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Original Paper

TPPP3 Promotes Cell Proliferation, Invasion and Tumor Metastasis via STAT3/Twist1 Pathway in Non-Small-Cell Lung Carcinoma

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Key Words
TPPP3 • Twist1 • NSCLC • Metastasis • STAT3 • Prognosis

Abstract
Background/Aims: Non-small-cell lung carcinoma (NSCLC) is the leading cause of cancer death, with tumor metastasis being mainly responsible for lung cancer-associated mortality. Our previous studies have found that tubulin polymerization promoting protein family member 3 (TPPP3) acted as a potential oncogene in NSCLC. Little is known about the function of TPPP3 in tumor metastasis. ***Methods:*** RT-qPCR and IHC were used to investigate the expression of TPPP3 in NSCLC tissues. CCK8 assay and transwell assay were used to measure proliferation and migration of NSCLC cells *in vitro* and xenograft model was performed to assess the tumor growth and metastasis *in vivo*. ***Results:*** In the present study, upregulation of TPPP3 was found to correlate with an increased metastasis capability of NSCLC. Ectopic expression of TPPP3 significantly enhanced cell proliferation *in vitro* and promoted tumor growth *in vivo*. Furthermore, overexpression of TPPP3 remarkably promoted NSCLC cell migration and invasion along with the upregulation of Twist1 both *in vitro* and *in vivo*. Further investigations showed that activation of STAT3 was required for TPPP3-mediated upregulation of Twist1, cell migration and invasion. A strong positive correlation between TPPP3 and Twist1 expression was identified in NSCLC tissues. Patients with low TPPP3 or low Twist1 in NSCLC tissues had a better prognosis with longer overall survival (OS) and disease-free survival (DFS). ***Conclusion:*** Overall, this study demonstrates that TPPP3 promotes the metastasis of NSCLC through the STAT3/Twist1 pathway.

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Introduction

Non-small-cell lung cancer (NSCLC) accounts for 85% of primary lung cancer and is the leading cause of cancer-associated death around the world [1]. Despite advances in therapeutic approaches, including curative resection, radiotherapy, targeted agents and increasingly immunotherapy, the overall survival (OS) rate remains poor due to diagnosed at advanced stages [2, 3]. Well-established environmental factors (including smoking, environmental and occupational exposures) are associated with NSCLC risk [4]. Apart from environmental factors, there are various known genetic factors (EGFR, KRAS, ALK and TITF1) contributing to the initiation and development of NSCLC [5-7]. Though a great improvement in multiple oncogenes and tumor-suppressive genes have been elaborated in NSCLC, the precise molecular mechanisms involved in the development of NSCLC are still remaining to be further illustrated.

Tubulin polymerization promoting protein family member 3 (TPPP3) is a member of a family of tubulin polymerization promoting proteins (TPPPs), which mediated the dynamics and stability of the microtubules [8, 9]. We have previously found that TPPP3 was significantly over-expressed in human NSCLC and associated with short overall survival [10]. Knockdown of TPPP3 expression by RNA interference results in Lewis lung carcinoma (LLC) cell growth inhibition and cell cycle arrest demonstrating a lineage-specific dependency of lung cancer on TPPP3 [11]. Further investigation showed that inhibition of TPPP3 attenuated cell proliferation by inducing cell apoptosis and arresting cell cycle [10]. However, the potential role of TPPP3 in NSCLC development and metastasis remains largely unknown.

Twist1, a basic helix-loop-helix (bHLH) domain-containing transcription factor, has been reported to increase in various cancers, including lung cancer [12]. Emerging evidences have indicated that Twist1 played a crucial role in cancer initiation and progression through its ability to inhibit DNA damage-induced apoptosis and induction of the epithelial-mesenchymal transition (EMT) [13-16]. Suppression of Twist1 resulted in significant growth inhibition of multiple defined oncogenic drives-induced NSCLC [17]. In addition, silencing of Twist1 sensitized NSCLC cells to cisplatin via stimulating AMPK-induced mTOR/S6K1 inhibition which led to a decrease of Mcl-1 protein [18]. Our previous study showed that TPPP3 modulates the cell growth by activating phosphorylation of STAT3 [10]. It was previously shown that STAT3 directly binds to the promoter of Twist1 and activated its transcriptional activity in human cancer cells [19, 20]. We hypothesized that a strategy to silence expression of Twist1 in NSCLC cells would abolish tumor aggressiveness induced by TPPP3.

In our present study, enforced TPPP3 expression promoted NSCLC cell growth and metastasis by STAT3/Twist1 signaling axis *in vitro* and *in vivo*. Further investigation indicated that Twist1, a key oncogenic transcription factor, was required for TPPP3-induced cell cancer phenotype in STAT3 dependent manner. Our findings provide a deeper understanding of the aggressive mechanisms of TPPP3.

Materials and Methods

Patients and Specimens

A total of 84 pairs of primary NSCLC specimens and adjacent non-tumor tissue specimens were collected from patients who had undergone primary surgical NSCLC resection at Shandong Cancer Hospital between May 2007 and November 2010. None of the patients received chemotherapy or radiotherapy prior to surgical resection. Written informed consent was obtained from all patients enrolled. Following resection, fresh tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until use. The research was approved by the Ethics Committee of Shandong Cancer Hospital and conducted in accordance with the Declaration of Helsinki was signed by the patients.

Real-time PCR

Total RNA was extracted from tissue specimens and collected cells using Trizol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription and quantitative real-time PCR were performed as our previously described [10].

Immunohistochemistry (IHC)

IHC staining for TPPP3 was carried out for NSCLC specimens and xenograft tumor tissues have been previously described [10].

Cell lines and cell culture

Four human NSCLC cell lines (95-C, SPC-A1, A549, and H1299) and human embryonic kidney 293T cells (HEK293T) were obtained from cell banks of the Shanghai Institutes of Biological Sciences (Shanghai, China) and maintained according to the supplier's instructions. Cryptotanshinone (CPT) was purchased from Selleckchem (Houston, TX, USA).

