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Downregulated TIPE2 is associated with poor prognosis and promotes cell proliferation in non-small cell lung cancer

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ABSTRACT

The present study aims to investigate the expression pattern of TIPE2 protein and its clinical significance in human non-small cell lung cancer (NSCLC). We investigated the expression levels of TIPE2 in 96 NSCLC tumor samples by immunohistochemistry and then analyzed its clinical significance. Furthermore, the role of TIPE2 on the biological properties of the NSCLC cell line H1299 and A549 was experimentally tested *in vitro* and *in vivo*. We found that the expression level of TIPE2 was significantly higher in normal lung tissues compared with NSCLC tissues ($P < 0.001$), and TIPE2 downregulation was significantly correlated with advanced TNM stage ($P = 0.006$). TIPE2 expression was lower in lung cancer cell lines than normal bronchial cell line HBE. Transfection of TIPE2 plasmid was performed in H1299 and A549 cells. TIPE2 overexpression inhibited lung cancer cell proliferation, colony formation and cell invasive *in vitro*, and prevented lung tumor growth *in vivo*. In addition, TIPE2 transfection reduced the anti-apoptotic Bcl-XL protein and mesenchymal marker N-cadherin expression. Taken together, our results demonstrate that TIPE2 might serve as a tumor suppressor in NSCLC progression.

1. Introduction

Currently, lung cancer is one of the leading causes of all cancer related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer [2]. In recent years, there has been substantial progress in the treatment of lung cancer, however, the long-term survival after surgical resection remains low due to the complex biological characteristics and high recurrence and metastasis of lung cancer [3]. Therefore, better understanding of the molecular mechanisms and identification of tumor suppressors is essential for the development of diagnostic markers that aid novel effective therapies for lung cancer.

Tumor necrosis factor-α-induced protein-8 like-2 (TIPE2) is recently identified as an immune negative regulator and mediates the maintenance of immune homeostasis, it belongs to tumor necrosis factor-α-induced protein-8 (TNFAIP8) family, which are thought to regulate cellular and immune homeostasis [4]. TIPE2 was first isolated and obtained from inflamed spinal cord of experimental autoimmune encephalomyelitis (EAE) mice through high-throughput microarray [4]. Human TIPE2 gene is located on chromosome 1q21.2–1q21.3; which shares 94% amino acid sequence identity with murine TIPE2 [4]. TIPE2 is predominantly expressed in lymphoid tissues, such as spleen, thymus, and lymph node immune cells. However, unlike murine TIPE2, human TIPE2 is also expressed on many kinds of non-immune cells, such as hepatocytes and neurons [5]. Animal studies showed that TIPE2 can inhibit activating protein 1 and NF-κB activation and TIPE2-deficient cells are hyperresponsive to Toll-like receptor (TLR) and T-cell receptor (TCR) activation [4]. In addition, TIPE2 binds to Ras-interacting domain of the RalGDS family of proteins to prevent Ras from forming an active complex.TIPE2 overexpression induces cell death and significantly inhibits Ras-induced tumorigenesis in mice [6]. These results suggest that TIPE2 is not only involved in inflammation but also in cancer.

However, the expression pattern of TIPE2 protein and its clinical significance in human cancer are not entirely clear to date. Cao et al. found that human TIPE2 was downregulated in tumor tissues of HCC compared with the paired adjacent non-tumorous tissues, loss of TIPE2 expression in HCC tissues was significantly associated with tumor metastasis. Overexpression of TIPE2 in HCC-derived cell lines inhibits tumor cell growth, migration and invasion *in vitro* and suppresses growth and metastasis of HCC *in vivo*.

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Mechanically, TIPE2 inhibits the migration and invasion of HCC cells through targeting Rac1 and reduces F-actin polymerization and expression of matrix metallopeptidase 9 (MMP9) [6,7]. Li et al. found that TIPE2 was expressed in the cytoplasm of colon cancer tissues and HT-29 cells. TIPE2 expression was more pronounced in colon cancer tissues compared to normal controls and it was related with lymph node metastasis and Dukes stage. TIPE2 can regulate TLR4 inflammatory effect through inhibit Caspase-8 activity, which prevent tumor progress [8]. Very recently, Zhang et al. reported that TIPE2 can inhibit TNF-α-induced hepatocellular carcinoma cell metastasis via Erk1/2 downregulation and NF-κB activation [9]. However, the role and underlining mechanisms of human TIPE2 in development and progression of NSCLC remain largely unknown.

