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RESEARCH ARTICLE

# Inhibitory Effect of Immune Checkpoint Cytotoxic T-Lymphocyte-Associated Antigen-4 Inhibitor Combined with Curcumin on Tumor Growth in Mice with Lung Cancer

Weiwei TANG [1,†] Hailan WU [2,†] Tangfeng LV [2 (*)]

† *The two authors contributed equally to this study*

[1] Nanjing University of Chinese Medicine, Nanjing 210029, Jiangsu Province, CHINA
[2] Jinling Clinical Medical College, Nanjing University of Chinese Medicine, Nanjing 210002, Jiangsu Province, CHINA

(*) **Corresponding author:**
Tangfeng LV
Phone: +86-15103624213
E-mail: lvtfnucm@csc-edu.cn

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**Abstract**

We aimed to investigate whether immune checkpoint cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) inhibitor plus curcumin can inhibit tumor growth in mice with lung cancer. A mouse model of lung cancer was established by injecting Lewis lung cancer cells. The mice were allocated to an anti-CTLA-4 group, a curcumin group, an anti-CTLA-4+curcumin group, and a Model group in random (n=10). The tumor inhibition rate and metastasis inhibition rate were calculated. Hematoxylin-eosin staining was performed on tumor tissues for their pathological changes. The apoptosis rate was measured by TUNEL assay. In comparison with the Model group, the tumor volume, tumor mass, number of lung metastatic nodules, and the protein and relative mRNA expressions of Bcl-2 in tumor tissues significantly decreased in anti-CTLA-4, curcumin, and anti-CTLA-4+curcumin groups, while the tumor inhibition rate, metastasis inhibition rate, apoptosis rate of tumor cells, and Caspase-3 and Bax contents in tumor tissues increased (P<0.0001). CTLA-4 inhibitor plus curcumin can inhibit tumor growth in mice with lung cancer, which may be associated with the promotion of lung cancer cell apoptosis by regulating the Caspase-3/Bcl-2/Bax signaling pathway.

**Keywords:** Curcumin, Tumor growth, Inhibitor, Lung cancer

## Introduction

As a leading frequently occurring malignancy in clinical practice, lung carcinoma is dominated by NSCLC, taking a proportion of nearly 85% [1]. Based on a statistical report in 2020 [2], there are about 2.207 million confirmed cases and about 1.796 million death cases of pulmonary carcinoma each year. Surgery and radiotherapy have been commonly adopted for treating this carcinoma over the past few years, but the survival rate of patients within 5 years is still below 15% due to adverse reactions such as chemotherapy resistance. Hence, exploiting efficacious therapeutic drugs is of particular importance for lung cancer for ameliorating the prognosis of patients [3].

Recently, immunotherapy has demonstrated significant survival benefits among patients with malignant solid tumors, such as malignant melanoma and NSCLC. As a novel anti-tumor drug for immunotherapy, immune checkpoint inhibitors (ICIs) have been widely studied [4]. Currently, the most representative ICIs in clinical practice range from PD-1 inhibitors, PD-L1 inhibitors to CTLA-4 inhibitors. Of note, CTLA-4 inhibitor can be applied in dual-immunotherapy, which greatly enriches the modes of tumor immunotherapy [5,6]. Specifically, the combination of nivolumab with anti-CTLA-4 demonstrates better therapeutic efficacy than chemotherapy alone on advanced NSCLC tumors [7].

Curcumin, a diketone compound extracted from Chinese herbal medicine, is supported to obstruct the malignant phenotypes of cancer cells and demonstrates optimal efficacy in preclinical as well as clinical investigations [8]. Previous research has illuminated that curcumin can facilitate apoptosis in liver cancer cells, coupled with descended Bcl-2 content, as well as ascended Bax and Caspase-3 contents [9]. Intriguingly, Endo et al. have

corroborated that curcumin can stimulate apoptosis in lung cancer [10]. Nevertheless, the therapeutic impacts of CTLA-4 inhibitor plus curcumin on lung cancer cell apoptotic level remain vague.

Collectively, this paper was conceived to plumb the efficacy of CTLA-4 inhibitor plus curcumin on the tumor growth in lung cancer mice models, which might preliminarily underscore the potential of CTLA-4 inhibitor plus curcumin as a prospective candidate for lung cancer relieve.

## Materials and Methods

### Ethical Approval

This study has been approved by the ethic committee of our university (No. JSNJM2022104), date: 13th/5/2022, and great efforts have been made to minimize the animals' suffering.