Stable cell lines construction and transfection

Lentivirus-mediated TPPP3-expressing vector and control plasmids were constructed by cloning the coding sequence of TPPP3 into pCDH-CMV-MCS-EF1-Puro vector. The TPPP3-expressing vectors or control vector were co-transfected with the pPACKH1 Packaging Plasmid Mix into HEK293T cells using TurboFect™ (Fermentas, Glen Burnie, MD, USA). Stable cell lines expressing TPPP3 were selected for one week with 1ug/ml puromycin (Sigma, Saint Louis, MI, USA) 48 h after infection. Small interfering RNA (siRNA) targeting Twist1 was designed and synthesized by GenePharm (Shanghai, China). Western blotting was performed to determine the expression and knockdown efficiencies.

Cell proliferation and colony formation assay

Cell proliferation and colony formation were performed as we previously described [10].

In vitro migration and invasion assay

Cell migration and invasion ability were assessed used transwell chambers (Corning, Tewksbury, MA, USA) coated with or without Matrigel (BD Biosciences, Bedford, MA, USA). Approximately 4×10^4 cells in 100 μL serum-free media were seeded into upper chamber of the Transwell chambers. 600 μL media containing 10 % FBS were added to the lower chamber as a chemokine. After 24 h of incubation, cells remaining on the upper membrane were removed with cotton swab, whereas cells that had invaded through the membrane were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Images were photographed under an inverted microscope (Olympus, Tokyo, Japan) and quantified in 6 random high-powered fields.

Confocal laser scanning microscopy

Logarithmic growth cells were seeded on coverslips in 96-well plates for 24 h. Then cells were fixed, blocked and incubated with primary antibody against Twist1 and E-cadherin. Following incubated with a FITC-conjugated secondary antibody and nuclear counterstaining with DAPI, the cells were photographed on an oil-immersion 60 × 1.4 Nikon APO objective (Nikon Instruments, Amsterdam, Netherlands).

Animal study

All animal experiments were performed in accordance with the guidelines for animal experiments of Shandong Cancer Hospital (permit number of 201601AE). Stable overexpression TPPP3 cells and control vector cells were used to assess tumor growth in mouse model according to our previously described method [10]. In order to explore the effects of TPPP3 on metastasis of NSCLC cells *in vivo*, cells (1×10^6 95-C-vector, 1×10^6 95-C-TPPP3) in 150μL serum-free media were injected into tail vein of 6 week-old BALB/c nude mice (eight mice in each group). The mice were sacrificed 5 weeks post-injection. All the lungs are removed, paraffin-embedded, formal infixed and stained by Hematoxylin and Eosin (HE) for further metastatic tumor nodules analysis.

Western blot analysis

Cells were lysed with RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with a protease inhibitor cocktail (Roche, CA, USA). Approximately 40 μg protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45 μm polyvinylidene fluoride membranes (PVDF, Bio-Rad, CA, USA). The membranes were then incubated with primary antibodies of TPPP3, STAT3, p-STAT3 (Tyr705), Twist1, c-Myc, CyclinD1, MMP-2, MMP-9 and E-cadherin (Cell Signaling Technology) overnight at 4°C. GAPDH (Cwbiotech, Beijing, China) was used as the loading control. Then the blots were incubated with HRP-conjugated second antibody and were visualized using an ImageQuant™ LAS 4000 (GE Healthcare, Chicago, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 Demo (GraphPad Software Inc., CA, USA) software. The correlation between TPPP3 expression and clinicopathological factors was evaluated using Chi-square test. Correlation of TPPP3 with Twist1 was assessed by the Pearson correlation test. Kaplan-Meier analysis was used to determine the survival analysis. All data were presented as the mean ± standard deviation (SD) of three independent experiments. A two-sided p-value of <0.05 was considered statistically significant.

Results

TPPP3 is overexpressed in NSCLC and correlates with aggressive and metastatic disease

We have previously reported that TPPP3 was increased in NSCLC tissues and that TPPP3 silencing inhibited cell proliferation [10]. However, it was still not well-recognized whether TPPP3 stimulated tumor metastasis in human cancer cells, especially in NSCLC cells. To further explore the role of TPPP3 in the progression of NSCLC, we compared the mRNA levels of TPPP3 in NSCLC tissues and para-carcinoma tissues from 84 individual patients and TPPP3 expression was significantly increased in tumor samples (Fig. 1A). Interestingly, in comparison to primary NSCLC tissues without metastasis, TPPP3 was significantly higher in primary NSCLC tissues with metastasis (Fig. 1B). Furthermore, IHC analyses demonstrated significantly increased expression of TPPP3 in NSCLC tissues, especially in primary NSCLC tissues with metastasis (Fig. 1C). These data indicated that TPPP3 might play a crucial role in tumor progress and metastasis.

