Tumorigenesis and Neoplastic Progression

Decreased TIP30 Expression Promotes Tumor Metastasis in Lung Cancer

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The HIV Tat-interacting protein (TIP30), also called CC3 or HTIP2, is encoded by *Tip30*, a putative tumor-suppressor gene located on human chromosome 11p15.1. In this study, we investigated the role of TIP30 in the progression and metastasis of lung cancer. TIP30 expression was analyzed in 206 paired lung cancers and adjacent non-tumor tissues, as well as in 70 matched lymph node metastases using a high-density tissue microarray. Results were compared with the clinicopathologic features of the patients from whom the tissues were taken. Low TIP30 expression levels were found in all 9 cases of small cell lung cancer and in 36.5% (72/197) of non-small cell lung cancer, which were correlated with lymph node metastasis in non-small cell lung cancer and with poor differentiation and advanced stage of tumor cells in squamous cell carcinoma. The immunostaining scores were significantly lower in the metastatic lesions than in the primary lesions. Down-regulation of TIP30 by a short hairpin RNA enhanced cell survival, migration, and invasion through Matrigel *in vitro*, and promoted lung metastasis and vascularization in nude mice. Further studies revealed that the down-regulation of TIP30 enhanced the expression of osteopontin, as well as matrix metalloproteinase-2 and vascular endothelial growth factor. Our results suggest that the down-regulation of TIP30 promotes metastatic progression of lung cancer, hence it could serve as a potential target for the development of lung cancer therapies. (Am J Pathol 2009, 174:1931–1939; DOI: 10.2353/ajpath.2009.080846)

Lung cancer is one of the leading causes of death worldwide.^{1,2} In China, about 300,000 new cases of lung cancer, causing more than 250,000 deaths, occur annually.² The prognosis for lung cancer remains poor despite of the improvements made over the years to its management and treatment. The overall survival of patients with lung cancer remains at a dismal 15% because of metastasis and the development of resistance to chemotherapy.¹ Therefore, it is important to better understand the molecular mechanisms of lung cancer progression and find more effective targets for improving the outcomes for patients with lung cancer.

The 30-kDa HIV Tat-interacting protein (TIP30), also called CC3 or HTIP2, is a tumor suppressor with proapoptotic and anti-metastasis properties.^{3–5} The *Tip30* gene is located on human chromosome 11p15.1. It was originally identified by a differential display analysis of mRNA from the highly metastatic human variant small cell lung cancer (SCLC) versus less metastatic classic SCLC cell line.³ The expression of TIP30 is mainly located in cytoplasm.⁶ Decreased TIP30 expression has been detected in some tumor cells, such as melanoma, breast cancer, neuroblastoma, glioblastoma, colon cancer, and hepatocellular carcinoma.^{3,6–9} About 24% of various types of cancer cells had *Tip30* missense mutation in exon 3 by comparing the *Tip30* cDNA sequences in National Center for Biotechnology Information databases.⁶ G134V mutation derived from liver cancer significantly shortened the half-life of TIP30 protein. Besides gene mutations, TIP30 expression was recently found

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frequently down-regulated by promoter hypermethylation in liver cancer.¹⁰

TIP30 was considered to have tumor suppressor activity by inhibiting tumor growth,¹¹ invasion,^{3,11} and angiogenesis,⁹ and by inducing apoptosis.^{3,11,12} Depletion of *Tip30* predisposed mammary epithelial cells to neoplastic transformation,¹³ and *Tip30*-deficient mice spontaneously developed various tumors at high incidence as compared with wild-type mice.⁶ G134V and R106H mutations identified in liver cancer not only abrogated the tumor suppressor potential but also gained oncogenic activities and promoted cell growth and invasion, and inhibited cisplatin-induced apoptosis through up-regulation the expression of N-cadherin and c-MYC, and down-regulation the expression of p53 and E-cadherin.^{6,14} It has been proved that TIP30 acts as a transcription cofactor and regulates expressions of genes involved in apoptosis, cell growth, and metastasis. TIP30 was shown to interact with human immunodeficiency virus-1 Tat and enhance Tat-activated transcription.⁴ TIP30 was also found to interact with estrogen receptor α -interacting coactivator and negatively regulate estrogen receptor α -mediated *c-myc* expression.¹⁵ Recently TIP30 was found to interact with Ets-1 and inhibit osteopontin (OPN) transcription.¹⁶