### Animals

SPF-grade BALB/c mice sourced from Chengdu Dossy Experimental Animals Co., Ltd. (China) [18-22 g, n=40, male, animal license No. SCCK (Sichuan) 2020-030]. All mice were adaptively raised for one week in temperature-controlled (23-25°C) cages with 12/12 h light-dark cycle, together with relative humidity of 50-60%, allowed for freely drinking water and eating fixed food.

### Reagents and Apparatus

Mouse Lewis lung cancer cell lines were purchased from ATCC (USA), curcumin (purity: 98%) was purchased from Zhengzhou Linuo Biotechnology Co., Ltd. (China). Anti-CTLA-4 was offered by Shanghai Yihui Biotechnology Co., Ltd. (China). Antibodies against Caspase-3, Bcl-2, GAPDH, and Bax sourced from Proteintech (USA). TUNEL was provided by Shanghai Beyotime Biotechnology Co., Ltd. (China). A microplate reader (Model: PT-3502C) was bought from Beijing Putian Xinqiao Technology Co., Ltd. (China). An optical microscope was provided by Shenzhen OSWare Medical Instrument Co., Ltd. (China).

### Tumor Model and Experimental Grouping

Mouse Lewis lung cancer cells were subjected to 10 min of 3000 r/min centrifugation, collection and preparation into a suspension. The resulting suspension was inoculated into the armpit of the right forelimb of mice at $1x10^6$ cells/mL (0.2 mL/mouse) for seven consecutive days. Modeling was considered successful if a soybean-sized tissue mass could be obviously palpated at the inoculation site. Then a Model group, an anti-CTLA-4 group, a curcumin group, and an anti-CTLA-4+curcumin group were established for random assignment of forty successfully modeled mice, each of which contained 10 mice. As for the anti-CTLA-4 group, anti-CTLA-4 was injected once every two days at 0.5 mg/kg into the mice *via* the tail vein for four times. In the curcumin group, the mice received intraperitoneal injection of 0.5 mL/mouse curcumin suspension once daily for eight days in a row. Mice in anti-CTLA-4+curcumin group received 0.5 mg/kg anti-CTLA-4 administration via the tail vein and intraperitoneal injection of 0.5 mL curcumin suspension. An equal volume of normal saline was intraperitoneally injected for eight days in the Model group.

### Detection of Tumor Inhibition Rate

Twenty-four h post the final administration, the mice underwent euthanasia and were subjected to dissection, and the tumor was harvested to measure its maximum diameter *a* plus short diameter *b*. Then the tumor volume was calculated: $V=0.52a\times b2$, the tumor mass was weighed and the tumor inhibition rate was finally calculated.

### Detection of Lung Metastasis Inhibition Rate

A portion of tumor tissues was frozen by liquid nitrogen rapidly for preservation in a -80°C refrigerator. The remaining tumor tissues underwent fixation using 4% paraformaldehyde solution. Then the two lungs were harvested, fixed with Buoin's solution, and washed 24 h later. Lung metastases were quantified under a light microscope, and the lung metastasis inhibition rate was finally calculated.

### Hematoxylin-Eosin (HE) Staining for Pathological Changes in Tumor Tissues

The paraformaldehyde solution (4%)-fixed tumor tissues were taken out, treated by paraffin embedding, sliced into sections (thickness: 5 μm) by a microtome, and attached to glass slides, followed by deparaffinization and hydration. Finally, HE staining and observation using the microscope were performed on the sections.

### TUNEL Assay for Detecting Apoptosis Rate

The resulting sections in 1.6 were subjected to dehydration, added dropwise with proteinase K (100 μL) solution and left to stand. 30 min later, the sections were added dropwise with TUNEL solution (50 μL) and left to stand away from light. 1 h later, stop buffer was supplemented for reaction termination. After 15 min, the sections were added dropwise with converter-POD solution (50 μL), left to stand away from light, and added with DAB solution (100 μL) for 10-min reaction 30 min later, followed by hematoxylin counterstaining and dehydration. Finally, the sections were transparentized with xylene and mounted with neutral resin, followed by microscope observation. Apoptosis index (%) = (number of apoptotic cells/total number of tumor cells) ×100%.

