

## Original Article

# Correlations between Fas/FasL expression and apoptosis as well as clinicopathological features in non-small cell lung cancer

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Received October 26, 2018; Accepted February 12, 2019; Epub June 15, 2020; Published June 30, 2020

**Abstract:** Lung cancer is a common malignant tumor with rapidly increased incidence. Fas and FasL are important protein molecules inducing cell apoptosis. This study tested Fas/FasL expression in non-small cell lung cancer (NSCLC) and analyzed the correlation with cell apoptosis and clinical/pathological features. NSCLC patients were recruited in our hospital, in parallel with healthy individuals. Enzyme linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) were used to test serum and tissue expression of Fas and FasL. Number of tumor infiltrative lymphocytes (TILs) was measured, in addition to TUNEL assay for lung cancer cell apoptosis. The correlation between Fas/FasL expression and cell apoptosis or clinical pathological features was analyzed. Compared to the control group, patients had lower serum or tissue Fas positive rate, and elevated FasL positive rate ( $P < 0.05$ ). Those individuals with high TIL had potent Fas expression than those with low TIL level, plus decreased FasL expression and elevated tumor cell apoptosis rate ( $P < 0.05$ ). FasL was negatively correlated with cell apoptosis, while Fas was positively correlated with cell apoptosis ( $P < 0.05$ ). With more advanced TNM stage, lower differentiation grade and occurrence of lymph node metastasis, FasL expression was increased while Fas expression was decreased ( $P < 0.05$ ). NSCLC patients have lower Fas expression and higher FasL expression, both of which participate in occurrence and progression of NSCLC.

**Keywords:** Non-small cell lung cancer, Fas, FasL

## Introduction

Apoptosis is programmed cell death that can maintain body homeostasis, and is mainly regulated by genetics. Previous studies suggested a correlation between malignant tumor genesis and immune escape from the perspective of immunology [1, 2]. Pathways for cell apoptosis include death receptor pathway, mitochondrial pathway. Fas is highly expressed in various cell types such as fibroblast or lymphocytes. As one important death receptor, Fas has the ligand, FasL, which is one type II transmembrane glycoprotein that is distributed on the surface of multiple cells. After binding between Fas and FasL, cell apoptosis is initiated after receptor activation [3, 4]. Tumor-specific antigen can regulate tumor infiltrative T lymphocytes (TIL) to induce their up-regulation of Fas, which enhances sensitivity of T cells for apoptosis. In-

duction is then achieved via ligand FasL, which produces cell apoptosis in those T lymphocytes with high expression of Fas [5, 6]. Previous study indicated lower Fas expression or even silencing during occurrence and progression of various malignant tumors such as colorectal carcinoma, rectal cancer and cervical cancer, leading to inhibited lymphocyte-induced apoptosis of tumor cells [7, 8]. This study thus recruited non-small cell lung cancer (NSCLC) patients to quantify Fas/FasL expression in blood and tissues, followed by the correlation analysis with cell apoptosis and clinical pathology features.

## Information and methods

### General information

A total of 40 NSCLC patients who received treatment in Inner Mongolia Peoples Hospital

**Table 1.** Blood Fas/FasL contents in the patient group

Group	N	Fas (ng/ml)	FasL (ng/ml)
Patients	40	0.16 ± 0.04*	1.47 ± 0.07*
Control	20	1.03 ± 0.01	0.02 ± 0.01

Note: \*, P < 0.05 compared to the control group.

from January 2015 to January 2016 were recruited. All patients were confirmed as NSCLC by pathology examination during surgery or in biopsy. There were 20 males and 20 females in the patient group, with ages between 20-70 years (average age = 45.2 ± 8.4 years). Another cohort of 20 healthy individuals in our hospital were recruited as the control group, including 12 males and 8 females, aging between 20 and 70 years (average age = 45.1 ± 3.4 years). No significant difference of sex or age existed between two groups (P > 0.05), which were thus comparable.

The study protocol was approved by the Research Ethics Committee of Inner Mongolia Peoples Hospital, and all patients gave their informed consent before study commencement.

#### Reagents and equipment

ELISA kits for Fas and FasL; Primary antibody for Fas and FasL; Rabbit anti-mouse secondary antibody; DAB test kit (Maixin Biotech, China); Mouse anti-human CD45RO monoclonal antibody (MAB-0039).

#### ELISA for blood Fas and FasL contents in patients

Fasted venous blood samples were collected from all patients. Blood sample was centrifuged to collect the supernatant (serum). ELISA was used to test blood Fas/FasL contents. In brief, patients' serum and standard samples were diluted and added into 96-well plate. After washing, developing and quenching, the absorbance value at 450 nm was measured.