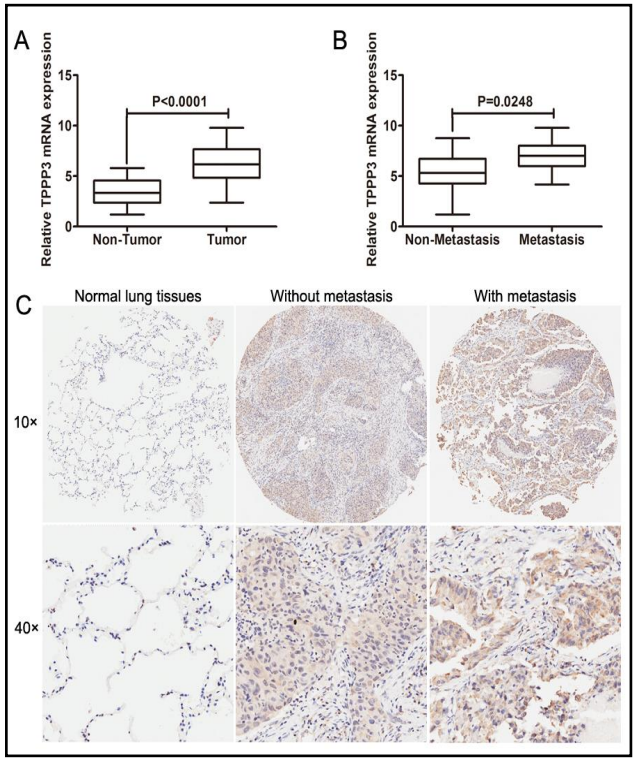

Fig. 1. TPPP3 mRNA and protein expression levels were elevated in NSCLC tissues. (A) Expression levels of TPPP3 mRNA in 84 pair NSCLC patients. (B) Higher expression of TPPP3 mRNA in primary NSCLC tissues with metastasis than the primary NSCLC tissues without metastasis. (C) Immunohistochemical analysis of TPPP3 in normal lung tissues (left), primary NSCLC tissues without metastasis (middle) or with metastasis (right).

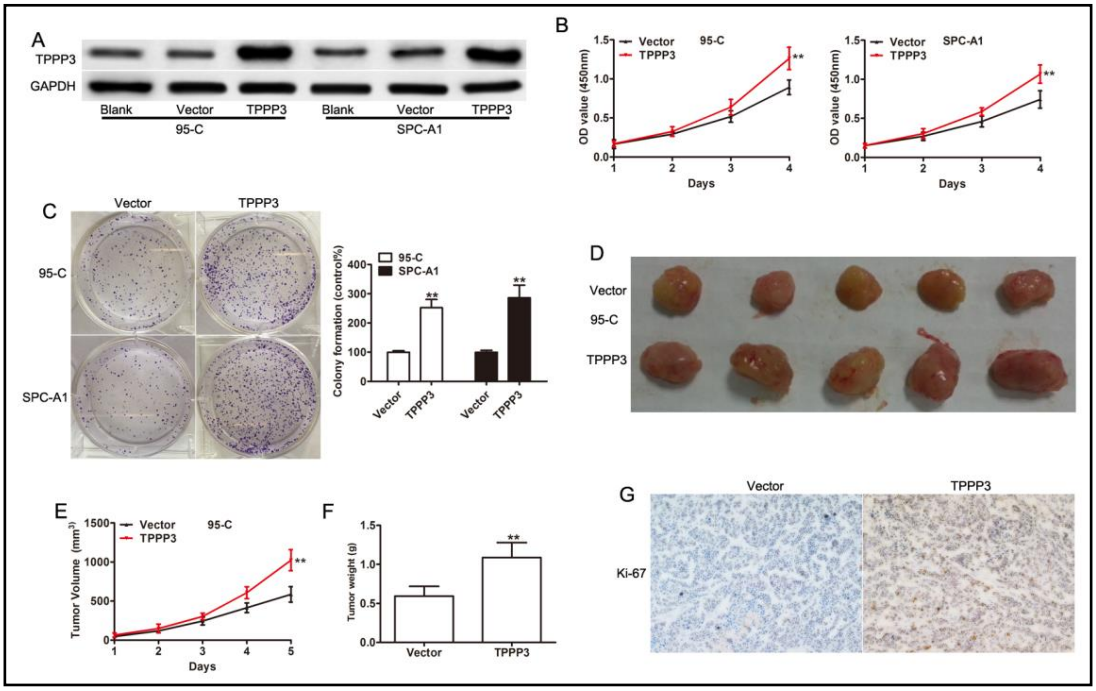

Fig. 2. Overexpression of TPPP3 contributed to NSCLC progression in vitro and in vivo. (A) Western blot of TPPP3 expression in 95-C and SPC-A1 cells stably overexpressing TPPP3. (B) Overexpression of TPPP3 promoted cell growth by CCK8 assays. (C) Monolayer colony formation was performed to determine the anchorage-independent growth of 95-C and SPC-A1 cells stably overexpressing TPPP3. (D) Representative images of xenografted tumors from mice injected with 95-C vector group or 95-C TPPP3 group. Quantitative analysis tumor weight (E) and tumor volume growth curves (F). (G) Representative immunohistochemical staining for Ki-67 in the xenografted tumors.

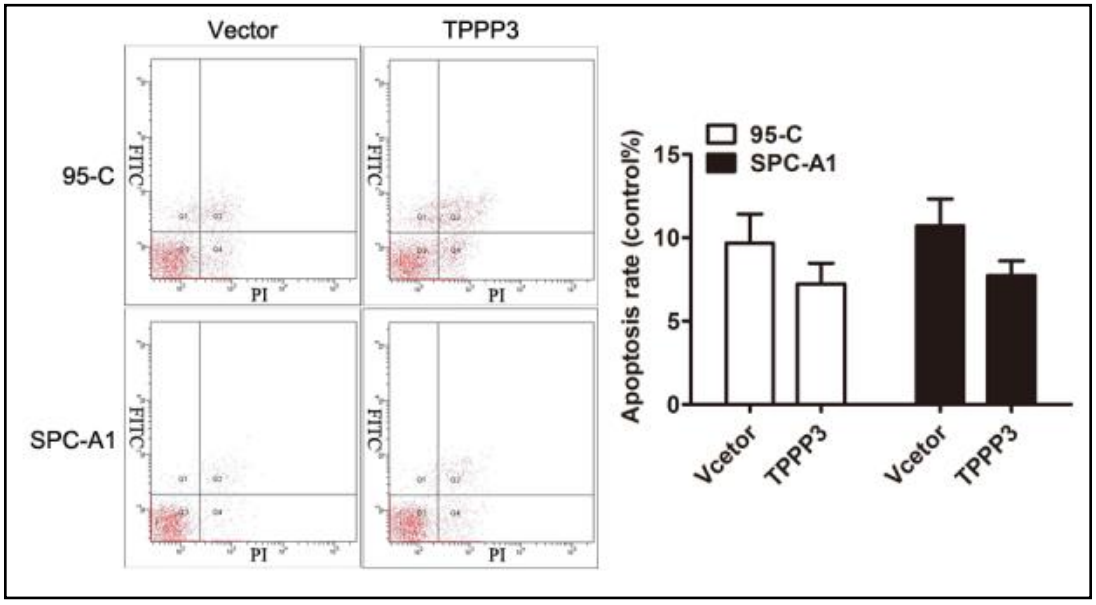

Fig. 3. Representative images and quantification of flow cytometry analysis of 95-C and SPC-A1 cells after TPPP3 overexpression.

