ORIGINAL ARTICLE

Expression of TNF-related Apoptosis-inducing Ligand (TRAIL) and TRAIL Receptor 1 on Cancer and Normal Tissues in Patients with Non-small Cell Lung Cancer

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ABSTRACT —— ***Objective.*** Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in various *in vitro* tumor studies. However *de novo* expression of TRAIL and TRAIL-receptor 1 (TRAIL-R1) in cancer tissues is not well documented, and few data are available on their effect on the clinical outcome of non-small cell lung cancer (NSCLC) patients. The aim of this study was to evaluate the expression of TRAIL and TRAIL-R1 proteins in cancer tissues surgically resected from patients with NSCLC by scoring the immunoreactivity, and to examine the correlation between the expression of these proteins and clinical findings of NSCLC. ***Methods.*** Cancer tissues and normal tissues were obtained from patients with NSCLC. The expression of TRAIL and TRAIL-R1 proteins was examined by immunohistochemical studies. ***Results.*** Significantly higher immunostaining scores for TRAIL and TRAIL-R1 were observed in cancer tissues compared with normal tissues of patients with NSCLC ($P < 0.0001$ for both). There was a significantly positive correlation between the immunostaining score of TRAIL or TRAIL-R1 and pathological T-factor in these patients ($P = 0.021$ and $P = 0.009$, respectively). ***Conclusion.*** The majority of NSCLCs expressed TRAIL and TRAIL-R1, which may play a role in regulating the progression of NSCLC. (*JJLC.* 2006;46:321-327)

KEY WORDS —— Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), Tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), Non-small cell lung cancer (NSCLC), Immunohistochemistry

INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a recently identified type II integral membrane protein belonging to the TNF family. This factor induces apoptosis in various tumor cell lines.[1,2] Among members of the TNF family, TRAIL exhibits high homology to Fas L/CD95L, which has been implicated in T-cell cytotoxicity and immune regulation.[3] TRAIL mRNA is found in a variety of tissues and cells,[1] but its physiological role remains largely unknown.[4]

Recent molecular cloning of the TRAIL receptors (TRAIL-Rs) has elucidated that TRAIL binds to at least four receptors (TRAIL-R1/DR4, TRAIL-R2/DR5/TRICK2, TRAIL-R3/TRID/DcR1/LIT and TRAIL-R4/TRUNDD/DcR2) with similar affinities.[5-9] These receptors can be classified into two groups; death-inducing receptors (TRAIL-R1 and TRAIL-R2) and death-inhibiting receptors (TRAIL-R3 and TRAIL-R4). Both TRAIL-R1 and TRAIL-R2 contain a death domain homologous to that in TNFR-I, DR3/TRAMP/APO-3/WSL-1, and Fas. Oligomerization of the death domain in TRAIL-R1 and TRAIL-R2 recruits caspase-8 or -10 via the Fas-associated death domain (FADD) or FADD-like adapter molecules, and activates the subsequent caspase cascade, resulting in apoptotic cell death.[5-7,9] In contrast to these death-inducing receptors, TRAIL-R3 is devoid of a cytoplasmic domain and exists as an aglycophospholipid-anchored