#### IHC method for detecting Fas, FasL and CD45RO expression in tissues

Tissues were fixed, dehydrated, and embedded in paraffin for preparing serial slices. Tissue slices were de-waxed, re-hydrated, processed in heated antigen retrieval and blocking solu-

tion. Primary antibody and secondary antibody were sequentially added into slices for 1 hour and 10 minute incubation. The slices were developed, quenched, counter-stained, and mounted for observation.

Fas/FasL positive cells were defined as no staining in nucleus and brown-yellow granules in cytoplasm. Staining was classified based on percentage of positively stained cells: negative (-), less than 10%; weak positive (+), 11%~25%; positive (++) , 26%~50%; strong positive (+++) , higher than 50%.

In evaluating CD45RO level, a total of 25 fields (200×) were counted. Low TIL was defined as less than 150 cells, while high TIL was defined as higher than 150 cells.

#### TUNEL for apoptotic cells

Tissues were dehydrated, embedded, and sectioned. Slices were rinsed in xylene and gradient ethanol, followed by Proteinase K incubation. A total of 50 µl TUNEL mixture was added for counting apoptotic cells. Also, 50 µl converter-POD was added, followed by 50 µl DAB substrate. The slice was counter-stained by hematoxylin and counted for those cells with brown granules in nucleus.

#### Data processing

SPSS17.0 software was used for data processing. Enumeration data were tested by Chi-square test, while measurements were analyzed by ANOVA and are presented as mean ± standard deviation (SD). Multi-variate analysis was performed using Logistic regression model. A statistical significance was defined when P < 0.05.

## Results

#### ELISA for Fas/FasL contents in patient serum

Tests for serum Fas/FasL contents revealed 0.16 ± 0.04 ng/ml Fas in patients group, which was higher than the control group, and 1.47 ± 0.07 ng/ml FasL, which was higher than the control group (P < 0.05, **Table 1**).

#### IHC for tissue Fas/FasL expression in patients

Further assays of Fas/FasL expression in patient tissues, using no nuclear staining plus

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**Table 2.** Fas/FasL expression in patients

Group	N	Fas expression level			Positive rate (%)	FasL expression level			Positive rate (%)
		-	+--+	+++		-	+--+	+++	
Tumor	40	25	14	1	37.5*#	10	19	11	75*#
Adjacent	40	5	25	10	87.5#	30	10	0	25#
Control	20	1	9	10	95	17	3	0	15

Note: \*, P < 0.05 compared to adjacent tissues; #, P < 0.05 compared to the control group.

brown/brown-yellow granules in cytoplasm as positive staining, revealed 37.5% positive rate of Fas in patient group, while tumor adjacent tissues showed abundant brown granules with 87.5% Fas-positive rate, which was significantly higher than tumor tissues (P < 0.05) (Table 2; Figure 1). In FasL staining, tumor tissues had abundant brown granules (positive rate = 75%), while adjacent tissues had minor signals (positive rate = 25%).

### *Fas and FasL expression in patient with different TIL grades*

Fas and FasL expression levels in patient's tumor tissues were tested with various TIL infiltration grade reagents. Results showed 87.5% Fas-positive rate in NSCLC cases with high TIL level, as higher than low-TIL group. FasL positive rate was 56.3% in high-TIL groups, as lower than low-TIL group (P < 0.05, Table 3).

### *TUNEL for cell apoptosis*

Apoptotic cells were further analyzed, which are presented as brown granules in lymphocyte nucleus. In pulmonary tissues, only minor brown granules were observed in cancer tissues, with (3.37 ± 1.50)%. Abundant brown granules were observed in both adjacent tissues and normal tissues, with apoptotic rates at 6.59 ± 7.46% and 31.47 ± 17.99%, respectively. Tumor tissues had lower cell apoptotic rate than adjacent tissues or the control group (P < 0.05, Figures 2 and 3).

### *Correlation between Fas/FasL expression and cell apoptosis*

FasL is negatively correlated with cell apoptotic rate (r = 0.84, P < 0.05). Fas is positively correlated with cell apoptotic rate (r = -0.65, P < 0.05). FasL is negatively correlated with Fas (r = -0.27, P < 0.05).

### *Correlation between Fas/FasL expression and clinical pathology features of lung cancer*

Analysis for the correlation between Fas/FasL expression and pathology features was then performed. Results show significantly decreased Fas expression and elevated FasL expression in NSCLC tumor tissues (P < 0.05). In

those with more advanced TNM stage, lower differentiation grade, or occurrence of lymph node metastasis, FasL expression was up-regulated while Fas expression was down-regulated (P < 0.05, Table 4).