Therefore, in current study, the expression of TIPE2 in cancer tissue samples from NSCLC patients and lung cancer cell lines were detected by immunohistochemistry and western blotting, and then analyzed its clinical significance. Additionally, the role of TIPE2 on the biological properties of the NSCLC lines H1299 and A549 was experimentally tested *in vitro* and *in vivo* to confirm the clinical observations.

2. Material and methods

2.1. Human subjects

96 cases of NSCLC samples and 40 adjacent normal lung tissues were obtained from The First Affiliated Hospital of Zhengzhou University during the period of 2010–2013. All 96 specimens were reevaluated with respect to their histological types, differentiation status, smoking status, and tumor TNM stages. Tumor stages were determined by TNM classification according to the 2002 International Union against Cancer guidelines. The histological diagnosis and grade of differentiation of the tumors were defined by evaluation of the hematoxylin and eosin-stained tissue sections, according to the World Health Organization guidelines of classification (2004). This study was approved by the local ethics committee and written informed consent was obtained from each patient.

2.2. Immunohistochemistry

Surgically excised tumor specimens and were fixed with 10% neutral formalin and embedded in paraffin, and 4-μm-thick sections were prepared. Immunohistochemistry streptavidin peroxidase conjugated method was used to detect the expression of TIPE2. Briefly, tissues were treated with 3% H_2O_2 methanol at room temperature for 10 min and then incubated in 5% goat antiserum for 15 min at 37 °C. After rinsing with PBS, rabbit polyclonal antibody against human TIPE2 (1:300; BOSTER, Wuhan, China) was added to tissues and incubated overnight at 4 °C. The sections were then incubated with goat anti-rabbit IgG at 37 °C for 15 min. After washing in PBS, Biotin-labeled goat anti-rabbit IgG was added to the sections and incubated at 37 °C for 10 min. SP complex was added and the sections were visualized by incubating with DAB–H_2O_2 for 5–10 min, desired color reaction was observed when monitored with the microscope. All of the slides were counterstained with hematoxylin. As negative controls, adjacent sections were processed as described above, except that they were incubated overnight at 4 °C in blocking solution without the primary antibody.

The intensity of positive staining was measured using a computerized image system (Leica Microsystems Imaging Solutions, United Kingdom). Five fields were randomly selected, and three slides for each specimen were counted. The staining extent was scored from 0 to 3 based on the percentage of positive cells (0, <1%; 1, 1–33%; 2, 34–66%; 3, 67–100%). The intensity of staining was classified as follows: 0 point, no staining; 1 point, weak staining (light yellow); 2 points, moderate staining (brown); and 3 points, strong staining (yellowish brown). The final score of CCNG2 expression was the product of the CCNG2 expression rate and intensity, graded as 0 for negative, + for 1–3 points, ++ for 4–6 points, and +++ for 7–9 points. As for the negative control, the primary antibody was replaced with PBS. When there were discrepancies between the two pathologists, the average score was used.

2.3. Animals and cell lines

Female BALB/c nu/nu mice (6–8 weeks old, Shanghai Institute of Material Medicine, Chinese Academy of Science) were housed in specific pathogen-free conditions. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). Human lung cancer cell lines (H1299, A549, HCC827 and H157) and normal bronchial epithelial HBE cell line were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator containing 5% CO_2.

2.4. Construction of TIPE2 overexpressing cell lines

Full-length human TIPE2 was generated from human PBMC cDNA by PCR and cloned into pcDNA3.1 vector. H1299 cells or A549 cells were seeded equably in a six well plate at the density of 5×10^5/mL. After cell attachment, the recombinant plasmid pcDNA3.1-TIPE2 and Mock plasmid were transferred into HCC827 or A549 cells using Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were diluted at the ratio of 1:15 into 12-well plate and the medium was changed with medium containing G418 (700 μg/mL). The stable cell lines were obtained after 28 days screening by G418. The effects of pcDNA3.1-TIPE2 were confirmed by the expression of TIPE2 protein using western blot.