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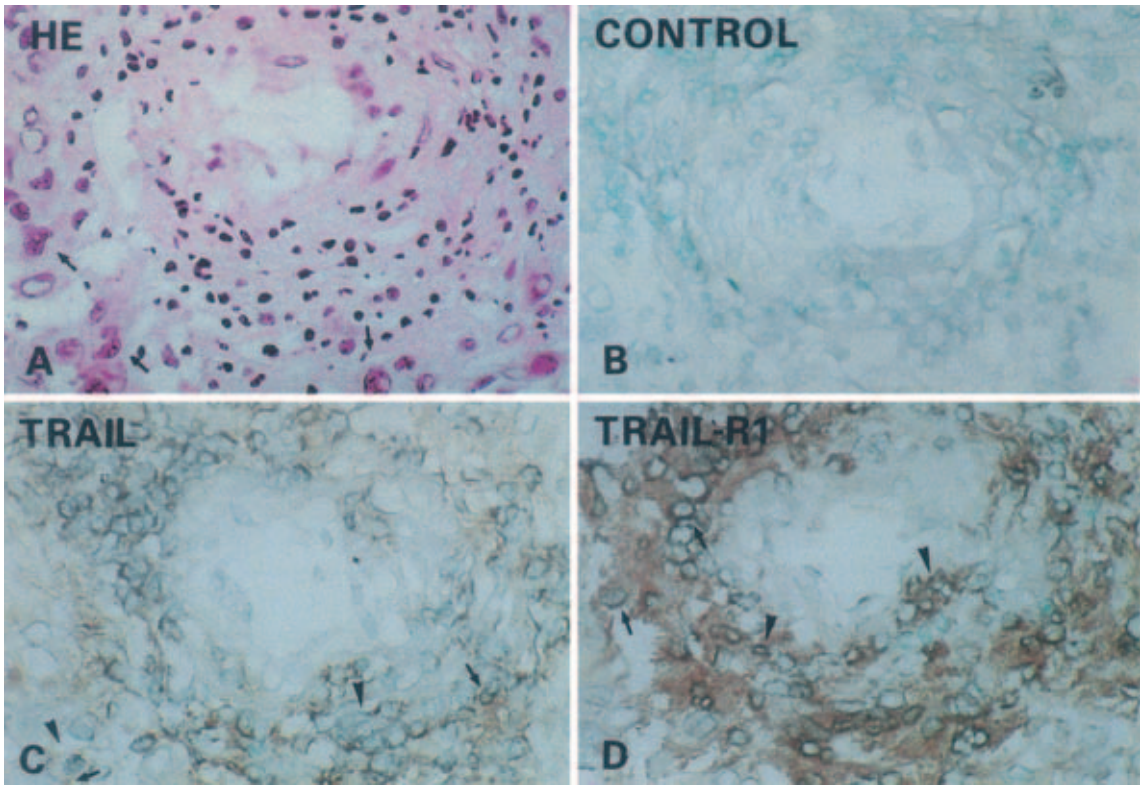

Figure 1. Cancer tissue.
Histopathology and immunohistochemical staining of TRAIL and TRAIL-R1 in cancer tissue from a patient with non-small cell lung cancer. **A:** Many cancer cells are observed (arrows). **B:** Control without primary antibody. **C:** Some TRAIL-positive infiltrating mononuclear cells (arrows) are seen around cancer cells (arrowheads). **D:** TRAIL-R1 expression is observed on some cancer cells (arrows) and a few mononuclear cells (arrowheads). **A:** H & E×200, **B-D:**×200.

protein on the cell surface. Previous reports have suggested that TRAIL-R 3 competes with the death-inducing TRAIL-Rs for binding sites and may function as a decoy receptor.[6,7] TRAIL-R3 may be responsible for the cellular resistance of normal cells to TRAIL-mediated cytotoxicity.[7,9] TRAIL-R4 has a cytoplasmic domain containing a truncated death domain that cannot transmit a death signal but can activate NF-κB, which may protect the cells from TRAIL-mediated apoptosis.[10,11] These findings suggest complex regulatory mechanisms of cellular susceptibility to TRAIL-mediated apoptosis through multiple receptor expression pathways. In contrast to the progress made in the studies of TRAIL receptors, little is known about the expression of TRAIL at the protein level and its physiological functions in lung cancer.[4] The present study aimed to explore the expression of TRAIL and TRAIL-R1 proteins on tissues in non-small cell lung cancer (NSCLC) and the relationship between the level of TRAIL/TRAIL-R1 expression and clinical findings in patients with NSCLC.

PATIENTS and METHODS

LUNG SPECIMENS

Twenty-nine patients with primary NSCLC who underwent curative surgery between February 2000 and May 2001 at the National Chiba-higashi Hospital were studied. No patient received preoperative chemotherapy or radiotherapy. Informed consent was obtained from all patients. There were 20 men and 9 women, with a mean age of 63 years (range, 38-78 years). Tumor and normal tissues were obtained from all 29 cases immediately after removing the resected lung specimens from the operating room. Histological classification and differentiation were assessed by light microscopy independently, prior to immunohistochemical staining. Tumors were

