A Polymorphic −844T/C in FasL Promoter Predicts Survival and Relapse in Non–Small Cell Lung Cancer

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Abstract

Purpose: Fas ligand (FasL) −844T/C polymorphism (rs763110) has a demonstrated association with lung cancer risk. FasL −844CC with higher FasL expression has been suggested to contribute to tumor progression via immune escape. However, the impact of FasL −844T/C polymorphism on the clinical outcome of non–small cell lung cancer (NSCLC) remains to be identified.

Experimental Design: A total of 385 adjacent normal lung tissues from patients with NSCLC were collected to determine FasL −844T/C polymorphism by PCR-based restriction fragment length polymorphism. FasL mRNA and protein expression in lung tumors were evaluated by real-time PCR and immunohistochemistry. The prognostic value of FasL −844T/C polymorphism on survival and relapse was determined by Kaplan–Meier analysis and Cox proportional hazards models.

Results: The FasL −844CC genotype had higher prevalence in those with advanced tumors than in those with early tumors (P = 0.008). In addition, patients with the FasL −844CC genotype were more prone to tumor relapse than those with the FasL −844TT+TC genotype (62.1% vs. 37.9%, P = 0.001). Multivariate Cox regression analysis showed that patients with the FasL −844CC genotype had poorer survival in terms of overall survival (OS) and relapse-free survival (RFS) than those with the FasL −844TT+TC genotype (24.1 vs. 42.8 months for OS, HR = 1.455, P = 0.004; 15.4 vs. 31.4 months for RFS, HR = 1.710, P < 0.001).

Conclusions: FasL −844T/C polymorphism may predict survival and relapse in NSCLC. We suggest that Fasl may be a molecular target for immunotherapeutic interventions to improve the clinical outcome of patients with NSCLC. This finding should be validated by another investigative group. Clin Cancer Res; 17(18); 5991–9. ©2011 AACR.

Introduction

FasL (Fas ligand: TNF receptor superfamily, member 6) is the key molecule in normal immune development, homeostasis, modulation, and function. FasL ligates to its receptor Fas (CD95), resulting in receptor oligomerization and subsequent recruitment of the adaptor protein FADD and caspases (procaspase-8 or -10). Together, these form the death-inducing signaling complex and, in turn, activate the apoptosis signaling pathway (1). Tumor cells express FasL to counterattack tumor-infiltrating T cells, which results in immune escape (2–5). However, many cancer cells resist Fas-mediated apoptosis via overexpression of apoptosis-signaling inhibitors, such as c-FLIPL, which is highly competitive and homologous to procaspases-8 and -10 but does not induce further apoptotic signaling by procaspases (6–8). Both in vitro and in vivo studies have provided overwhelming evidence of activation of tumor progression by tumor-derived Fasl via the AKT, NF-xB, and c-jun signaling pathway (8–16). Tumor size and the number of tumor nodules have also been significantly reduced in mice injected with FasL antibody (9). This suggests that tumor-derived Fasl may counterattack T cells to reduce anticancer ability, thus promoting tumor malignancy.

Functional single-nucleotide polymorphism of Fasl −844T/C (rs763110) lies within a binding motif for a CAAT/enhancer-binding protein β element as evidenced by the observation that T cells with the Fasl −844CC genotype have higher transcriptional activity than those with the Fasl −844TT+TC genotype (17). A series of
To improve the outcome of patients with resectable lung cancer, a reliable indicator is needed to predict disease recurrence and survival. Here, we showed that the Fas ligand (Fasl) –844CC carrier had higher Fasl mRNA and protein levels than the Fasl –844TT+TC carrier. In addition, patients with the Fasl –844CC genotype were more prone to tumor relapse and shorter survival than patients with the Fasl –844TT+TC genotype. Therefore, Fasl –844 polymorphism evaluated by a simple PCR-based restriction fragment length polymorphism technique in bloods may potentially be used to predict the disease relapse and prognosis in resected lung cancer, and once this technique is validated, it would be helpful for the clinician to choose a better therapeutic strategy.

case-controlled studies from Lin’s group indicated that the functional Fasl –844T/C polymorphism in a Chinese population was associated with an increased risk of different human cancers, including those of the cervix, lung, esophagus, breast, and pancreas (18–22).

