

*Tumorigenesis and Neoplastic Progression*

# Loss of TIMP-3 Promotes Tumor Invasion via Elevated IL-6 Production and Predicts Poor Survival and Relapse in HPV-Infected Non-Small Cell Lung Cancer

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**Human papillomavirus (HPV) 16/18 E6 oncoprotein is expressed in lung tumors and is associated with p53 inactivation. The tissue inhibitor of metalloproteinase 3 (TIMP-3) is essential for limiting inflammation; therefore, we expected that TIMP-3 loss might induce chronic inflammation, thereby promoting tumor malignancy as well as poor survival and relapse in patients with HPV-infected non-small cell lung cancer. In this study, the loss of TIMP-3 by loss of heterozygosity and/or promoter hypermethylation was more frequent in HPV16/18 E6-positive tumors than in E6-negative tumors. To explore the possible underlying mechanism, E6-negative TL4 and CL1-0 cells were transfected with an E6 cDNA plasmid. A marked decrease in TIMP-3 expression was caused by promoter hypermethylation via increased DNA (cytosine-5)-methyltransferase 1 (DNMT1) expression. Mechanistic studies indicated that TIMP-3 loss promoted interleukin-6 (IL-6) production, which led to cell invasion and anchorage-independent growth on soft agar plates. Kaplan-Meier and Cox regression models showed that patients with low-TIMP-3/high-IL-6 tumors had shorter overall survival and relapse-free survival periods when compared with patients with high-TIMP-3/low-IL-6 tumors. In summary, loss of TIMP-3 may increase IL-6 production via the tumor necrosis factor  $\alpha$ /nuclear factor  $\kappa$ B axis, thereby promoting tumor malignancy and subsequent relapse and poor survival in patients with HPV-infected non-small cell**

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Lung cancer is the leading cause of cancer death worldwide, and cigarette smoking is a predominant factor for lung cancer incidence; however, 25% of lung cancer patients are nonsmokers.<sup>1</sup> In fact, lung cancer in nonsmokers ranks as the seventh most common cause of cancer death worldwide, before cancers of the cervix, pancreas, and prostate.<sup>1</sup> In Taiwan, more than 50% of lung cancer patients are nonsmokers, and in female patients, more than 90% are nonsmokers. Unexpectedly, lung cancer has been the leading cause of cancer death in Taiwanese women since 1982. Major clinicopathological, molecular and sex differences in lung cancers between nonsmokers and smokers have been recognized.<sup>1</sup> Because the increasing body of evidence refers only to driver mutations and not HPV infection in lung cancer development, very few studies have found a significant role of HPV infection in non-Taiwanese patients.<sup>2–7</sup>

Our previous case-control study indicated that human papillomavirus (HPV) 16/18 infection was associated with lung cancer development in female Taiwanese nonsmokers.<sup>5</sup> We further reported that E6 oncoprotein is expressed in HPV-positive lung tumors and promotes tumor growth via inactivation of p53, DDX3, and miR-218<sup>8–10</sup> and upregulation of human telomerase reverse transcriptase (hTERT), IL-6, and Mcl-1.<sup>11,12</sup> Therefore, HPV infection conceivably may play a role in lung tumorigenesis, at least in Taiwanese nonsmokers.

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The tissue inhibitor of metalloproteinase 3 (TIMP-3) gene is located at 22q12.3. TIMP3 is an insoluble 24-kDa glycoprotein that is produced by most cell types and is sequestered at the cell surface, where it is bound by components of the extracellular matrix.<sup>13</sup> TIMP-3 acts as a tumor suppressor to inhibit tumor growth, invasion, and angiogenesis.<sup>14,15</sup> Loss of heterozygosity (LOH) of TIMP-3 has frequently been reported in meningiomas,<sup>16</sup> secondary glioblastomas,<sup>17</sup> and pancreatic endocrine carcinomas,<sup>18</sup> but TIMP-3 LOH has not yet been reported in cervical or lung cancers. Promoter methylation is the key pathway for TIMP-3 inactivation in cancers of the kidney, brain, breast, colon, esophagus, gastric, head and neck, and lung.<sup>19–22</sup> However, the level of promoter methylation of the TIMP-3 for cervical and ovarian carcinomas was reported as less than 10%.<sup>23,24</sup> Reduced TIMP-3 expression was associated with poor outcomes in esophageal adenocarcinoma and lung cancer patients,<sup>25,26</sup> but an early report indicated an association between TIMP-3 promoter methylation and better survival in lung cancer patients.<sup>27</sup> In addition, TIMP-3 expression has been linked to favorable outcomes in head and neck cancer.<sup>22</sup> The prognostic value of TIMP-3 in human cancers therefore needs to be further clarified.

Elevated levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) have been reported in TIMP-3–knockout mice. This activates NF- $\kappa$ B and IL-6 production and subsequently leads to severe inflammation of the liver.<sup>28</sup> Our genome-wide analysis revealed a TIMP-3 allelic imbalance at 22q12.3, which occurred more frequently in HPV-infected lung tumors than in non-HPV-infected lung tumors. This preliminary finding prompted us to investigate whether TIMP-3 loss by LOH and/or promoter hypermethylation could play a role in HPV-infected lung tumorigenesis that was mediated by IL-6. The association between TIMP-3 and IL-6 expression in lung tumors was statistically analyzed to determine whether TIMP-3 and/or IL-6 expression could predict overall survival (OS) and relapse-free survival (RFS) in lung cancer patients. Mechanistic studies using HPV E6–positive and –negative lung cancer cells were conducted to elucidate whether HPV E6 could induce TIMP-3 promoter hypermethylation and could promote the capability for cell invasion and anchorage-independent growth on soft-agar plates via elevated IL-6 production.

## Materials and Methods

### Study Subjects

Lung tumor specimens were collected from 165 patients with primary lung cancer at the Department of Thoracic Surgery, Taichung Veterans General Hospital (Taichung, Taiwan), between 1998 and 2004. Patients were asked to submit written informed consent; the study was approved by the Institutional Review Board. The tumor type and stage of each collected specimen were histologically determined according to the World Health Organization classification system. Cancer relapse data were obtained by chart review and confirmed by thoracic surgeons.