Overexpression of TPPP3 promotes cell proliferation and tumor growth of NSCLC

In order to determine the biological role of TPPP3 on NSCLC cells growth, 95-C and SPC-A1 cells stably overexpressing TPPP3 were established (Fig. 2A). CCK-8 assay demonstrated that the overexpression of TPPP3 dramatically increased the proliferation of 95-C and SPC-A1 cells (Fig. 2B). Furthermore, enhanced TPPP3 expression displayed a substantial increase in clone capacity compared with control cells (Fig. 2C). The subcutaneous xenograft tumor models were performed to assess the effect of TPPP3 on 95-C cell growth *in vivo*. As shown in Fig. 2D, overexpression of TPPP3 promoted the tumor growth. The volumes or weights of xenografted tumors were larger or higher in the TPPP3-overexpressing group than in the control group (Fig. 2E and F). To further confirm the role of TPPP3 in cell proliferation, immunohistochemical analysis of Ki67 was performed in xenografted tumors and our results showed that the percentage of Ki67-positive tumor cells was significantly increased in tumors from the TPPP3-overexpressing group (Fig. 2G). However, overexpression of TPPP3 did not affect cell apoptosis (Fig. 3). These results strongly suggested that TPPP3 promoted tumor growth *in vivo* and played a proto-oncogene.

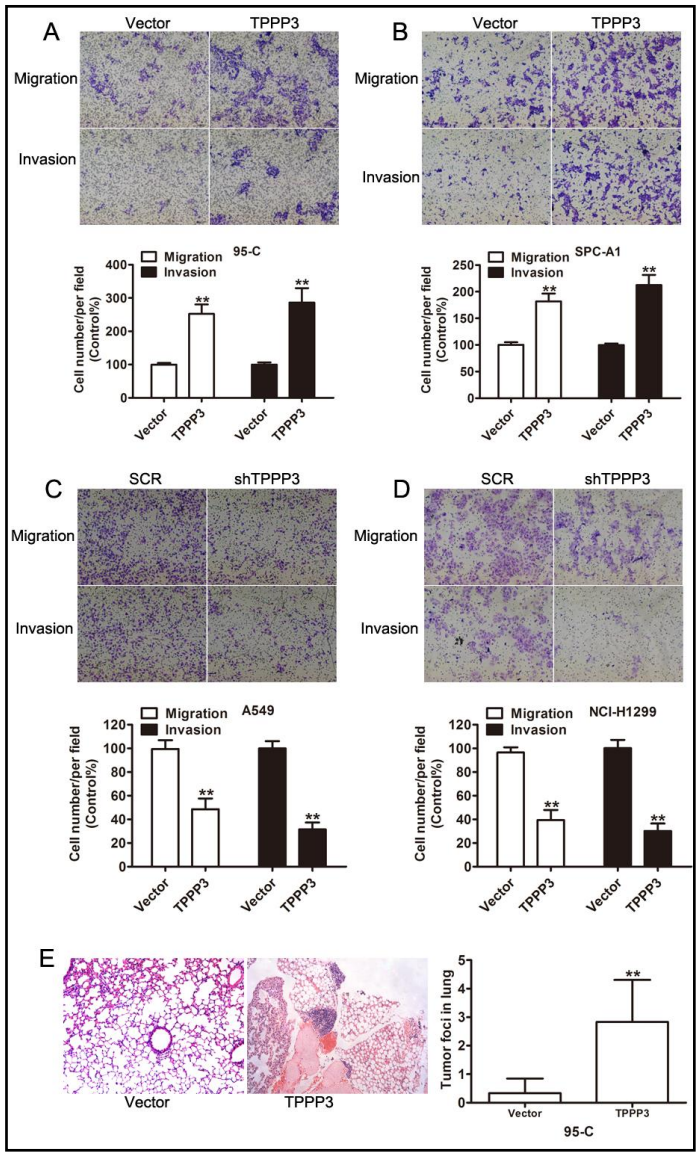

Fig. 4. Effects of TPPP3 on the invasive abilities of NSCLC cells. (A and B) Representative images (upper panel) and quantification (lower panel) of invading 95-C and SPC-A1 cells stably overexpressing TPPP3. (C and D) The cell invasion ability was determined in TPPP3 silencing A549 and H1299 cells. (E) Overexpression of TPPP3 in 95-C cells promoted tumor nodules in vivo.