A higher prevalence of T-cell apoptosis in the Fasl –844CC carrier was expected to cause a greater enhancement of tumor progression and metastasis via immune escape (19, 21). In fact, the Fasl protein was overexpressed in different tumors, including colon, breast, liver, gastric, ovary, and lung carcinomas (2, 5, 23–29). Among these, Fasl expression was more prevalent in advanced ovarian tumors but was not related to the patients’ clinical outcome (4). In cervical cancers, Fasl expression tended to be related to tumor stage and patients had a poorer clinical outcome (30). Therefore, the prognostic significance of Fasl polymorphism in non–small cell lung cancer (NSCLC) should be further identified.

In this study, we hypothesized that lung tumors with Fasl –844CC genotype might have higher Fasl expression than those with Fasl –844TT+TC genotype. High Fasl expression in tumor cells might induce more T-cell apoptosis. However, tumors with high Fasl expression might have high c-FLIPL expression, which could prevent Fasl-induced apoptosis. We therefore suspected that patients with Fasl –844CC genotype might have poorer overall survival (OS) and relapse-free survival (RFS) than those with Fasl –844TT+TC genotype.

**Materials and Methods**

**Patients**

This study consisted of 385 patients with NSCLC. All patients were unrelated ethnic Chinese and residents of central Taiwan. Patients were diagnosed with adenocarcinoma (194; 50.4%) or squamous cell carcinoma (191; 49.6%) and treated with surgical resection at the Division of Thoracic Surgery, Taichung Veterans General Hospital. Patients were consecutively recruited between 1993 and 2004. Samples were immediately frozen at surgery and kept at –80°C until processed. All patients were informed and had provided written consent. The study was approved by the Institutional Review Board. Cancer relapse data were obtained by chart review and confirmed by thoracic surgeons. Clinical parameters and OS and RFS data were collected from chart reviews (32 patients had no relapse data) and the Taiwan Cancer Registry, Department of Health, Executive Yuan, ROC. For coculture experiments, blood samples were obtained from 11 healthy male volunteers working in our university between the ages of 22 and 32 years (mean ± SD = 26.6 ± 3.1).

**Genomic DNA extraction, RNA extraction, and cDNA synthesis**

Genomic DNA was extracted by conventional methods. Surgically resected normal tissues adjacent to the lung tumor were prepared by proteinase K digestion and phenol–chloroform extraction, followed by ethanol precipitation.

Total RNA was extracted from 174 available lung tumor tissues using TRIzol reagent (Invitrogen). First-strand cDNA synthesis in the presence of random primers was carried out using a high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer’s instructions.

**PCR-based restriction fragment length polymorphism analysis for Fasl –844T/C genetic polymorphism**

Genotypes of Fasl –844T/C were determined by PCR-based restriction fragment length polymorphism (PCR-RFLP) as described by Sun and colleagues (20). PCR amplification products from 100 samples were randomly selected for direct sequencing to confirm the genotype analysis by PCR-RFLP.

**Real-time PCR**

Real-time PCR amplification of cDNA samples was carried out with the ABI 7500 Real-Time PCR System (Applied Biosystems) and SYBR Green dye to quantify Fasl mRNA transcripts. Real-time PCR primers were as follows: for Fasl transcripts, 5’-CAGTCCACCCCCCTGAAAAAA-3’ (forward) and 5’-TCCATAGGCTCITCCCATTC-3’ (reverse); for 18S gene transcripts, 5’-TCCGAACTGAGGCGCTGATA-3’ (forward) and 5’-CCGGTCGGCATCTTGA-3’ (reverse). The products amplified by Fasl primers were checked by direct sequencing. The amounts of Fasl mRNA transcripts were quantified relative to the 18S internal control and expressed as 2\(^{-\Delta\Delta C_{t}}\) (where \(C_{t}\) represents the fluorescence threshold cycle, \(\Delta C_{t} = C_{\text{Fasl}} - C_{\text{18S}}\)) according to the manufacturer’s instructions (Applied Biosystems). The cutoff value was defined by the medium value for high and low mRNA expression.