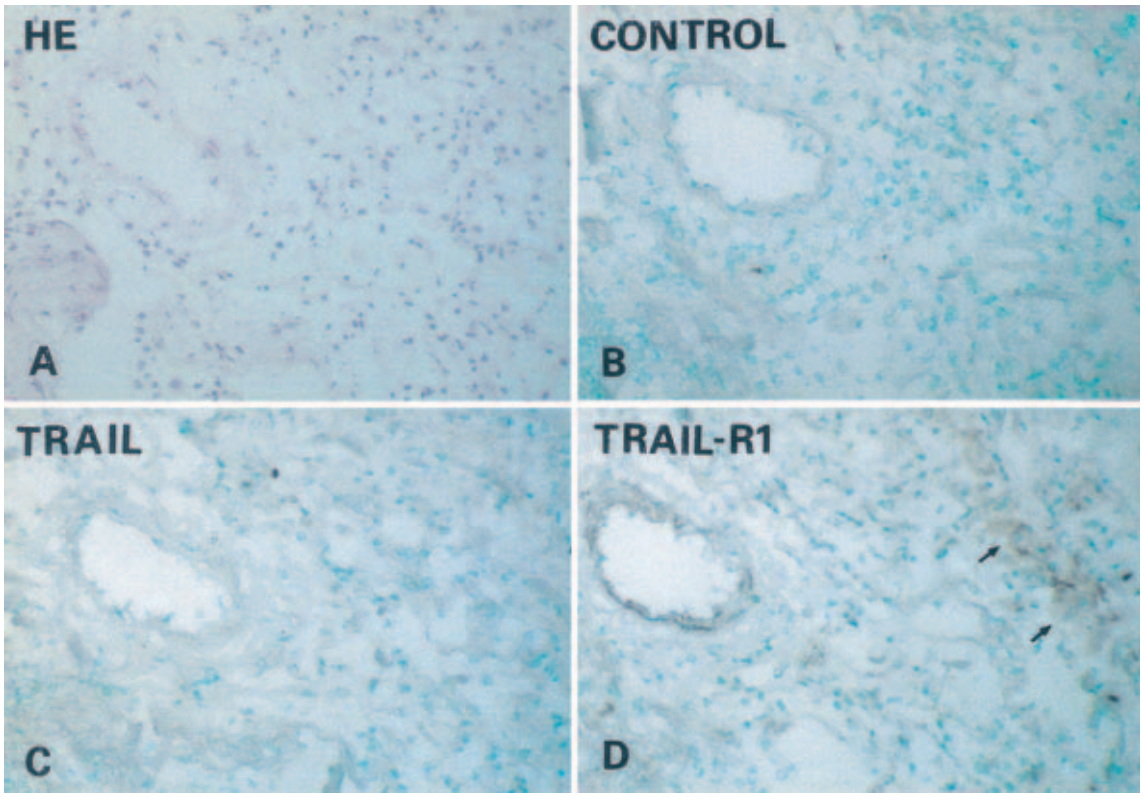

Figure 2. Normal tissue.
Histopathology and immunohistochemical staining of TRAIL and TRAIL-R1 in normal tissue from a patient with non-small cell lung cancer. **A:** No cancer cell is observed. **B:** Control without primary antibody. **C:** No TRAIL-positive cell is observed. **D:** TRAIL-R1 is found on a few alveolar macrophages (arrows). **A:** H & E × 100, **B-D:** × 100.

classified histologically according to the World Health Organization criteria,[12] and included adenocarcinoma (15 cases), squamous cell carcinoma (12 cases), adenosquamous carcinoma (1 case), and large cell carcinoma (1 case). The disease status was determined according to the tumor-node-metastasis staging system[13,14] and classified as stageI (14 cases), stageII (4 cases), stageIII (10 cases), or stage IV (1 case). All tissues were frozen in liquid nitrogen and stored at −80℃ until required.

IMMUNOHISTOCHEMICAL STAINING

Samples of cancer tissue and normal tissue were obtained from each patient during surgical resection of the lung. Tissue sections were prepared for immunoperoxidase staining as follows. Six-μm thick cryostat sections were dried, fixed in acetone for 15 min. at 4℃, and rinsed with phosphate buffered saline (PBS). To detect TRAIL, the sections were treated with normal goat serum, and then incubated successively in the following solutions (washed with PBS between transfer): monoclonal antibody to human TRAIL (RIK-1, a gift from Drs. Kayagaki and Okumura[15]), Envision solution$^+$ (goat anti-mouse immunoglobulins conjugated to peroxidase labelled-dextran polymer; Dako-Japan, Kyoto, Japan), and 0.02% 3,3'-diaminobenzidine (DAB) containing 0.03% H_2O_2 and 10 mM sodium azide. The sections were counterstained with methyl green. To detect TRAIL-R1, the sections were treated with normal goat serum, and then incubated successively in polyclonal antibodies to human TRAIL-R1 (Pharmingen; Fujisawa, Japan), and Envision solution$^+$ (goat anti-rabbit immunogulobulins conjugated to peroxidase labelled-dextran polymer; Dako-Japan). Negative controls were prepared by replacing the primary antibody with buffer.

