PDGFRβ-antagonistic affibody mediated tumor-targeted TNFα for enhanced radiotherapy in lung cancer

Xiaohui Tang1, Jie Chen2, Zhenxiong Zhao3, Jie Liu1, Ranfei Yu1, Kunlong Zhao1, Fei Wang1, Yang Li1, Baoqing Tian1, Dandan Yuan1, Qin Wei4, Yuguo Liu4, Zhong Feng Gao4, and Qing Fan1

1Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences
2NHC Key Lab of Transplant Engineering and Immunology, West China Hospital, Sichuan University, Chengdu 610041, PR China
3Key Laboratory of Interfacial Reaction & Sensing Analysis in Universities of Shandong, School of Chemistry and Chemical Engineering, University of Jinan, Jinan 250022, PR China

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Abstract

The morbidity and mortality of lung cancer are still the highest among all malignant tumors. Radiotherapy plays an important role in clinical treatment of lung cancer. But the effect of radiotherapy is not ideal due to the radiation resistance of tumor tissues. Abnormalities in tumor vascular structure and function affect blood perfusion and oxygen transport is impeded, making tumor microenvironment hypoxic. Tumor hypoxia is the major cause of radiotherapy resistance. By promoting tumor vessels normalization and enhancing vascular transport function, tumor hypoxia can be relieved to reduce radiotherapy resistance and increase tumor radiotherapy sensitivity. In our previous study, a pericytes-targeted tumor necrosis factor alpha (named Z-TNFα) was firstly constructed and produced by genetically fusing the platelet-derived growth factor receptor β (PDGFRβ)-antagonistic affibody (ZPDGFRβ) to the TNFα, and the Z-TNFα induced normalization of tumor vessels and improved the delivery of doxorubicin, enhancing tumor chemotherapy. In this study, the tumor vessel normalization effect of Z-TNFα in lung cancer was further clarified. Moreover, the tumor hypoxia improvement and radiosensitizing effect of Z-TNFα were emphatically explored in vivo. Inspiring, Z-TNFα specifically accumulated in Lewis lung carcinoma tumor graft, and relieved tumor hypoxia as well as inhibited HIF-1α expression. As expected, Z-TNFα significantly increased the effect of radiotherapy in mice bearing Lewis lung carcinoma tumor graft. In conclusion, these results demonstrated that Z-TNFα is also a promising radiosensitizer for lung cancer radiotherapy.
The morbidity and mortality of lung cancer are still the highest among all malignant tumors. Radiotherapy plays an important role in clinical treatment of lung cancer. But the effect of radiotherapy is not ideal due to the radiation resistance of tumor tissues. Abnormalities in tumor vascular structure and function affect blood perfusion and oxygen transport is impeded, making tumor microenvironment hypoxic. Tumor hypoxia is the major cause of radiotherapy resistance. By promoting tumor vessels normalization and enhancing vascular transport function, tumor hypoxia can be relieved to reduce radiotherapy resistance and increase tumor radiotherapy sensitivity. In our previous study, a pericytes-targeted tumor necrosis factor alpha (named Z-TNFα) was firstly constructed and produced by genetically fusing the platelet-derived growth factor receptor β (PDGFRβ)-antagonistic affibody (ZΠΔΓΦΡβ) to the TNFα, and the Z-TNFα induced normalization of tumor vessels and improved the delivery of doxorubicin, enhancing tumor chemotherapy. In this study, the tumor vessel normalization effect of Z-TNFα in lung cancer was further clarified. Moreover, the tumor hypoxia improvement and radiosensitizing effect of Z-TNFα were emphatically explored in vivo. Inspiring, Z-TNFα specifically accumulated in Lewis lung carcinoma tumor graft, and relieved tumor hypoxia as well as inhibited HIF-1α expression. As expected, Z-TNFα significantly increased the effect of radiotherapy in mice bearing Lewis lung carcinoma tumor graft. In conclusion, these results demonstrated that Z-TNFα is also a promising radiosensitizer for lung cancer radiotherapy.