**Immunohistochemical staining**

Immunohistochemical staining to evaluate Fasl and c-FLIPL expression in tumor tissue was carried out on 87 whole-mount paraffin sections of lung cancer specimens.
Anti-Fasl (1/500) polyclonal primary antibody (N-20; Santa Cruz Biotechnology) and anti-c-FLIP₅ (1/100) polyclonal primary antibody (C-19; Santa Cruz Biotechnology) were used (26, 31). An immunohistochemistry detection kit for in vitro diagnostic use (Invitrogen) was used according to the standard protocol. Colon cancer specimens and Fasl-expressed immune cells were used as positive controls. Immunohistochemical staining scores were defined as previously described (32).

**Direct sequencing**
Tumor DNA was amplified and used for determining p53 mutation by direct sequencing with the Applied Biosystems 3100 Avant Genetic Analyzer, as described by Cheng and colleagues (32).

**Cell culture**
H1355 and A427 lung cancer cells were purchased from the American Type Culture Collection. TL4 and TL13 lung cancer cells were established from pleural effusions as described previously (32). The pathologic diagnosis of TL13 was lung adenocarcinoma of a 79-year-old male nonsmoker with T₂N₂M₁. H1355 and A427 were maintained in Dulbecco’s Modified Eagle’s Medium (Gibco) with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. TL4 and TL13 were maintained in RPMI medium (Gibco) with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

**Plasmid construction and transfection reaction**
RNA interference was carried out by expression of small hairpin RNA (shRNA) to target Fasl mRNA in lung cancer cell lines. The shRNA control was kindly provided by Dr. J.H. Chang Tsai from the Institute of Medicine at Chung Shan Medical University, Taichung, Taiwan. The shRNA contain 21 nucleotides from the target site was AGGCCCTGTGTCTCCTTGTGAT. The procedures and methods were as described previously (32).

**Peripheral blood monocyte isolation and coculture experiments**
Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by Ficoll-Paque (GE Healthcare) density-gradient centrifugation as described previously (21). The PBMCs were used for the determination of cancer cell–induced T-cell apoptosis by coculture with lung cancer cells (TL4, A427, H1355, and TL13) at a ratio of 10:1 for 36 hours. The PBMCs were then collected for analysis of T-cell apoptosis by flow cytometry.

**Flow cytometric analysis**
A flow cytometer (FACSCalibur; BD Biosciences) was used to determine the weight cell population size and apoptosis percentage. PBMCs were stained with PE-Cy₅.7 mouse anti-human CD3 (BD Pharmingen), and CD3-positive cells in the lymphocyte gate were identified as T cells. Annexin V–fluorescein isothiocyanate (BD Pharmingen) was used to mark cell apoptosis. After staining the cells according to the recommended protocol, the samples were analyzed within 1 hour. For T-cell apoptosis, we gated the lymphocyte gate in forward scatter–side scatter FSC-SSC, and the population with CD3⁺ and Annexin V⁺ was calculated.

**Statistical analysis**
The Student t test and the χ² test were applied for continuous or discrete data analysis. The associations between the Fasl –844T/C genotype and patient survival were estimated using the Kaplan–Meier method and assessed using the log-rank test. Potential confounders were adjusted by Cox regression models, with the Fasl –844T/C genotype fitted as indicator variables. All statistical analyses were done using the SPSS statistical software program (version 11.0; SPSS, Inc.). All statistical tests were 2-sided, and the values of P < 0.050 were considered to be statistically significant.

**Results**

**Advanced tumors and tumor recurrence are more common in Fasl –844CC carriers than in Fasl –844TT+TC carriers**
The Fasl –844T/C genotype of 385 normal lung tissues surgically resected from patients with NSCLC was analyzed by PCR-RFLP. The results of PCR-RFLP for Fasl –844 genotype (100 of 385 samples) have been confirmed by a direct sequencing. The relationships between the Fasl –844T/C genotype and clinicopathologic parameters were examined. The Fasl –844CC genotype was more prevalent in advanced tumors (stage II and III) than in early tumors (stage I; 56.6% for stage III, 52.4% for stage II vs. 39.6% for stage I, P = 0.008; Table 1). Patients with the Fasl –844CC genotype were found to be more prone to tumor relapse than those with the Fasl –844TT+TC genotype (62.1% for CC vs. 44.1% for TT + TC, P = 0.001). Fasl –844CC was not associated with other clinicopathologic parameters including age, gender, smoking status, and tumor type (Table 1). These results suggest that the Fasl –844CC genotype may confer greater tumor progression and relapse than the Fasl –844TT+TC genotype.