### Cell Lines and Cell Culture

The TL-1 and TL-4 lung cancer cells were established from patients' pleural effusions, as described previously.<sup>8</sup> A549 and H1299 cells were obtained from the ATCC and cultured as previously described (<http://www.atcc.org>). CL1-0 and CL1-5 cells were kindly provided by Dr. P.-C. Yang (Department of Internal Medicine, National Taiwan University Hospital, Taiwan, ROC). A549 and H1299 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. TL1, TL4, CL1-0, and CL1-5 cells were grown in RPMI-1640 medium with 10% fetal bovine serum. All of these cell lines were grown at 37°C in a 5% carbon dioxide atmosphere.

### LOH Assay

In all, two highly polymorphic markers were selected from the Ensembl Genome Browser (<http://www.ensembl.org/index.html>) and the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).<sup>29–36</sup> Their selection was based on the published chromosome 22q sequence and on the frequency of heterozygosity, as well as by coverage and flanking of the region of interest. The GenBank accession number for D22S280 is Z17028 and for D22S283 is Z17176 (<http://www.ncbi.nlm.nih.gov/geo>). For each marker, the sense primer was labeled with a fluorescent dye, and paired normal and tumor DNA samples from each patient were amplified for 30 cycles with an annealing temperature of 55°C. Aliquots of the PCR reactions were then mixed with a size standard and formamide, denatured, and subjected to capillary electrophoresis on a Genetic Analyzer 310 (ABI, Foster City, CA). Collected data were analyzed with GENESCAN software (ABI). Analyses of each marker were repeated independently at least twice and showed a variation of no more than 3% in allelic ratios. Only samples heterozygous for a given locus were regarded to be informative; locus homozygosity and/or microsatellite instability rendered any particular sample noninformative. Samples were considered to show LOH when a peak allele signal from the tumor DNA was reduced by 50% compared to the normal tissue counterpart.

### Real-Time Quantitative Methylation-Specific PCR

Sodium bisulfite–treated genomic DNA was amplified using fluorescence-based real-time methylation-specific PCR<sup>37</sup> using SYBR Green qPCR MasterMix (Life Technologies, Carlsbad, CA). The methylation status of the TIMP-3 gene was examined using actin as the internal control for DNA quantification. Actin contains no CpG dinucleotides and is not affected by DNA methylation status or sodium bisulfite treatment. The following primers were used: methylated (TIMP-3) forward primer, 5'-TCGGGTTGTAGTAGTTTCGTC-3' and methylated (TIMP-3) reverse primer, 5'-ACGATAAACCCGAACCAA-3'. actin-forward, 5'-TGGTGATGGAGGAGGTTTAGTAGT-3' and actin-reverse, 5'-AACCAATAAACCTACTCTCCCTTAA-3'. The bisulfite-treated *in vitro* methylated

DNA (SssI methyltransferase, New England Biolabs) was used as a positive control. Each reaction was performed in triplicate. The bisulfite-treated *in vitro* methylated DNA was included in each run to serve as the 100% methylated reference for calculating the relative methylation percentages of DNA samples based on the relative  $2^{(-\Delta\Delta C_T)}$  quantitation approach.<sup>38,39</sup> Samples were considered to show positive methylation when the percentage of methylation was more than 50%, whereas a finding of less than 50% was considered as negative.

### Immunohistochemistry

Immunohistochemistry was used to detect HPV16/18 E6, IL-6, and TIMP-3 expression. HPV16/18 E6 and IL-6 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-TIMP-3 antibody was obtained from R&D Systems (Minneapolis, MN). The immunohistochemical procedures were conducted as previously described.<sup>8</sup> Negative controls were obtained by leaving out the primary antibody. The intensities of signals were evaluated independently by three observers. The immunostaining results for HPV16/18 E6 and IL-6 expression in lung tumors were partially obtained from previous reports.<sup>8,11,12</sup> The immunohistochemical staining scores were defined as described previously<sup>40,41</sup> and the intensities of signals were evaluated independently by three observers. Immunostaining scores were defined as the cell staining intensity (0 = nil; 1 = weak; 2 = moderate; and 3 = strong) multiplied by the percentage of labeled cells (0% to 100%), leading to scores from 0 to 300. A score of more than 150 and include 150 itself were rated as "high" immunostaining, whereas a score of less than 150 was rated as "low."

### RNA Isolation and Real-Time Quantitative Reverse Transcription-PCR Analysis

Total RNA was extracted by homogenization in 1 mL of TRIzol reagent, followed by chloroform extraction and isopropanol precipitation. A 3- $\mu$ g sample of total RNA from lung tumor tissues was reverse transcribed using SuperScript II Reverse Transcriptase (Life Technologies) and oligo d(T)<sub>15</sub> primer. The following primer sequences were used for amplification of the TIMP-3 gene: the forward primer, 5'-TGCAACTCCGACATCGTGAT-3' and the reverse primer, 5'-TCTTCATCTGCTTGATGGTGTAGAC-3'. The TIMP-3 mRNA levels in lung tumors that were higher than the median value were defined as "high," whereas levels lower than the median value were defined as "low."

### Western Blot Assay

The cells were lysed with lysis buffer containing 0.5% NP-40, 50 mmol/L Tris-Cl (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), and protease inhibitor cocktail (Roche, Indianapolis, IN). After 3 minutes of lysis, the cell debris was removed by centrifugation, and the protein concentration was determined using a Bradford

protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred from the gel onto a polyvinylidene difluoride membrane (PerkinElmer, Norwalk, CT). After blocking, the membranes were reacted with antibody at 4°C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 hour. The blots were observed using an enhanced chemiluminescence kit (PerkinElmer).

### Plasmid Construct and Transfection Assays

The TIMP-3-overexpressed plasmid was constructed in pCDNA3.1A (-). RNA interference was performed by expression of small hairpin RNA to target TIMP-3 in lung cancer cell lines. The small hairpin RNA template was constructed from two oligonucleotides with a complementary sequence in the loop region. The following primer sequences were used for construction of the TIMP-3si gene: the forward primer, 5'-GATCGCAAGATCAAGTCTGCTACTTTCAAGAGAAGTAGCAGGACTTGATCTTGCTTTTT-3' and the reverse primer, 5'-AGCTAAAAGCAAGATCAAGTCTGCTACTTCTTGAAAGTAGCAGGACTTGATCTTGC-3'. The NF- $\kappa$ B luciferase construct was kindly provided by Dr. Tsui-Chun Tsou (Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Zhunan, Miaoli, Taiwan). The different concentrations of expression plasmids were transiently transfected into lung cancer cells ( $1 \times 10^6$ ) using the Turbofect reagent (Fermentas, Glen Burnie, MD). After 48 hours, cells were harvested, and whole-cell extracts were assayed in subsequent experiments.