**KEYWORDS** Tumor necrosis factor α, Affibody delivery, Vessel normalization, Tumor hypoxia, Radiosensitization

**Graphical Abstract**

**1 | INTRODUCTION**

The World Health Organization’s Global Cancer Report shows that there were 19.3 million new cases and 10 million deaths from cancer worldwide in 2022, and the lung cancer is the leading cause of cancer-related
Lung cancer is also the cancer with the highest morbidity and mortality in China, with approximately 942,000 new cases and 766,000 deaths annually. Currently, clinical treatments for lung cancer primarily include chemotherapy, immunotherapy, targeted therapy, interventional therapy, and radiation therapy. Radiation therapy (RT) plays a critical role in the treatment of lung cancer, with approximately 70% of lung cancer patients requiring radiation therapy at some point during their treatment. Besides, RT is the only treatment that is suitable for all stages and categories in lung cancer patients.

RT delivers ionizing radiation to tumor site, directly causing DNA breaks and cell death in tumors. Additionally, RT can also generate reactive oxygen species (ROS) through ionization and decomposition of water molecules in tumor microenvironment under well-oxygenated conditions, inducing irreversible biological oxidative damage to proteins and DNA and indirectly killing tumor cells. Yet, the rapid proliferation of tumor cells consumes a significant amount of oxygen, and the abnormal structure of tumor blood vessels hinders efficient oxygen delivery to tumor site, creating a hypoxic microenvironment that impedes ROS generation. Hypoxic tumor cells, which make up 10%-50% of tumor tissue, are approximately only one-third to half as sensitive to radiation as aerobic cells, leading to radiation resistance and unsatisfactory therapeutic effect with conventional radiation doses. To address radiation therapy resistance caused by tumor hypoxia, one conventional solution was to increase the radiation dose. However, as the radiation dose increases, the incidence of adverse reactions in normal tissues and organs, such as radiation dermatitis and pneumonia, also significantly rises. Another approach involves increasing oxygen levels in tumor tissues through high-pressure oxygen inhalation, but the abnormal tumor blood vessels impede oxygen delivery and potential oxygen poisoning, barometric injury as well as decompression disease severely limit clinical use. Therefore, adjusting tumor blood vessel structure and function to increase oxygen delivery and alleviate tumor hypoxia is a strategy for enhance tumor radiosensitivity.

The persistent abnormal angiogenesis is a critical hallmark of tumor progression. In tumor tissues, a large amount of pro-angiogenic factors are secreted, promoting abnormal blood vessel formation. Compared with normal tissue blood vessels, these excessively proliferating tumor blood vessels exhibit irregular morphologies, disorganized structures, and dysfunctional functions. Jain et al. proposed the theory of "vascular normalization," which suggests that anti-angiogenic therapy can reconstruct the balance of angiogenesis, making tumor blood vessels more regular, increasing blood perfusion, reducing tumor vessel density and enhancing vessel coverage of pericytes. After tumor vessel normalization, blood flow perfusion and transportation function are superior to those of untreated tumor vessels. Therefore, tumor vessel normalization represents a novel strategy to relieve tumor hypoxia for enhancing radiosensitivity. Tumor vessels contain endothelial cells (ECs) that are irregularly lined along with a few pericytes (PCs) and smooth muscle cells (SMCs) covering them. Inhibition of the proliferation, migration and metabolism of ECs could promote tumor vessel normalization. Furthermore, despite the unclear underlying mechanism, the connection between immune cells and vascular cells has been demonstrated to play a role in tumor vessel normalization. Nevertheless, the entry of immune cells into the tumor is typically hindered because anergic tumor vascular cells lack adhesion molecules.

Tumor necrosis factor-alpha (TNFα), a member of the tumor necrosis factor family, plays a role in maintaining immune system homeostasis, inflammation, and host defense. TNFα can inhibit tumor cell growth and disrupt tumor neangiogenesis by inducing inflammation and immune responses. However, clinical application of TNFα is limited to local therapy due to systemic administration being associated with serious toxicity. Interestingly, low-dose EC-targeted TNFα treatment could normalize tumor vessel through inducing adhesion molecule expression and recruiting immune cells, making TNFα a promising cytokine for targeted tumor therapy. Although tumor-targeted delivery of TNFα to ECs could promote tumor vessel normalization, this delivery method poses a risk of damaging normal tissue vessels during prolonged treatment. PCs, also known as mural cells, interact with ECs through physical contact and paracrine signaling pathways, regulating vascular morphology and functional stability. In tumor tissues, abnormal proliferation and disorganized arrangements of ECs lead to PCs being frequently exposed to the vessel lumen. Conversely, normal tissues vessels pose well-organized and tight arrangements, keeping PCs hidden. Platelet-
derived growth factor receptor β (PDGFRβ) is overexpressed on tumor-associated PCs in different types of tumors, suggesting PC-targeted delivery of TNFα through fusion of PDGFRβ-binding molecules might be a new strategy to promote tumor vessel normalization. Developed using the Z-domain of Protein A, affibody is a unique type of scaffold protein that is not based on immunoglobulins. It is a small molecule with excellent thermal tolerance, remarkable specificity, nanomolar affinity, and the ability to effectively penetrate tumors. In our previous study, Z09591 (named ZθΔΓΦΡβ), an affibody that antagonizes PDGFRβ with a strong and specific affinity, was fused to the N-terminus of mouse TNFα and expressed using an Escherichia coli (E. coli) expression system to produce a fusion protein, Z-TNFα. As expected, Z-TNFα effectively restored the structure and function of tumor blood vessels primarily by decreasing the secretion of VEGF by PCs, increasing the expression of intercellular cell adhesion molecule-1 (ICAM-1), attracting macrophages to tumor vessels, and enhanced the delivery of doxorubicin (DOX) to improve chemotherapy in mice bearing S180 or B16F1 tumor grafts. These results sparked our curiosity to investigate whether Z-TNFα could be used as an effective radiosensitizer for lung cancer radiotherapy through alleviating tumor hypoxia induced by tumor vessel normalization.

