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

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Running head: FasL -844CC predicts poor prognosis in NSCLC

Key words: FasL; lung cancer; clinical outcome

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C-Y Chen and H Lee equally contribute to this work.
Translational Relevance

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 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-RFLP technique in bloods may potentially be used to predict the disease relapse and prognosis in resected lung cancer, and once validated, it would be helpful for the clinician to choose a better therapeutic strategy.
Abstract

**Purpose:** 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-RFLP. 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). Additionally, patients with the FasL -844CC genotype were more prone to tumor relapse when compared with 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 validation by other investigative group.
Introduction

FasL (Fas Ligand: TNF receptor superfamily, member 6) is 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 (pro-caspase-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-FLIP_L, which is highly competitive and homologous to pro-caspases-8 and -10 but does not induce further apoptotic signaling by pro-caspases (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-κB, and JNK 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 anti-cancer 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 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 over-expressed 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 the present 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-FLIP_L expression that 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, 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 co-culture 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 using 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, USA). First-strand cDNA synthesis in the presence of random primers was performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer’s instructions.

**PCR-RFLP Analysis for FasL -844T/C Genetic Polymorphism**

Genotypes of FasL -844T/C were determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) as described by Sun et al. (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 performed with an ABI 7500 Real time PCR System (Applied Biosystems, USA) and SYBR Green dye to quantify FasL mRNA transcripts. Real-time PCR primers were as follows: for FasL transcripts, 5'-CAGTCCACCCCCTGAAAAAA-3' (forward) and 5'-TCCATAGGTGTCTTCCCATTCC-3' (reverse); for 18S gene transcripts, 5'-TCGGAACTGAGGCCATGA-3' (forward) and 5'-CCGGTCGGCATCGTTTA-3' (reverse) were used. 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 $2^{-\Delta CT}$ (where CT represents the fluorescence threshold cycle, $\Delta CT = CT_{FasL} - CT_{18S}$) according to the manufacturer’s instructions (Applied...
Biosystems, USA). The cut-off 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 performed on 87 whole-mount paraffin sections of lung cancer specimens. Anti-FasL (1/500) polyclonal primary antibody (N-20, Santa Cruz Biotechnology, USA) and anti-c-FLIPL (1/100) polyclonal primary antibody (C-19, Santa Cruz Biotechnology, USA) were used (26, 31). An immunohistochemistry (IHC) detection kit for in vitro diagnostic use (Invitrogen, USA) 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 (26).

**Direct sequencing**

Tumor DNA was amplified and used for determining p53 mutation by direct sequencing with an Applied Biosystems 3100 Avant Genetic Analyzer (Applied Biosystems, USA) as described by Cheng et al. (32).

**Cell culture**

H1355 and A427 lung cancer cells were purchased from The American Type Culture Collection (ATCC, USA). TL4 and TL13 lung cancer cells were established from pleural effusions as described previously (32). The pathological diagnosis of TL13 was lung adenocarcinoma of a 79-year-old male nonsmoker with T₄N₃M₁. H1355 and A427 were
maintained in DMEM medium (Gibco™, USA) with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. TL4 and TL13 were maintained in RPMI medium (Gibco™, USA) with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Plasmid construction and transfection reaction**

RNA interference was performed 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 Medical at Chung Shan Medical University, Taichung, Taiwan. The shRNA contain 21 nucleotides from the FasL sequence as target site was AGGCCTGTGTCTCCTTGTGAT. The procedures and methods were as described previously (32).

**Peripheral blood monocyte isolation and co-culture experiments**

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

**Flow cytometry analysis**
A flow cytometer (FACSCalibur; BD Biosciences, Canada) was used to determine the cell population size and apoptosis percentage. PBMCs were stained with PE-Cy™7 mouse anti-human CD3 (BD Pharmingen, Canada) and the CD3 positive cells in the lymphocyte gate were identified as T cells. Annexin V-FITC (BD Pharmingen, Canada) 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 FSC-SSC, and the population with CD3⁺ and Annexin V⁺ was calculated.