### Luciferase Assay

For the luciferase reporter assay, appropriate numbers of cells were transfected with sufficient reporter plasmid, NF- $\kappa$ B-Luc or its derivatives, and either the control vector or the TIMP-3si and TIMP-3 cDNA plasmid. For normalization of transfection efficiency,  $\beta$ -gal was also co-transfected. Transfected cells were harvested at 48 hours posttransfection, and a luciferase assay was performed according to the manufacturer instructions. The luciferase activity was measured with an AutoLumat LB953 luminometer (Berthold, Bad Wildbad, Germany) and normalized with the co-transfected  $\beta$ -gal activity.

### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) analysis was performed as described in a previous report<sup>9</sup> with the following modifications: Immunoprecipitated DNA was precipitated with ethanol and resuspended in 20  $\mu$ l of ddH<sub>2</sub>O. Samples were resuspended in 100  $\mu$ l of ddH<sub>2</sub>O and diluted 1:100 before PCR analysis. PCR amplification of immunoprecipitated DNA was performed with diluted aliquots, using the primers consisting of the oligonucleotides that encompass the promoter region of TIMP-3. The following primer sequences were used for TIMP-3

ChIP: the forward primer, 5'-GCGCCGGAGGCCAAGGT-TGC-3' and the reverse primer, 5'-CAGTCCCCCAG-GCTCCAGCTGC-3'. The PCR products were separated on 2% agarose gels and analyzed using ethidium bromide staining. All ChIP assays were performed at least twice with similar results.

**Bisulfite Sequencing**

For bisulfite sequencing analysis, the endpoint PCR products from the primers (TIMP-3-Forward, 5'-TTGT-TATTGGTTGAGGGG-3' and TIMP3-Reverse, 5'-TC-CCCCAACTCCAACACTAC-3') were purified with the PCR purification kit (Qiagen, Valencia, CA). The purified PCR products were cloned into the TA cloning vector according to the manufacturer protocol (Yeastern Biotech, Taiwan). For each DNA sample, 5 bacterial clones were randomly selected and their respective plasmids were extracted. The plasmid DNA was subjected to sequencing analysis using an Applied Biosystems automated fluorescent sequencer (Applied Biosystems, Foster, CA) using vector primers according to the manufacturer instructions.

**Anchorage-Independent Soft-Agar Colony Formation**

Anchorage-independent growth was assayed by the ability of cells to form colonies in soft agar. The bottom agar consisted of growth medium containing 10% fetal bovine serum and 0.75% agarose in 60-mm tissue culture dishes. 500 cells were re-suspended in growth medium containing 10% fetal bovine serum and 0.75% agarose and plated on top of the bottom agar. The cells were incubated at 37°C in 5% CO<sub>2</sub>. Colonies were visualized

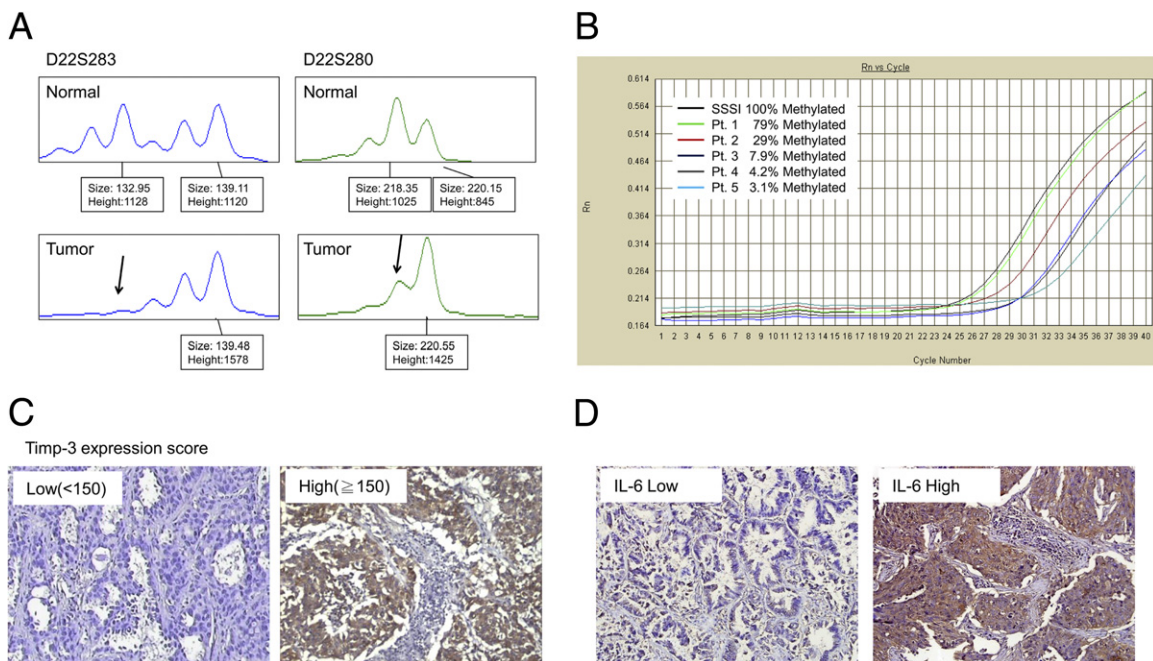
and quantified under a microscope after 18 day cultivation, and the numbers of colonies larger than 100 micrometers in diameter were counted.