*Table 1. Tumor growth and metastasis in mice (X±s, n=10)*

| Group | Tumor Volume ($cm^3$) | Tumor Mass (g) | Tumor Inhibition Rate ($\times10^{-2}$) | Number of Lung Metastatic Nodules | Metastasis Inhibition Rate ($\times10^{-2}$) |
|---|---|---|---|---|---|
| Model | 6.85±0.72 | 6.48±0.67 | 0 | 15.21±1.67 | 0 |
| Anti-CTLA-4 | 5.71±0.60* | 5.39±0.56* | 19.86 | 9.33±1.02* | 31.54 |
| Curcumin | 5.63±0.59* | 5.21±0.55* | 20.07* | 9.06±1.00* | 32.67* |
| Anti-CTLA-4 + curcumin | 4.26±0.45*# | 3.46±0.48*# | 41.21*# | 5.23±0.55*# | 65.18*# |
| *F* | 31.45 | 48.53 | | 132.2 | |
| *P* | <0.0001 | <0.0001 | | <0.0001 | |

** P<0.05 vs. Model group, # P<0.05 vs. Anti-CTLA-4 group and Curcumin group*

### Western Blotting

The tumor tissues stored in the refrigerator at -80°C in 1.5 were taken out, added with 100 μL of pre-prepared lysate mixture, mixed well in a tissue homogenizer, and subjected to 15 min of 15,000 r/min centrifugation at 4°C. An EP tube was employed to acquire the supernatant, and BCA approach was adopted for concentration quantification. Next, protein samples (50 μg) were denatured *via* boiling, segregated utilizing SDS-PAGE, and translocated to PVDF membranes. Then 5% skim milk was added for 1-h membrane blocking, TBST solution was used for membrane washing, and primary antibodies (1:500) were supplemented for 4°C overnight membrane incubation. Subsequently, the membrane underwent 2 h of conventional incubation again under HRP-labeled secondary antibodies (1:1000). Next, the sample was added dropwise with ECL solution for development. Finally, images were acquired using a camera and the protein bands were analyzed for the gray value using Image Lab.

### RT-qPCR

The tumor tissues were lysed on ice into a suspension, and lysed with TRIzol reagent. By reference to the kit instructions, total RNA obtained from tissues or cells was converted into cDNA through reverse transcription. In accordance with the PCR kit instructions, a 10 μL reaction system was prepared (5 μL of 2×SYBR Mix, 0.4 μL of cDNA, forward and reverse primers in a single volume of 0.4 μL, 0.4 μL of reference dye Rhodamine X, and 3.4 μL of DEPC-treated water), and quantitative RT-PCR was implemented. With the internal reference determined as GAPDH, 2-△△CT method was adopted for the calculation of gene expression level. The primers used were as follows:
Caspase-3 F: 5'-GGTGGCATCTCCTGTGATTGTG-3',
R: 5'-CAGGAGCTTCTGATCTGG-3'.
Bcl-2 F: 5'-CGGGAGATCGTGATGAAGTAC-3',
R: 5'-CTCAGGCTGGAAGGAGAAGA-3'.
Bax F: 5'-GCTACAGGGTTTCATCCAGGGT-3',
R: 5'-TGTTGTCCAGTTCATCGC-3'.
GAPDH F: 5'-CAAGGAGTAAGAAACCCTGGA-3',
R: 5'-CCCTGTTGTTATGGGGTCTGG-3'.

### Statistical Analysis

GraphPad Prism 8.0 for statistics was employed. The format of mean ± standard deviation (X±s) was applied to present the measurement data. The normally distributed data underwent one-way ANOVA and comparison *via* the LSD-*t* test between two groups. The abnormally distributed data were subjected to the Kruskal-Wallis rank sum test. A difference of statistical significance was denoted with P lowering than 0.05.

## Results

### Tumor Growth and Metastasis in Mice

Relative to the Model group, the tumor volume, tumor mass and number of lung metastatic nodules were smaller in mice received the treatment of anti-CTLA-4 or/and curcumin, while the tumor inhibition rate and metastasis inhibition rate were significantly higher (P<0.0001). The anti-CTLA-4+curcumin group had significantly smaller tumor volume, tumor mass and number of lung metastatic nodules, and significantly higher tumor inhibition rate and metastasis inhibition rate than the anti-CTLA-4 plus curcumin groups (P<0.0001) *(Table 1)*.

### Pathological Changes in Tumor Tissues

The lung cancer cells had an intact structure, and exhibited tumor cell morphology, without obvious necrosis, in the Model group. In the anti-CTLA-4, curcumin and anti-CTLA-4+curcumin groups, a lot of necrotic tumor cells were observed, most of the cells showed karyopyknosis or karyorrhexis, but karyokinesis was rare, they were arranged irregularly, and the intercellular space around the necrotic region increased and was distributed unevenly, especially in the anti-CTLA-4+curcumin group *(Fig. 1)*.