In this study, the fusion protein of Z-TNFα was prepared and cell binding was investigated. Subsequently, cell radiotherapy in vitro and tumor-targeting assay in vivo were further performed.

The effects of Z-TNFα in tumor vessel normalization and alleviating tumor hypoxia were emphatically evaluated in mice bearing lung cancer tumor grafts. Finally, the radiosensitizing effect of Z-TNFα for lung cancer radiotherapy was measured.

2 | MATERIALS AND METHODS

2.1 | Πρεπαρατιον οφ Z-TNFα

The expression and purification of Z-TNFα were performed in accordance with our previous study. Briefly, the fusion protein consisting of ZθΔΓΦΡβ and mouse TNFα (77-233aa) was created by attaching ZθΔΓΦΡβ to the N terminus of mouse TNFα using a flexible linker (G4S). The fusion protein was then expressed by E. coli M15 under the induction of isopropyl-L-thio-β-D-galactopyranoside (IPTG). Subsequently, the fusion protein was purified using a high-affinity Ni-NTA resin (GenScript, Nanjing, China) owing to an additional His-tag at the N-terminus. The presence of the protein was identified through the utilization of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), while the quantification of protein concentration was performed using a Bradford protein assay kit (Beyotime, Jiangsu, China).

2.2 | Protein labeling

For cell-binding and optical imaging assay, TNFα and Z-TNFα were respectively labeled with 6-carboxyfluorescein (FAM) NHS ester or Sulfo-Cy7 NHS ester (Ruixi Biological Technology, Xi’an, China). Briefly, the pH of protein solution (1 mg/mL) was adjusted to 8.0 using 1 M NaHCO3. Then the fluorescent dye was added into the protein solution at a 5:1 molar ratio of dye to protein. The mixture was dialyzed against PBS with several changes and verified by SDS-PAGE after reaction at room temperature for 1 h.

2.3 | Cell culture

Lewis lung carcinoma (LLC) cell line was purchased from Cell Bank of Chinese Academy Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Pericytes derived from human brain vasculature were purchased from ScienCell (CA, USA) and cultured in their specific medium. All cells were cultured at 37 °C in a 5% CO2 humidified atmosphere.

2.4 | Flow cytometry

To detect the expression of PDGFRβ in the LLC cells and PCs, cells were respectively trypsinized, resuspended and incubated with PE-anti-PDGFRβ antibody (BioLegend, CA, USA) in DMEM at room temperature for 45 min. Following incubation, the cells were rinsed three times with PBS and then suspended in PBS (0.5 mL). Subsequently, flow cytometry analysis was performed (BD, LSD Fortessa). In the cell binding
experiments, a total of $2 \times 10^5$ cells were cultured with FAM-tagged protein (100 nM) at room temperature for one hour. After being rinsed thrice with PBS, the cells were examined using flow cytometry. To conduct the experiments blocking the PDGFRβ receptor, PCs were first incubated with rabbit anti-human PDGFRβ (Abcam, MA, USA) at room temperature for one hour. Subsequently, they were incubated with FAM-Z-TNFα. Afterward, the cells were rinsed thrice with PBS and then resuspended in PBS (0.5 mL) for flow cytometry. The decrease in the rate of binding indicates the receptor’s involvement in mediating cell binding to Z-TNFα.