**Statistical Analysis**

The Student’s t test and Chi-square 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., Chicago, IL). All statistical tests were two-sided and P values < 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 result of PCR-RFLP for FasL -844 genotype (100 of 385 samples) has been confirmed by a direct sequencing. The relationships between the FasL -844T/C genotype and clinic-pathological 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 clinic-pathological 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 \( \beta \) 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 IHC 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% vs. 40.6%, P = 0.004 for FasL genotype vs. mRNA; 77.8% vs 35.7%, P < 0.001 for FasL mRNA vs. protein; Table 2). These results suggest that FasL -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 -844CC carriers**

Previous reports have shown that PBMCs with the FasL -844CC genotype undergo more T cell apoptosis than those with FasL -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 -844T/C genotype in a panel of lung cancer cells by a direct sequencing. We selected H1355 and TL13 cells harbored FasL -844CC genotype and TL4 and A427 cells that harbored the FasL -844TT for this study. As expected, H1355 and TL13 cells carrying FasL -844CC genotype had a higher level of FasL expression than did TL4 and A427 cells carrying the FasL -844TT genotype (Figure 1A). We also verified whether lung cancer cells carrying different FasL -844 genotypes could induce different degree of T cell apoptosis. PBMCs from 11 healthy donors were co-cultured with the four 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
co-cultured with H1355 and TL13 cells than in PBMCs co-cultured 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 a small interfering RNA (RNAi). We found that the prevalence of T cells apoptosis decreased significantly in PBMCs co-cultured with FasL-knockdown cells when compared with control cells expressing a non-specific RNAi control (NC cells) (Figure 1C and D, P = 0.003 for H1355; P = 0.012 for TL13). Lung cancer cells carrying the FasL -844CC genotype clearly induced greater level of T cell apoptosis than did lung cancer cells carrying the FasL -844TT genotype. PBMCs carrying the FasL -844CC genotype also shown higher T cell apoptosis than PBMCs carrying FasL -844TT genotype when PBMCs were co-cultured with A549 lung cancer cells or treated with Phytohemagglutinin (PHA, Sigma) (P = 0.046 for A549 cells; P = 0.025 for PHA; Supplementary Figure 1B). The cytotoxicity of A549 cells against PBMCs carrying different FasL -844 genotypes was evaluated by a flow cytometry with Annexin V under PHA stimulation. Lower cytotoxicity to A549 cells was seen for PBMCs with the FasL -844CC genotype than with the FasL -844TT+TC genotype (Supplementary Figure 1C, P = 0.020). Considering this evidence, we suggest that the FasL -844CC carrier appeared to be less capable of immune surveillance.

Tumor evasion to FasL-induced apoptosis may be due to the concomitant expression of FasL and c-FLIP_L 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-FLIP_L
expression by IHC. Up to 80% of the tumors with high FasL expression had high c-FLIP_L, while only 26.2% of tumors with low FasL expression showed high c-FLIP_L (P < 0.001; Table 3), suggesting that tumors resist FasL-induced apoptosis via overexpression of c-FLIP_L. Expression of c-FLIP_L 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 over-expressed c-FLIP_L, resulting in evasion of FasL-induced apoptosis.

**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 appeared 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. Among 353 patients with tumor relapse follow-up data, 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 (17 cases), liver (17 cases), chest wall (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 one organ. All 385 patients were available for OS and 353
were available for RFS analysis. Among the studied population, patients with adenocarcinoma, late-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 compared with those with FasL -844TT+TC genotype (Supplementary Figure. 2). Multivariate Cox-regression analysis further indicated that patients with the FasL -844CC genotype had a shorter median survival than did those with the FasL -844TT+TC genotype (24.1 months vs. 42.8 months for OS, 15.4 months 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% 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 epidemiological 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 -844T/C 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). Surprisingly, the distribution of FasL -844 genotypes in two studies reported by Park’s group was quite different (TT/TC/CC: 51.6%/41.8%/6.6% vs. 8.1%/39.2%/52.8%) (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 compared with those with the FasL -844TT+TC genotype, which results in a high rate of T cell apoptosis when these are co-cultured with cervical and breast cancer cells (19, 21). In the present study, co-culture of PBMCs from healthy donors with lung cancer cells
gave a similar result (Figure. 1). Previous reports indicated that significantly higher FasL expression 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.

Aside 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 anti-cancer 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
analysis. 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 ≥ 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.

