Tumor necrosis factor-related apoptosis-inducing ligand modulates angiogenesis and apoptosis to inhibit non-small cell lung carcinoma tumor growth in mice

Yuanmei Chen1,*, Yuanji Xu2,*, Kunshou Zhu1, Zhiyun Cao3 and Zhengrong Huang4

Abstract
Objective: To investigate the anti-tumor effect and mechanism of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in non-small cell lung carcinoma (NSCLC) in mice.
Methods: We first established NSCLC animal models using 20 BALB/c nude mice that were randomly divided into two equal groups ($n = 10$): TRAIL-treated and control untreated groups. We measured expression levels of B cell leukemia/lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), vascular endothelial growth factor (VEGF), and VEGF receptor (VEGFR). We also performed microvessel density, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and immunohistochemical assays to determine the effect of TRAIL on apoptosis and angiogenesis in NSCLC tumors in vitro.
Results: TRAIL inhibited tumor growth in the NSCLC mouse model, and the TUNEL assay showed that it induced tumor cell apoptosis. Immunohistochemical staining revealed that TRAIL induced Bcl-2 protein downregulation, suggesting that the mitochondrial apoptotic pathway is involved in regulating NSCLC apoptosis. However, TRAIL did not affect Bax protein expression. Immunohistochemical staining also revealed significantly reduced VEGF and VEGFR protein

1Department of Thoracic Surgery, Fujian Cancer Hospital, Fujian Medical University Cancer Hospital, Fuzhou, Fujian, P.R. China
2Department of Radiation Oncology, Fujian Cancer Hospital, Fujian Medical University Cancer Hospital, Fuzhou, Fujian, P.R. China
3Fujian Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian, P.R. China
4Department of Integrative Traditional Chinese and Western Medicine, Fujian Cancer Hospital, Fujian Medical University Cancer Hospital, Fuzhou, Fujian, P.R. China

*These authors contributed equally to this work.

Corresponding author:
Zhengrong Huang, Department of Integrative Traditional Chinese and Western Medicine, Fujian Cancer Hospital, Fujian Medical University Cancer Hospital, Fuzhou, Fujian 350014, P.R. China.
Email: hzr1999@sohu.com

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (http://www.creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).
expression in the TRAIL group, indicating that TRAIL limits angiogenesis in NSCLC tumor tissues.

**Conclusions:** In conclusion, TRAIL inhibits NSCLC growth both by inducing tumor cell apoptosis and restricting angiogenesis in tumors.

**Keywords**
Tumor necrosis factor-related apoptosis-inducing ligand, non-small cell lung carcinoma, apoptosis, angiogenesis, mouse model, Bcl-2, vascular endothelial growth factor

*Date received: 9 December 2018; accepted: 10 May 2019*

**Introduction**

Lung cancer is the leading cause of cancer-related deaths in both men and women worldwide, with non-small cell lung cancer (NSCLC) accounting for approximately 85% of all cases. Systemic chemotherapy is currently the primary treatment method for early-stage NSCLC. However, therapy is often unsatisfactory because most chemotherapy drugs are toxic to healthy somatic cells, and drug resistance has become increasingly problematic. Hence, there is an urgent need to develop non-cytotoxic specific agents and measures to overcome chemotherapy resistance in lung cancer therapy. Activation of death receptors was found to be effective in NSCLC therapy through directly inducing apoptosis and preventing the development of cellular drug resistance.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a new member of the TNF family, has shown promise as an anti-cancer drug because of its ability to specifically target tumor cells for apoptosis. TRAIL mediates apoptosis by binding directly to the TRAIL DR4/5 death receptors on the plasma membrane, activating the death signaling pathway, inducing caspase-8 and its downstream effector caspases, and resulting in apoptosis. TRAIL has also been implicated in disease development, and targeting TRAIL death receptors on tumor cells, either alone or in combination with other chemotherapeutic drugs, was reported to be an effective therapeutic strategy. Hence, we hypothesized that targeting TRAIL may be similarly effective in lung cancer therapy.

Because the underlying mechanism of TRAIL in lung carcinoma has not yet been characterized, we designed this study to clarify the role of TRAIL in mediating apoptosis and angiogenesis of lung tumors using *in vivo* mouse models and the *in vitro* NSCLC A549 lung carcinoma cell line. Specifically, we investigated the expression levels of the apoptotic markers B cell leukemia/lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax), as well as angiogenesis markers vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR). We also performed microvessel density (MVD), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and immunohistochemical assays.