Overexpression of TPPP3 enhances cell invasion and metastasis, while TPPP3 silencing suppresses cell migration and invasion

Since TPPP3 was significantly upregulated in primary NSCLC tissues with metastasis, transwell assay was used to investigate the effect of TPPP3 on NSCLC cell invasion. 95-C and SPC-A1 cells with low endogenous TPPP3 transduced with TPPP3 showed increased cell invasion (Fig. 4A). In contrast, transfection with TPPP3 shRNA in A549 and H1299 cells with high endogenous TPPP3 had the opposite effect on cell migration (Fig. 4B). Furthermore, the

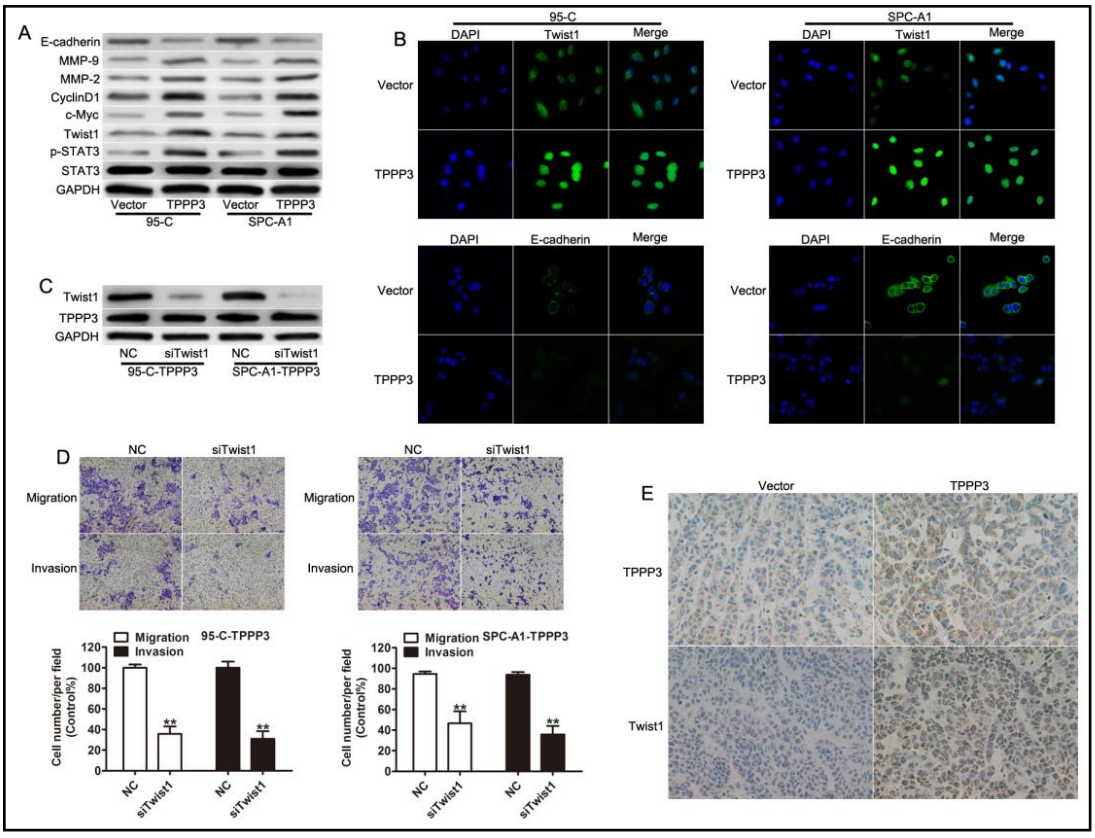

Fig. 5. Twist1 was involved in oncogenic effects of TPPP3 on NSCLC cell migration and invasion. (A) The STAT3, p- STAT3, Twist1, c-Myc, CyclinD1, MMP-2, MMP-9 and E-cadherin protein levels were measured in 95-C and SPC-A1 cells after TPPP3 overexpression using western blot. (B) The subcellular localization of Twist1 and E-cadherin were examined by immunofluorescence staining and nuclei were stained with DAPI in 95-C and SPC-A1 cells stably overexpressing TPPP3. (C) The protein expression of Twist1 determined in stable overexpressing TPPP3 95-C and SPC-A1 cells transfected with Twist1 siRNA by western blot. (D) Transwell assays of the invasive abilities of stable overexpressing TPPP3 95-C and SPC-A1 cells transfected with Twist1 siRNA. (E) IHC staining of TPPP3 and Twist1 in xenograft tumors.

possible effect of TPPP3 on metastatic potential was explored *in vivo*. Our results showed that the mice which were injected with TPPP3-overexpressing 95-C cells had significantly higher levels of metastatic tumor nodules in the lungs as compared to control group (Fig. 4C). Taken together, these results indicated that TPPP3 enhanced aggressive metastatic phenotype of NSCLC cells *in vitro* and *in vivo*.

Twist1 is involved in TPPP3-induced cell invasion and migration in NSCLC

To explore potential mechanisms by which TPPP3-medicated cell migration and invasion, we analyzed the expression of markers of cell proliferation- and invasion-related protein. Interestingly, western blot analyses showed that a decrease in the expression levels of E-cadherin and an increase in the expression levels of the p-STAT3, Twist1, c-Myc, CyclinD1, MMP-2 and MMP-9 in 95-C and SPC-A1 cells expressing TPPP3 (Fig. 5A). It has been reported that Twist1 was one of the master transcription factors that induce cell migration and invasion in many types of aggressive cancers [13, 21, 22]. The immunofluorescence results revealed that increased expression of Twist1 and reduced expression of E-cadherin in 95-C and SPC-A1 cells after TPPP3 overexpression (Fig. 5B). Furthermore, to investigate whether Twist1 was involved in TPPP3-mediated cell migration and invasion, RNAi-mediated gene knockdown was performed to diminish expression of Twist1 in 95-C and SPC-A1 cells expressing TPPP3 (Fig. 5C). As shown in Fig. 5D, TPPP3-induced cell migration and invasion

could be inhibited by Twist1 siRNA transfection. IHC staining confirmed that TPPP3 enhanced the expression of Twist1 *in vivo*. These data suggest that TPPP3 promotes cell migration and invasion by upregulatuion of Twist1 expression.