**Invasion Assay**

A Boyden chamber with a pore size of 8 μm was used for the *in vitro* cell invasion assay. Cells (1 × 10<sup>4</sup>) in 0.5% serum containing culture medium (HyClone, Ogden, UT) were plated in the upper chamber and 10% fetal bovine serum was added to culture medium in the lower chamber as a chemoattractant. The upper side of the filter was covered with 0.2% Matrigel (Collaborative Research, Boston, USA) diluted in RPMI-1640. After 16 h, cells on the upper side of the filter were removed and cells that adhered to the underside of the membrane were fixed in 95% ethanol and stained with 10% Giemsa dye. The number of invasive cells was counted. Ten contiguous fields of each sample were examined to obtain a representative number of cells that invaded across the membrane.

**Statistical Analysis**

Statistical analysis was performed using the SPSS statistical software program Version 15.0 (SPSS Inc., Chicago, IL). The associations between HPV16/18 E6 and TIMP-3 expressions were analyzed by a χ<sup>2</sup> test. Survival plots were generated using the Kaplan-Meier method, and differences between patient groups were determined by a log-rank test. A multivariate Cox regression analysis was performed for overall survival and relapse-free survival. The analysis was stratified for all known prognostic variables (age, sex, smoking status, tumor type, and stage) and protein expression.



**Figure 1.** Representative LOH (A), promoter methylation (B), and immunostaining results of TIMP-3 (C) and IL-6 (D) in lung tumors.

**Table 1.** Relationships of LOH, Promoter Methylation, and Immunostaining Level of TIMP-3 with Clinicopathological Parameters in Lung Cancer

Parameter	LOH*				TIMP-3 methylation				TIMP-3 protein <sup>†</sup>		
	Case no.	Retention	Deletion	P	Case no.	Absence	Presence	P	Low (<150)	High (≥150)	P
Age											
≤66	77	54 (70)	23 (30)	0.494	81	48 (59)	33 (41)	0.728	42 (52)	39 (48)	0.395
>66	80	60 (75)	20 (25)		84	52 (62)	32 (38)		38 (45)	46 (55)	
Sex											
Female	51	30 (59)	21 (42)	0.007	55	30 (55)	25 (45)	0.260	27 (49)	28 (51)	0.912
Male	106	84 (79)	22 (21)		110	70 (64)	40 (36)		53 (48)	57 (52)	
Smoking status											
Nonsmoker	93	61 (66)	32 (34)	0.017	97	50 (52)	47 (48)	0.004	50 (51)	47 (49)	0.347
Smoker	64	53 (83)	11(17)		68	50 (74)	18 (36)		30 (44)	38 (56)	
Tumor type											
Adenocarcinoma	93	60 (64)	33 (36)	0.006	98	52 (53)	46 (47)	0.016	54 (55)	44 (45)	0.040
Squamous cell carcinoma	64	54 (84)	10 (16)		67	48 (72)	19 (38)		26 (39)	41 (61)	
Stage											
I, II	86	60 (70)	26 (30)	0.379	91	64 (70)	27 (30)	0.005	35 (39)	56 (62)	0.004
III	71	54 (76)	17 (24)		74	36 (49)	38 (51)		45 (61)	29 (39)	
TIMP-3 LOH											
Retention	114					77 (68)	37 (32)	0.031	45 (40)	69 (60)	0.001
Deletion	43					21 (49)	22 (51)		30 (70)	13 (30)	
TIMP-3 methylation											
Presence	65								46 (71)	19 (29)	<0.001
Absence	100								34 (34)	66 (66)	
LOH/methylation											
Deletion/presence	22								19 (86)	3 (14)	<0.001
Deletion/absence	21								11 (52)	10 (48)	
Retention/presence	37								23 (62)	14 (38)	
Retention/absence	77								22 (29)	55 (71)	

\*Eight of 165 tumors in the TIMP-3 LOH analysis were noninformative.

<sup>†</sup>TIMP-3 expression score: [percentage of stained cells × staining intensity (0 to -3)]. A score of more than 150 and include 150 itself were regarded as "high" immunostaining, whereas a finding of less than 150 was considered as "low."

## Results

### Loss of TIMP-3 by LOH and Promoter Hypermethylation Are Frequent in Female, Adenocarcinoma, and Nonsmoker Patients

To verify whether TIMP-3 loss could be mediated by LOH and promoter methylation, two markers (D22S283 and D22S280) and quantitative methylation-specific PCR were used to determine TIMP-3 LOH and promoter hypermethylation, respectively, of 165 lung tumors. The representative TIMP-3 LOH and promoter methylation in the lung tumors are shown in Figure 1, A and B. The relationships between TIMP-3 and clinicopathological features in the lung cancer patients are shown in Table 1. In total, 157 patients had an informative TIMP-3 LOH, which was more common in female, nonsmoker, and adenocarcinoma patients than in male, smoker, and squamous cell carcinoma patients. However, TIMP-3 promoter methylation was only associated with smoking status (51% for nonsmokers versus 32% for smokers,  $P = 0.017$ ), not with sex or tumor histology. Immunohistochemistry analysis showed that low TIMP-3 protein expression was more common in tumors with TIMP-3 LOH (70% versus 40%,  $P = 0.001$ ), promoter methylation (71% versus 34%,  $P < 0.001$ ), and both combinations (86% for deletion/presence, 52% for deletion/absence, 62% for retention/presence, and 29% for retention/ab-

sence,  $P < 0.001$ ) than their high expression counterparts.

### TIMP-3 Expression Is Negatively Associated with Tumors Showing HPV16/18 E6 Expression

We tested whether TIMP-3 LOH and promoter hypermethylation were associated with HPV16/18 E6 expression in lung tumors. E6 immunostaining results of lung tumors were obtained from our previous reports.<sup>8,11</sup> As shown in Table 2, TIMP-3 LOH, promoter hypermethylation, and protein expression in lung tumors were significantly associated with HPV16/18 infection and E6 oncoprotein expression. Specifically, TIMP-3 LOH and promoter hypermethylation were more prevalent in HPV16/18-infected tumors than in noninfected tumors (40% versus 17%,  $P = 0.001$  for LOH; 51% versus 30%,  $P = 0.005$  for promoter methylation). TIMP-3 expression was less common in E6-positive tumors than in E6-negative tumors (39% versus 61%,  $P = 0.004$ ). As expected, TIMP-3 LOH, promoter methylation, and low TIMP-3 expression were more prevalent in HPV 16/18 E6 expression tumors. These results strongly suggest that high frequency of TIMP-3 loss by LOH and promoter hypermethylation may be linked to HPV16/18 infection.