**Materials and methods**

**Animals**

Twenty 4–7-week-old male BALB/c nude mice (Shanghai SLAC Laboratory Animal
Co., Ltd., Shanghai, China) weighing between 20–22 g were bred in pathogen-free conditions under a 12-hour light/dark cycle. Mice were allowed food and water ad libitum. All animal experiments were performed according to International Ethical Guidelines and National Institutes of Health Guidelines on the Care and Use of Laboratory Animals, and with the approval of the Institutional Animal Care and Use Committee of Fujian Cancer Hospital, Fujian Medical University Cancer Hospital, China (reference number K201427).

Cell culture
Human NSCLC A549 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and penicillin–streptomycin (100 U/mL; 100 µg/mL) at 37°C in a 5% CO₂ humidified incubator. DMEM, FBS, and penicillin–streptomycin were purchased from Maixin Bio (Fuzhou, China).

Animal model and grouping
Subcutaneous injection of human NSCLC A549 cells into the right flank of BALB/c nude mice was performed to establish our NSCLC animal model. Tumor-bearing mice were randomly divided into two equal groups (n = 10) after 7 days: the control group (treated with normal saline) and the TRAIL-treated group (treated with 0.08 mg/kg TRAIL). Recombinant human TRAIL Apo II ligand was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). According to our previous study,¹² TRAIL was injected once daily into the mouse abdominal cavity for 15 days. Mouse body weights were measured once every 5 days from experimental Day 1, and the tumor size was measured once every 5 days from experimental Day 7. Tumors were excised from mice and weighed on Day 22. Tumor volume (mm³) was calculated as \(d^2 \times D/2\), where \(d\) and \(D\) represent the shortest and longest diameter, respectively.

In situ TUNEL assay
Tumor sections were treated with formalin for 48 hours at 4°C, then with 4% formaldehyde in phosphate-buffered saline (PBS) for 25 minutes at 4°C, and finally immersed in 0.2% TritonX-100 in PBS for 5 minutes. Equilibration Buffer (100 µl) was added to the slides at room temperature for 5–10 minutes, and staining was performed using the TUNEL assay kit (Promega Systems, Madison, WI, USA). Apoptotic cells that stained pale brown were visualized at ×100 magnification under light microscopy (Olympus Optical Co., Tokyo, Japan) equipped with the Moticam 5000 C camera (Richmond, BC, Canada). Five randomly selected fields were used to count apoptotic cells, and image analysis was performed using Motic Med 6.0 software (Xiamen Motic Software Engineering Co., Ltd., Xiamen, China). The apoptosis index was calculated as the number of apoptotic cells/total number of cells ×100%. All other chemicals including xylene and ethanol were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

Immunohistochemistry
Immunohistochemistry was performed to assess protein levels of Bcl-2, Bax, VEGF, and VEGFR2, as well as MVD in tumor tissues. Tissue sections were incubated with Bax, Bcl-2, VEGF, and VEGFR2 primary antibodies (Maixin Bio, Fuzhou, China) for 1 hour at 37°C. Yellow or tan particles at the cell membrane or cytoplasm
were indicative of Bcl-2, Bax, VEGF, and VEGFR2 expression. Staining intensities and areas were quantified as the average integral absorbance. To determine MVD, two areas with a relatively high number of new vessels were selected by preliminary scanning at $\times 10–100$ magnification. Five random fields of each selected area at $\times 400$ magnification were then analyzed.

**Statistical analysis**

Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Armonk, NY, USA). Data are presented as the mean ± standard deviation (SD). Comparisons between two groups were performed using the Student’s $t$-test. Values of $P<0.05$ were deemed statistically significant.

**Results**

**TRAIL inhibits tumor growth in mice**

There was no significant change in the body weight of tumor-bearing mice in the TRAIL group compared with the control group during the 15-day experiment (Figure 1a). However, there was a significant increase in both tumor volume ($P<0.05$; Figure 1b) and tumor weight ($P<0.05$; Figure 1c) in tumor-bearing mice in the TRAIL group compared with the control group, indicating that TRAIL inhibits tumor growth.

**TRAIL promotes tumor cell apoptosis via the Bcl-2 pathway**

Following the characterization of the effect of TRAIL on tumor growth in NSCLC

![Figure 1](https://example.com/f1.png)

**Figure 1.** The effect of TRAIL on tumor growth *in vivo*. Tumor-bearing BALB/c nude mice were treated with TRAIL 0.08 mg/kg once daily for 15 days. The same volume of NS was administered to the control group. Body weight (a), tumor volume (b), and final tumor weight (c) were then measured. Values are shown as mean ± SD, $n = 10$, $^*P < 0.05$ versus the control group.
mouse models, we next determined the underlying mechanism by which TRAIL regulates tumor cell apoptosis. We observed a significant increase in apoptotic A549 cells in the TRAIL group compared with the control group (TRAIL vs. control: 15.2% vs. 10.5%; \( P < 0.05 \); Figure 2), indicating that TRAIL induces tumor cell apoptosis. This observation was consistent with the significant downregulation of Bcl-2 protein expression (TRAIL vs. control: 16.5% vs. 24.1%; \( P < 0.05 \)). No significant change was detected in Bax protein expression (TRAIL vs. control: 24.6% vs. 26%; Figure 3).

