Thymidine phosphorylase promotes metastasis and serves as a marker of poor prognosis in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) ranks as one of the most common and lethal malignancies worldwide. A better understanding of the mechanism responsible for HCC metastasis will be helpful for the treatment of HCC patients. Thymidine phosphorylase (TP), a key enzyme that catalyzes the conversion of thymidine to thymine and deoxyribose-1-phosphate, was demonstrated to promote the invasion and metastasis of HCC in our study. Clinical retrospective analysis revealed that metastatic HCC tumor tissues have higher TP expression, and TP expression was significantly correlated with matrix metalloproteinase (MMP) 2 and 9 expression. Survival analysis revealed that TP expression was negatively correlated with the prognosis of HCC patients. Moreover, *in vitro* cell experiments confirmed that TP could promote the migration and invasion of HCC cells. In addition, MMP2 and MMP9 were activated by TP overexpression. Overall, this study suggests that TP promotes metastasis and may serve as a marker of poor prognosis in HCC. Thus, TP is a potential target for the treatment of HCC.

Hepatocellular carcinoma (HCC) ranks as one of the top causes of cancer-related deaths worldwide and has a poor prognosis.[1,2] Metastasis of HCC is one of the top reasons for the death of HCC patients.[3] Thymidine phosphorylase (TP), an enzyme that catalyzes the reversible phosphorolysis of thymidine and other pyrimidine-2′-deoxyribonucleosides, is more highly expressed in colorectal, pancreatic, breast, lung, and liver cancers than in adjacent non-neoplastic tissues.[4–7] Also, TP plays an important role in tumor angiogenesis, apoptosis, and proliferation.[8,9] Although TP was shown to be associated with the metastasis of some tumors such as gastric and colorectal cancers, whether TP could promote the metastasis of HCC remains unclear; this subject therefore requires further exploration.[10,11]

In this study, specimens from 75 cases of clinical HCC were used for clinicopathologic analysis. Results revealed that TP expression was correlated with tumor size, pathologic grading, and metastasis and high TP expression meant a poor prognosis. An *in vitro* study further confirmed that TP

promoted HCC cell invasion and migration. Additionally, TP was able to promote the expression and activity of MMP2 and MMP9. As both TP and MMPs are important components of the metastasis cascade, we hypothesized that TP depends on MMP2 and MMP9 to promote the metastasis of HCC.[10,12]

This study reveals the relationship between TP and metastasis, and prognosis of HCC for the first time. Our findings will provide a basis for the treatment of HCC by targeting TP and taking TP as a prognostic marker.

MATERIALS AND METHODS
Patient Selection and Clinical Data
An HCC tissue microarray that contains 75 cases was obtained from US Biomax for IHC. Tissue blocks for creating a tissue microarray were collected within 5 years. Each single tissue spot on every array slide was individually examined by pathologists and certified according to standards of diagnosis, classification, and pathological grade published by the World Health Organization. Each specimen collected from any clinic

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Table 1 Correlation between TP and primary tumor characteristics

Variant	TP		χ^2	P-value
	Low (n)	High (n)		
Gender				
Male	6	8	0.127	0.721
Female	23	38		
Age (years)				
≤ 50	16	25	0.005	0.944
> 50	13	21		
Tumor size (cm)				
D ≤ 5	27	15	26.417	< **0.001**[**]
D > 5	2	31		
Stage				
< III	27	14	28.187	< **0.001**[**]
≥ III	2	32		
Grade				
≤ II	23	31	1.253	0.263
> II	6	15		
Metastasis				
Negative	27	37	9.45	**0.002**[**]
Positive	2	9		
Family history				
No	25	34	1.602	0.206
Yes	4	12		
Wine drinking				
No	13	24	0.384	0.535
Yes	16	22		
Smoking				
No	16	26	0.013	0.909
Yes	13	20		
Cirrhosis				
No	20	27	0.802	0.371
Yes	9	19		

Table 1 Continued

Variant	TP		χ^2	P-value
	Low (n)	High (n)		
AFP (µg/l)				
≤ 200	16	19	1.374	0.241
> 200	13	27		
ALT (U/l)				
< 40	13	12	2.811	0.094
≥ 40	16	34		

Grade means the histological grade, and stage means the clinical stage. Bold entries statistically significant. $**P < 0.01$.

was consented to by both the hospital and the individual. All tissues were collected under the highest ethical standards with the donor being informed completely and with their consent. All human tissues were collected under HIPAA-approved protocols. In this study, the age of patients ranged from 17 to 79 years. The patients consisted of 14 females and 61 males, and the survival time was between 1 and 116 months.

Cell Culture and Transfection

Human HCC cell lines HepG2 and PLC-PRF-5 were purchased from KeyGen Biotech (Nanjing, China). Cells were separately cultured with DMEM and RPMI-1640 medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and penicillin (50 µg/ml)/streptomycin (50 µg/ml). All cultures were maintained at 37 °C in a humidified atmosphere that contained 5% CO_2. When cells reached a density of ~ 80%, the overexpression and interference plasmids of M02-TYMP and psiU6-TYMP purchased from GeneCopoeia (Guangzhou, China) were transfected into cells according to the instructions of the Roche transfection reagent (Roche, Switzerland).