SCORING

For each specimen, we counted the percentage of TRAIL-positive mononuclear cells relative to all mononuclear cells, and TRAIL-R1-positive cancer cells rela-

Table 1. Immunostaining Scores of TRAIL and TRAIL-R1 in 29 Cases

Case no.	Score of TRAIL*		Score of TRAIL-R1†	
	normal tissue	cancer tissue	normal tissue	cancer tissue
1	1	0	0	1
2	0	1	0	2
3	1	1	1	1
4	0	1	1	1
5	0	1	1	1
6	0	1	0	2
7	0	1	2	2
8	0	1	0	2
9	0	1	1	2
10	0	1	0	2
11	0	1	0	1
12	1	1	2	2
13	0	1	1	1
14	0	1	0	1
15	1	0	1	1
16	0	1	0	2
17	1	0	1	1
18	1	1	1	2
19	1	2	1	2
20	0	1	0	1
21	0	2	1	2
22	0	0	1	1
23	0	0	0	1
24	0	0	0	1
25	0	1	1	2
26	0	1	0	2
27	0	2	1	2
28	1	1	1	2
29	0	2	1	2
Total score	8	27	19	45

*: Percentage of immunoreactive mononuclear cells, 0: no reactive cell, 1: less than 20%, 2: more than 20%.

†: Percentage of immunoreactive cancer cells, 0: no reactive cell, 1: less than 20%, 2: more than 20%.

tive to all cancer cells. Then we scored TRAIL or TRAIL-R1 expression according to the following criteria: Score 0, none; score 1, less than 20%; Score 2, more than 20% TRAIL or TRAIL-R1 expression.

STATISTICAL ANALYSIS

Statistical analysis was done by nonparametric tests. Differences between two groups were analyzed by the Mann-Whitney U-test and differences between more than three groups were analyzed by Kruskal-Wallis rank test. A p-value less than 0.5 was considered to indicated a statistically significant difference.

Table 2. Comparison of Total Immunostaining Scores of TRAIL and TRAIL-R1 in Normal and Cancer Tissues Obtained from 29 Cases of NSCLC

	Normal tissue	Cancer tissue	p-value
TRAIL	8	27	< 0.0001
TRAIL-R1	19	45	< 0.0001

RESULTS

TRAIL-positive cells were detected in 23 of the 29 cancer tissue samples examined (79.3%) (Figure 1C), and the total immunostaining score of TRAIL was 27. On the other hand, TRAIL expression was detected in 8 of the 29 normal tissue samples examined (27.6%) (Figure 2C), and the total immunostaining score of TRAIL was 8 (Table 1). The total immunostaining score of TRAIL was significantly higher in cancer tissues than in normal tissues ($p = 0.0001$) (Table 2). TRAIL-R1 expression was detected in all cancer tissues examined (100%) (Figure 1D), and the total immunostaining score of TRAIL-R1 was 45. On the other hand, TRAIL-R1 expression was detected in 17 of the 29 in normal tissue samples examined (58.6%) (Figure 2D), and the total immunostaining score of TRAIL-R1 was 19 (Table 1). The total immunostaining score of TRAIL-R1 was significantly higher in cancer tissues than in normal tissues ($p = 0.0001$) (Table 2).

The clinical and histopathological characteristics of the patients are shown in Table 3. A significantly positive correlation was observed between the immunostaining score of TRAIL or TRAIL-R1 and pathological T-factor ($p = 0.021$, $p = 0.009$, respectively). However there was no significant correlation between the immunostaining score of TRAIL or TRAIL-R1 and sex, age, histological type, stage, N-factor or differentiation.

DISCUSSION

Multiple genetic lesions have been described in lung cancer.[16] Mutations of both dominant oncogenes and tumor suppressor genes have been reported. Allelic loss (LOH) at chromosomal regions 3p and 9p has been noted in both invasive lung cancer and preneoplastic lesions.[16-18] Allelic loss of chromosome 8p21-22 is a frequent event in various cancers including lung, prostate and colon cancers.[18-20] Deletions of 8p21-23 have been de-

Table 3. Clinicopathological Data and Immunostaining Scores of TRAIL and TRAIL-R1 in 29 Cases of NSCLC