Recent studies have linked TIP30 to metastatic progression of several different cancer types. Introduction of TIP30 into v-SCLC cells suppressed metastasis in *SCID-hu-L* mice.³ The expression of TIP30 was inversely associated with axillary lymph node metastasis and vascular invasion in breast cancer.¹⁷ The conditioned media from CC3-expressing tumor cells greatly inhibited the proliferation and migration of endothelial cells *in vitro*.⁹ Our recent studies showed that TIP30 inhibited tumor metastasis through suppressing the expression of OPN in human hepatocellular carcinoma.¹⁶

The role of TIP30 in the development and progression of lung cancer has not been fully characterized. In the present study, TIP30 expression was examined by immunohistochemistry in 206 lung carcinoma tissues, including 197 non-small cell lung cancers (NSCLC) and 9 SCLCs, and adjacent non-tumor tissues, as well as 70 matched lymph node metastases using a high-density tissue microarray. The correlations of TIP30 expression with tumor stage, histological grade, and lymph node metastasis were evaluated. The effects of TIP30 on tumor cell survival, invasion, and angiogenesis were assessed both *in vitro* and in nude mice.

Materials and Methods

Patients and Tissue Microarray

Tissue samples were obtained from 206 patients with lung cancer without chemotherapy in Changhai Hospital, Shanghai, P. R. China, from January 2001 to June 2006. The use of all of the human samples and the experimental procedures for this study were reviewed and approved by the university and hospital ethics committees. The specimens consisted of 197 tumors of NSCLC and 9 SCLC, as well as corresponding adjacent non-tumor lung

tissues taken from the same patients; 70 (62 NSCLC and 8 SCLC) matched lymph nodes with metastatic tumors were also collected.

Tissue microarrays were constructed as previously described.¹⁸ Briefly, tissues were stained with H&E to identify viable and representative areas of the specimen. From the defined areas, core biopsies were taken with a Tissue Arrayer (Beecher Instruments, Silver Spring, MD). Duplicate 2-mm tissue cores were used to construct the tissue microarrays. Four tissue microarray blocks were constructed. Array blocks were sectioned to produce serial 4- μ m sections, and the first section was stained with H&E to assess adequacy.

Antibody Generation

Bacterially produced glutathione S-transferase fusion proteins were used in the production of anti-human TIP30 antibody. The fusion proteins were injected subcutaneously into rabbits to get antiserum and the antibody purification was performed by using Protein A Sepharose CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ), according to manufacturer's instructions. The specificity of the polyclonal antibody was demonstrated by Western blot and immunohistochemical analysis.

Immunohistochemical Staining

The expressions of TIP30 protein in the specimens were detected by immunohistochemistry assay with a polyclonal antibody against human TIP30 as described previously.¹⁷ For antibody control, one set of samples was incubated with non-immune rabbit IgG (1:150) instead of primary antibody.

Evaluation of TIP30 staining was independently performed by two experienced pathologists. The intensity of TIP30 immunostaining was semiquantitatively estimated according to the signal intensity and distribution. Briefly, a mean percentage of positive tumor cells was determined in at least five areas $\times 400$ magnification and assigned to one of the five following categories: 0, $<5\%$; 1, 5% to 25% ; 2, 25% to 50% ; 3, 50% to 75% , and 4, $>75\%$. The intensity of immunostaining was scored as follows: 1, weak; 2, moderate and 3, intense. For tumors that showed heterogeneous staining, the predominant pattern was taken into account for scoring. The percentage of positive tumor cells and the staining intensity were multiplied to produce a weighted score for each case. Tissues with immunohistochemical scoring ≤ 4 were considered as having low expression, and scores of 5 to 12 were considered high expression.