2.5. Western blotting

About 20 μg of total protein was extracted and separated by 10% SDS–PAGE, transferred onto polyvinylidene fluoride membranes, and then reacted with primary antibodies against TIPE2 (1:200), Bcl-2 (1:500), Bcl-XL (1:500), Bax (1:500), Bak (1:500), Caspase-9 (1:500), Caspase-3 (1:500), N-cadherin (1:500), E-cadherin (1:500) and β-actin (1:1000) (all from Santa Cruz, CA, USA). After being extensively washed with PBS containing 0.1% Triton X-100, the membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody for 30 min at room temperature. The bands were visualized using 1-step TM NBT/BCIP reagents (Thermo Fisher Scientific, Rockford, IL) and detected by an Alpha Imager (Alpha Innotech, San Leandro, CA).

2.6. Cell viability assay

Cells were seeded into 96-well plates at 5×10^3 cells per well and cultured for indicated time points. Cell viability was evaluated using CCK8 (Beyotime, Haimen, China) according to manufacturer's instructions. OD values were read using a microplate reader (Bio-Tek Company, Winooski, VT, USA) at the 450-nm wavelength. Each time point was repeated in three wells and the experiment was independently performed for three times.

2.7. Colony formation assay

Cells were seeded in six-well plates at a density of 1500 cells per well for 12 days and then fixed with 20% methanol and stained with 1% crystal violet. Colonies that is consisted of more than 50 cells were counted and calculated as a percentage of that of the control group. The experiment was independently performed for three times.

2.8. Transwell assay

Cell invasion were analyzed in 24-well Boyden chambers with 8-μm pore size polycarbonate membranes (Costar, Acton, USA). The membranes were precoated with 50 μg Matrigel (BD Biosciences, San Diego, USA) to form matrix barriers. 1×10^5 cells were resuspended in 100 μl serum-free DMEM and placed in the upper chamber, and the lower compartments were filled with 700 μl DMEM with 10% FBS. After 24 h incubation, the cells on the upper surface of the membrane were removed with a sterile cotton swab, and cells on the lower surface of the membrane were fixed and stained with crystal violet and counted under a light microscope at 200× magnification. Six random screens were selected for each sample, and the experiment was repeated three times.

2.9. In vivo assays for tumor growth

For evaluation of the tumor growth *in vivo*, 1×10^6/0.1 mL cells were injected subcutaneous into the flank region of 6–8 week-old female nude mice. Tumor growth was monitored every 5 days and tumors were measured with fine digital calipers and tumor volume was calculated by the following formula: tumor volume = $0.5 \times \text{width}^2 \times \text{length}$.

2.10. Statistical analysis

Statistical analysis was performed with SPSS 16.0 for Windows (SPSS, Chicago, IL). Pearson chi-square test was used to examine possible correlations between TIPE2 expression and clinicopathological factors. Student's *t* test was used to compare other data.