**Table 2.** Association of HPV16/18 DNA and E6 Expression with LOH, Promoter Methylation, and Immunostaining Level of TIMP-3 in Lung Tumors

	Case no.	LOH			P	Case no.	TIMP-3 methylation		P	TIMP-3 protein <sup>†</sup>		P
		Retention	Deletion	Negative			Positive	Low (<150)		High (≥150)		
HPV16/18 DNA*												
Negative	87	72 (83)	15 (17)	0.001	91	64 (70)	27 (30)	0.005	35 (39)	56 (61)	0.004	
Positive	70	42 (60)	28 (40)		74	36 (49)	38 (51)		45 (61)	29 (39)		
HPV16/18 E6												
Negative	109	87 (80)	22 (20)	0.002	114	79 (69)	35 (31)	0.001	45 (40)	69 (60)	0.001	
Positive	48	27 (56)	21 (44)		51	21 (41)	30 (59)		35 (69)	16 (31)		

\*P value for HPV16/18 DNA and E6 protein was <0.001.

<sup>†</sup>TIMP-3 expression score: [percentage of stained cells × staining intensity (0 to -3)]. A score of more than 150 and include 150 itself were regarded as "high" immunostaining, whereas a finding of less than 150 was considered "low".

### E6-Positive and TIMP-3-Loss Tumors Have Higher IL-6 Expression than Their Counterparts

Chronic hepatic inflammation induced by IL-6 production has been shown in TIMP-3 knockdown mice.<sup>28</sup> Therefore, we tested whether IL-6 expression could be related to HPV16/18 infection and E6 and TIMP-3 expression in lung tumors. Immunohistochemical data showed that tumors with high IL-6 expression were more prevalent in HPV16/18-positive patients than in HPV16/18-negative patients (85% versus 60%, *P* = 0.008; Table 3). As expected, high IL-6 expression was more prevalent in

HPV16/18 E6-positive than in HPV16/18 E6-negative tumors (Table 3). IL-6 expression was negatively associated with TIMP-3 expression (62% versus 82%, *P* = 0.024). We also observed that the prevalence of high IL-6 expression in patients with low TIMP-3 plus E6 positivity was greater than for the other three combinations (*P* = 0.001; Table 3). The same phenomenon was seen in patients with low TIMP-3 plus E6 positivity (*P* = 0.012; Table 3). These results indicate that IL-6 expression is positively correlated with E6 positivity and TIMP-3 loss in lung tumors.

### TIMP-3 Combined with IL-6 May Predict OS and RFS in Lung Cancer

We examined whether E6, TIMP-3, and IL-6 could be associated with OS and RFS in lung cancer. In this study population, no patients received adjuvant chemotherapy before surgical resection, and 147 patients were available for estimation of relapse-free survival. The mean follow-up period after surgery was 59.3 months. The median survival times were 27.2 months for OS and 21.2 months for RFS. During the survey, 121 patients died. Among the 147 patients with tumor relapse follow-up data, 59 patients experienced relapse. As expected, the prognostic value of tumor stage on OS and RFS, determined by Kaplan-Meier and multivariate Cox regression models, was observed in this study population (see Supplemental Tables S1 and S2 at <http://ajp.amjpathol.org>). In addition, a poorer OS and RFS in lung adenocarcinoma compared with squamous cell carcinoma was revealed after multivariate Cox regression analysis (*P* = 0.016) (see Supplemental Table S2 available at <http://ajp.amjpathol.org>).

Kaplan-Meier analysis showed that HPV16/18 infection and E6 expression was not associated with OS or RFS in this study population (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Patients with low TIMP-3 expression had shorter OS and RFS than those with high TIMP-3 expression (*P* < 0.001 for OS; *P* < 0.001 for RFS; Figure 2A). We also observed that patients with high IL-6 expression had poorer OS and RFS than did patients with low IL-6 expression (*P* = 0.034 for OS; *P* = 0.020 for RFS; Figure 2B). The prognostic signifi-

**Table 3.** Association of IL-6 Expression with HPV16/18 E6, TIMP-3, and Both Combinations in Lung Tumors

	Case no.	IL-6*		P
		Low	High	
HPV16/18 DNA				
Negative	72	29 (40)	43 (60)	0.001
Positive	62	9 (15)	53 (85)	
HPV16/18 E6				
Negative	90	32 (36)	58 (64)	0.008
Positive	44	6 (14)	38 (86)	
TIMP-3 protein <sup>†</sup>				
Low	66	12 (18)	54 (82)	0.010
High	68	26 (38)	42 (62)	
HPV16/18 DNA/TIMP-3				
Negative/high	44	19 (43)	25 (57)	0.001
Positive/high	24	7 (29)	17 (71)	
Negative/low	28	10 (36)	18 (64)	
Positive/low	38	2 (5)	36 (95)	
HPV16/18 E6/TIMP-3				
Negative/high	54	22 (41)	32 (59)	0.012
Positive/high	14	4 (29)	10 (71)	
Negative/low	36	10 (28)	26 (72)	
Positive/low	30	2 (7)	28 (93)	

\*Thirty-one of 165 tumors were unavailable for IL-6 immunostaining. E6 and IL-6 immunostaining results were partially obtained from previous reports.

<sup>†</sup>TIMP-3 expression score: [percentage of stained cells × staining intensity (0 to -3)]. A score of more than 150 and include 150 itself were regarded as "high" immunostaining, whereas a finding of less than 150 was considered as "low."

**Figure 2.** Kaplan-Meier analysis for the influence of TIMP-3 (A), IL-6 (B), both combinations between HPV16/18 infection (C), HPV16/18 E6 expression (D), IL-6 (E) with TIMP-3 on overall survival (OS), and relapse-free survival (RFS) in lung cancer patients.