We also examined whether the greater T cell apoptosis seen in FasL -844CC carriers could be induced by a lower c-FLIP_L expression in tumor-infiltrating lymphocytes than in lung tumors. We compared the number of tumor-infiltrating lymphocytes and c-FLIP_L 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%) demonstrated the presence of tumor-infiltrating lymphocytes. Among these, 6 of 58 (10.3%) tumor-infiltrating lymphocytes showed high c-FLIP_L immunostaining. Representative tumor-infiltrating lymphocytes found in lung tumor paraffin sections and the expression of c-FLIP_L in tumor-infiltrating lymphocytes was shown in the supplementary figure 3. Overall, these findings support our hypothesis that c-FLIP_L is more commonly expressed in lung tumors (47 of 87, 54%) than in tumor-infiltrating lymphocytes (10.3%) and that it may more confer lung tumors with the ability to escape FasL-induced apoptosis.

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

The limitation of the current study was patients to be enrolled from a hospital, not confirmed by the other study population. Therefore, the findings need further validation by other 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-FLIP\textsubscript{L} 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 appears to be a promising potential target for immunotherapeutic interventions to reduce the disease recurrence rate and to improve the outcome in resected NSCLC.
ACKNOWLEDGMENT

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Figure legend

Figure 1. Greater T cell apoptosis in PBMCs co-cultured with lung cancer cells carrying the FasL -844CC genotype than co-cultured with lung cancer cells carrying the FasL -844TT genotype. T cell apoptosis was defined as Annexin V+/CD3+. (A) Flow cytometry evaluation of FasL expression in TL4, A427, H1355, and TL13 cells. (B) Higher level of T cell apoptosis in PBMCs from 11 healthy male volunteers co-cultured with H1355 and TL13 cells which harbored the FasL -844CC genotype, compared with cells co-cultured with TL4 and A427 cells which harbored FasL -844TT genotype. The mean values of T cell apoptosis in PBMCs co-cultured with TL4, A427, H1355, and TL13 were 5.2 ± 2.1, 11.7 ± 2.4, 18.9 ± 2.2, and 14.5 ± 3.4 (mean ± SD), respectively. (C) FasL expression in FasL-knockdown H1355 and TL13 cells was evaluated by flow cytometry and compared with parental and non-specific RNAi control cells. (D) T cell apoptosis decreased markedly in PBMCs from 11 healthy male volunteers co-cultured with FasL-knockdown H1355 and TL13 cells compared with PBMCs co-cultured with non-specific RNAi control cells. The mean value of T cell apoptosis in PBMCs co-cultured with H1355 non-specific RNAi control, H1355 FasL knock down, TL13 non-specific RNAi control, and TL13 FasL knock down cells were 18.1±4.0, 11.5±2.2, 13.5±4.6, and 8.5±3.8 (mean ± SD), respectively.
Figure 1.

(A)

(B)

(C)

(D)

Quantified Analysis of Cell Membrane

P < 0.001

P < 0.001

P < 0.001

P < 0.001

TL4  A27  H155  TL13

Quantified Analysis of Cell Membrane

P = 0.003

P = 0.012

H155  TL13
Table 1. Relationships of FasL -844T/C polymorphism with clinical parameters in non-small cell lung cancer patients.

<table>
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<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 (49.0)</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>
</tbody>
</table>
Table 1. (continued)

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>63 (16.4)</td>
<td>30 (47.6)</td>
<td>33 (52.4)</td>
</tr>
<tr>
<td></td>
<td>173 (44.9)</td>
<td>75 (43.4)</td>
<td>98 (56.6)</td>
</tr>
</tbody>
</table>

**Tumor size**

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<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>
</tbody>
</table>

**Nodal involvement**

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<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>
</tbody>
</table>

**Tumor relapse**

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<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>