TPPP3 upregulates Twist1 in STAT3-dependent manner

Our previous studies have demonstrated that TPPP3-STAT3 signaling was critical to promote aggressive behaviors [10]. In addition, it has been shown that p-STAT3 could directly activate expression of Twist1 to drive human cancer progression [20, 23]. To assay if p-STAT3 was involved in TPPP3-mediated Twist1 upregulation and cell migration and invasion, Cryptotanshinone (CPT), a STAT3 inhibitor, was used to treat TPPP3-overexpressing 95-C and SPC-A1 cells. Our results showed that inhibition of STAT3 reduced the protein expression of Twist1, c-Myc, CyclinD1, MMP-2 and MMP-9, whereas increased the protein expression of E-cadherin compared with the control (Fig. 6A). Moreover, invasion assays on 95-C-TPPP3 and SPC-A1-TPPP3 cells treated with CPT resulted in a significant decrease in invasive activity (Fig. 6B). Collectively, these results suggest that p-STAT3 was required for TPPP3-mediated Twist1 upregulation and cell migration and invasion.

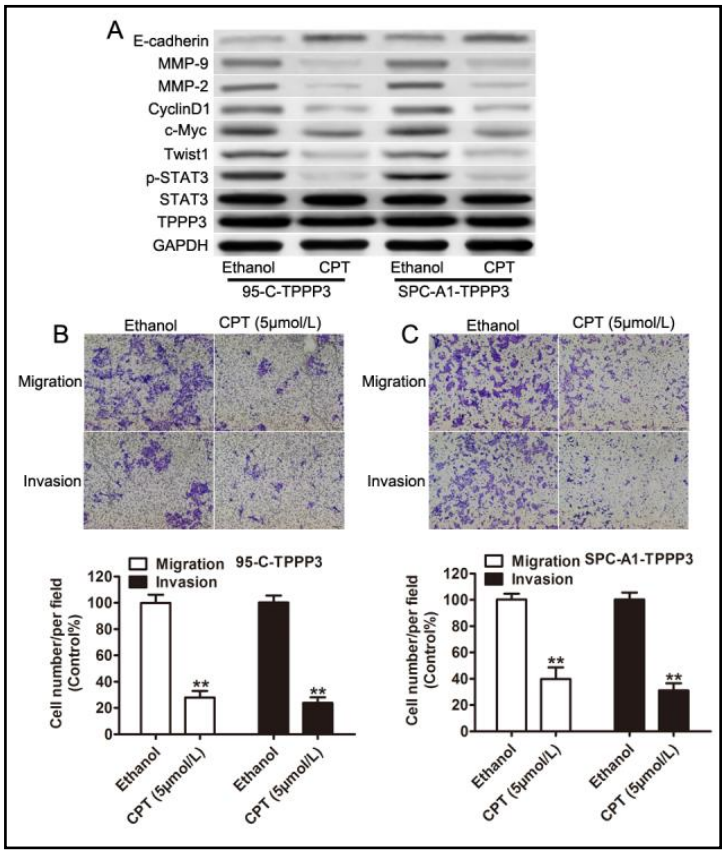

Fig. 6. P-STAT3 was essential for TPPP3 to promote cell invasion and upregulation of Twist1.(A) Western blot analysis showed STAT3, p-STAT3, Twist1, c-Myc, CyclinD1, MMP-2, MMP-9 and E-cadherin expression levels in stable overexpressing TPPP3 95-C and SPC-A1 cells treated with CPT (5 μM for 24 h). The invasion ability of 95-C-TPPP3 cells (B) and SPC-A1-TPPP3 (C) was assessed with CPT treatment.

The prognostic values of TPPP3 and Twist1 for NSCLC patients

We also evaluated the expression of Twist1 across our cohort of NSCLC specimens and confirmed that Twist1 was upregulated in NSCLC specimens (Fig. 7A). To investigate the roles of

Table 1. Associations between PPP3/Twist1 expression level and clinicopathologic features of NSCLC patients (n =84). Abbreviations: TNM, tumor-node-metastasis; *Fisher's exact tests, and X^2 tests for all the other analysis

Variables	TPPP3 Expression, n		P	Twist1 Expression, n		P
	Low n=42	High n=42		Low n=42	High n=42	
Sex						
Female	3	2	1*	2	3	1*
Male	38	39		39	38	
Age,y						
≤60	23	22	0.824	24	21	0.506
>60	18	19		17	20	
Histology						
Squamous cell carcinoma	34	29	0.314	28	35	0.126
Adenocarcinoma	4	9		8	5	
Others	3	3		5	1	
Smoking						
Yes	36	39	0.432	39	36	0.432*
No	5	2		2	5	
Histology grade						
Well	13	18	0.283	20	11	0.060
Moderate	28	22		20	30	
Poor and Unknown	0	1		1	0	
TNM stage						
Ⅰ-Ⅱ	36	16	<0.001*	34	18	<0.001
Ⅲ-Ⅳ	5	25		7	23	
Tumor progression						
Metastasis	4	20	<0.001*	3	21	<0.001
No metastasis	37	21		38	20	