Cell Culture and Lentiviral Infection

Human lung cancer cells (A549, NCI-H460, SK-MES-1, LTP-a-2, H1299) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified incubator at 37°C in $5\% \text{CO}_2$ atmosphere. Infection of lentivirus encoding *Tip30* cDNA and short hairpin (sh)RNA targeting *Tip30* was performed

as reported previously.¹⁶ Briefly, the double-strand oligo DNAs for *Tip30* were as follows: top strand, 5'-CACCGAT-GGAAGTCTGGAGAACAATCAAGAGTTGTTCTCCAGCA-GTTCCATC-3', and bottom strand, 5'-AAAAGATGGA-ACTGCTGGAGAACAACCTCTTGATTGTTCTCCAGCAGTTC-CATC-3'). For negative control, we used scramble shRNA, top strand, 5'-CACCGAATTCTCCGAACGTGTCACGTTCAAGAGACGTGACACGTTCCGAGAATT-3' and bottom strand, 5'-AAAAAATTCTCCGAACGTGTCACGTCTCTTG-AACGTGACACGTTCCGAGAATT-3'. pENTR-U6-shRNA plasmid was recombined with destination vectors pLenti6/BLOCKiT-DEST vector to generate the shRNA constructs. For construction of lentiviral vector expressing human *Tip30* gene, *Tip30* cDNA was amplified and subcloned to pLenti6/V5-TOPO vectors (Invitrogen, Carlsbad, CA).

For virus production, HEK-293T cells were co-transfected with the resulting vector described above and ViraPower Packaging Mix (Invitrogen) using Lipofectamine 2000 according to the manufacturer's guidelines. Infectious lentiviruses were harvested and concentrated and the infectious titer was determined by counting the blue-stained colonies after crystal violet staining in 293 cells.

Lung cancer cells were infected with concentrated virus at a multiplicity of infection of 20 in the presence of 8 μ g/ml polybrene (Sigma-Aldrich, St. Louis, MO). Supernatant was removed after 24 hours and replaced with complete culture medium. Seventy-two hours after infection, the expressions of TIP30 were confirmed by Western blot.

Quantitative Reverse-Transcription PCR and Western Blot

Expression of *Tip30* mRNA was determined by quantitative reverse-transcription (qRT-PCR) using the LightCycler system (Roche, Mannheim, Germany) as described previously.¹⁷ *Actin* was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The primers used for PCR were as follows: *Tip30*: sense 5'-TCACCTTCGACGAGGAAGCT-3'; anti-sense 5'-GCTCTGCAGACTTCAGACCA-3'; *Actin*: sense 5'-CGTGGACATCCGTAAAGACC-3'; antisense 5'-ACATCTGCTGGAAGGTGGAC-3'.

For Western blot, the cells were lysed in RIPA buffer. Proteins at the same amount were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After probing with antibodies, the signals were visualized by Supersignal enhanced chemiluminescence reagent (Pierce, Rockford, IL). The antibodies used were anti-osteopontin (R&D Systems, Minneapolis, MN), anti-matrix metalloprotein (MMP)-2, and anti-vascular endothelial growth factor (VEGF) (Boster Biotechnology, Wuhan, P. R. China), and anti-glyceraldehyde-3-phosphate dehydrogenase (KangChen Bio-tech, Shanghai, P. R. China).

Cell Growth Assays

Cell growth was measured by 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

(MTS) assay (Promega, Madison, WI) in 96-well plates (1000 cells per well) following the instructions of the manufacturer. Each experiment was done in triplicate and repeated three times.

For anchorage-independent growth assays, the cells in single-cell suspension were plated in 0.3% agarose over a 0.6% agarose bottom layer at a density of 200 cells per well in 24-well plates and incubated for 14 days and stained. Colonies with a diameter greater than 100 μ m were counted.