**TRAIL inhibits angiogenesis in an NSCLC mouse model**

Next, we investigated the effect of TRAIL on modulating angiogenesis in tumors by assessing MVD and angiogenesis markers including VEGF and VEGFR2. Immunohistochemical staining revealed significantly reduced MVD (TRAIL vs. control: 14.5% vs. 26.3%; \( P < 0.05 \); Figure 4) and VEGF

![Figure 2](image1.jpg)

**Figure 2.** The effects of TRAIL on apoptotic cells. The tumor apoptotic index was detected by the TUNEL assay. Quantification of the results was performed as a percentage of positively-stained cells. Data are presented as mean ± SD from six individual mice per group. \( * P < 0.05 \) versus the control group. Positive apoptotic cells are those stained pale brown.
TRAIL vs. control: 14.0% vs. 36.8%; $P < 0.05$; Figure 5), and reduced VEGFR2 (TRAIL vs. control: 33.1% vs. 37.6%; Figure 5) expression in tumor-bearing mice compared with controls. Taken together, TRAIL treatment effectively inhibited angiogenesis in our NSCLC mouse model.

**Discussion**

TRAIL is a well-studied cytokine with a promising anti-cancer function because of its ability to directly induce tumor cell apoptosis through the activation of its death-inducing receptors (TRAIL-R1 and...
TRAIL-R2).\textsuperscript{14,15} However, the role of TRAIL in lung carcinoma and the underlying mechanism are poorly characterized. Hence, we performed this study to clarify how TRAIL regulates apoptosis and angiogenesis in NSCLC. We found that tumor growth was inhibited in our TRAIL-treated NSCLC mouse model, but that there was no significant change in the body weight of tumor-bearing mice during the 15-day experiment, indicating that TRAIL treatment did not induce side effects \textit{in vivo}. We also observed increased apoptosis using the TUNEL assay and an increased Bax/Bcl-2 ratio, as determined by immunohistochemical staining, in the TRAIL group. Additionally, significantly reduced MVD and angiogenesis marker expression were detected in TRAIL-treated mice. Taken together, our findings suggest that increased apoptosis and decreased angiogenesis are the underlying mechanisms of TRAIL-mediated inhibition of NSCLC tumor growth \textit{in vivo}.

Apoptosis is regulated by several signaling cascades including the mitochondrial death pathway. Bcl-2 and Bax are factors in this pathway and form heterodimers; Bcl-2 preserves the mitochondrial membrane integrity while Bax promotes cytochrome C release from the mitochondria into the cytosol to induce apoptosis.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.png}
\caption{The effects of TRAIL on the expression of microvessel density (MVD). MVD was detected by immunohistochemical staining for CD31 as a marker. MVD is represented as a percentage of CD31-positive tumor cells. Values are shown as mean ± SD, n = 6; *P < 0.05 versus the control group.}
\end{figure}
The Bax/Bcl-2 ratio determines the response of cells to various apoptotic stimuli.\textsuperscript{16,17} Hence, the increased Bax/Bcl-2 ratio observed in this study parallels the detected increase in apoptosis, suggesting that NSCLC A549 cell apoptosis may be mediated via the mitochondrial death pathway. Because the modulation of MVD and changes in VEGF and VEGFR2 levels affect tumor angiogenesis, invasion, and metastasis,\textsuperscript{18–20} these are ideal markers of tumor angiogenesis. The observed changes in MVD, VEGF, and VEGFR2 levels upon TRAIL treatment of NSCLC mice in the present study demonstrate the effect of TRAIL on angiogenesis.

In conclusion, we demonstrated that TRAIL inhibits NSCLC tumor growth by inducing tumor cell apoptosis potentially via the Bcl-2/Bax mitochondrial death pathway and restricting tumor angiogenesis. Our results suggest that TRAIL could be targeted in clinical NSCLC therapy.

**Figure 5.** The effects of TRAIL on VEGF and VEGFR2 protein expression evaluated using immunohistochemical staining. Data are presented as mean ± SD from six individual mice per group. *\textit{p} < 0.05 versus the control group.
Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: We thank the Natural Science Foundation of Fujian Province, China (Nos. 2011J01035 and 2019J05140) and the Fujian Provincial Health and Family Planning Research Talent Training Program, China (No. 2016-ZQN-20) for funding this study.