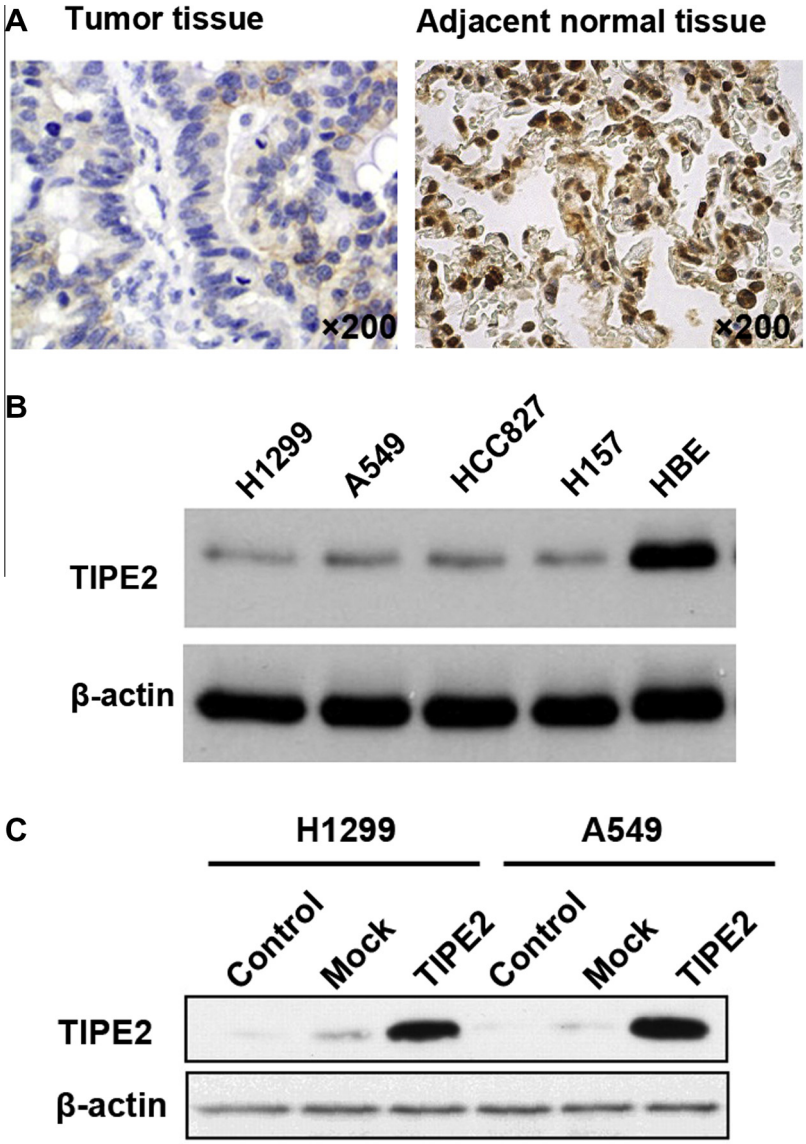

Fig. 1. Expression of TIPE2 on NSCLC patients and lung cancer cell lines. (A) Representative photographs of TIPE2 protein expression in NSCLC tissues and adjacent normal lung tissues (Magnification 200×). (B) TIPE2 protein expression in lung cancer cell lines. (C) TIPE2 protein expression in two NSCLC cell lines (H1299, A549) were examined by western blot after transient transfection with Mock and TIPE2 plasmid.

Table 1
Expressions of TIPE2 in NSCLC tissues and in normal lung tissues.

Groups	Cases	Expression of TIPE2				χ^2	*P* value
		–	+	++	+++		
Tumor tissues	96	70	14	8	4	19.393	0.000
Normal tissues	40	13	7	8	12		

All *P* values were two sided and $P < 0.05$ was considered as statistical significance.

3. Results

3.1. TIPE2 is down-regulated in lung tumor tissues and correlates with poor prognosis in NSCLC patients

We first analyzed TIPE2 protein expression in 96 NSCLC specimens and 40 corresponding normal tissues by immunohistochemistry. In NSCLC tissues, TIPE2 staining was negative or weak. In normal lung tissues, TIPE2 staining ranged from light yellow to brown. Statistically, TIPE2 was expressed in 27.08% (26/96) of NSCLC tissues, which was significantly lower than the 67.5% (27/40) in normal tissues ($P < 0.001$) (Fig. 1A and Table 1). We then analyzed the relationship between the TIPE2 expression and clinical characteristics. As shown in Table 2, there was no significant correlation of TIPE2 expression to age ($P = 0.906$), gender ($P = 0.155$), histological type ($P = 0.942$) and differentiation ($P = 0.853$). However, low expression TRIM31 was significantly correlated with primary tumor status ($P = 0.043$) and advanced TNM stage (I versus II + III, $P = 0.006$).

3.2. TIPE2 inhibits cell proliferation, colony formation and invasion in lung cancer cell lines

We first examined the expression of TIPE2 in a panel of human lung cancer cell lines by western blotting. As shown in Fig. 1B, the TIPE2 protein expression in lung cancer cell lines (H1299, A549, HCC827 and H157) was lower than that in normal HBE cells. We then generated TIPE2 overexpression cell lines by transfecting TIPE2 plasmid into H1299 and A549 cell lines. As shown in Fig. 1C, western blotting analysis showed that TIPE2 protein displayed significant upregulation in overexpression group compared to the control and Mock group.