Wound-Healing and Matrigel Invasion Assays

For wound-healing assays, the cells were first seeded in 6-well culture plates. A wound was made in the confluent monolayer with a plastic pipette tip and the migration of the cells at the wound front was photographed using an inverted microscope at indicated times after the scratch.

Cell invasion assays were quantified *in vitro* using Transwell chambers with polycarbonate membrane filters (8 μ m pore size; Corning, NY) coated with a Matrigel (Sigma) according to the manufacturer's instructions. In brief, the lower chamber was filled with 0.6 ml medium containing 20% fetal bovine serum, and 0.2 ml of medium that contained 3×10^5 cells under serum-starved conditions was plated in the upper chamber in triplicate wells and incubated at 37°C for 72 hours. Then cells attached to the upper side of the membrane were removed gently with a cotton swab and rinsed. The cells that migrated through the membrane and attached to the bottom of the membrane were fixed and stained with crystal violet. The number of cells invading through Matrigel was counted by randomly selecting five visual fields, and the extent of invasion was expressed as the average number of cells per microscopic field at a magnification of $\times 200$. All experiments were performed for three times. Two independent investigators were blinded when reading the assays for wound-healing and Matrigel invasion.

Detection of Apoptosis

Nuclear morphology was assessed using Hoechst staining. Four days after lentivirus infection, cells were deprived of serum for 72 hours and fixed with 70% ethanol and labeled with Hoechst 33342 (Sigma) for 10 minutes. Apoptotic cells were distinguished by their characteristic patterns of nuclear condensation, cytoplasmic rounding, and membrane blebbing. The morphological aspect of nuclei was observed with Olympus IX71 fluorescence microscopy (Olympus, Shinjuku Monolith, Tokyo, Japan) by using UV light excitation.

In situ apoptosis assay was performed with the Fluorescein FragEL DNA Fragmentation Detection Kit (Calbiochem, San Diego, CA). Formalin-fixed paraffin sections were deparaffinized and incubated with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) reaction mixture. Apoptotic cells carrying DNA labeled with fluorescein isothiocyanate-dUTP were observed under fluorescence microscope.

Figure 1. Immunohistochemistry analysis of TIP30 expression in alveolar epithelial cells (A), ciliated and basal epithelial cells (B), matched pairs of lung cancer (upper) and metastatic lesions (lower) in SCC (C and D), AC (E and F), and SCLC (G and H). Positive control and negative control were shown in I and J. (original magnification $\times 400$).

Animal Experiments

Female BALB/c nude mice and female severe combined immunodeficient/beige mice at 6 weeks old, were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, P. R. China). A549 cells were infected with lentiviral (LV)-sh*Tip30* or LV-shNon, whereas H1299 cells were infected with LV-*Tip30* or LV-green fluorescent protein (GFP). Viability of cells was determined by trypan blue exclusion staining 4 days after infection. For the tumorigenicity assay, viable cells (1×10^7) were injected subcutaneously into the right flank of each mouse (6 mice/group). The tumor volume was calculated using the equation $V (\text{mm}^3) = a \times b^2/2$, where a is the largest dimension and b is the perpendicular diameter. For tumor metastasis analysis, female severe combined immunodeficient/beige mice (for H1299) and female nude mice (for A549) were inoculated with 1×10^6 viable cells in 200 μl of phosphate buffered saline via tail vein injection as described previously.¹⁹ Four weeks later, mice were sacrificed and examined for development of pulmonary metastasis under microscopy.

Analysis of Tumor Vascular Density

Tumor microvessel density (MVD) was quantified using sections immunostained for CD31 (BioLegend, San Diego, CA) by two investigators independently and in a blinded manner according to the method described previously.²⁰ The areas with the greatest density of CD31-positive endothelial cells were designated "hot spots." The whole section was scanned at low power ($\times 40$) to identify the best fields for counting. Counting was performed on five separate fields within a hot spot at $\times 200$ magnification. Each stained endothelial cell or cell cluster was counted as one microvessel. If two or more CD31-positive foci appeared to belong to a single continuous vessel, this was counted as one microvessel. The mean vessel count from these fields was used for MVD scoring.

