells were seeded into Transwell chambers for 6 h before being stained and imaged. Magnification, x200. Data are presented as the mean ± standard deviation. *P<0.05 vs. Scramble. TPPP, tubulin polymerization promoting protein; BC, bladder cancer; siRNA, small interfering RNA.

and NLR (P<0.05). However, no association was revealed between TPPP gain and sex or tumor size (Table III). These results suggested that the copy number amplification of TPPP may be associated with BC progression. FISH results indicated that 34.6% BC patients had >4 copies of the TPPP gene.

Knockdown of TPPP reduces cell proliferation and suppresses migration in MGH-U1R and MGH-U4 cells. The effects of TPPP on cell viability and migration were next determined using CCK-8 and Transwell migration assays. The expression of TPPP in the MGH-U1, MGH-U1R, and MGH-U4 cells was measured after siTPPP transfection (Fig. 3A). The results showed that the expression of TPPP was reduced by TPPP siRNA compared with a scrambled sequence at 24, 48 and 72 h, while the expression of TPPP was recovered at 96 h. At 96 h, the growth rates of the MGH-U1R and MGH-U4 cells after TPPP knockdown were significantly lower compared with those in the scramble control groups. However, the growth rates of the MGH-U1 cells did not differ between those transfected with siTPPP or scrambled control. The role of TPPP in BC cell migration was subsequently examined. The degree of cell migration of cells following TPPP knockdown was revealed to be significantly lower compared with that in the scramble control groups in the MGH-U1R and MGH-U4 cell lines 72 h after siTPPP transfection (Fig. 3B). The results of the present study suggested that TPPP knockdown reduced the levels of cell proliferation and migration in BC cell lines.

Discussion

A multicolor FISH-based urine assay has been developed for BC screening in clinical practice, which has been documented to confer higher sensitivity compared with urine cytology (35). Previous studies have revealed that copy number alterations detected via FISH assays can aid in the identification and localization of genes associated with cancer (32,36,37). Initially, the present study demonstrated that the occurrence of TPPP copy number amplification in the four human BC epithelial cell lines was higher compared with that in the two normal human uroepithelial cell lines tested. In addition, the occurrence of copy number amplification of TPPP was also demonstrated to be associated with age, histological type, NLR, advanced histological grade and pathological stage of

the patients with BC. FISH analysis subsequently revealed that tissue samples obtained from 34.6% of the patients with BC contained ≥ four copies of the TPPP gene.

The present study confirmed that the copy number of TPPP was higher in BC cells compared with that in normal uroepithelial cells. Accumulating evidence has highlighted the role of inflammation as a critical component in processes associated with tumor progression, including angiogenesis, cell proliferation and metastasis (38,39). Neutrophil and lymphocyte counts serve crucial roles in systemic inflammation. The neutrophil count increases in response to tumor growth and proangiogenic factors, regulated by the upregulation of mediators such as NF-κB and vascular endothelial growth factor (40-42). Therefore, a high NLR has been considered to be a potential prognostic factor in various cancer types, including pancreatic cancer, gastric cancer and BC (34,42,43). Previous studies have indicated that preoperative NLR served a vital role in predicting the recurrence of high-grade bladder tumors consisting of the superficial transitional cell type and in distinguishing between MIBC and NMIBC (34,37). The present study indicated that gain of TPPP copy numbers is associated with higher NLR in patients with BC. Therefore, the potential interaction between TPPP and NLR in the pathogenesis of BC warrants further future study.

Transitional cell carcinomas (TCCs) represent >90% of all BCs, where most BCs exhibit papillary features, particularly in the noninvasive types (44). Polesel *et al* (45) previously reported that 57.7% non-papillary TCCs were muscle invasive, whilst 85.4% papillary TCCs were low-grade superficial tumors of BC. The results of the present study indicated that 62% non-papillary TCCs were muscle invasive, while 91% papillary TCCs were low-grade tumors. Results from the present study were similar to those reported by Polesel *et al* Low-grade papillary urothelial carcinoma is characterized by an orderly overall appearance, minimal variability in the cellular architecture and the lack of significant atypia in terms of cytological and mitotic activity (44). Additionally, the present study revealed that the gain of TPPP copy numbers was strongly associated with non-papillary urothelial carcinomas, suggesting that amplification of TPPP promoted the aberrance of tubulin assemblies in BCs.

Chromosomal locus 5p15.33 amplification has been previously reported to be a predictor for disease progression in BC (17). Yamamoto *et al* (17) examined the number of aberrations in the 5p15.33 locus via CGH array and FISH, but did not directly focus on the evaluation of TPPP genetic variants (17). Notably, the present study demonstrated that in patients with BC, the gain of TPPP occurrence associated strongly with histological type, though this association was not observed in the report by Yamamoto *et al* (17) Furthermore, another previous study also revealed that gains at the chromosome loci 5p, 7 and 19q and losses at loci 4q, 9p and 15q were prevalent in UTUC samples of patients with ESRD (16). Therefore, genetic alterations are likely to serve an important role in the development of urothelial carcinoma from the observations of the present study.

Metastasis is the cause of the majority of mortalities associated with cancer (46). Microtubules regulate cell shape maintenance, cell migration and cell division via the activity of MAPs (47). In addition, agents targeting the microtubule system have been identified to confer among the most effective anticancer effects, which have also been recognized to exhibit potent anti-mitotic and anti-proliferative properties (48). As TPPP is a MAP, the present study demonstrated that knockdown of TPPP attenuated cell migration and proliferation in MGH-U1R and MGH-U4 cells, which may have resulted from disrupted microtubule dynamics. However, previous studies have reported that TPPP overexpression also reduced cell proliferation and migration in U2SO osteosarcoma cells (49,50). Downregulation of LIM-kinase 2 has been reported to sensitize neuroblastoma cell lines BE(2)-C and SHEP, to chemotherapeutic drugs, enhancing mitotic arrest and cell apoptosis via reducing TPPP and acetylated-tubulin expression levels (51). Chen *et al* (52) demonstrated that TPPP promoted the migration, invasion and angiogenesis via the p38/MAPK and PI3K/AKT signaling pathways in pancreatic cancer. Although results from the present study also supported these aforementioned findings (data not shown), the underlying mechanisms of TPPP in the regulation of BC metastasis require further exploration.

In summary, the present study highlighted the crucial role of TPPP in cell viability, cell migration and BC progression, suggesting TPPP to be a novel therapeutic target for BC treatment. In addition, it emphasized the possibility of expanding preoperative urinary cytology, the current gold standard, to the clinical examination of TPPP copy numbers for the detection of urothelial neoplasia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YHC and STP provided the initial concept and arranged the study design. PHL and CCC executed the major experiments and wrote the majority of the manuscript. WHW made substantial intellectual contributions to the study design and technical support. KJY, CYL and CKC performed clinical specimen collection and clinicopathological analysis. CHH performed western blot assay. THC, IHS and HCK performed data analysis and figure preparation. CKC and STP edited the final manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Human Subject Research Ethics Committee/Institutional Review Board at Chang Gung Memorial Hospital at Linkou (approval no. IRB No.103-1999C; Taoyuan, Taiwan). All tissue samples were retrieved from Biobank and Tissue Bank Core Lab at Chang Gung Memorial Hospital. Written informed consent was obtained from all participants before surgical samples were deposited to Biobank and Tissue Bank Core Lab of Chang Gung Memorial Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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