Using these stably transfection cell lines, we next evaluated the role of TIPE2 on lung cancer cells proliferation, colony formation and invasion *in vitro*. As shown in Fig. 2A and B, Time course CCK-8 assay revealed that overexpression of TIPE2 led to a significant reduction of the proliferation rate from 72 h and colony formation compared with Mock cell in both H1299 and A549 cells. In addition, overexpression of TIPE2 markedly reduced the invasion of H1299 and A549 cells compared with that of Mock (Fig. 2C).

3.3. TIPE2 inhibits NSCLC progression in vivo

We next used a subcutaneous xenograft NSCLC model to elucidate the effects of TIPE2 on NSCLC progression *in vivo*. We found that treatment with TIPE2 plasmid remarkably attenuated the subcutaneous tumor growth (Fig. 3A and B). The mean tumor weight in TIPE2 group was also significantly lower than those of Mock group (Fig. 3C and D).

3.4. TIPE2 inhibits expression of Bcl-XL and N-cadherin

To investigate the potential mechanism by which TIPE2 affected cell proliferation and invasion, we explored the change of cell proliferation and invasion-related molecules in cells with TIPE2 transfection. We observed a remarkable decrease in Bcl-XL and significant increase in Bax, cleaved Caspase-9 and Caspase-3 protein levels after TIPE2 transfection, while no significant change were observed in Bcl-2 and Bak level (Fig. 3E). Moreover, TIPE2 transfection significantly reduced N-cadherin level and increased E-cadherin protein levels compared to Mock cells (Fig. 3E).

Table 2
Association between TIPE2 expression and clinicopathological characteristics of 96 NSCLC patients.

Characteristics	Cases (96)	TIPE2 expression		*P* value*
		+ ~ +++ (26)	– (70)	
Age (years)				
⩽60	60	16	44	
>60	36	10	26	0.906
Gender				
Male	52	11	41	
Female	44	15	29	0.155
Histological type				
Adenocarcinoma	67	18	49	
Squamous cell carcinoma	29	8	21	0.942
Tumor status				
T1–T2	62	21	41	
T3–T4	34	5	29	**0.043**
Differentiation				
Well-moderate	41	12	29	
Poor	55	14	31	0.853
TNM stage				
I	41	17	24	
II–III	55	9	46	**0.006**

Abbreviations and note: TNM, tumor-node-metastasis.
* Pearson's χ^2 test.

4. Discussion

Immune homeostasis is maintained by multiple immune regulation genes that are unable to compensate for each other [10]. Two classes of immune homeostasis-maintaining genes have been identified. The first class genes have functions in limiting the strength of immune cell activation and expansion, such as TGF-β, IL-10, CTLA4 and FoxP3. The second class controls cell death and apoptosis, including Fas, Bim, Bax and Caspases 8 and 10. Increasing evidences have found that breakdown of genes that maintain immune homeostasis by controlling cell death contributed to tumor pathogenesis [4]. TIPE2 was reported as an potent negative regulator of innate and cellular immunity, shared considerable sequence homology with members of the TNFAIP8 family. Moreover, similar to other regulators of cell death and apoptosis, TIPE2 also contains a death effector domain (DED) or DED-like domain, and is able to regulate caspase-mediated apoptosis [11]. In addition, the other TNFAIP8 family members, TNFAIP8 or TIPE1, were described as an oncogene in human cancers, such as breast cancer, lung cancer and HCC [12,13]. Taken together, we hypothesized that the immune regulation gene TIPE2 may also involve in tumor carcinogenesis.

In the present study, by using tissue immunohistochemistry, we confirmed for the first time that the expression of TIPE2 was markedly downregulated in NSCLC samples compared with normal lung tissues, TIPE2 downregulation was significantly correlated with advanced TNM stage, suggesting that low expression of TIPE2 may be associated with metastasis of NSCLC. Moreover, we confirmed that TIPE2 expression was lower in lung cancer cell lines compared with normal bronchial epithelial cell line, which was in accord with our immunohistochemical results.