Western Blot Analysis

Immunoblots were performed with samples that contain total protein (30 µg) through sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membrane was probed with primary antibody of TP (1:1000; Abcam) or MAPK2, extracellular signal-regulated kinase (Erk2), GAPDH purchased from Affinity Bioreagents (Colorado, USA), followed by incubation with a second antibody (1:2000; Santa Cruz Biotechnology). Blots were detected by using an enhanced chemiluminescence detection kit (Millipore). Densitometric analysis was performed using the ImageJ software.

Wound Healing Assay

The transfected cells were seeded into 24-well culture plates at a density of 5×10^5 cells per well. When the cells adhered to the plate, a 200 μl pipette tip was used to scratch the wound and the cells were incubated with serum-free medium. Images of the wounds were taken at the time of 0, 24, and 48 h. Each experiment was repeated in triplicate, and the mean values (mean ± s.d.) were presented.

Matrigel Invasion Assay

Cell invasion assay was performed using Transwell cell culture inserts (Corning, USA) and Matrigel (BD, USA). The transfected cells were cultured for 48 h and allowed to invade for another 24 h. The passed cells were fixed in 4% paraformaldehyde, stained with crystal violet solution, and counted with a light microscope (Nikon, Japan).

Gelatin Zymography Assay

The transfected cells were grown in 12-well plates and cultured in serum-free medium at a density of 1×10^6 cells per well for 24 h. The conditioned medium was harvested and subjected to 10% SDS-polyacrylamide gel electrophoresis that contained 0.1% gelatin. The gel was soaked in 2.5% Triton X-100 for 40 min at room temperature and incubated in
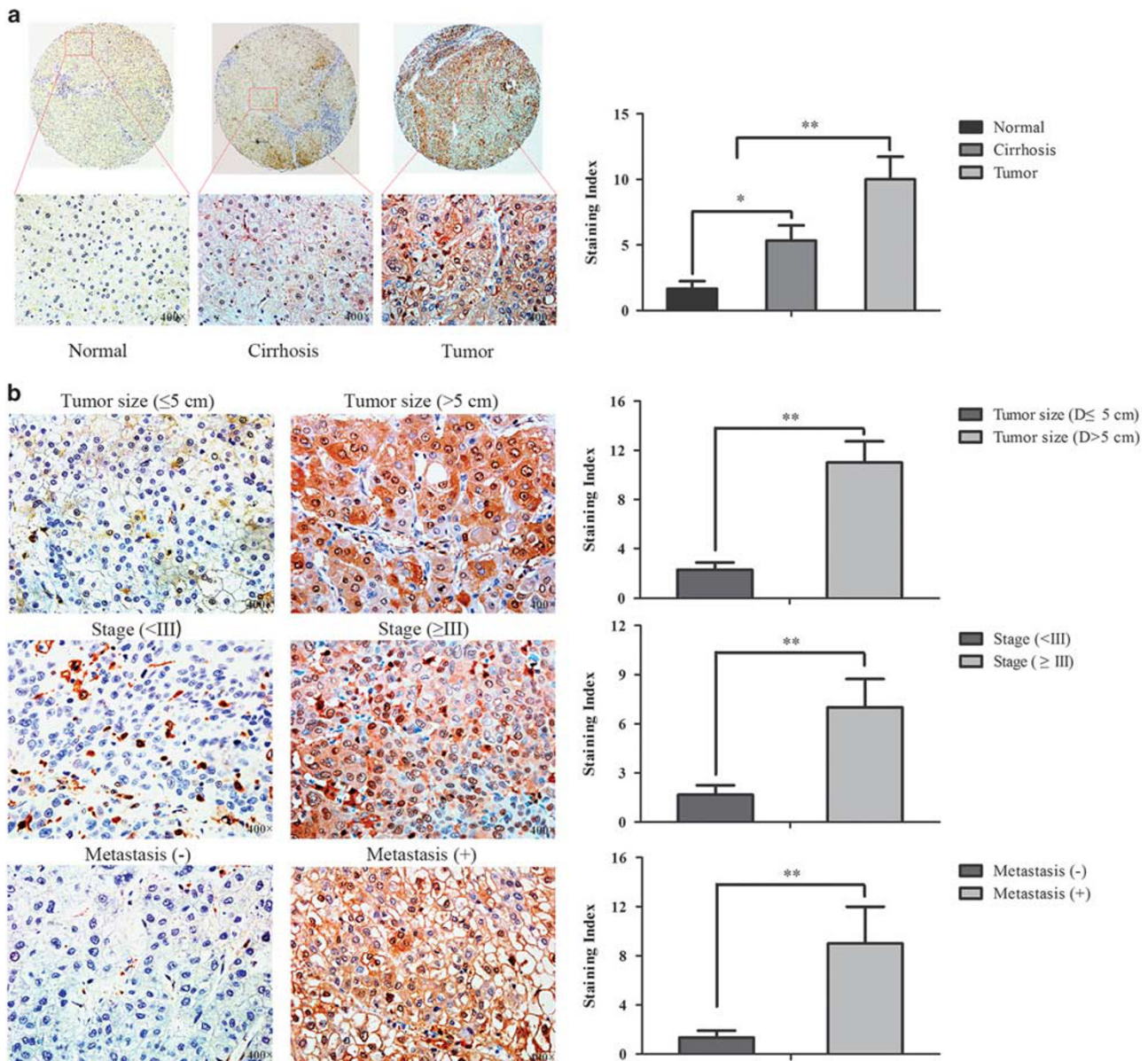

Figure 1 Correlation of TP expression with HCC tumor characteristics in clinical samples. (**a**) TP staining was negative, weakly positive, and strongly positive in normal, cirrhosis, and tumor tissues, respectively. (**b**) The first column represents staining of negative expression of TP in liver cancers of stage (<III), grade (≤II), and metastasis (−), while the second column indicates that tumors with stage (≥III), grade (>II), and metastasis highly express TP. Images were taken at ×400 magnification. (*$P < 0.05$, **$P < 0.01$). HCC, hepatocellular carcinoma; TP, thymidine phosphorylase.

substrate buffer (50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.02% NaN_3) for 48 h at 37 °C. The gel was stained with Coomassie R250 and destained in water with 10% acetic acid and 30% methanol until the apparent cleared zones appeared associated with MMP activity. The zones were quantified with the ImageJ software.