**Expression of Fasl mRNA and protein in lung tumors was higher in Fasl –844CC carriers than in Fasl –844TT+TC carriers**
To verify whether the polymorphic CAAT/enhancer-binding protein β element in the Fasl gene –844T/C promoter could predict Fasl expression in lung tumors, 174 and 87 of 385 lung tumors were randomly selected to evaluate Fasl mRNA and protein expression by real-time PCR and immunohistochemical staining, respectively. Positive Fasl immunostaining of immune cells was used as a positive control, and the number of tumor cells with high immunostaining was scored under a light microscope. Higher Fasl mRNA and protein expression levels were typically seen in tumors with the Fasl –844CC genotype than in those with the Fasl –844TT+TC genotype (63.0%...
These results suggest that FasL/C0 844T/C polymorphism in the promoter region may be responsible for FasL expression in lung tumors. Greater T-cell apoptosis induced by FasL expression may result in greater immune escape in FasL/C0 844CC carriers Previous reports have shown that PBMCs with the FasL/C0 844CC genotype undergo more T-cell apoptosis than those with FasL/C0 844 TT+TC genotype (19, 21). Therefore, the immune evasion induced by FasL overexpression in tumor tissues may promote tumor progression and metastasis, leading to poor clinical outcomes for the patients. We tested this possibility by first determining the FasL/C0 844T/C genotype in a panel of lung cancer cells by direct sequencing. We selected H1355 and TL13 cells that harbored FasL/C0 844CC genotype and TL4 and A427 cells that harbored FasL/C0 844TT for this study. As expected, H1355 and TL13 cells carrying FasL/C0 844CC genotype had a higher level of FasL expression than did TL4 and A427 cells carrying the FasL/C0 844TT genotype (Fig. 1A). We also verified whether lung cancer cells carrying different FasL/C0 844 genotypes could induce different degrees of T-cell apoptosis. PBMCs from 11 healthy donors were cocultured with the 4 lung cancer cell types, and T cells were then gated for apoptotic analysis. As shown in Fig. 1B, a greater degree of T-cell apoptosis was found in PBMCs cocultured with H1355 and TL13 cells than in PBMCs cocultured with TL4 and A427 cells. We further examined whether FasL was responsible for T-cell apoptosis by knockdown of FasL expression in H1355 and TL13 cells with shRNA. We found that the prevalence of T-cell

### Table 1. Relationships of FasL/C0 844T/C polymorphism with clinical parameters in NSCLC patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases, n (%)</th>
<th>FasL/C0 844T/C polymorphism</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TT + TC, n (%)</td>
<td>CC, n (%)</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>163 (42.3)</td>
<td>85 (52.1)</td>
<td>78 (47.9)</td>
</tr>
<tr>
<td>≥65</td>
<td>222 (57.7)</td>
<td>110 (49.5)</td>
<td>112 (50.5)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>117 (30.4)</td>
<td>62 (53.0)</td>
<td>55 (47.0)</td>
</tr>
<tr>
<td>Male</td>
<td>268 (69.6)</td>
<td>133 (49.6)</td>
<td>135 (50.4)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoking</td>
<td>197 (51.2)</td>
<td>102 (51.8)</td>
<td>95 (48.2)</td>
</tr>
<tr>
<td>Smoking</td>
<td>188 (48.8)</td>
<td>93 (49.5)</td>
<td>95 (50.5)</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SQ</td>
<td>191 (49.6)</td>
<td>96 (50.3)</td>
<td>95 (49.7)</td>
</tr>
<tr>
<td>AD</td>
<td>194 (50.4)</td>
<td>99 (51.0)</td>
<td>95 (49.9)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>149 (38.7)</td>
<td>90 (60.4)</td>
<td>59 (39.6)</td>
</tr>
<tr>
<td>II</td>
<td>63 (16.4)</td>
<td>30 (47.6)</td>
<td>33 (62.4)</td>
</tr>
<tr>
<td>III</td>
<td>173 (44.9)</td>
<td>75 (43.4)</td>
<td>98 (56.6)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>17 (4.4)</td>
<td>7 (41.2)</td>
<td>10 (58.8)</td>
</tr>
<tr>
<td>T2</td>
<td>273 (70.9)</td>
<td>149 (54.6)</td>
<td>124 (45.4)</td>
</tr>
<tr>
<td>T3</td>
<td>66 (17.1)</td>
<td>26 (39.4)</td>
<td>40 (60.6)</td>
</tr>
<tr>
<td>T4</td>
<td>29 (7.5)</td>
<td>13 (44.8)</td>
<td>16 (55.2)</td>
</tr>
<tr>
<td>Nodal involvement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>185 (48.1)</td>
<td>105 (56.8)</td>
<td>80 (43.2)</td>
</tr>
<tr>
<td>N1</td>
<td>77 (20.0)</td>
<td>37 (48.1)</td>
<td>40 (51.9)</td>
</tr>
<tr>
<td>N2</td>
<td>123 (31.9)</td>
<td>53 (43.1)</td>
<td>70 (56.9)</td>
</tr>
<tr>
<td>Tumor relapse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>229 (64.9)</td>
<td>128 (55.9)</td>
<td>101 (44.1)</td>
</tr>
<tr>
<td>Positive</td>
<td>124 (35.1)</td>
<td>47 (37.9)</td>
<td>77 (62.1)</td>
</tr>
</tbody>
</table>