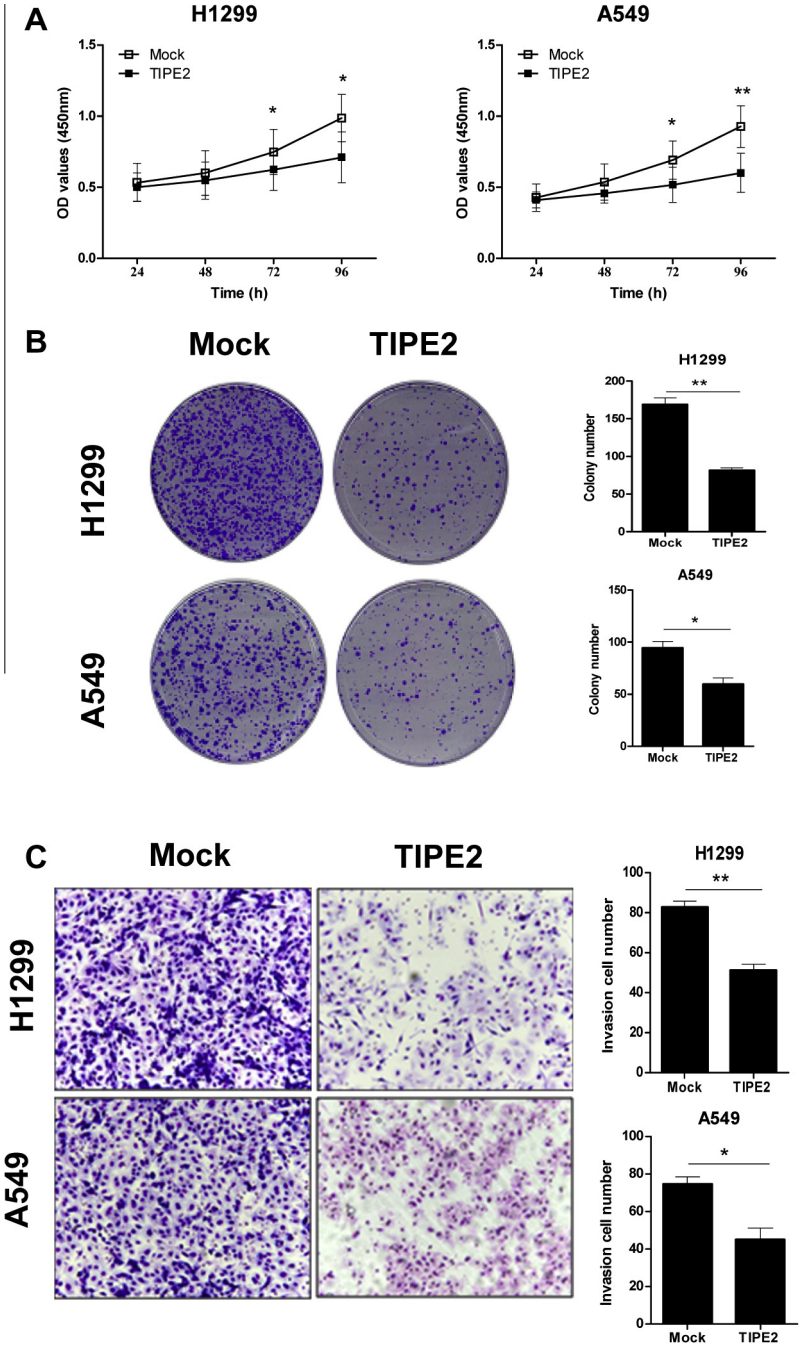

Fig. 2. The role of TIPE2 on cell proliferation, colony formation and cell invasion of NSCLC cell lines. (A) Cell viability was assessed by CCK8 assay at indicated time points. (B) Colony formation assay was performed and colonies that consisted of more than 50 cells were counted. (C) The effect of TIPE2 on cell invasive ability was analyzed by Transwell invasion experiment. Data shown are mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Mock cells.