MMP Activity Assays

The MMP activity assay kit was purchased from Abcam. The collecting medium was incubated with APMA working solution for 10–15 min at room temperature. Then, an equal volume of MMP Green Substrate solution was added to the samples for another 1 h incubation at 37 °C. Fluorescence detection was mediated by the Microplate Reader (Thermo Scientific) at wavelengths of 490 and 525 nm.

Reporter Gene Assays

MMP2 and MMP9 reporter gene plasmids were purchased from GeneCopoeia. After cotransfection, the cells were incubated for another 48 h. Fluorescence detection was performed according to the Dual-Luciferase Assays System (Promega) by using the Luminoskan Ascent reader system (Thermo Scientific).

Animal Studies

Sixteen BALB/c nu/nu mice that were 5–6 weeks old, with an equal ratio of male and female, were divided randomly into four groups. Xenografts of tumors were established by subcutaneous injection of 1×10^7 cells of HepG2 (C/psiU6-TYMP) or PLC-PRF-5 (C/M02-TYMP) in different groups into the flank. The mice were maintained in a specific pathogen-free animal care facility according to institutional

guidelines. On the 10th day after tumor inoculation, body weights and tumor diameters were measured every 2 days, and tumor volumes were calculated according to the formula $V = ab^2/2$ (a = length, b = width). Four weeks after treatment, all mice were killed, and xenografts and lungs were collected. Lung tissues were harvested for histologic examination to measure the metastasis, and the xenografts were further analyzed by IHC.

Immunohistochemical Analysis

Tumor tissues from mice were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 4 μm thick slices, and placed on slides. IHC staining was used to verify the expression of different proteins in the HCC tissue microarray and the slides. In the progress, tissues were deparaffinized in xylene prior and then rehydrated by gradient alcohol. Then, 3% hydrogen peroxide in methanol was used to block the endogenous peroxidase activity for 30 min. Then the chips were soaked in 0.01 M citric acid buffer for 10 min at 95 °C in a microwave oven. After blocking with 10% normal goat serum for 20 min, the chips were incubated with primary antibodies for 30 min at room temperature and then 4° C overnight. Antibody dilutions were as follows: mouse monoclonal anti-TP (Abcam, dilution 1:100); rabbit polyclonal anti-MMP2 (Abcam, 1:200), and rabbit polyclonal anti-MMP9 (Abcam, 1:100). The next morning, chips were washed with phosphate buffered solution three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG for 1 h at

Table 2 Cox proportional hazards analysis of the effects of different factors on overall survival rates

Factor	Cox proportional hazards analysis		
	Relative risk	95% CI	*P*-value
TP	2.557	1.288–5.074	**0.007**[**]
Gender	0.707	0.234–2.139	0.539
Age	1.002	0.978–1.028	0.852
Tumor size (cm)	2.332	1.288–4.220	**0.005**[**]
Clinical stage (I:II:III:IV)	1.850	1.135–3.017	**0.014**[*]
Grade	1.392	0.922–2.101	0.116
Metastasis	3.078	1.673–5.661	<**0.001**[**]
Family history	0.764	0.387–1.509	0.439
Wine drinking	1.172	0.653–2.104	0.595
Smoking	1.170	0.647–2.115	0.603
Cirrhosis	1.011	0.553–1.848	0.972
AFP (μg/l)	0.872	0.484–1.571	0.648
ALT (U/l)	0.839	0.440–1.600	0.595

Abbreviation: CI, confidence interval. Bold entries statistically significant. *$P < 0.05$, **$P < 0.01$.
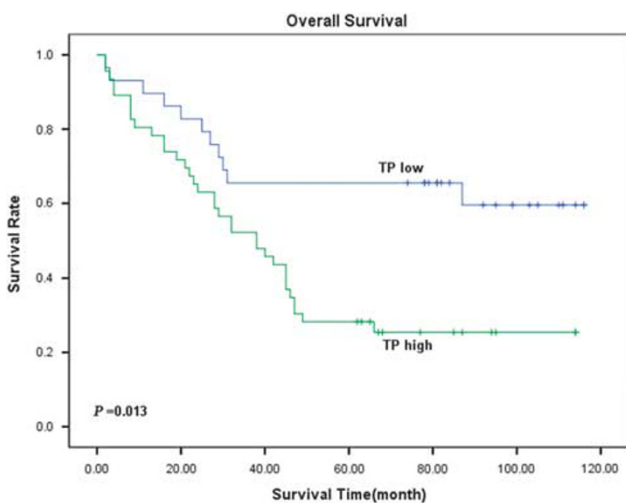

Figure 2 Overall survival analysis for TP expression. Kaplan–Meier curves for overall survival in 28 patients with high TP expression and 47 patients with low TP expression. Survival rate sharply decreased in patients with high TP expression in the first 4 years when significantly compared with those expressing low TP ($P = 0.013$). TP, thymidine phosphorylase.

room temperature. 3, 3′-diaminobenzidine was used for staining. Chips were then counterstained with haematoxylin, dehydrated, and mounted.