NOTE: P values were obtained by 2-sided χ² test. P = 0.002 for stage I versus stage II + III; P = 0.031 for T1 + T2 versus T3 + T4; P = 0.021 for N0 versus N1 + N2. Patients with local regional recurrence and distant metastasis after surgical therapy were identified to have tumor relapse.

Abbreviations: SQ, squamous cell carcinoma; AD, adenocarcinoma.
These results suggest that tumors with higher FasL expression scores showed significantly higher cytotoxicity of A549 cells to the concomitant expression of FasL and c-FLIPL in lung tumors. Tumor evasion to FasL-induced apoptosis may be due to the concomitant expression of FasL and c-FLIPL in lung tumors.

To verify whether lung tumors with higher FasL expression can evade FasL-induced apoptosis, 87 tumors in our studied population were selected to evaluate c-FLIPL expression by immunohistochemistry. Up to 80% of the tumors with high FasL expression had high c-FLIPL expression, whereas only 26.2% of tumors with low FasL expression showed high c-FLIPL expression (P < 0.001; Table 3), suggesting that tumors resist FasL-induced apoptosis via overexpression of c-FLIPL. Expression of c-FLIPL in p53 wild-type tumors was significantly higher than in p53-mutated tumors (61.7% for p53 wild type vs. 37.0% for p53 mutation, P = 0.033; Table 3). These results suggest that tumors with higher FasL expression concomitantly overexpressed c-FLIPL, resulting in evasion of FasL-induced apoptosis.

### Table 2. Association of FasL mRNA expression levels with FasL −844T/C polymorphism and FasL protein expression in NSCLC patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases, n (%)</th>
<th>FasL mRNA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>FasL genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT + TC</td>
<td>101 (58.0)</td>
<td>60 (59.4)</td>
<td>41 (40.6)</td>
</tr>
<tr>
<td>CC</td>
<td>73 (42.0)</td>
<td>27 (37.0)</td>
<td>46 (63.0)</td>
</tr>
<tr>
<td>FasL expression score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100</td>
<td>42 (48.3)</td>
<td>27 (64.3)</td>
<td>15 (35.7)</td>
</tr>
<tr>
<td>≥100</td>
<td>45 (51.7)</td>
<td>10 (22.2)</td>
<td>35 (77.8)</td>
</tr>
</tbody>
</table>

NOTE: FasL expression score: [percentage of stained cells × staining intensity (0–3)]. Staining intensity: 0–1 (undetectable to low) and 2–3 (moderate to high); N = 87. P = 0.066 for FasL −844T/C polymorphism versus FasL protein expression.

Poorer OS and RFS were seen in NSCLC with a FasL −844CC genotype than with a FasL −844TT+TC genotype.