Statistic Analysis

Pearson χ^2 tests were used to evaluate the relationship between the expression of TIP30 and clinicopathologic

variables, as well as the TIP30 expression levels between primary and metastatic lesions. Comparisons between groups of related samples were assessed with the Wilcoxon paired-sample test. All other *in vitro* assay results represent the arithmetic mean $\pm$ SE of triplicate determinations of at least two independent experiments done under the same conditions. Student's *t*-test was used to determine the differences between groups and $P < 0.05$ was considered as statistically significant. All statistical tests were two-sided. Calculations were done with the Statistical Package for the Social Sciences version 13.0 (SPSS, Inc., Chicago, IL).

Results

Expression of TIP30 in Lung Cancers and the Correlations with Clinicopathologic Features

The expressions of TIP30 were examined in 206 lung cancers and adjacent non-tumor tissues in a tissue microarray using anti-human TIP30 polyclonal antibody. In the non-tumor tissues, immunopositive staining of TIP30 was detected in the cytoplasm of alveolar epithelial cells (Figure 1A), as well as in ciliated and basal epithelial cells in bronchial mucosa (Figure 1B). Weak immunostaining pictures of TIP30 was found in 72 of 197 (36.5%) cases of NSCLC, with 33/90 (36.7%) in squamous cell carcinoma (SCC) (Figure 1C) and 39/107 (36.4%) in adenocarcinoma (AC) (Figure 1E). Low expression of TIP30 was found in all of the 9 cases of SCLC (Figure 1G). Statistical analysis indicated that the immunostaining scores in the adjacent non-tumor tissues were significantly higher than the scores in tumor tissues in lung cancer ($P < 0.001$).

Lymph node metastases were found in 8 out of 9 cases of SCLC, in which TIP30 expressions were almost undetectable (Figure 1, H–J). The expression levels of TIP30 in the primary lesions were inversely associated with lymph node metastasis in SCC and AC ($P = 0.033$; $P = 0.038$) (Table 1). Moreover, when TIP30 expression in the lymph node metastatic lesions was compared with that in the paired primary lesions, in 62 cases of NSCLC, 43 cases showed equal TIP30 expression, 4 cases showed increased TIP30 expression, and 15 cases showed de-

Table 1. The Association between TIP30 Expression and Clinicopathological Characteristics

Clinicopathological characteristics	AC (n = 107)			SCC (n = 90)		
	Low expression	High expression	P value	Low expression	High expression	P value
Age (years)						
<60	21	34	0.702	14	20	0.489
≥60	18	34		19	37	
Sex						
Male	39	68	0.985			0.827
Female	19	33		30	51	
Tumor size(cm)						
<3	39	68	0.555			0.921
≥3	16	24		9	15	
Lymph node involvement						
-	23	44	0.038*	24	42	0.033*
+	16	42		17	42	
Stage						
I-II	23	26	0.411	16	15	0.012*
III-IV	39	68		20	48	
Differentiation						
Well differentiated	18	37	0.123	13	9	0.005**
Moderately differentiated	3	9		3	18	
Poorly differentiated	15	36		21	35	
	21	23		9	4	

* $P < 0.05$; ** $P < 0.01$.

creased TIP30 expression in metastatic lesions (Figure 1, D and F). The immunostaining scores in the metastatic lesions were significantly lower than the scores in the primary lesions ($P = 0.001$, Table 2). These data suggest that decreased expression of TIP30 may promote tumor metastasis in lung cancer patients.