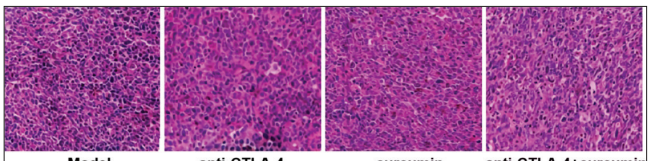


**Fig 1.** HE staining images of tumor tissues (×200)

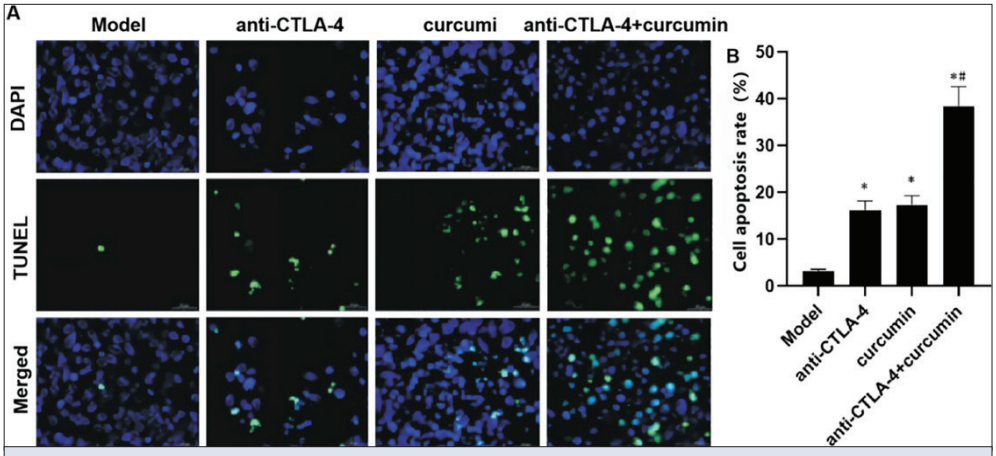


**Fig 2.** Tumor cell apoptosis rate. A: TUNEL assay for tumor cell apoptosis. B: The quantitative analysis of cell apoptosis. *P<0.05 *vs.* Model group, #P<0.05 *vs.* Anti-CTLA-4 group and Curcumin group

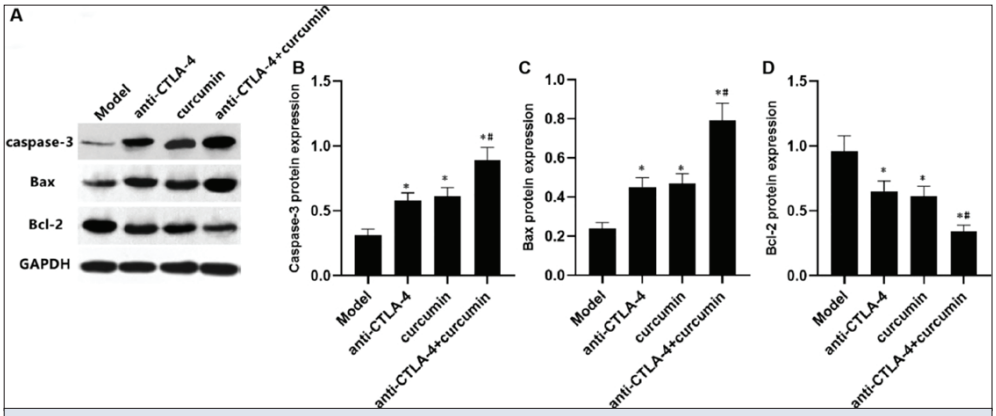


**Fig 3.** Protein contents in Caspase-3, Bcl-2 and Bax in tumor tissues. A: Protein bands of Caspase-3, Bcl-2 and Bax. B-D: Analysis of Caspase-3, Bax and Bcl-2 protein contents in tumor tissues. *P<0.05 *vs.* Model group, #P<0.05 *vs.* Anti-CTLA-4 group and Curcumin group