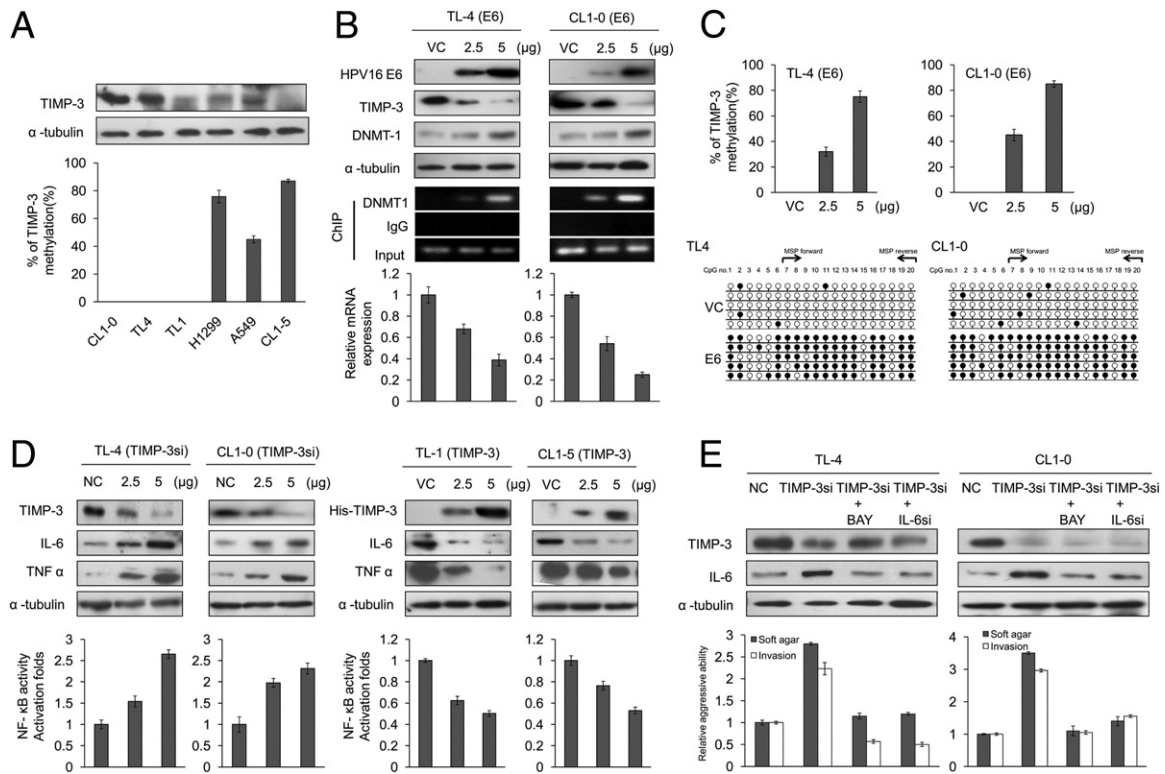
cance of the combination of HPV16/18 DNA and TIMP-3 was indicated by Kaplan-Meier analysis (HPV DNA-negative/high-TIMP-3 versus HPV DNA-positive/low-TIMP-3,  $P < 0.001$  for OS,  $P < 0.001$  for RFS; Figure 2C). Interestingly, the prognostic significance of the combination of E6 and TIMP-3 was also significant ( $P = 0.002$  for OS,  $P < 0.001$  for RFS; Figure 2B). In addition, patients with low-TIMP-3/high-IL-6 tumors had shorter OS and RFS than did those with high-TIMP-3/low-IL-6 expression ( $P < 0.001$  for OS;  $P < 0.001$  for RFS; Figure 2E).

We further determined whether TIMP-3, IL-6, or a combination of both could independently predict clinical outcome. Multivariate analysis was conducted after adjusting for the parameters of age, sex, smoking, tumor type, and tumor stage. In this studied population, patients with low-TIMP-3 tumors had hazard ratios (HRs) of 1.97 and 2.18 for OS and RFS, respectively, when compared with patients with high-TIMP-3 tumors (95% confidence interval [CI] = 1.34 to 2.92,  $P = 0.001$  for OS; 95% CI = 1.47 to 3.23,  $P < 0.001$  for RFS; Table 4). The 5-year survival rates were lower and the mean

**Table 4.** Cox Regression Analysis for the Influence of TIMP-3, IL-6, and Both Combinations on OS and RFS in Lung Cancer Patients

Variable	Case no.	OS					RFS					
		5-Year survival, %	Median survival, months	HR*	95% CI	<i>P</i>	Case no.	5-Year survival, %	Median survival, months	HR*	95% CI	<i>P</i>
TIMP-3 protein												
High ( $\geq 150$ )	85	51.8	49.9	1			78	44.9	40.2	1		
Low ( $< 150$ )	80	17.5	21.3	1.97	1.34–2.92	0.001	69	8.7	13.8	2.18	1.47–3.23	<0.001
IL-6												
Low	38	44.7	44.5	1			35	40.0	39.8	1		
High	96	29.2	24.2	1.44	0.90–2.32	0.133	85	21.2	16.0	1.59	0.98–2.57	0.063
TIMP-3/IL-6												
High/low	26	61.5	59.9	1			24	54.2	45.9	1		
High/high	42	42.9	36.0	1.50	0.75–2.97	0.300	39	35.9	27.6	1.55	0.78–3.07	0.208
Low/low	12	8.3	23.2	2.36	1.00–5.56	0.049	11	9.1	18.0	2.38	1.01–5.65	0.048
Low/high	54	18.5	19.8	2.67	1.37–5.18	0.004	46	8.7	11.1	3.07	1.58–5.95	0.001

\*HR was adjusted for age, sex, smoking status, tumor type, and tumor stage.