The correlation of TIP30 expression in the primary lesions and clinicopathologic features was further analyzed in NSCLC. There was no significant correlation between TIP30 expression and age, sex of patients, and tumor size, as determined by χ^2 test (Table 1). However, TIP30 expression was inversely associated with the stage of the disease ($P = 0.012$) and positively associated with the degree of differentiation of the tumor ($P = 0.005$) in SCC, which indicates low expression of TIP30 may be associated with the progression of SCC.

Down-Regulation of TIP30 Promotes Lung Cancer Cell Survival

Previous data suggest that decreased expression of TIP30 may play an important role in the development of lung cancer. To more rigorously explore this possibility, we detected the levels of *Tip30* mRNA and protein in five lung cancer cell lines (Figure 2A). The level of *Tip30* mRNA and protein was relatively high in the AC cell line

A549 and SCC cell line SK-MES-1, and decreased in the AC cell line H1299. We used lentivirus to knockdown the expression of TIP30 by shRNA targeting *Tip30* (sh*Tip30*) in A549 and SK-MES-1 cells or elevate it by lentivirus encoding *Tip30* cDNA (LV-*Tip30*) in H1299 cells (Figure 2B). LV-sh*Tip30* significantly promoted cell growth and anchorage-independent growth in A549 and SK-MES-1 cells as compared with LV-shNon or mock treatment, while LV-*Tip30* inhibited cell growth and anchorage-independent growth in H1299 cells (Figure 3A). The suppressive effect of TIP30 on lung cancer cell growth was confirmed by *ex vivo* assay. As shown in Figure 3B, down-regulation of TIP30 in A549 cells dramatically promoted tumor development in nude mice compared with LV-shNon control group, whereas introduction of TIP30 into H1299 significantly inhibited tumor growth.

The inhibitory effects of TIP30 on tumor growth were likely the result of induction of apoptosis as revealed by Hoechst staining. The percentage of apoptotic cells was significantly increased by LV-*Tip30* in H1299 cells, and decreased by LV-sh*Tip30* in A549 and SK-MES-1 cells under serum starvation (Figure 3C). Apoptosis was further examined by *in situ* TUNEL assay on A549 and

Table 2. Comparative Analysis of TIP30 Expression in Primary and Lymph Node Metastatic NSCLC Tissues

TIP30 expression in primary tumor	TIP30 expression in metastatic lymph node		P value
	Low expression	High expression	
Low expression	19	4	0.001**
High expression	15	24	

** $P < 0.01$.

Figure 2. A: Expression of TIP30 in lung cancer cells was analyzed by real-time PCR (qRT-PCR) (top) and Western blot (bottom). B: A549 and SK-MES-1 cells were infected with LV-sh*Tip30* and LV-shNon as control (top). H1299 cells were infected with LV-*Tip30* and LV-GFP as control (bottom). Expressions of TIP30 were detected by Western blot.

Figure 3. Down-regulation of TIP30 promotes lung cancer cell growth. **A:** Four days after virus infection, cells were seeded in 96-well plates for MTS assay (a) or in semisolid soft agar medium to monitor anchorage-independent growth (b). The numbers represented the mean of three independent experiments $\pm$ SD (* $P < 0.05$, ** $P < 0.01$). **B:** A549 cells infected with LV-shTip30 or LV-shNon and H1299 cells with LV-Tip30 or LV-GFP were injected subcutaneously into nude mice. Tumor volume of each group was scored every 4 days. Data represented the means $\pm$ SD (* $P < 0.05$, ** $P < 0.01$). **C:** Four days after lentivirus infection, cells were deprived of serum for 72 hours. Apoptosis was determined with Hoechst staining. Photographs are representative images from each group as indicated under fluorescence microscopy (left). The bar graph shows the average number of Hoechst staining cells for A549, SK-MES-1 and H1299 (right) from five microscopy views (* $P < 0.05$, ** $P < 0.01$). **D:** *In situ* TUNEL apoptosis analysis was performed in tumor sections derived from the same mice as in (B) at day 24 after cell inoculation. The apoptotic nuclei were seen as green color under fluorescence microscopy. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole and seen as blue (magnification = original $\times 400$). The percentage of apoptotic cells was calculated by counting green-stained nuclei versus blue-stained nuclei from six randomly chosen fields in each section. Data are presented as means $\pm$ SD (* $P < 0.05$).