Scoring and Statistical Analysis

Immunohistochemical stains were scored based on positive degree and positive rate. The degree was divided into four classes: none for 0, weak brown for 1, moderate brown for 2, and strong brown for 3. The positive rate includes four classes: 0–25% for 1, 25–50% for 2, 50–75% for 3, and 75–100% for 4. Multiplication of the degree and rate scores was utilized as the staining index to judge the results. A staining index >6 was the value of a high-expression immunohistochemical assay.

We tested the relation between TP, MMP2, and MMP9 expression, and clinic pathological parameters using Pearson χ^2-test. Overall survival analyses were conducted using the Kaplan–Meier method with the log-rank test. P-values <0.05 were deemed statistically significant. All statistical analyses were performed using IBM SPSS Statistics 22.0 software (Chicago, IL, USA).

Proteomics

The control and M02-TYMP transfected PLC-PRF-5 cell lines were incubated for 48 h. Proteins were digested with trypsin for 20 h at 37 °C. The sample was evaporated and resuspended in water/0.1% formic acid for LC-MS/MS analysis. The database search was performed using Trans-Proteomic Pipeline software, and the results were imported into Microsoft Excel for further DAVID analysis.

Results

Characteristics of Patients and TP Expression in HCC

As shown in Table 1, characteristics of the HCC in the microarray were presented. A total of 75 patients consisted of 14 (18.7%) men and 61 (81.3%) women with ages from 17 to 79 years (median, 50 years). Forty-one (54.7%) patients were diagnosed with lower than stage III HCC, and 34 (45.3%) patients were diagnosed with higher than stage III HCC. Fifty-four (72.0%) patients had lower than grade II HCC, and 21 (28.0%) patients had higher than grade II HCC. Eleven (14.7%) patients had lymph and/or distant metastasis, and 64 (85.3%) patients had no metastasis. In the 75 patients,
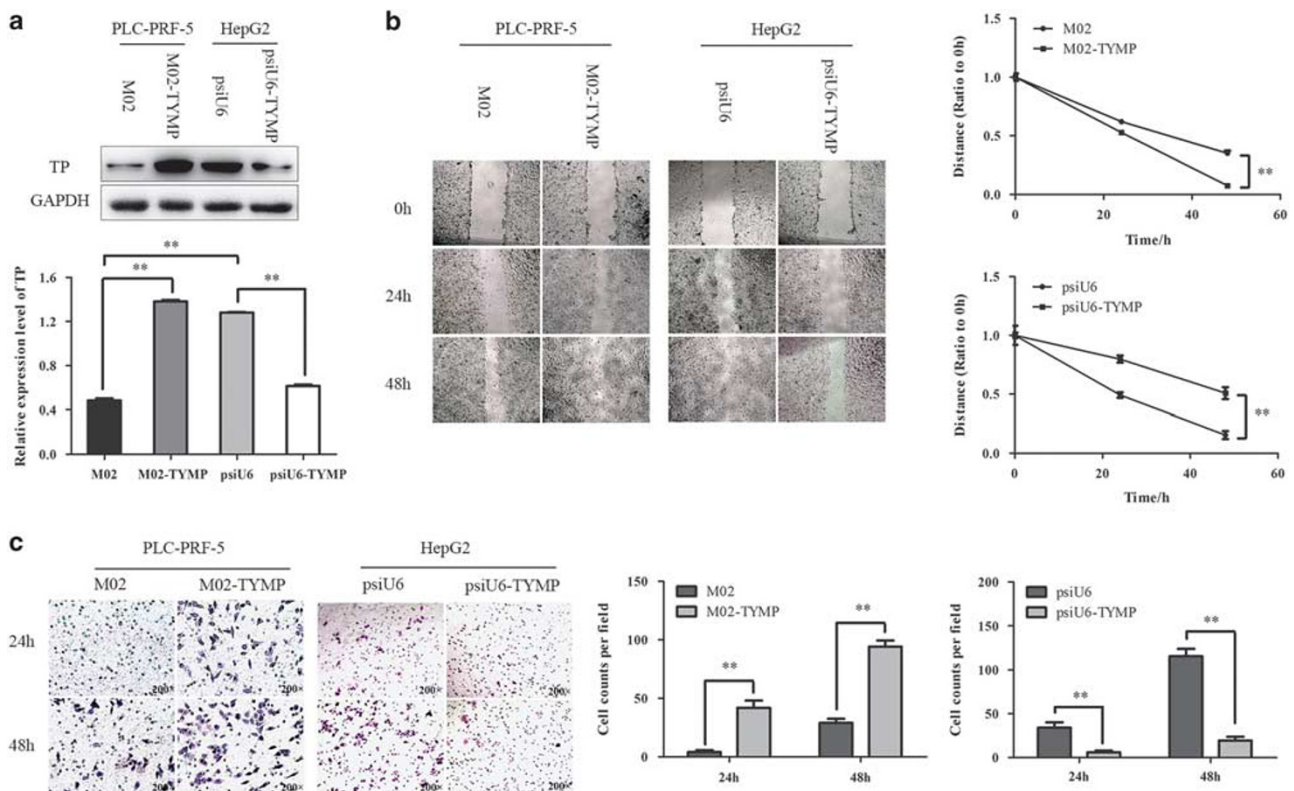