P values were obtained by two sided Chi-square test; SQ: Squamous cell carcinoma; AD: Adenocarcinoma; P = 0.002 for stage I vs. stage II+III; P = 0.031 for T1+T2 vs. T3+T4; P = 0.021 for N0 vs. N1+N2.
Patients with local regional recurrence and distant metastasis after surgical therapy were identified to have tumor relapse.
Table 2. Association of FasL mRNA expression levels with FasL -844T/C polymorphism and FasL protein expression in non-small cell lung cancer patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case No. (%)</th>
<th>FasL mRNA</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td></td>
<td>P value</td>
</tr>
<tr>
<td>FasL genotype</td>
<td></td>
<td></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>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>73 (42.0)</td>
<td>27 (37.0)</td>
<td>46 (63.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FasL expression score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score &lt; 100</td>
<td>42 (48.3)</td>
<td>27 (64.3)</td>
<td>15 (35.7)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Score ≥ 100</td>
<td>45 (51.7)</td>
<td>10 (22.2)</td>
<td>35 (77.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 vs. FasL protein expression.
Table 3. Association of FasL expression and p53 status with c-FLIP<sub>L</sub> in non-small cell lung cancer patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Case No. (%)</th>
<th>c-FLIP&lt;sub&gt;L&lt;/sub&gt;</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Score &lt; 100</td>
<td>Score ≥ 100</td>
</tr>
<tr>
<td>FasL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Score &lt; 100</td>
<td>42 (48.3)</td>
<td>31 (73.8)</td>
<td>11 (26.2)</td>
</tr>
<tr>
<td>Score ≥ 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>

c-FLIP<sub>L</sub> 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.
Table 4. Univariate and multivariate analysis of the influence of various parameters on overall survival (OS) and relapse free survival (RFS) in NSCLC patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Category</th>
<th>Median OS (Month)</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
<th>Median RFS (Month)</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>≥65/&lt;65</td>
<td>28.8/39.0</td>
<td>1.250</td>
<td>0.968-1.615</td>
<td>0.087</td>
<td>21.2/23.2</td>
<td>1.219</td>
<td>0.945-1.573</td>
<td>0.127</td>
</tr>
<tr>
<td>Gender</td>
<td>Male/Female</td>
<td>30.6/33.1</td>
<td>1.231</td>
<td>0.930-1.629</td>
<td>0.146</td>
<td>20.9/24.0</td>
<td>1.243</td>
<td>0.941-1.641</td>
<td>0.125</td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes/No</td>
<td>28.8/30.6</td>
<td>1.079</td>
<td>0.841-1.383</td>
<td>0.551</td>
<td>21.9/22.1</td>
<td>1.037</td>
<td>0.810-1.327</td>
<td>0.772</td>
</tr>
<tr>
<td>Tumor type</td>
<td>AD/SQ</td>
<td>26.4/41.4</td>
<td>1.337</td>
<td>1.041-1.716</td>
<td>0.023</td>
<td>19.9/26.9</td>
<td>1.302</td>
<td>1.016-1.669</td>
<td>0.037</td>
</tr>
<tr>
<td>Stage</td>
<td>II+III/I</td>
<td>22.1/56.7</td>
<td>2.110</td>
<td>1.608-2.768</td>
<td>&lt;0.001</td>
<td>14.4/39.0</td>
<td>1.970</td>
<td>1.517-2.559</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>II+III/I</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>-------------------</td>
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<td>---</td>
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</tr>
<tr>
<td>Stage*</td>
<td>II+III/I</td>
<td>22.1/56.7</td>
<td>2.343</td>
<td>1.774-3.095</td>
<td>&lt;0.001</td>
<td>14.4/39.0</td>
<td>2.163</td>
<td>1.562-2.833</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FasL -844T/C</td>
<td>CC/TT +TC</td>
<td>24.1/42.8</td>
<td>1.628</td>
<td>1.268-2.091</td>
<td>&lt;0.001</td>
<td>15.4/31.4</td>
<td>1.843</td>
<td>1.435-2.367</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FasL -844T/C*</td>
<td>CC/TT +TC</td>
<td>24.1/42.8</td>
<td>1.455</td>
<td>1.129-1.874</td>
<td>0.004</td>
<td>15.4/31.4</td>
<td>1.710</td>
<td>1.327-2.205</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, smoking, tumor type, and stage.
Clinical Cancer Research

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

Wen-Wei Sung, Yao-Chen Wang, Ya-Wen Cheng, et al.

Clin Cancer Res  Published OnlineFirst August 1, 2011.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-0227

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2011/08/01/1078-0432.CCR-11-0227.DC1

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