**Figure 3.** TIMP-3 loss promotes lung cancer cell invasion capability via elevation of TNF $\alpha$ -mediated IL-6 production. **A:** Reduced TIMP-3 protein expression in a panel of lung cancer cells was predominantly mediated through promoter methylation. TIMP-3 promoter methylation and protein expression levels were determined by quantitative methylation-specific PCR and Western blot analysis, respectively. **B:** Ectopic E6 expression in TL-4 and CL1-0 cells reduced TIMP-3 protein and mRNA expression via promoter methylation. The degree of TIMP-3 promoter methylation induced by E6 was consistent with an increase in DNMT1 expression in both cells. E6, TIMP-3, and DNMT1 expressions were determined by Western blot analysis. E6 induced DNMT1 binding to the TIMP-3 promoter was evaluated by ChIP assay; E6-induced reduction in TIMP-3 mRNA levels was evaluated by real-time reverse transcription-PCR (**lower panel**); E6-induced reduction in TIMP-3 protein and mRNA expression via promoter methylation (**C**). TIMP-3 promoter methylation and methylated CpG island status were determined by quantitative methylation-specific PCR and bisulfite sequencing analysis, respectively. Five independent clones were sequenced; each row represents one sequenced allele. Each circle represents a CpG dinucleotide. Filled circle indicates methylation; open circle indicates no methylation. **D:** TIMP-3-knockdown TL-4 and CL1-0 cells transfected with a small interference RNA (siRNA) show increased capability for cell invasion and soft-agar growth via elevation of TNF $\alpha$ -mediated IL-6 production. The expression of TIMP-3, IL-6, and TNF $\alpha$  of both TIMP-3-knockdown cells was determined by Western blot analysis (**upper panel**).  $\alpha$ -Tubulin was used as a loading control. Luciferase reporter analysis was used to evaluate NF- $\kappa$ B reporter activity of both cells after treated with two doses of TIMP-3si (**lower panel**). **E:** IL-6 elevated by TIMP-3 knockdown is responsible for the invasion and soft-agar growth capability in both cells. TIMP-3 and IL-6 expression was determined by Western blot analysis. NC, nonspecific control; VC, vector control. In these experiments, the relative expression level in the VC or NC controls was arbitrarily assigned as 1.

survival periods were shorter for OS and RFS in patients with low-TIMP-3 tumors than for patients with high-TIMP-3 tumors (17.5% versus 51.8%, 21.3 months versus 49.9 months for OS; 8.7% versus 44.9%, 13.8 months versus 40.2 months for RFS). A prognostic significance for IL-6 on OS and RFS was not seen in this study population. However, patients with low-TIMP-3/high-IL-6 tumors had the highest HR value for OS (HR = 2.67, 95% CI = 1.37 to 5.18,  $P = 0.004$ ), followed by patients with low-TIMP-3/low-IL-6 tumors (HR = 2.36, 95% CI = 1.00 to 5.56,  $P = 0.049$ ), but the prognostic value of patients with high-TIMP-3/high-IL-6 tumors did not reach statistical significance when compared with those with high-TIMP-3/low-IL-6 tumors and low-IL-6 tumors. A similar phenomenon was also seen for the combined effects of TIMP-3 and IL-6 on RFS (HR = 3.07, 95% CI = 1.58 to 5.95,  $P = 0.001$  for low-TIMP-3/high-IL-6 tumors; HR = 2.38, 95% CI = 1.01 to 5.65,  $P = 0.048$  for low-TIMP-3/low-IL-6 tumors; **Table 4**). These results clearly suggest that patients with low-TIMP-3 plus high-IL-6 tumors may in-

dependently predict the poorest survival and relapse in lung cancer.

### Loss of TIMP-3 by E6 Is Mediated through Promoter Methylation in Lung Cancer Cells

DNA (cytosine-5)-methyltransferase 1 (DNMT1) overexpression dysregulated by p53/Sp1 leads to gene promoter hypermethylation.<sup>42</sup> Therefore, we expected that DNMT1 overexpression might be induced by E6 via p53/Sp1 alteration, resulting in TIMP-3 promoter hypermethylation. To test this possibility, TIMP-3 promoter methylation and protein expression levels were evaluated by quantitative methylation-specific PCR and Western blot analysis, respectively. As shown in **Figure 3A**, the expression levels of TIMP-3 protein were inversely correlated with TIMP-3 promoter methylation in all of these cell lines except the TL-1 cells. We next examined whether TIMP-3 may be altered by E6 via promoter methylation. TIMP-3-methylated TL-4 and CL1-0 cells were transfected with



two doses of E6 cDNA plasmid. Western blot examination showed that TIMP-3 expression levels in both cell types were decreased in a dose-dependent manner by ectopic E6 expression. Interestingly, the decrease in TIMP-3 protein expression by ectopic E6 expression was consistent with TIMP-3 mRNA expression, but was inversely correlated with DNMT1 protein expression (Figure 3B, upper panel). ChIP analysis showed that the DNA binding activity of DNMT1 onto the TIMP-3 promoter region was increased by ectopic E6 expression in TL-4 and CL1-0 cells (Figure 3B, middle panel). Quantitative methylation-specific PCR analysis further indicated that the frequency of TIMP-3 promoter methylation was elevated in a dose-dependent manner in E6 ectopic expression TL-4 and CL1-0 cells (Figure 3C, upper panel). A higher frequency of methylated CpG islands in the TIMP-3 promoter region was further confirmed by bisulfite sequencing in ectopic E6 expressing TL-4 and CL1-0 cells to compare with the vector control cells (Figure 3C, lower panel). These results clearly indicate that ectopic E6 expression in lung cancer cells may promote TIMP-3 promoter methylation via increased DNA binding activity of DNMT1 on TIMP-3 promoter.

### Elevation of IL-6 by TIMP-3 Loss May Promote Cell Invasion Capability

IL-6 production via the TNF $\alpha$ /NF- $\kappa$ B axis has been shown to lead to chronic liver inflammation in TIMP-3 knockout mice.<sup>28</sup> In the present study, we explored whether TIMP-3 loss could increase TNF $\alpha$  and IL-6 expression in lung cancer cells. Two high-TIMP-3 (TL-4 and CL1-0) and low-TIMP-3 expression (TL-1 and CL1-5) cell lines were selected for modulation of TIMP-3 expression by knockdown and ectopic expression. Western blot analysis showed that IL-6 and TNF $\alpha$  expression increased concomitantly in TIMP-3-knockdown TL-4 and CL1-0 cells (Figure 3D, left panel). Conversely, IL-6 and TNF $\alpha$  expression were reduced by ectopic TIMP-3 expression in TL-1 and CL1-5 cells (Figure 3D, right panel). As expected, NF- $\kappa$ B reporter activity, evaluated by a luciferase reporter assay, increased in TIMP-3-knockdown and decreased in TIMP-3-overexpressed cells (Fig. 3D, lower panel).