Figure 3 Effects of TP expression on cell migration and invasion. (**a**) PLC-PRF-5 and HepG2 cells were overexpressed or knocked down TP with plasmids of M02-TYMP or psiU6-TYMP, separately. After 48 h of transfection, the proteins were examined by western blot. (**b**) Following the wound healing assay, quantitative analysis showed a significant difference in the speed of wound healing among the TP overexpression or knockingdown groups compared with the negative control ($n = 3$). (**c**) Cell invasion assay was performed using Transwell filters; when TP was overexpressed, the passed cells increased nearly three-fold compared with the control group in PLC-PRF-5; in the interference group of HepG2, knockingdown of TP significantly reduced the invasion ($n = 3$). Images were taken at $\times 200$ magnification. Results show the means of the three experiments, and the error bars represent s.d. (**$P < 0.01$). TP, thymidine phosphorylase.
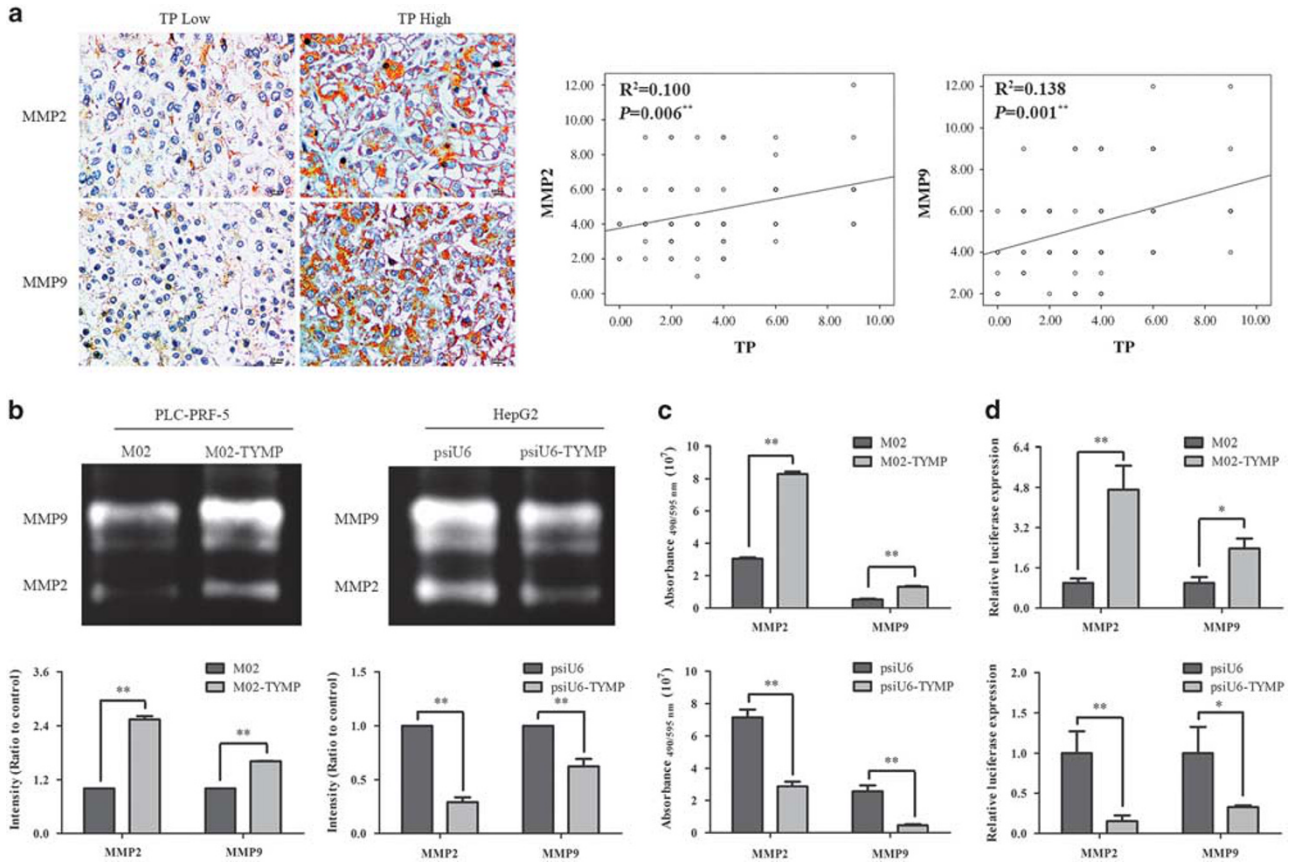
Figure 4 TP promoted the expression and activity of MMP2 and MMP9. (**a**) Clinical samples with high TP expression showed strong positive staining of MMP2 ($P = 0.006$) and MMP9 ($P = 0.001$). (**b** and **c**) The activities of MMP2 and MMP9 were detected by gelatin zymography assay and activity assay. Both MMP2 and MMP9 were significantly higher in the TP overexpression group in the PLC-PRF-5 cell line compared with the control group. Similarly, in the interference group, the activity of MMP2 and MMP9 was significantly decreased when TP was knocked down. (**d**) Reporter gene assay results for PLC-PRF-5 and HepG2 cells suggested that TP promoted the transcriptional activity of MMP2 and MMP9. Images were taken at × 200 magnification. Results show the means of the three experiments, and the error bars represent s.d. (*$P < 0.05$, **$P < 0.01$). MMP, matrix metalloproteinase; TP, thymidine phosphorylase.