2.5 | Cell cytotoxicity and radiotherapy in vitro

Around $1 \times 10^4$ LLC cells were added to a 96-well plate and incubated overnight. Then, they were exposed to various protein concentrations ranging from 0.05 to 10 μM. Following a 24-hour treatment, the quantity of viable cells was assessed utilizing a Cell Counting Kit-8 (CCK-8, Dojindo, Japan). The cells treated with PBS were deemed to have 100% viability.

Cell cloning experiment was used to perform the radiotherapy in vitro. Briefly, approximately $8 \times 10^2$ LLC cells were inoculated into a 6-well plate and cultured overnight. To examine the radiosensitizing effect of Z-TNFα in vitro, the cells were incubated with Z-TNFα (100 nM) for 45 min prior to the X-ray radiation (1 Gy). After radiation, the cells continued to be cultured for 10 days. After cultivation, cells were washed three times with PBS, followed by the addition of fixative solution (methanol: glacial acetic acid = 1:3) to fix for 30 min. Then the 800 μl of 10% crystal violet staining solution was added to each well and allowed to stain for another 30 min. After drying, the area ratio of cells was calculated and analyzed using Image J software.

2.6 | Tumor imaging assay in vivo

The experiments involving animals were conducted following the guidelines provided by the National Institute for the Care and Use of Laboratory Animals. Approval for the experimental protocols was granted by the Ethics Committee of Shandong First Medical University and Shandong Academy of Medical Sciences (NO.2021003014). Female C57BL/6 mice weighing 14-16 g (n=3) were subcutaneously implanted with LLC cells ($2 \times 10^5$ cells/mouse). The daily measurements of the tumor grafts’ longitudinal (L) and transverse (W) diameters were used to calculate tumor volumes (V) using the formula: $V = L \times W^2/2$. Upon reaching a volume of around 200 mm$^3$, the mice received an intravenous injection of Cy7-Z-TNFα (2.5mg/kg, 100 μl) or an equivalent amount of TNFα to examine the in vivo tumor-targeting efficacy of Z-TNFα. The IVIS optical imaging system was utilized to conduct dynamic scanning. Following the final scan, the mice were euthanized, and the primary organs/tumors were gathered and subjected to scanning. The fluorescence intensities were analyzed by the imaging system software.

To examine the co-localization of protein and PDGFRβ-positive cells in tumors, mice with LLC tumor xenografts were injected intravenously with either FAM-labeled TNFα or Z-TNFα at an equivalent molar concentration. Afterwards, the tumor xenografts were extracted 60 min after injection, frozen, cut into sections, and then stained with an anti-PDGFRβ antibody and its corresponding secondary antibody.

2.7 | Evaluation of tumor vessel normalization

To clarify the tumor vessel normalization effect of Z-TNFα, especially on the function of tumor blood vessels, $2 \times 10^5$ LLC cells were subcutaneously implanted into C57BL/6 mice. When the tumor volumes reached approximately 50 mm$^3$, mice were intravenously injected with 1 μg of Z-TNFα every two days for three times. The mice injected with the same volume of PBS were used as control group. To evaluate the vascular perfusion, 50 μg FITC-labeled tomato lectin was injected into the tail vein on the second day after the third administration. Moreover, mice were also intravenously injected with FITC-labeled dextran (70 kDa, Sigma, MA, USA, 1 mg/mouse) to assess the vascular permeability of tumor. After circulation of 10 and 20 min for tomato lectin and dextran in vivo, mice were perfused with 20 ml PBS and 2% paraformaldehyde successively under anesthesia to remove free tomato or dextran. Then the tumor tissues were obtained and sectioned under frozen conditions. The blood vessels were displayed with rat anti-mouse CD31 antibody.
(Biolegend, CA, USA) to evaluate the morphology and density of tumor blood vessels. The slides were observed under a fluorescence microscope and the images were collected. The software used for measuring the fluorescence intensity was Image-J or Image-Pro Plus 6.0.

### 2.8 | Tumor hypoxia assessment

To detect tumor hypoxia, a Hypoxyprobe-1 Plus kit (Millipore, MA, USA) was used according to the manufacturer’s protocol. Mice bearing LLC tumor grafts were intravenously injected with Hypoxyprobe-1 (60 mg/kg) after the treatment of Z-TNFα like the experiment above. After 90 min, tumor grafts were extracted and divided while being kept frozen. Subsequently, they were stained using a FITC labeled antibody against Hypoxyprobe-1. Besides, the expression level of hypoxia-inducible factor 1α (HIF-1α) was also evaluated in tumor tissue by an immunofluorescence assay with rabbit anti-mouse HIF-1α (Abcam, MA, USA).