Because FasL expression associated with the FasL −844T/C genotype seemed to contribute to tumor progression and disease relapse (Tables 1 and 2), we next questioned whether the FasL −844T/C genotype could be associated with clinical outcome. Univariate and multivariate Cox regression models were used to test this hypothesis in 385 patients. The median follow-up after surgery was 30.8 months, and the median OS of all patients was 31.4 months. During the survey, 138 patients died. Based on follow-up data from 353 patients with tumor relapse, 124 patients relapsed (22 had local recurrence, 72 had distant metastasis, and 30 had local and distant metastasis). In our studied cases, tumors frequently relapsed in the lung (41 cases) and metastasized in the bone (30 cases), brain (20 cases), pleura (4 cases), lymph node (4 cases), adrenal gland (4 cases), and kidney (3 cases). One patient had tumor metastases in the rectum, mediastinum, chest tube, and vertebral body. In total, 32 patients had tumors that metastasized to more than 1 organ. All 385 patients were available for OS, and 353 patients were available for RFS analysis. Among the studied population, patients with adenocarcinoma, early-stage (II−III), and FasL −844CC genotype had poorer OS and RFS than those with squamous cell carcinoma, early-stage (I), and FasL −844TT+TC genotype (tumor type: HR = 1.337, P = 0.023 for OS, HR = 1.302, P = 0.037 for RFS; stage: HR = 2.110, P < 0.001 for OS, HR = 1.970, P < 0.001 for RFS; FasL −844T/C: HR = 1.628, P < 0.001 for OS, HR = 1.843, P < 0.001 for RFS; Table 4). Kaplan–Meier analysis showed that patients with the FasL −844CC genotype had poorer OS and RFS than those with FasL −844TT+TC genotype (Supplementary Fig. S2). Multivariate Cox regression analysis further indicated that patients with the FasL −844CC genotype had a shorter median survival than those with the FasL −844TT+TC genotype (24.1 vs. 42.8 months for OS, 15.4 vs. 31.4 months for RFS;
Table 4). Therefore, the Fasl −844CC genotype may act as an independent negative indicator of OS and RFS in NSCLC compared with the Fasl −844TT+TC genotype [HR = 1.455, 95% confidence interval (CI), 1.129–1.874; \( P = 0.004 \) for OS; HR = 1.710, 95% CI, 1.327–2.205, \( P < 0.001 \) for RFS; Table 4]. The Fasl −844 CC genotype may predict poor survival outcome and increased likelihood of NSCLC relapse.

Discussion

A series of epidemiologic studies conducted by Lin’s group indicated that subjects with the Fasl −844CC genotype have a higher cancer risk than those with the Fasl −844TT+TC genotype, including increased risk of esophageal, cervical, lung, breast, and pancreatic carcinomas (18–22). However, the Fasl −844TC genotype was not associated with lung cancer risk in our case-control study (385 cases and 308 controls), which might be explained by differences in the studied case populations (Fasl −844CC genotype: 61.2% for Chinese vs. 46.4% for Taiwanese). Nevertheless, a similar distribution of the Fasl −844CC genotype was observed in control subjects (47.3% for Chinese vs. 46.4% for Taiwanese). In the present study, we investigated a large population and showed that Fasl −844T/C polymorphism may predict both OS and RFS in NSCLC. However, the prognostic significance of Fasl −844T/C polymorphism in NSCLC was not shown in the Korean population (33). Interestingly, the distribution of Fasl −844 genotypes in the 2 studies reported by Park and colleagues was quite different (TT/TC/CC: 51.6%/41.8%/6.6% vs. 8.1%/39.2%/52.8%; refs. 33, 34). The Fasl −844T/C polymorphism in the promoter region was associated with Fasl expression in lung tumors (Table 2). Therefore, higher Fasl expression levels in T cells and lung cancer cells with the Fasl −844CC genotype were expected to contribute to immune escape and tumor malignancy. Previous reports have indicated that T cells with the Fasl −844CC genotype had higher Fasl expression than those with the Fasl −844TT+TC genotype, which results in a high rate of T-cell apoptosis when these are cocultured with cervical and breast cancer cells (19, 21). In the present study, coculture of PBMCs from healthy donors with lung cancer cells gave a similar result (Fig. 1). Previous reports indicated that significantly higher Fasl expression