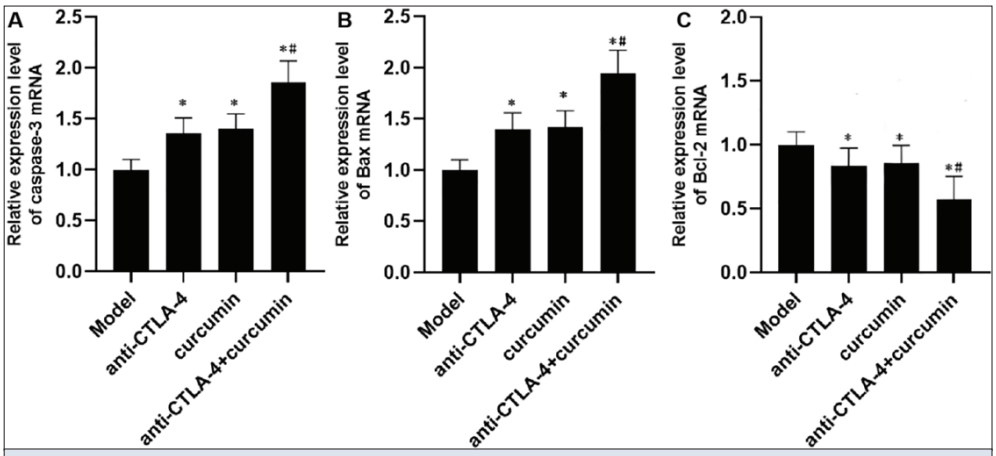


**Fig 4.** mRNA expressions of Caspase-3, Bcl-2 and Bax. A: Caspase-3 mRNA expression in tumor tissues. B: Bax mRNA expression in tumor tissues. C: Bcl-2 mRNA expression in tumor tissues. *P<0.05 *vs.* Model group, #P<0.05 *vs.* Anti-CTLA-4 group and Curcumin group

## Tumor Cell Apoptosis Rate

Relative to the Model group, apoptosis in mice treated with anti-CTLA-4 or curcumin was conspicuously enhanced. Noticeably, the combination of anti-CTLA-4 and curcumin demonstrated more promotive impacts than anti-CTLA-4 or curcumin treatment alone (P<0.0001) *(Fig. 2)*.

## Protein Expressions of Caspase-3, Bcl-2 and Bax in Tumor Tissues

Compared with the Model group, the anti-CTLA-4, curcumin and anti-CTLA-4+curcumin groups had significantly increased Caspase-3 plus Bax contents and a significantly reduced Bcl-2 content in tumor tissues

(P<0.0001). In the anti-CTLA-4+curcumin group, Caspase-3 and Bax contents were markedly ascended, whereas Bcl-2 descended significantly at the protein expression level in tumor tissues by contrast to the anti-CTLA-4 and curcumin groups (P<0.0001) *(Fig. 3)*.

### mRNA Expression of Caspase-3, Bcl-2 and Bax in Tumor Tissues

The mRNA expressions of Caspase-3 plus Bax were distinctly increased, but that of Bcl-2 in tumor tissues significantly declined following the administration of anti-CTLA-4 or/and curcumin relative to the Model group (P<0.0001). Moreover, an enhancement in mRNA expressions of Caspase-3 and Bax together with a decline in mRNA expression of Bcl-2 in tumor tissues could be inspected in anti-CTLA-4+curcumin group relative with the anti-CTLA-4 and curcumin groups (P<0.0001) *(Fig. 4)*.

## Discussion

Pulmonary carcinoma ranks among the malignant tumors featured by the highest incidence and death rates in China. Due to its high degree of malignancy and difficulty in early diagnosis, most patients have been in the mid-late stage or developed metastasis at the time of diagnosis, resulting in ineffective surgical treatment, a poor prognosis and an unsatisfactory overall survival rate [11]. Therefore, searching for effective treatment means is urgently needed so far.

Immune checkpoints are an immunoregulatory factor that can regulate the immune response by maintaining autoimmune homeostasis [12]. Under normal circumstances, tumor cells can cause disorders of antitumor immune response by inactivating some immune checkpoints, thus contributing to tumor growth and proliferation. ICIs can promote immune cell activity by suppressing immune checkpoints and inhibit tumor cells by activating autoimmune responses [13]. CTLA-4 is an immune checkpoint commonly used in recent years. As a transmembrane protein, CTLA-4 presents widespread expressions in activated T cells and conjugates with B7 ligand to suppress T lymphocyte activation, thus preventing T cells from attacking tumor cells [14]. It can be seen that blocking the CTLA-4 pathway can achieve an antitumor immune response by promoting immune cell proliferation. The ability of CTLA-4 inhibitor to inhibit the proliferation of childhood acute lymphoblastic leukemia cells, induce apoptosis, and hinder the growth of $ALDH^+$ stem-like tumor cells has been verified [15]. Curcumin, a phenolic pigment, possesses many advantages such as low toxicity, a variety of sources and low price, which can exert a good antitumor effect. It has been proved that curcumin inhibits various tumor cells from the aspect of propagation and movement [16]. Nevertheless, the effect of CTLA-4 inhibitor plus curcumin on tumor growth in mice with lung cancer remains unclear.