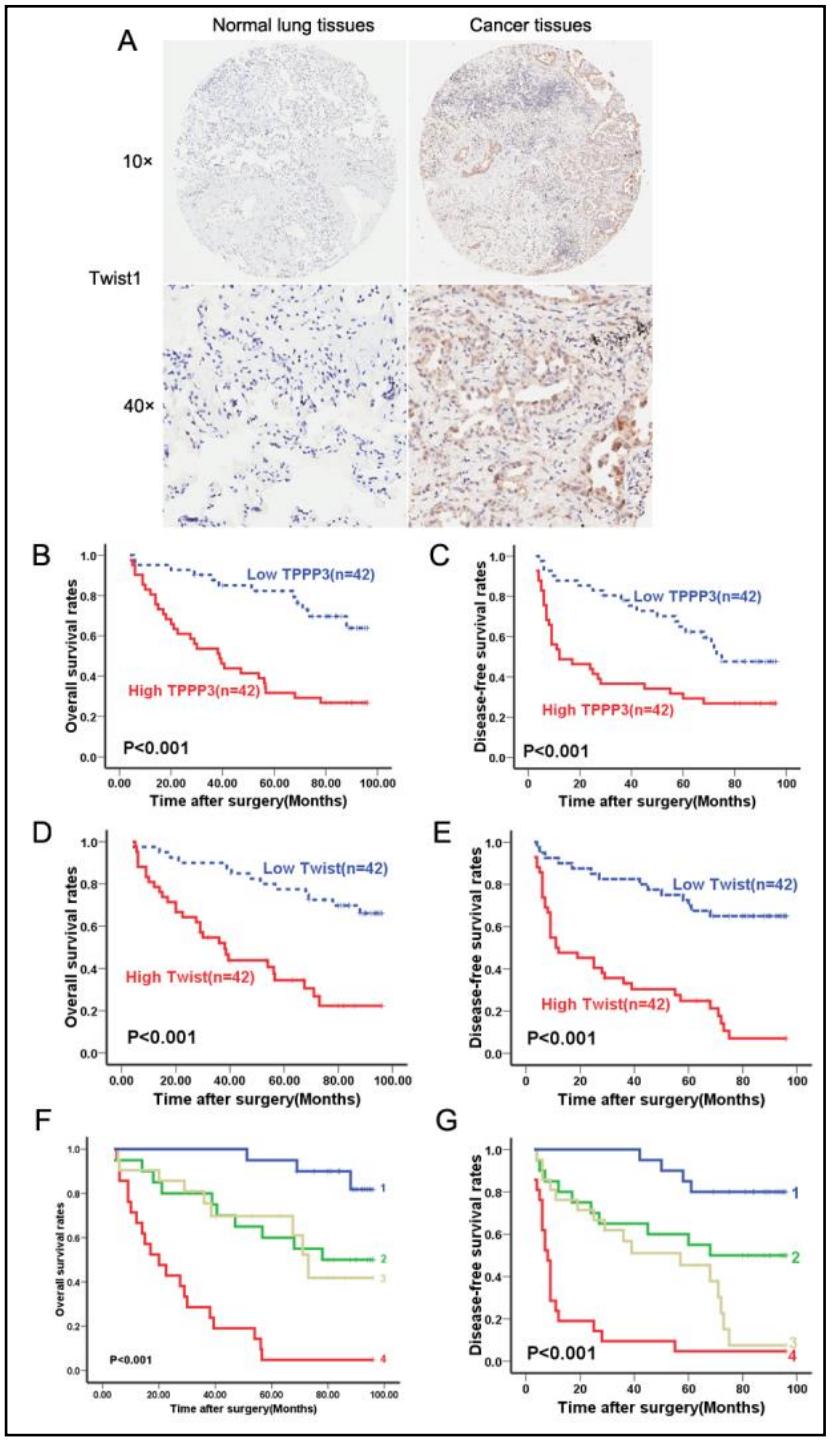

Fig. 7. Clinical prognoses of TPPP3 expression with Twist1 expression in human NSCLC tissues. (A) Immunohistochemical analysis of Twist1 expression in the same panel NSCLC tumor specimens. (B and C) Kaplan-Meier analysis of the correlation between TPPP3 expression and overall survival (B) and disease free survival (C) in NSCLC patients. (D and E) Kaplan-Meier analysis of the correlation between Twist1 expression and overall survival (D) and disease free survival (E) in NSCLC patients. (F and G) Patients with low TPPP3 and low Twist1 had the longest OS (F) and lowest possibility of disease free survival (G) according to the cut-off values of TPPP3 and Twist1, which were defined as the median of the cohort. 1, low TPPP3 and low Twist1; 2, low TPPP3 and high Twist1; 3, high TPPP3 and low Twist1; 4, high TPPP3 and high Twist1.

TPPP3 and Twist1 in NSCLC patients, we analyzed the association between TPPP3 and Twist1 expression and clinical pathological characteristics of NSCLC patients. Our results showed that higher protein levels of TPPP3 were significantly associated with TNM stage and Tumor progression (Table 1). In consistent with TPPP3, high Twist1 expression was also significantly associated with TNM stage and tumor progression (Table 1). The prognostic value of TPPP3 and Twist1 was analyzed by comparing the overall survival (OS) and disease-free survival (DFS) of patients with high and low TPPP3 or Twist1 expression (n=84). Kaplan-Meier analysis showed that patients with high TPPP3 expression had a significantly shorter

OS and DFS than patients with low TPPP3 expression (Fig. 7B and C). When patients were divided according to the level of Twist1 expression, those with low Twist1 expression had a significantly higher OS and DFS than those with high Twist1 expression (Fig. 7D and E). Univariate analysis showed that TPPP3, Twist1, TNM stage, and tumor progression were significantly associated with OS and DFS in NSCLC patients (Table 2). However, sex, age, smoking, histology, and histology grade did not significantly associate with OS and DFS (Table 2). In addition, multivariate analysis showed that TPPP3 and Twist1 were independent prognostic indicators for both OS and DFS (Table 3).

Table 2. Univariate Analyses of Factors Associated with Overall Survival and Disease Free Survival. NOTE: Univariate analysis, Cox proportional hazards regression model. Abbreviation: 95% CI, 95% confidence interval; NS, not significant(Cox proportional hazards regression model); TNM, tumor-node-metastasis

	Overall Survival		Disease Free Survival	
Variable	Hazard ratio (95% CI)	P Value	Hazard ratio (95% CI)	P Value
Sex (male vs. female)	0.043(0.000-5.049)	NS	0.416(0.101-1.715)	NS
Age, y (>60 vs. ≤60)	0.954(0.519-1.752)	NS	0.944(0.540-1.651)	NS
Smoking (yes vs. no)	24.327(0.378-1566.855)	NS	2.152(0.669-6.925)	NS
Histology (Adenocarcimoma vs Squamous cell carcinoma)	0.485 (0.235-0.998)	0.050	0.382(0.184-0.795)	0.010
Histology grade (Poor vs. well)	0.618(0.344-1.110)	NS	0.701(0.409-1.200)	NS
TNM stage (III/IV vs. I/II)	2.539 (1.377-4.681)	0.003	2.288(1.303-4.020)	0.004
Tumor progression (Metastasis vs. No metastasis)	2.463(1.319-4.596)	0.005	2.142(1.194-3.843)	0.011
TPPP3 (high vs. low)	3.652(1.863-7.158)	<0.001	2.317(1.311-4.097)	0.004
Twist1 (high vs. low)	4.347(2.211-8.547)	<0.001	5.333(2.820-10.085)	<0.001