16 (21.3%) patients had a family history of the disease, 38 (50.7%) patients had a history of wine consumption, 33 (44.0%) patients had a history of smoking, 28 (37.3%) patients had liver cirrhosis, 40 (53.3%) patients had an AFP higher than 200 µg/l, and 50 (66.7%) patients had an ALT higher than 40 U/l.

By IHC staining of TP, 29 (38.7%) patients were diagnosed as low expression, while 46 (61.3%) patients had high expression.

Association of TP Expression with Tumor Characteristics
To evaluate the clinical significance of TP in HCC, the TP expression in HCC tissues of the microarray was compared on the basis of 12 characteristics, including gender, age, and tumor size (Table 1). TP expression was significantly increased in HCC and cirrhotic liver tissues than in normal liver tissues (Figure 1a). TP was highly expressed in primary tumors with lymph and/or distant metastasis, and it had a significant correlation with metastasis ($P = 0.002$)

(Table 1; Figure 1b). Similarly, significant associations among the TP expression and tumor size ($P < 0.001$) and stage ($P < 0.001$) were observed (Table 1; Figure 1b). However, high TP expression had no significant relationship with gender, age, grade, family history, wine consumption, smoking, cirrhosis, and AFP and ALT contents (all $P > 0.05$, Table 1).

Association between Survival Status and TP Expression
To investigate the correlation between TP expression and survival time, overall survival analysis and Cox proportional hazards analysis were used. The median survival time was 45 months (ranging from 1 to 116 months). Overall survival analysis revealed that patients who suffered from high TP expression had a significantly lower survival time compared with those with a low TP expression ($P = 0.013$, Figure 2). The Cox proportional hazards analysis revealed that the TP expression, tumor size, clinical stage, and metastasis were significantly correlated with survival time in HCC patients
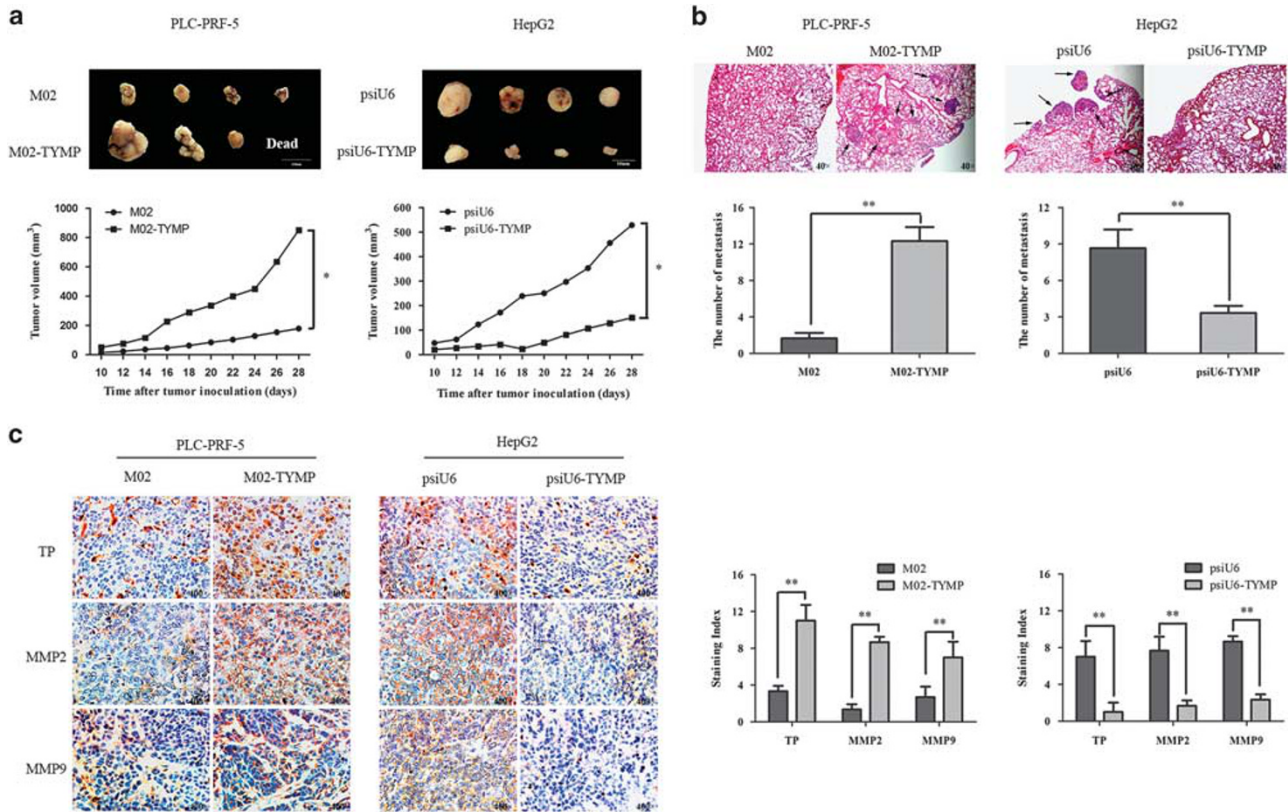
Figure 5 Effects of TP in HCC metastasis *in vivo*. (**a**) Overexpression of TP promoted PLC-PRF-5 xenograft growth, and knockingdown of TP inhibited HepG2 xenografts growth. (**b**) The numbers of tumor formation of lung metastasis in different groups, images were taken at ×40 magnification. (**c**) TP, MMP2, and MMP9 expression in xenografts; images were taken at ×400 magnification. Results show the means of the three experiments, and the error bars represent s.d. (*$P < 0.05$, **$P < 0.01$). HCC, hepatocellular carcinoma; MMP, matrix metalloproteinase; TP, thymidine phosphorylase.

with relative risk coefficients of 2.557, 2.332, 1.850, and 3.078, respectively (Table 2).