Lung carcinoma animal models have important significance for human studies on this disease, and appropriate animal models are particularly essential for research on the pathogenesis and diagnosis of this cancer as well as screening of drugs [17]. Heterotopic subcutaneous transplantation characterized by simple operation and easy observation of tumor formation has been widely used in clinic. In this study, Lewis lung cancer cell suspension was subcutaneously injected to establish the mouse models of lung cancer. It was uncovered that the tumor volume, tumor mass and number of lung metastatic nodules significantly decreased, while both tumor inhibition rate and metastasis inhibition rate significantly increased in the anti-CTLA-4, curcumin and anti-CTLA-4+curcumin groups. As observed by HE staining, there were a lot of necrotic tumor cells, and most of the cells showed karyopyknosis or karyorrhexis, but karyokinesis was rare in the above three groups, suggesting that CTLA-4 inhibitor or curcumin can inhibit tumor growth and metastasis in mice with lung cancer, and CTLA-4 inhibitor plus curcumin have a more significant effect.

Cell death can be classified into physiological and pathological death according to its cause, and apoptosis functions as a common cell death mode [18]. Apoptosis is an active and programmed death process that can maintain healthy development and metabolism by eliminating damaged cells in time [19]. A study has shown that apoptosis may be affected by the relative balance between cellular pro-apoptotic and anti-apoptotic proteins [20], among which Bcl-2 and Bax have been widely studied as an antitumor and pro-tumor gene, respectively. Both Bcl-2 and Bax belong to the Bcl-2 family, in which activated Bcl-2 can bind to Bax to form a heterodimer, thus inhibiting Bax activity and apoptosis. In addition, activated Bax can bind to Bax to form a homodimer, thus activating Caspase-3. Then activated Caspase-3 induces apoptosis by initiating a caspase cascade [21]. Research suggests that activating the Bcl-2/Bax signaling pathway serves as one of the possible mechanisms of inducing apoptosis in HepG2 cells [22]. Wen et al. [23] also confirmed that apoptosis of breast cancer cells was enhanced by up-regulation of Caspase-3 plus Bax protein expressions as well as inhibition of Bcl-2 protein expression. Our results presented that anti-CTLA-4 or curcumin treatment could evidently facilitate cell apoptosis, coupled with declined Bcl-2 content as well as enhanced Bax and Caspase-3 contents. Noticeably, the combination of anti-CTLA-4 and curcumin exhibited better therapeutic effects than anti-CTLA-4 or curcumin alone.

Nevertheless, this study has limitations. This study focused on the Bcl-2/Bax/Caspase-3 axis as a representative

apoptotic signaling pathway. However, the apoptosis cascade is highly complex and involves multiple other regulators and signaling modules, such as cytochrome c release, Apaf-1-mediated apoptosome formation, caspase-9 activation, and extrinsic death receptor pathways. These components were not comprehensively examined in the current work. Further investigations are warranted to explore these additional mechanisms and their potential crosstalk in the regulation of apoptosis, which may enrich our understanding of the cellular response to apoptotic stimuli.

In conclusion, CTLA-4 inhibitor plus curcumin can obstruct tumor growth in mice with lung cancer, the protective mechanism of which possible is related to the regulation of the Caspase-3/Bcl-2/Bax signaling pathway for promoting lung cancer cell apoptosis.

## Declarations

**Availability of Data and Materials:** The datasets used and/or analyzed during the current study are available from the corresponding author (T. Lv) on reasonable request.

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**Competing Interests:** The authors declared that there is no competing interest.

**Declaration of Generative Artificial Intelligence:** The article, tables and figures were not written by AI and AI-assisted technologies.

**Authors Contributions:** W.T. designed the study and drafted the paper; H.W. performed and analyzed the experiments; T.L. supervised the study and significantly revised the paper. W.T. and H.W. contributed equally to this study.

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