ORCID iD
Zhengrong Huang  https://orcid.org/0000-0002-7194-0433

References

1. Torre LA, Bray F, Siegel RL, et al. Global cancer statistics, 2012. *Ca Cancer J Clin* 2015; 65: 87–108.
2. Ramalingam S and Belani C. Systemic chemotherapy for advanced non-small cell lung cancer: recent advances and future directions. *Oncologist* 2008; 13: 5–13.
3. Kim ES. Chemotherapy resistance in lung cancer. *Adv Exp Med Biol* 2016; 893: 189–209.
4. Cohen EE, Subramanian J, Gao F, et al. Targeted and cytotoxic therapy in coordinated sequence (TACTICS): erlotinib, bevacizumab, and standard chemotherapy for non-small-cell lung cancer, a phase II trial. *Clin Lung Cancer* 2012; 13: 123–128.
5. Kenmotsu H, Naito T, Kimura M, et al. The risk of cytotoxic chemotherapy-related exacerbation of interstitial lung disease with lung cancer. *J Thorac Oncol* 2011; 6: 1242–1246.
6. Singh TR, Shankar S, Chen X, et al. Synergistic interactions of chemotherapeutic drugs and tumor necrosis factor-related apoptosis-inducing ligand/Apo-2 ligand on apoptosis and on regression of breast carcinoma in vivo. *Cancer Res* 2003; 63: 5390–5400.
7. Refaat A, Abd-Rabou A and Reda A. TRAIL combinations: the new ‘trail’ for cancer therapy (Review). *Oncol Lett* 2014; 7: 1327–1332.
8. You M, Savaraj N, Wangpaichitr M, et al. The combination of ADI-PEG20 and TRAIL effectively increases cell death in melanoma cell lines. *Biochem Biophys Res Commun* 2010; 394: 760–766.
9. Oh Y, Swierczewska M, Kim TH, et al. Delivery of tumor-homing TRAIL sensitizer with long-acting TRAIL as a therapy for TRAIL-resistant tumors. *J Control Release* 2015; 220: 671–681.
10. Sharma R1, Buitrago S, Pitoniak R, et al. Influence of the implantation site on the sensitivity of patient ancreatic tumor xenografts to Apo2L/TRAIL therapy. *Pancreas* 2014; 43: 298–305.
11. Szliszka E, Czuba ZP, Kawczyk-Krupka A, et al. Chlorin-based photodynamic therapy enhances the effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in bladder cancer cells. *Med Sci Monit* 2012; 18: R47–R53.
12. Zhu K, Fang W, Chen Y, et al. TNF-related apoptosis-inducing ligand enhances vinorelbine-induced apoptosis and antitumor activity in a preclinical model of non-small cell lung cancer. *Oncol Rep* 2014; 32: 1234–1242.
13. Perri P, Zauli G, Gonelli A, et al. TNF-related apoptosis inducing ligand in ocular cancers and ocular diabetic complications. *Biomed Res Int* 2015; 2015: 424019.
14. Dai X, Zhang J, Arfuso F, et al. Targeting TNF-related apoptosis-inducing ligand (TRAIL) receptor by natural products as a potential therapeutic approach for cancer therapy. *Exp Biol Med (Maywood)* 2015; 240: 760–773.
15. Kim TH, Jiang HH, Park CW, et al. PEGylated TNF-related apoptosis-inducing ligand (TRAIL)-loaded sustained release PLGA microspheres for enhanced stability and antitumor activity. *J Control Release* 2011; 150: 63–69.
16. Al-Fatlawi AA, Al-Fatlawi AA, Irshad M, et al. Rice bran phytic acid induced apoptosis through regulation of Bcl-2/Bax and p53 genes in HepG2 human hepatocellular carcinoma cells. *Asian Pac J Cancer Prev* 2014; 15: 3731–3736.
17. Buranabunwong N, Ruangrungsi N, Chansriniyom C, et al. Ethyl acetate extract from *Glycosmis parva* leaf induces apoptosis and cell-cycle arrest by decreasing expression of COX-2 and altering BCL-2 family gene expression in human colorectal cancer HT-29 cells. *Pharm Biol* 2015; 53: 540–547.

18. Lu QL, Liu J, Zhu XL, et al. Expression of nerve growth factor and hypoxia inducible factor-1alpha and its correlation with angiogenesis in non-small cell lung cancer. *J Huazhong Univ Sci Technolog Med Sci* 2014; 34: 359–362.

19. Qi L, Zhu F, Li SH, et al. Retinoblastoma binding protein 2 (RBP2) promotes HIF-1alpha-VEGF-induced angiogenesis of non-small cell lung cancer via the Akt pathway. *PLoS One* 2014; 9: e106032.

20. Yao L, Dong H, Luo Y, et al. Net platelet angiogenic activity (NPAA) correlates with progression and prognosis of non-small cell lung cancer. *PLoS One* 2014; 9: e96206.