### 2.9 | Radiotherapy in vivo

In order to assess the radiosensitizing effect of Z-TNFα in vivo, mice bearing LLC tumor grafts (approximately 70 mm³) were divided randomly into four treatment groups (n=4) at Day 9 post-inoculation: PBS, Z-TNFα, RT and Z-TNFα+RT. Mice were intravenously injected with PBS (100 µl) or Z-TNFα (1 µg, 100 µl) every two days for five times. For RT, mice received a X-ray radiation (12 Gy) 2 h post-injection of Z-TNFα. Tumor volumes and body weights in all groups were recorded every other day. At Day 19 post-inoculation, all the mice were sacrificed and the blood, tissues/organs (brain, heart, liver, spleen, lung, kidney and tumor) were collected. Tumor tissues were weighed and they were examined using a TUNEL assay (Promega, WI, USA) along with DAPI staining. Glutamic-pyruvic transaminase (ALT), glutamic-oxaloacetic transaminase (AST), urea, creatinine (CREA), uric acid (UA) and creatine kinase (CK) levels were determined through the analysis of blood samples. Histological analysis was performed on paraffin tissue sections of the brain, heart, liver, spleen, lung, and kidney after H&E staining.

### 2.10 | Statistical analysis

Between-group comparisons were conducted using SPSS software (version 13.0) through one-way analysis of variance (ANOVA) for multiple comparisons. The mean ± standard deviation (SD) is used to present all results. The threshold for statistical significance was established at P < 0.05. Data are denoted as (*) when P < 0.05, (**) when P < 0.01, (***) when P < 0.001, and (ns) when not significant.

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Z-TNFα βινδς το ΠΔΓΦΡβ-ποσιτιvε ΠΓζ ντ ενη παρ

Z-TNFα was designed by fusing ZΠΔΓΦΡβ to the N terminus of mouse TNFα (77-233aa). To reduce the steric hindrance of ZΠΔΓΦΡβ and TNFα, a flexible linker (G4S)3 was inserted between the two functional domain. Then the fusion protein underwent expression through an E. coli expression system and was subsequently purified using Ni-NTA affinity chromatography. As shown in Figure 1A, the gel electrophoresis indicated that the fusion protein of Z-TNFα was eluted as a single protein band, and the molecular weights of Z-TNFα was approximately 7 kDa larger than that of TNFα. These results demonstrate that the Z-TNFα was successfully prepared with correct molecular weight.

The flow cytometry was used to detect PDGFRβ expression in cells. As shown in Figure 1B, PDGFRβ was highly expressed in PCs. However, a negligible fluorescence signal was detected in LLC cells which were PDGFRβ-negative (PDGFRβ positive rate < 5%). Then the cells were incubated with FAM-labeled TNFα or Z-TNFα to perform the cell-binding assay, followed by analysis with flow cytometry. Z-TNFα could bind to PDGFRβ-positive PCs cells but bot to PDGFRβ-negative LLC cells (Figure 1C), and TNFα didn’t bind to both of these cells (PDGFRβ positive rate < 5%). Moreover, the preincubation with an antibody targeting PDGFRβ resulted in a substantial decrease in binding rates, lowering them to less than 10% (Figure 1C), indicating that the cellular binding of Z-TNFα is PDGFRβ-dependent. These results demonstrated that Z-TNFα had a high PDGFRβ-dependent cellular binding activity due to the fusion to ZΠΔΓΦΡβ.
3.2 | Radiotherapy in vitro

In order to investigate the radiosensitizing effect of Z-TNFα in vitro, the cytotoxicity of Z-TNFα was firstly tested using CCK-8 kit. Although LLC cells were incubated with 10 μM of Z-TNFα for 24 h, cell viability was not affected (Figure 2A), indicating no obvious toxicity to cells in vitro. Then the cell cloning experiment was used to explore the cytotoxicity of Z-TNFα combined with X-Radiation. As shown in Figure 2B, there was no significant change in the number of cell clones of Z-TNFα treatment group compared with that of PBS group. This result was consistent with the CCK-8 assay. After the treatment of RT or RT combined with Z-TNFα, the numbers of cell clones were significantly reduced (Figure 2B-C). However, there was no significant difference between the two groups with cell area of 15.4±0.6% and 13.7±0.9%, respectively (Figure 2C). The cell areas in PBS and Z-TNFα group were respectively 23.3±2.3% and 21.6±1.9%. From the above experimental results, Z-TNFα has no obvious cytotoxic effect as well as radiosensitization effect in vitro.