Characteristics	No. of cases	Score of TRAIL	P-value	Score of TRAIL-R1	P-value
Sex					
Male	20	18	NS	31	NS
Female	9	9		14	
Age					
≧ 60	18	14	NS	28	NS
< 60	11	13		17	
Histology					
adeno	15	17		24	
squamous	12	8	NS	18	NS
adenosquamous	1	1		1	
large	1	1		2	
pStage					
I	14	14		24	
II	4	5	NS	6	NS
III	10	7		13	
IV	1	1		2	
pT factor					
pT 1	6	2	P = 0.021	6	P = 0.009
pT 2, 3, 4	23	25		39	
pN factor					
pN 0	19	19	NS	29	NS
pN 1, 2	10	8		16	
Differentiation					
well	6	8		10	
mod	17	15	NS	28	NS
poor	4	2		5	

NS, Not significant; adeno, adenocarcinoma; squamous, squamous cell carcinoma; adenosquamous, adenosquamous cell carcinoma; large, large cell carcinoma; well, well differentiated; mod, moderately differentiated; poor, poorly differentiated; Excluding 2 cases of unknown differentiation.

tected at high frequencies as an early event in both small cell lung cancer (SCLC) and NSCLC cell lines, as well as in primary tumors.[21]

The family of TRAIL receptors, including the proapoptotic TRAIL-R1 and TRAIL-R2 as well as the decoy receptors TRAIL-R3 and TRAIL-R4, are all located on the human chromosome 8p21-22.[8,10,22,23] TRAIL receptors are excellent candidate tumor suppressor genes, because inactivation of these receptors would be expected to result in deficient apoptotic signaling. To date, mutations in the death domain (DD) of TRAIL-R1 and TRAIL-R2 have been reported in lung cancer.[24,25]

Xu et al[26] showed that adenovirus vector-induced overexpression of the cytoplasmic domain (CD) of TRAIL-R1 in human lung, breast and colon cancer cell lines led to p53-independent apoptotic cell death involving cleavage of CASP8 and 10 proximally and CASP3, 6 and 7 distally. On the other hand, normal lung fibroblasts were resistant to TRAIL-R1 overexpression and showed no evidence of CASP8 or CASP3 cleavage despite possessing similar levels of adenovirus-delivered TRAIL-R1-CD protein as the cancer cells.

In contrast to these advances in research of TRAIL receptors, little is known about the expression of TRAIL at the protein level and its physiological functions in lung cancer.

This report presents the *de novo* expression of TRAIL and TRAIL-R1 at the protein level in human NSCLC. We also observed significantly greater positive staining of TRAIL and TRAIL-R1 in cancer tissues compared with normal tissues from patients with NSCLC. In cancer tissues, TRAIL expression was observed on mono-

nucler cells (Figure 1C) and TRAIL-R1 expression was found on cancer cells and mononucler cells (Figure 1D). TRAIL was reported to be expressed on T-cells[2] and NK-cells[27] and to exhibit effector mechanisms of cytotoxicity against tumor cells. Kim et al[28] reported that TRAIL-sensitive cancer cell lines expressed high levels of TRAIL-R1 mRNA, whereas TRAIL-resistant cell lines expressed low or undetectable levels of TRAIL-R1, and that the levels of TRAIL-R2, TRAIL-R3 and TRAIL-R4 expression did not correlate with TRAIL sensitivity. Our result seems to agree with Kim's data, although we studied only the level of TRAIL-R1 expression. Lee et al[24] reported that somatic mutation of TRAIL-R2 may play a role in the pathogenesis of some NSCLCs. Concerning the physiological functions of TRAIL in NSCLC, we found a significant positive correlation between the immunostaining score of TRAIL, TRAIL-R1 and pathological T-factor. However, there was no significant correlation between the immunostaining score of TRAIL or TRAIL-R1 and sex, age, histological type, stage, N-factor or differentiation. This result may suggest that the TRAIL/TRAIL-R1 system is induced by the expansion of tumor cells, and the system plays a role to block the expansion of tumor cells by inducing tumor apoptosis. Recent studies showed that apoptotic deficiency would be expected to contribute to transformed phenotype and tumor expansion,[24] and repeated intravenous injection of recombinant and biologically active TRAIL induced tumor cell apoptosis, suppressed tumor progression, and improved survival in mice bearing solid tumors.[29,30] Recently Spierings et al[4] reported that the death receptor/ligand systems may have an important role in tumorigenesis by providing an autocrine growth factor and thus promoting tumor growth.

In summary, we demonstrated *de novo* expression of TRAIL and TRAIL-R1 in cancer tissues of NSCLC, correlating with pathological staging. An important question remains as to why such tumor-suppressing molecules fail to function in cancer cells. One possible reason is that even though these molecules are expressed in cancer cells, they do not function normally due to gene mutation. Further analysis at the gene level is required to elucidate this issue.

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