In addition, we employed plasmid to upregulate TIPE2 expression in both H1299 and A549 cell lines with low endogenous TIPE2 levels to explore the biological roles of TIPE2 in lung cancer cells. We found that decreased cell growth rate, impaired colony formation ability and reduced cell invasive capacity of both H1299 and A549 cells after TIPE2 transfection. More importantly, the restoration of TIPE2 expression in NSCLC cells markedly suppresses tumor growth in subcutaneous xenograft tumor model. These data was in accord with previous reports that TIPE2 could inhibit malignant cell proliferation, colony formation and metastasis in cancer cell lines [6,7,9]. Taken together, our results demonstrated that TIPE2, unlike other members of TNFAIP8 family as oncogene in various cancers, severs as a tumor suppressor in NSCLC. However, in patients with renal cell carcinoma (RCC), TIPE2 mRNA expression was significantly upregulated compared with the healthy controls, and TIPE2 mRNA overexpression was correlated with TNM staging [14], suggesting that the expression pattern of TIPE2 may depend on the cancer type and different pathological conditions, the correlation between TIPE2 expression and tumorigenesis in tumor patients requires further investigation.

The molecular mechanisms by which TIPE2 inhibits cancer cell proliferation and metastasis remain unclear. Previous study showed that murine TIPE2 is associated with Caspase-8 and inhibits activating protein (AP)-1 and nuclear factor (NF)-κB activation