harbored the Fasl −844CC genotype than cells cocultured with TL4 and A427 cells that harbored Fasl −844TT genotype. Mean ± SD values of T-cell apoptosis in PBMCs cocultured with TL4, A427, H1355, and TL13 cells were 5.2 ± 2.1, 11.7 ± 2.4, 18.9 ± 2.2, and 14.5 ± 3.4, respectively, whereas Fasl expression in Fasl-knockdown H1355 and TL13 cells was evaluated by flow cytometry and compared with parental and nonspecific shRNA control cells. D, T-cell apoptosis decreased markedly in PBMCs from 11 healthy male volunteers cocultured with Fasl−knockdown H1355 and TL13 cells compared with PBMCs cocultured with nonspecific shRNA control cells. NC, nonspecific RNAi control. Mean ± SD values of T-cell apoptosis in PBMCs cocultured with H1355 nonspecific shRNA control, H1355 Fasl−knockdown, TL13 nonspecific shRNA control, and TL13 Fasl−knockdown cells were 18.1 ± 4.0, 11.5 ± 2.2, 13.5 ± 4.6, and 8.5 ± 3.8, respectively.
levels were found in T cells with the FasL –844CC genotype than with the TC or TT genotype and that the FasL –844CC genotype also resulted in greater T-cell apoptosis after stimulation by PHA and cancer cell antigen (19, 21). The results obtained in the present study for FasL-induced T-cell apoptosis following the activation of PHA or antigen derived from A549 lung cancer cells were consistent with the results from previous studies (35–37). We therefore suggest that T cells activated by PHA or cancer cell antigen may induce FasL expression, thereby resulting in greater T-cell apoptosis via activation-induced cell death.

Apart from T cells, FasL was also expressed by tumor cells (5, 27, 28, 38). High FasL expression detected by IHC in tumor tissues has been linked to poor prognosis in various human carcinomas (23, 25, 26, 29, 30, 39). This observation seems to be explained by the suppression of T-cell immunity via increased apoptosis of tumor-infiltrating lymphocytes (2–5), which consequently contributes to tumor progression and lymph node metastasis (29, 40, 41). Interestingly, tumor-derived FasL has shown a tumor growth-promoting effect operating via the Fas receptor (8–16). A recent animal model study further indicated that human FasL, but not murine FasL neutralized by antibody, resulted in significantly reduced tumor load and colony formation efficacy (9). Collectively, these results indicate that tumor-derived FasL can suppress anticancer immunity and promote tumor growth.

The prognostic significance of FasL mRNA and protein expression was also observed in a subset of this study population after Kaplan–Meier survival and Cox regression analyses. Shorter OS and RFS periods were found for patients with higher FasL mRNA expression (HR = 2.223, 95% CI, 1.473–3.356, \( P < 0.001 \) for OS; HR = 2.028, 95% CI, 1.351–3.046, \( P = 0.001 \) for RFS). In addition, poorer OS and RFS were seen in patients with a high immunostaining score for FasL (score \( \geq 100 \)) than in those with low FasL immunostaining score (score <100; HR = 2.024, 95% CI, 1.135–3.612, \( P = 0.017 \) for OS; HR = 1.809, 95% CI, 1.010–3.242, \( P = 0.046 \) for RFS). These results further support our hypothesis that FasL –844T/C promoter polymorphism may be a helpful predictor of patient survival outcomes.

### Table 3. Association of FasL expression and p53 status with c-FLIPL in NSCLC patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case, n (%)</th>
<th>c-FLIPL</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Score &lt;100</td>
<td>Score ( \geq 100 )</td>
<td></td>
</tr>
<tr>
<td>FasL expression score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100</td>
<td>42 (48.3)</td>
<td>31 (73.8)</td>
<td>11 (26.2)</td>
</tr>
<tr>
<td>( \geq 100 )</td>
<td>45 (51.7)</td>
<td>9 (20.0)</td>
<td>36 (80.0)</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>60 (69.0)</td>
<td>23 (38.3)</td>
<td>37 (61.7)</td>
</tr>
<tr>
<td>Mutation</td>
<td>27 (31.0)</td>
<td>17 (63.0)</td>
<td>10 (37.0)</td>
</tr>
</tbody>
</table>

NOTE: c-FLIPL expression score: [percentage of stained cells \( \times \) staining intensity (0–3)]. Staining intensity: 0–1 (undetectable to low) and 2–3 (moderate to high); \( N = 87 \).