Promotion of Migration and Invasion of HCC Cells *In vitro* by TP

In this study, we selected PLC-PRF-5 cell lines that expressed low levels of TP and HepG2 cell lines with high TP expression as the pairing model to study the relationship between TP expression and HCC migration and invasion. The TP expression upregulated through transfection with the plasmid of M02-TYMP. TP was downregulated through transfection with the plasmid of psiU6-TYMP (Figure 3a). Wound healing assay was performed to verify the relation of TP with cell migration, and results showed that after 24 and 48 h treatment, the scratch wounds were thinner in the high TP expression groups (Figure 3b). In the Matrigel invasion assay, compared with the control groups after incubation of 24 and 48 h, the number of the cells that passed through the chamber significantly increased when TP was overexpressed in PLC-PRF-5 cells and significantly decreased when TP was knocked down in HepG2 cell lines (Figure 3c). PLC-PRF-5 cell lines were transfected with psiU6-TYMP and the HepG2 cell lines were transfected with M02-TYMP as the control group. The

results showed that there were no significant differences in TP expression, migration, and invasion compared with the empty vector groups (Supplementary Figure S1).

Promotion of the Expression and Activity of MMP2 and MMP9 by TP

During cancer invasion and metastasis, the extracellular matrix and basement membrane are degraded by proteolytic enzymes. When TP was overexpressed in KB cells, the expression and activity of MMP9 increased.[13] Thus, immunohistochemical analysis was performed to assess the TP, MMP2, and MMP9 expression. Results showed that the MMP2 and MMP9 expression were higher in cancers with high TP expression compared with those with low TP expression, and their expression was significantly correlated with Pearson coefficients of 0.006 and 0.001, respectively (Figure 4a). Gelatin zymography assay showed that when TP was overexpressed, the cancer cells obviously secreted MMP2 and MMP9 at a higher level than control, and with the knockdown of TP, the secreted level of MMP2 and MMP9 decreased (Figure 4b). MMP2 and MMP9 activity were significantly increased when TP was overexpressed in PLC-PRF-5 cell lines and decreased after TP was knocked down in
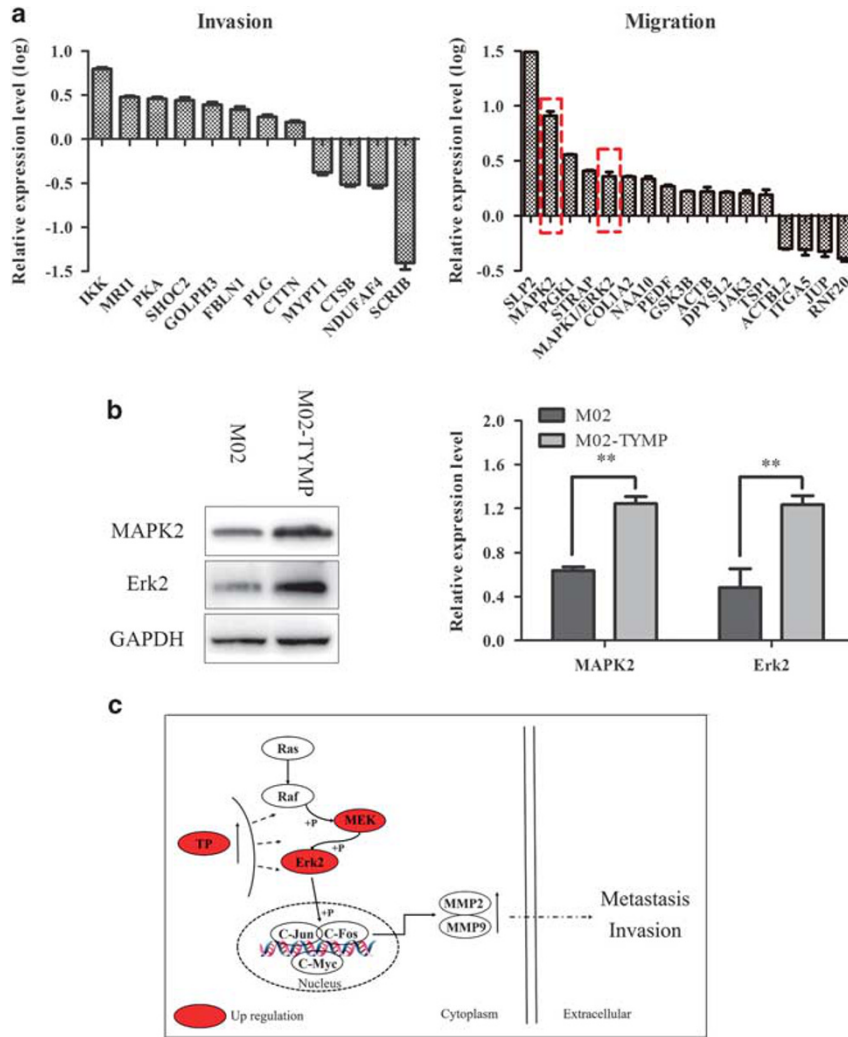
Figure 6 Proteomics analysis results. (**a**) Multidimensional liquid chromatography–tandem mass spectrometry was performed to analyze the differentially expressed proteins of PLC-PRF-5 cells. Among these proteins, 8 proteins were upregulated and 4 were downregulated, which were involved in invasion, and 13 proteins were upregulated and 4 were downregulated, which were involved in migration. (**b**) MAPK2 and Erk2 were upregulated when PLC-PRF-5 cells were transfected with M02-TYMP by western blot. (**c**) TP-Ras-MAPK-MMPs signaling pathway. Results show the means of the three experiments, and the error bars represent s.d. (**P < 0.01). Erk, extracellular signal-regulated kinase; MMP, matrix metalloproteinase.