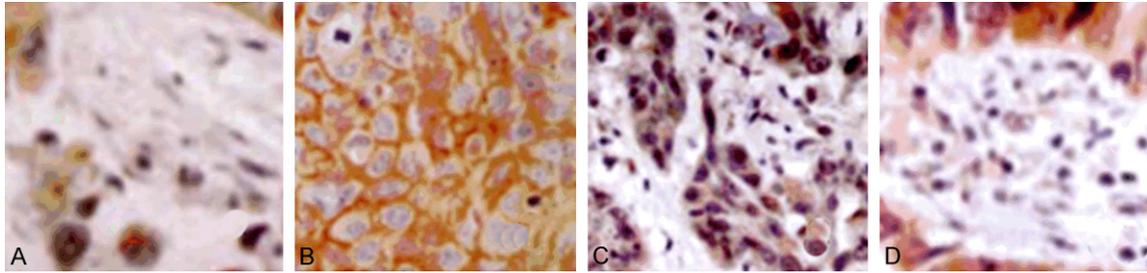
## Discussion

Fas is one transmembrane glycoprotein, and has FasL as its natural ligand. After specific binding, Fas can induce apoptotic related signal to stimulate apoptosis of Fas-positive target cells. Maintenance of Fas/FasL signal transduction pathway thus can guarantee homeostasis of cell proliferation and apoptosis, thereby playing a critical role in maintaining body homeostasis [9, 10]. Fas expression is expressed in various body cells and malignant tumors, while its ligand FasL is expressed in mature T cells and NK cells surface [11]. When both Fas and FasL are highly expressed in the surface of malignant tumors, binding between those factors can resist against incoming death signal, thus escaping from immune surveillance and achieving tumor proliferation and progression [12, 13].

In this study, NSCLC patients were recruited to collect fasted blood samples, in which serum Fas and FasL levels were quantified by ELISA. Experimental group had decreased Fas level and elevated FasL level. Further IHC staining showed decreased Fas and increased FasL expression in NSCLC tumor tissues compared to adjacent tissues or control group. These results indicated that NSCLC patients presented Fas down-regulation and FasL up-regulation.

Previous studies have shown abundant lymphocytes in the infiltration of various tumor tissues. These lymphocytes mainly induce cellular immunity to achieve effective immune surveillance and cytotoxicity [14]. Mouse anti-human CD45RO monoclonal antibody can recog-

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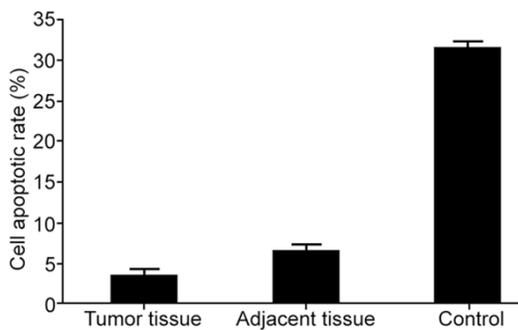


**Figure 1.** Fas and FasL expression in patient tissues ( $\times 400$ ). A. Fas expression in tumor adjacent tissues (+); B. Fas expression in tumor tissues (+++); C. FasL expression in adjacent tissues (+++); D. FasL in tumor tissues (+).

**Table 3.** Fas and FasL expression in NSCLC patients with different TIL infiltration grades

TIL grade	N	Fas expression			Positive (%)	FasL expression			Positive (%)
		-	+++	+++		-	+++	+++	
Low	24	15	8	1	37.5	4	9	11	83.3
High	16	2	5	9	87.5*	7	9	0	56.3*

Note: \*,  $P < 0.05$  compared to the low TIL group.



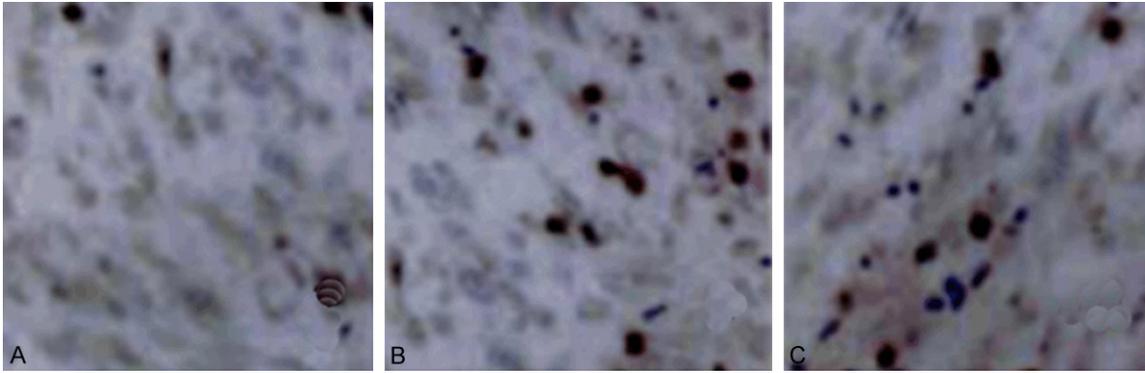
**Figure 2.** Apoptotic rate of tissues.