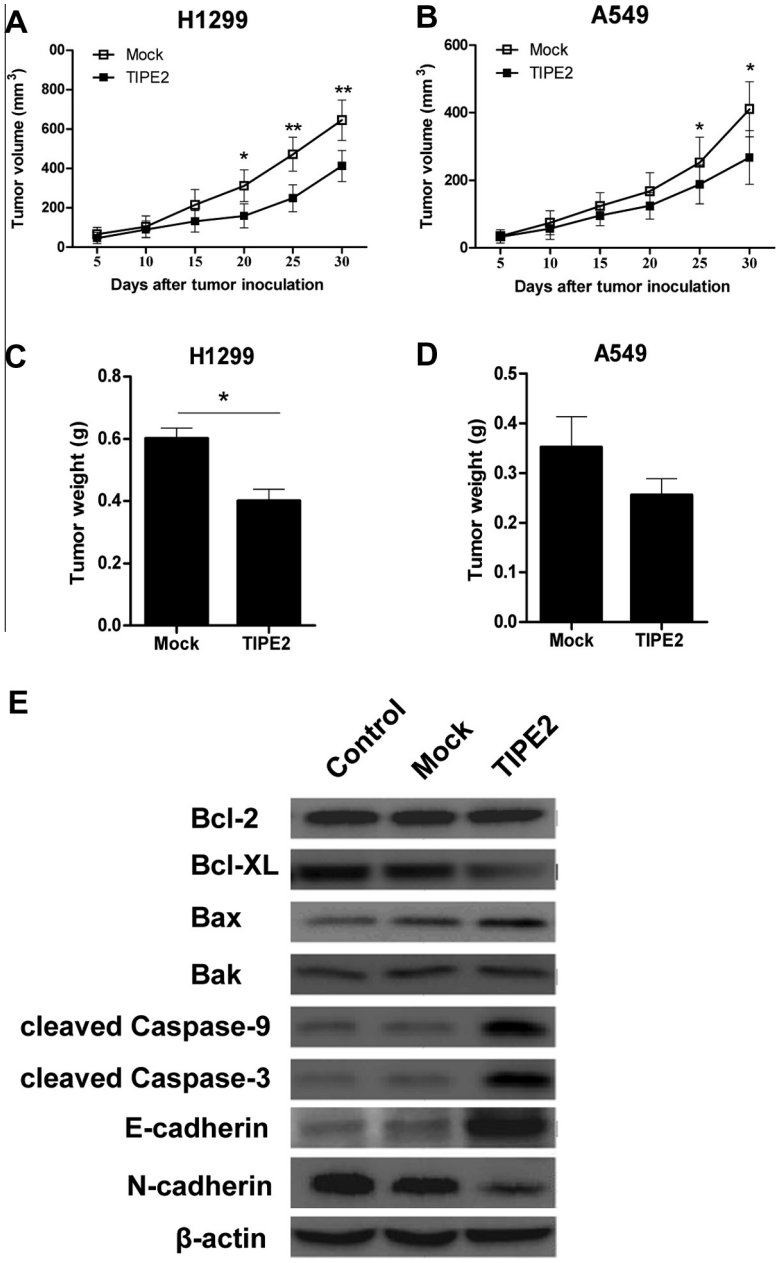

Fig. 3. The effects of TIPE2 on *in vivo* cell growth in subcutaneous xenograft model. (A and B) The growth curves of tumors in Mock and TIPE2 groups. (C and D) The mean tumor weight in Mock and TIPE2 groups. (E) The effect of TIPE2 on Bcl-2, Bcl-XL, Bax, Bak, Caspase-9, Caspase-3, E-cadherin and N-cadherin protein expression levels. Data are shown as mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Mock cells.

while it promotes Fas-induced apoptosis [4]. TIPE2 also binds to Ras-interacting domain of the RalGDS family of proteins to prevent Ras from forming an active complex [6]. In human tumor, TIPE2 was reported to inhibit the HCC migration and invasion through targeting Rac1 and reduces F-actin polymerization and expression of matrix metallopeptidase 9 (MMP9) and urokinase plasminogen activator (uPA) [7]. TIPE2 can also inhibit HCC metastasis via Erk1/2 downregulation and NF-κB activation [9]. However, the underlying mechanisms of TIPE2 in NSCLC progression remain largely unknown. Here, we examined the expression of cell apoptosis-related protein (Bcl-2, Bcl-XL, Bax, Bak, Caspase-9 and Caspase-3) and EMT (epithelial mesenchymal transition) related protein (E-cadherin and N-cadherin).

We found that TIPE2 overexpression decreased Bcl-XL protein expression and increased Bax expression, unregulated Caspase-9 and Caspase-3 cleavage and activity. Bcl-XL and Bax are both the members of Bcl-2 family, which has either pro-or anti-apoptotic activities by controlling the permeabilization of the outer mitochondrial membrane [15]. The ratio of BCL-XL protein, an inhibitor of apoptosis, to Bax protein, an inducer of apoptosis, determines survival or death after an apoptotic stimulus [16]. Our results indicated that the tumor suppressor role of TIPE2 may be partially responsible for the reduced levels of intrinsic anti-apoptotic signaling pathway. However, a question remains concerning the mechanisms involved in the tumor suppressor role of TIPE2 in tumor growth *in vivo*. In this study, we used xenograft tumor model performed in immune-incompetent mice to clarify the effects of TIPE2 on NSCLC progression *in vivo*. However, this tumor model could not tell us whether TIPE2 inhibits tumor development through regulating immune cell activation and expansion. Thus, further study is

required in the future to investigate the association between the immune system and TIPE2 in immune-competent mice.

Accumulating evidence shows that epithelial–mesenchymal transition (EMT) is involved in the progression and metastasis of various cancers [17,18]. The EMT is associated with the progressive redistribution or downregulation of apical and basolateral epithelial cell-specific tight, such as E-cadherin and cytokeratin, and the novel expression of mesenchymal molecules, such as N-cadherin and vimentin [19]. In the present, we found that TIPE2 transfection significantly reduced N-cadherin level and increased E-cadherin protein levels compared to Mock cells, suggesting that TIPE2 may inhibit the metastasis and invasion of NSCLC through a switch from N-cadherin to E-cadherin.

In conclusion, our study demonstrated that TIPE2 was downregulated in NSCLC and its downregulation was correlated with advanced TNM stage of NSCLC patients. Moreover, our results showed that TIPE2 overexpression inhibited lung cancer cell proliferation, colony formation and cell invasive *in vitro*, and prevented lung tumor growth *in vivo*, possibly through inhibiting Bcl-XL and N-cadherin expression. Given these findings, TIPE2 might serve as a potential tumor suppressor in lung cancer, and future studies are needed to investigate its molecular mechanisms.

Conflict of interest statement

The authors have no conflict of interest.

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