cell lines of HepG2 (Figure 4c). In TP overexpressed cells, the luciferase reporter plasmids of MMP2 and MMP9 were transfected, and TP promoted the transcription of MMP2 and MMP9. Correspondingly, in the interference group, the transcription level of MMP2 and MMP9 decreased (Figure 4d). Taken together, these results indicated that TP is a positive regulator of MMP2 and MMP9.

TP Affects the Tumor Growth in Mouse Xenograft Models
The mice xenograft model was used to measure the relationship between TP and metastasis *in vivo*. The model revealed that high TP expression promoted tumor growth and significantly increased the tumor formation of lung metastasis from 1.67 ± 0.57 to 12.33 ± 1.52. A higher mortality rate was observed in the TP overexpression group. Consistent with this

result, in the HepG2 xenografts, when TP was knocked down, tumor growth was inhibited and lung metastasis was also decreased from 8.67 ± 1.53 to 3.33 ± 0.58 (Figures 5a and b). Further IHC analysis of the xenografts showed that TP significantly promoted the expression of MMP2 and MMP9 *in vivo* (Figure 5c).

TP may Promote HCC Progression through MAPK-MMP Pathway
To elucidate the molecular mechanism of TP's promotion of HCC progression, proteomic analysis was used to compare the differential protein expression when TP was overexpressed. The proteomic analysis revealed that TP overexpression influenced the expression of a series of proteins related to invasion and migration (Figure 6a). The pathway analysis showed that the Ras-MAPK-MMPs signaling pathway

was activated by upregulation of the protein of MAPK2 and Erk2. Western blot analysis revealed that the expression of MAPK2 and Erk2 was significantly upregulated with the overexpression of TP (Figure 6b). These results may explain the mechanism of tumor metastasis and invasion mediated by TP (Figure 6c).

DISCUSSION

HCC ranks as the second most common cause of cancer-related mortality with 1.6 million global deaths annually.[14] HCC patients are usually diagnosed at advanced stages, and metastasis is a major reason for the high mortality and poor prognosis for HCC patients.[15] The mechanism of HCC metastasis remains unclear and requires further explanation. TP, also known as platelet-derived endothelial cell growth factor, is an enzyme involved in pyrimidine nucleoside metabolism, and it catalyzes the reaction of thymidine to thymine and deoxyribose-1-phosphate.[5] TP is upregulated in a wide variety of solid tumors and promotes tumor progression by preventing apoptosis.[16–18] Elevated TP levels are associated with tumor aggressiveness and poor prognosis. The presence and overexpression of TP in HCC have been reported. However, whether TP can promote the metastasis of HCC deserves further discussion.[7]

In this study, our results show that TP was correlated with HCC progression and significantly promoted HCC cell migration and invasion, and that high TP expression was positively correlated with poor prognosis of HCC patients. These results indicate that TP may be used as a marker of HCC. Further results showed that TP regulated HCC metastasis through upregulating the activity of MMPs. MMP2 and MMP9 usually have an upregulated expression in different metastatic tumors; the MMPs could degrade the extracellular matrix, thereby making it easy for the tumor cells to transfer.[19–21] However, TP is not a transcription factor, and the MMPs cannot be directly regulated; how TP influences MMPs needs further explanation.

Erk, a member of the MAPK family, may stimulate cell proliferation and restrain cell apoptosis by activating the Ras-Raf-Mek-Erk signal cascade pathway.[22] Erk also plays an important role in the metastasis of different tumors including HCC.[23,24] Phosphorylation of the Erk1/2 signaling pathway could affect the expression of some transcriptional coactivators, which could then regulate the gene transcription of MMPs.[25–27] Our current study does not examine whether TP directly induces the chemokines production or corresponding receptor expression. It is possible that increased invasion and migration might be due to increased generation of chemokines from tumor cells. In this study, proteomic analysis showed that with the overexpression of TP, a series of proteins related to invasion and migration was affected; the expression of the proteins of MAPK2 and Erk2 in the Ras/Raf pathway in particular increased significantly. These results indicate that TP is likely to affect the expression and activity of MMPs by regulating the MAPK pathway, which promotes

the metastasis of HCC. However, how TP affects the MAPK pathway is unclear and needs further exploration.

In summary, we provided quantitative data to clarify the important role of TP in the metastasis of HCC. We demonstrated that TP was correlated with MMP2 and MMP9 expression, and that TP mediated the migration of tumor cells through the activity of MMPs in HCC cell lines. TP affected the expression of MMPs through Erk2 in the Ras/Raf pathway. This study provides further understanding of the molecular mechanisms of metastasis in HCC by TP. Moreover, TP may be a potential marker of poor prognosis in HCC.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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