nize lymphocytes infiltrated around malignant tumors, mainly via reaction with CD45RO determinants [15]. This study thus tested Fas and FasL expression level in NSCLS patients with different TIL grade, and found 87.5% Fas-positive rate in NSCLC patients with high-TIL, which was higher than low-TIL group. While the FasL-positive rate was 56.3% in high-TIL group and was lower than the low-TIL group. These studies collectively show that higher TIL grade in NSCLC patients is correlated with elevated Fas and decreased FasL expression.

To further illustrate the relationship between Fas/FasL expression in NSCLC tissues and cell apoptosis, apoptosis of all cells was further tested and found to have a decreased apop-

otic rate in tumor tissues compared with adjacent tissues or control group. Previous study showed the existence of susceptible window of activated TIL for Fas-induced apoptosis [16]. Fas expression must be down-regulated for tumor cells in order to escape from TIL-induced apoptosis. This down-regulation, on the other hand, leads to highly expressed FasL to produce attacking roles for direct effects on TIL. At this time, TIL is more susceptible for apoptosis, making tumor cells escape from body immune surveillance, enhance their proliferation and accelerate growth velocity [16]. Among various studies regarding Fas and cell apoptosis, resistance of lung cancer cells against Fas-induced apoptosis is observed. This effect may be related to multiple factors, among which the most important one is p53 mutation. Wild-type p53 can enhance Fas expression. In contrast, the mutant form of p53 inhibited Fas expression [17-19]. Due to high frequency of p53 gene mutation in pulmonary carcinoma, it is thus common to observe inhibition of Fas/FasL-induced cell apoptosis pathway in lung cancer tissues.

To further analyze the correlation between Fas/FasL expression in NSCLC and clinical pathology features, lower Fas and higher FasL expression in NSCLC cases with advanced TNM stage was observed. This led to lower differentiation grade or even lymph node metastasis. Previous studies have shown higher distal metastasis probability in FasL-positive tumor cells [20]. Dai et al. found lower Fas level in breast cancer tissues [21]. Another clinical study for NSCLC also found lower Fas plus higher FasL expression in patients with lymph node metastasis [22], similar to this study.



**Figure 3.** TUNEL staining images. A. Tumor tissues; B. Tumor adjacent tissues; C. Control group.

**Table 4.** Correlation between Fas/FasL expression and apoptotic rate

Parameter	N	Fas	FasL
Control group	20	19	3
Tumor tissue	40	15	30
Adjacent tissue	40	35	10
P		P < 0.05	P < 0.05
Tumor subtype			
Squamous	25	10 (40)	19 (76)
Adenoma	15	5 (33.3)	11 (73.3)
P		P > 0.05	P > 0.05
TNM stage			
I	15	7 (46.7)	10 (66.7)
II	10	4 (40)	7 (70)
III	10	3 (30)	8 (80)
IV	5	1 (20)	5 (100)
P		P < 0.05	P < 0.05
Differentiation grade			
High	7	5 (71.4)	5 (71.4)
Moderate	11	6 (54.5)	7 (63.6)
Low	23	4 (17.4)	18 (78.3)
P		P < 0.05	P < 0.05
Lymph node metastasis			
Negative	24	11 (45.8)	17 (70.8)
Sum	16	4 (25)	13 (81.3)
P		P < 0.05	P < 0.05

**Conclusion**

In both serum and tissue samples of NSCLS patients, Fas is down-regulated while FasL is up-regulated. For those patients with higher TIL grade, positive rate is higher for Fas and lower for FasL. Moreover, cell apoptotic rate of tumor tissues is significantly decreased. With advanced TNM stage, lower differentiation gra-

de and lymph node metastasis, FasL positive rate is lower and FasL expression is higher. The lower Fas and higher FasL expression profile may achieve unlimited proliferation of tumor cells, causing unfavorable prognosis. In treating NSCLC, facilitation of Fas expression plus suppression of FasL expression may achieve certain treatment effects, although detailed mechanism remains to be elucidated.

**Disclosure of conflict of interest**

None.

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