Figure 4. Down-regulation of TIP30 enhances tumor metastasis and angiogenesis. **A:** Motilities of A549 and SK-MES-1 cells infected with LV-shTip30 or LV-shNon and H1299 cells infected with LV-Tip30 or LV-GFP were examined by an *in vitro* wound healing assay. Digital pictures were taken at 0 hours as well as at 24 hours, 48 hours, or 72 hours. **B:** Lung cancer cells were incubated with Matrigel for 72 hours. Invading cell numbers were determined by the average count of five random microscopic fields (**top**). Values shown are means $\pm$ SD (**bottom**). * $P < 0.05$, ** $P < 0.01$. **C:** (a) A549 cells infected with LV-shTip30 or LV-shNon and H1299 cells with LV-Tip30 or LV-shNon were injected into nude mice via tail vein. The numbers of lung metastatic foci in each group were calculated under microscope. Representative lung tissue sections (H&E stain; magnification = original $\times$ 200) from each group were presented. **Arrows** indicate metastatic tumors. (b) CD31 expression was examined in tumor sections derived from the same mice as in Figure 3B at day 24 after cell inoculation. **Arrows** indicate CD31-positive cells (magnification = original $\times$ 400) (**left**). The numbers of MVD were quantified (**right**). **D:** The expressions of OPN, MMP-2, and VEGF were examined in A549 or H1299 cells 4 days after lentivirus infection.

H1299 xenografted tumor tissues. The apoptotic nuclei, seen as green color under fluorescence microscopy, were greatly decreased in LV-shTip30 infected A549 xenografts than that in shNon controls. On the contrary, when elevating the level of TIP30 in H1299, the apoptotic cells were greatly increased (Figure 3D).

Down-Regulation of TIP30 Enhances Tumor Metastasis and Angiogenesis in Lung Cancer

To confirm the inhibitory effects of TIP30 on tumor metastasis observed in lung cancer tissues, the metastatic potentials of lung cancer cells were examined *in vitro* and in nude mice. A549 and SK-MES-1 cells infected with LV-shTip30 migrated rapidly and filled in the wound faster than LV-shNon infected cells. In contrast, H1299 cells infected with LV-Tip30 filled in the wound slower than LV-GFP infected cells (Figure 4A). Consistent with the data of wound-healing assay, the invasive abilities through Matrigel were significantly increased in LV-shTip30 infected A549 and SK-MES-1 cells, relative to that in LV-shNon infected cells, and decreased in LV-Tip30 infected H1299 cells relative to that in LV-GFP infected cells (Figure 4B).

The inhibitory effects of TIP30 on tumor metastasis in lung cancer cells were further examined in nude mice. Lung metastatic clusters presented in mice injected with LV-shTip30-infected A549 cells (117 ± 31.22 clusters per

mice) were significantly higher than that in LV-shNon group (73 ± 14.37 clusters per mice) ($P < 0.05$, Figure 4Ca). However, mice injected with H1299/LV-Tip30 cells exhibited a lower occurrence of lung metastases than those injected with H1299/LV-GFP cells (20 ± 13.28 vs. 59 ± 13.27 clusters per mice, $P < 0.01$, Figure 4Ca). These data suggest that loss of TIP30 may promote the motile and invasive abilities of lung cancer cells.

Angiogenesis is a prerequisite for advanced tumor growth and is logically believed to be an important factor in tumor metastasis. We evaluated tumor angiogenesis by down-regulation and overexpression of TIP30 in A549 and H1299 respectively. Tumor-associated neovascularization, as indicated by MVD, was determined by immunohistochemistry using anti-CD31 antibodies. As shown in Figure 4Cb, a significant increase in tumor MVD was observed by down-regulation of TIP30 in A549 xenografts as compared with control tumors. While in H1299 xenografts, a significant reduction of MVD was observed by introduction of TIP30. Thus, TIP30 negatively regulates tumor metastasis and angiogenesis.