Table 3. Multivariate Analyses of Factors Associated with Overall Survival and Disease Free Survival. NOTE: Multivariate analysis, Cox proportional hazards regression model. Abbreviation: 95% CI, 95% confidence interval; NS, not significant(Cox proportional hazards regression model)

	Overall Survival		Disease Free Survival	
Variable	Hazard ratio (95% CI)	P Value	Hazard ratio (95% CI)	P Value
Histology (Adenocarcimoma vs Squamous cell carcinoma)	0.620(0.301-1.279)	NS	0.458(0.221-0.949)	0.014
Tumor progression (Metastasis vs. No metastasis)	2.489(1.289-3.715)	0.028	2.225(1.189-2.901)	0.036
TPPP3 (high vs. low)	8.243(3.509-19.363)	<0.001	5.582(2.771-11.241)	<0.001
Twist1 (high vs. low)	10.081(3.946-25.753)	<0.001	9.295(4.218-20.485)	<0.001
Combination of PPP3 and Twist				
Overall		<0.001		<0.001
High TPPP3/High Twist1 vs. low TPPP3/low Twist1	60.048(13.741-262.415)	<0.001	47.592(13.255-170.878)	<0.001

In the combined analysis of TPPP3 and Twist1 expression, patients with low TPPP3 and low Twist1 expression had the highest OS and DFS, and the opposite pattern was observed in patients with high TPPP3 and Twist1 expression (Fig. 7F and G). Taken together, these results indicate that TPPP3 and Twist1 expression levels are positively correlated with poor OS and DFS in NSCLC patients, suggesting that TPPP3 and Twist1 are prognostic indicators in NSCLC.

Discussion

In our recent investigation, we have found that higher TPPP3 expression was associated with Lymph node metastasis, more advanced stage and worse prognosis in NSCLC [10]. However, the underlying molecular mechanism by which tumor cells acquire aggressive phenotype remains largely unknown. In this study, we demonstrated that the expression of TPPP3 was significantly abundant in primary NSCLC tissues with metastasis. We further showed that overexpression of TPPP3 promoted lung cancer cell growth and proliferation both *in vitro* and *in vivo*. Moreover, we revealed that promoted lung cancer migration and invasion depending on STAT3/Twist1 signaling pathway, suggesting that TPPP3 might be a potential diagnostic marker and therapeutic target of NSCLC.

Our earlier study has shown that TPPP3 silencing repressed cell growth *in vitro* and *in vivo* accompanying with inactivation of p-STAT3 [10]. In contrast, our results indicated that TPPP3 overexpression enhanced cell proliferation, migration and invasion and regulated the expression of Twist1, suggesting that aberrant expression of TPPP3 could induce a more metastatic phenotype in NSCLC cells. Twist1, a downstream effector of STAT3, is a transcription factor of inducing EMT to promote tumor metastasis [13, 14, 19]. Our experiments also showed that overexpression of TPPP3 decreased the expression of E-cadherin, which have shown to be repressed by Twist1 through directly bind to the

E-cadherin promoter [24]. Interestingly, Twist1 knockdown in TPPP3 stable overexpression NSCLC cells partially attenuated cell migration and invasion and strongly downregulate Twist1-induced the alteration of c-Myc, CyclinD1, MMP-2, MMP-9, and E-cadherin expression. Since MMP-2, MMP-9, and E-cadherin were markers of critical genes and indicator for NSCLC metastasis, it was conceivable that TPPP3-induced Twist1 expression played a crucial role in promoting NSCLC progression.

Drugs and inhibitors targeting STAT3 have shown promising clinical implications [25]. To better understand TPPP3-mediated Twist1 expression and tumor metastasis, CPT was used to inhibit STAT3 phosphorylation. STAT3 inhibition reversed TPPP3-mediated cell migration and invasion, upregulated epithelial markers, and decreased the expression of the mesenchymal markers. Thus, STAT3 appears to be critical for TPPP3-indueced cell migration and invasion and upregulation of Twist1.

Previous studies have demonstrated that Twist1 was significantly increased and associated with prognosis in many cancers, including colorectal cancer [26], breast cancer [27], melanoma [28], and esophageal squamous cell carcinoma [29]. By further univariate and multivariate analysis, although the expression of TPPP3 and Twist1 were demonstrated to be an independent prognostic factor for OS and DFS in NSCLC patients, the predictive range of TPPP3 combined with Twist1 was more sensitive than that of alone. Moreover, we made comparisons of prognosis among four subgroups ($TPPP3^{low}$/$Twist1^{low}$, $TPPP3^{low}$/$Twist1^{high}$, $TPPP3^{high}$/$Twist1^{low}$, and $TPPP3^{high}$/$Twist1^{high}$). The NSCLC patients with $TPPP3^{low}$/$Twist1^{low}$ had the most favorable prognosis.

Conclusion

In this research we provided the evidence for first time that TPPP3 and Twist1 expressions are positively correlated in NSCLC. Furthermore, we found that TPPP3 upregulated the expression of Twist1 in a STAT3 depend manner, providing a molecular basis for potential therapeutic implications in the treatment of patients with NSCLC.

Abbreviations

NSCLC (Non small cell lung cancer); OS (overall survival); DFS (disease-free survival); SD (Standard deviation).

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Disclosure Statement

The authors declare that they have no competing interests.

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