We next tested whether IL-6 production by TIMP-3 loss could promote cell invasion capability and soft-agar growth via TNF $\alpha$ /NF- $\kappa$ B activation. A Boyden chamber and soft-agar colony formation assay showed that the invasion capability and soft-agar growth of TIMP-3-knockdown TL-4 and CL1-0 cells increased 2.5- and 3.0-fold, respectively, when compared with their nonspecific small RNA interference control cells (Figure 3E). Interestingly, when TIMP-3-knockdown TL-4 and CL1-0 cells were treated with NF- $\kappa$ B inhibitor (BAY) or small RNA interference of IL-6 (IL-6si), the elevation of invasion capability and soft-agar growth by TIMP-3 knockdown was completely restored, compared with their nonspecific control cells (Figure 3E). These results clearly indicate that elevation of IL-6 expression by TIMP-3 loss via

TNF $\alpha$ /NF- $\kappa$ B axis may be responsible for lung cancer cell invasion and anchorage-independent soft-agar growth.

### Discussion

In this study population, we found a high frequency of TIMP-3 loss by the LOH or/and promoter hypermethylation in E6-positive lung tumors, compared with E6-negative lung tumors (Table 2). This observation was partially consistent with previous reports.<sup>19–22</sup> TIMP-3 promoter methylation was common in lung cancer but less frequent in TIMP-3 LOH. Surprisingly, loss of TIMP-3 by LOH and promoter methylation is not common in HPV-related cervical and head and neck cancers.<sup>22,23</sup> To our knowledge, this is the first study to reveal that TIMP-3 LOH and promoter hypermethylation are positively correlated with HPV16/18 E6 expression in lung tumors. Consistent with this, TIMP-3 LOH and/or its promoter methylation were associated with its protein expression in adenocarcinoma, but no similar association of TIMP-3 LOH with its protein expression was observed in squamous cell carcinoma (see Supplemental Tables S3 and S4 at <http://ajp.amjpathol.org>). In addition, the occurrence of TIMP-3 LOH and promoter methylation, and its protein expression, were significantly associated with the presence of HPV16/18 DNA and E6 oncoprotein in adenocarcinoma, but not in squamous cell carcinoma (see Supplemental Tables S5 and S6 at <http://ajp.amjpathol.org>). Similarly, an association of IL-6 expression with E6, TIMP-3, and both combinations was observed in lung adenocarcinoma, but not in squamous cell carcinoma (see Supplemental Tables S7 and S8 at <http://ajp.amjpathol.org>). Therefore, TIMP loss by LOH and/or promoter methylation was more common in HPV-infected tumors than in non-HPV-infected tumors. TIMP loss may play a more important role in lung adenocarcinoma than in squamous cell carcinoma. This observation was consistent with our previous reports indicating that HPV infection was more prevalent in lung adenocarcinoma than in squamous cell carcinoma.<sup>5,8</sup>

Accumulated evidence reveals a strong association among chronic infection, inflammation, and human cancers.<sup>43–45</sup> Infection with a high risk of HPV16/18 has been recognized to be associated with the development of cervical and head and neck cancers.<sup>46,47</sup> NF- $\kappa$ B is the key pathway link between inflammation and tumor progression, and NF- $\kappa$ B activation by HPV plays a crucial role in HPV-infected carcinogenesis.<sup>48,49</sup> In the present study, we addressed the *in vitro* cell evidence, indicating that loss of TIMP-3 by E6 increased IL-6 production via TNF $\alpha$ /NF- $\kappa$ B activation. The positive association of IL-6 with HPV16/18 E6 and the negative association with TIMP-3 expression in lung tumors (Table 3) appear to support the observation of cell models that indicate that a loss of TIMP-3 due to E6 leads to TNF $\alpha$  overexpression and elevation of IL-6 production via NF- $\kappa$ B activation. Loss of TIMP-3 affects innate immunity by dysregulating the cleavage of TNF $\alpha$ .<sup>13</sup> In TIMP-3-knockout mice, lipopolysaccharide causes serum levels of TNF $\alpha$  and IL-6 to rise higher than in wild-type mice; in addition, TIMP-3

knockout mice are more susceptible to lipopolysaccharide-induced mortality.<sup>28</sup> Thus, TIMP-3 is essential for limiting inflammation via normal innate immune function.<sup>50</sup> TNF $\alpha$  and IL-6 are potent pleiotropic, proinflammatory cytokines that are produced by many cells in response to inflammation, and both cytokines are also linked to inflammation-induced tumorigenesis.<sup>51,52</sup> For example, in human lung epithelial cells, TNF $\alpha$  and IL-6 increase the production of reactive oxygen species, which increases 8-oxo-deoxyguanosine, a marker of oxidative DNA damage.<sup>53</sup> This suggests a common mechanism by which inflammation from multiple sources can lead to the mutagenic changes necessary for the development and progression of lung cancer.

More than 50% of lung adenocarcinoma progression occurs predominantly through IL-6-induced STAT3 activation. This is especially the case for adenocarcinoma with epidermal growth factor receptor mutation, which suggests that IL-6 is a key player in lung adenocarcinoma tumorigenesis.<sup>54,55</sup> A recent report using IKK $\beta$  and IL-6 knockout mice demonstrated that IKK $\beta$ /NF- $\kappa$ B activation controls the development of liver metastasis by way of IL-6 expression.<sup>56</sup> Previously, the requirement for NF- $\kappa$ B activation has been shown in the progression and metastasis of lung adenocarcinoma.<sup>57</sup> NF- $\kappa$ B is constitutively activated in high-grade squamous intraepithelial lesions and squamous cell carcinoma of the human uterine cervix.<sup>58</sup> However, the underlying mechanism of NF- $\kappa$ B activation and IL-6 production during the development of liver, lung, and cervical cancers is not yet fully understood. In the present study, we provide evidence from a cell model to show that loss of TIMP-3 by E6 markedly elevates IL-6 production via the TNF $\alpha$ /NF- $\kappa$ B axis and consequently promotes cell invasion capability and soft-agar growth.

In summary, the possible mechanism of tumor invasion mediated by TIMP-3 loss, via the TNF $\alpha$ /NF- $\kappa$ B/IL-6 pathway, in E6-positive lung cancer cells seems to be consistent with the observations in lung tumors. Moreover, TIMP-3 expression may not only independently predict clinical outcome, but it may be a prognostic indicator for assessing the likelihood of tumor recurrence in patients after surgical resection.

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