We have previously reported that TIP30 inhibited tumor metastasis through inhibition of OPN expression, a key molecule involved in tumor metastasis in hepatocellular carcinoma.¹⁶ The expression of OPN on knock-down or introduction of TIP30 was examined in A549 and H1299, respectively. Consistent with the observation in hepatocellular carcinoma cells, TIP30 negatively regulated OPN

expression in lung cancer cells as well (Figure 4D). Previous studies have demonstrated that OPN enhanced tumor metastasis through induction of matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) expression.^{21,22} Concomitance with the expressions of OPN, the expressions of MMP-2 and VEGF were enhanced by down-regulation of TIP30 in A549 cells, and decreased by introduction of TIP30 in H1299 cells (Figure 4D). Thus, down-regulation of TIP30 might enhance tumor metastasis and angiogenesis through induction of OPN expression.

Discussion

Transformation of a normal phenotype into a malignant phenotype requires accumulation of multiple genetic and epigenetic changes, which result in growth and cellular survival advantage. Loss of tumor suppressor genes is one of the critical alterations in this multistep process.²³ In this study, the expression of TIP30, a novel tumor suppressor gene, was assessed for the first time in a large-scale study of clinical samples from lung cancers and adjacent non-tumor tissues. The expression of TIP30 was almost undetectable in all of the nine cases of SCLC, which is a deadly form of lung cancer and has the poorest survival rate of all histological types.^{24,25} Low expression of TIP30 was found in 36.5% of NSCLC, which was significantly associated with poor tumor differentiation. The significant reduction or absence of TIP30 protein expression in lung cancers underscored its importance in the development of lung cancer. *In vitro* experiments further demonstrated that down-regulation of TIP30 enhanced cell growth and anchorage-independent cell growth, which was related with inhibition of apoptosis.

The expression of TIP30 was found to be inversely associated with axillary lymph node metastasis and vascular invasion in breast cancer in our previous study.¹⁷ In this study, we found that low expression of TIP30 was associated with lymph node metastasis in lung cancer, and the expressions of TIP30 in the metastatic lesions were lower than that in the primary lesions. These data suggest that decreased expression of TIP30 may promote tumor metastasis in lung cancer patients. The inhibitory effects of TIP30 on lung cancer metastasis were further demonstrated *in vitro* and in nude mice. Inhibition of TIP30 expression significantly promoted tumor cell invasion through Matrigel and wound healing *in vitro*, and lung metastasis and angiogenesis in nude mice. Consistent with our previous observation in hepatocellular carcinoma,¹⁶ down-regulation of TIP30 might promote tumor metastasis through induction of OPN expression, which enhances tumor metastasis through induction of MMPs and VEGF expression.^{21,22} These data strongly suggest down-regulation of TIP30 promotes tumor metastasis and angiogenesis through induction of OPN expression in lung cancer.

However, it must be noted that there were certain patients with lymph node metastasis whose primary tumors were immunohistochemically TIP30 positive and in some cases TIP30 expression was increased relative to

that in adjacent benign tissues. These differences with respect to TIP30 expression pointed to several possible explanations: the anti-TIP30 polyclonal antibody cannot discriminate between a normal and a mutant protein, or a gene mutation could give rise to an altered protein with diminished normal function. Therefore, mutation analyses in TIP30-increased samples are needed to further answer these questions.

In conclusion, TIP30 may function as a tumor suppressor gene and play important roles in suppressing the progression and metastasis of lung cancer. Given that introduction of TIP30 into tumor cells with decreased TIP30 expression promotes tumor apoptosis and inhibits tumor metastasis, TIP30 may be a potent target for the development of therapeutic strategies for patients with lung cancer.

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