### Table 4. Univariate and multivariate analyses of the influence of various parameters on OS and RFS in NSCLC patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Category</th>
<th>OS</th>
<th>RFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median survival (mo)</td>
<td>HR</td>
</tr>
<tr>
<td>Age, y</td>
<td>( \geq 65/65 )</td>
<td>28.8/39.0</td>
<td>1.250</td>
</tr>
<tr>
<td>Gender</td>
<td>Male/female</td>
<td>30.6/33.1</td>
<td>1.231</td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes/no</td>
<td>28.8/30.6</td>
<td>1.079</td>
</tr>
<tr>
<td>Tumor type</td>
<td>AD/SQ</td>
<td>26.4/41.4</td>
<td>1.337</td>
</tr>
<tr>
<td>Stage</td>
<td>II + III/I</td>
<td>22.1/56.7</td>
<td>2.110</td>
</tr>
<tr>
<td>Stage( ^a )</td>
<td>II + III/I</td>
<td>22.1/56.7</td>
<td>2.343</td>
</tr>
<tr>
<td>FasL –844T/C</td>
<td>CC/TT + TC</td>
<td>24.1/42.8</td>
<td>1.628</td>
</tr>
<tr>
<td>FasL –844T/C( ^a )</td>
<td>CC/TT + TC</td>
<td>24.1/42.8</td>
<td>1.455</td>
</tr>
</tbody>
</table>

\( ^a \)Adjusted for age, gender, smoking, tumor type, and stage.

Abbreviations: SQ, squamous cell carcinoma; AD, adenocarcinoma.
We also examined whether the greater T-cell apoptosis seen in Fasl. –844CC carriers could be induced by a lower c-FLIPL expression in tumor-infiltrating lymphocytes than in lung tumors. We compared the number of tumor-infiltrating lymphocytes and c-FLIPL expression in 87 lung tumor paraffin sections from both Fasl. –844CC and Fasl. –844TT+TC. Fifty-eight of 87 lung tumor paraffin sections (66.6%) showed the presence of tumor-infiltrating lymphocytes. Among these, 6 of 58 (10.3%) tumor-infiltrating lymphocytes showed high c-FLIPL immunostaining. Representative tumor-infiltrating lymphocytes found in lung tumor paraffin sections and the expression of c-FLIPL in tumor-infiltrating lymphocytes are shown in Supplementary Fig. S3. Overall, these findings support our hypothesis that c-FLIPL may be more commonly expressed in lung tumors (47 of 87, 54%) than in tumor-infiltrating lymphocytes (10.3%) and that it may confer lung tumors with the ability to escape FasL-induced apoptosis.

Tumors evade Fas-mediated apoptosis via a mechanism mediated by c-FLIPL expression (42). Overexpression of c-FLIPL may result in reduction of FasL-induced apoptosis in tumor cells. In our cases, FasL expression was positively correlated with c-FLIPL, which suggests that c-FLIPL may be an important apoptotic inhibitor that blocks FasL-induced apoptosis in NSCLC (Table 3). In addition, c-FLIPL expression was higher in p53 wild-type tumors than in p53-mutated tumors (Table 3). A similar result was also reported for ovarian carcinoma, indicating that c-FLIPL overexpression was more common in p53 wild-type tumors than in p53-mutated tumors (31). The positive correlation between Fasl. and c-FLIPL in lung tumors and their prognostic significance were also observed in colorectal and bladder urothelial carcinomas (23, 43). Therefore, the occurrence of higher expression of c-FLIPL in tumor cells than in their normal counterparts suggests that c-FLIPL plays an important role in tumor survival and may serve as a potential molecular target for anticancer therapy (44–46).

In the current study, 385 patients were enrolled from 1 hospital, but these findings have not been confirmed in another study population. Therefore, the findings need further validation by another investigative group. Nevertheless, to our knowledge, this is the first report to indicate that the Fasl. –844CC carrier has a poorer OS and RFS than the Fasl. –844TT+TC carrier, due to the higher Fasl. expression in lung tumors in Fasl. –844CC carriers. Fasl. expression was positively correlated with c-FLIPL expression in lung tumors, which suggests that Fasl. –844CC carriers with high Fasl. expression had pronounced T-cell apoptosis that resulted in immune escape. Therefore, FasL seems to be a promising potential target for immunotherapeutic interventions to reduce the disease recurrence rate and to improve the outcome in resected NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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gene promoter alters Fas ligand expression: a candidate background 
polymorphisms in cell death pathway genes FAS and FASL contribute 
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