3.3 | Τυμορ-ταργετινγ αξιολόγηση του Ζ-ΤΝΦα τον ιο

The optical imaging assay was performed to investigate the tumor-targeting ability of Z-TNFα in vivo. After intravenous injection of Cy7-TNFα or Cy7-Z-TNFα, LLC tumor graft-bearing mice were anesthetized, followed by scanning using an optical imaging system. As Figure 3(A) showed, the tumor exhibited a passive accumulation of TNFα, resulting in a minimal fluorescence signal at the site of the tumor. In contrast, high-contrast images of tumor xenografts in mice injected with Z-TNFα were clearly visible within one and two hours after injection (Figure 3A). The Z-TNFα signal intensity was significantly greater than that in the TNFα group and exhibited an increase from 1 to 2 hours post-injection (Figure 3A).

The distribution of Z-TNFα in the body was additionally assessed using the optical imaging device. Following the final scan, mice were euthanized and major organs/tissues were gathered and imaged. The fluorescence signals of tumor xenografts in the Z-TNFα group were extremely notable (Figure 3B). Nevertheless, the tumor fluorescence signals of the TNFα group were delicate, indicating inadequate capacity to target tumors. As shown in Figure 3C, the tumors in the Z-TNFα group exhibited fluorescence signal intensities that were 2.9 times higher than those in the muscles. In contrast, the ratio of tumor uptake to muscle uptake was merely 1.5 in the TNFα group (Figure 3C). In co-location assay, intravenously injected FAM-Z-TNFα was well co-located with the PDGFRβ+ cells in tumor grafts (Figure 3D). These findings demonstrated that the fusion to ZΔΓΦΡβ endowed TNFα with good tumor-targeting ability in vivo.

3.4 | Ζ-ΤΝΦα προμήθεια της ΛΛ′ τυμορ εσσέλες νορμαλιστική

To confirm the tumor vessel normalization effect of Z-TNFα in vivo, mice with LLC tumor grafts were administered Z-TNFα (1 μg/mouse) every alternate day for a total of three injections. The examination of tumor vessels’ structure and function took place on the second day following the final injection. As shown in Figure 4A, in Z-TNFα-treated tumor grafts, the vessels exhibited a more uniform diameter and reduced tortuosity in comparison to the PBS-treated group. Moreover, the surface areas of vessels were respectively 13.6 ± 2.9% and 4.7 ± 0.8% in PBS and Z-TNFα group, demonstrating a significant decrease in the blood vessel count (Figure 4A). The results indicated that Z-TNFα induced a normalization of tumor vessels, making them less twisted and more consistent in size. To evaluate vessel integrity and permeability, the leakage of dextran assay was performed. As shown in Figure 4B, the percentage of dextran diffused into the tumor tissues in Z-TNFα group (0.12 ± 0.1%) was much lower than those in the PBS group (2.2 ± 0.6%). This result indicated that the prompted tumor vessels normalization as improving vessel integrity and reducing vessel permeability.

Moreover, the EC-binding tomato lectin perfusion assays were performed to evaluate the function of tumor vessels. The proportion of lectin-binding ECs was significantly increased from 4.6 ± 1.2% to 48.5 ± 5.6% after the treatment of Z-TNFα (Figure 5), indicating the improvement of vessel perfusion in LLC tumor. The outcomes of all these experiments indicated that Z-TNFα therapy led to the normalization of tumor vessel function in LLC tumor.

3.5 | Ζ-ΤΝΦα σημερισταντικός υπολογισμός της τυμορ ηψοξία
A Hypoxyprobe-1 Plus kit was used to detect tumor hypoxia. As shown in Figure 5A, the area of hypoxia (green) was significantly reduced after Z-TNFα treatment. Accordingly, the PIMO areas representing hypoxia in Z-TNFα group was 1.1 ± 0.1%, compared with 18.3 ± 2.9% of PBS-treated group (Figure 6A). Moreover, tumor hypoxic conditions can promote the expression of hypoxia-inducible factor 1α (HIF-1α), and further upregulates the genes involved in cell survival result in RT resistance. As a result, the HIF-1α expression level that is proportional to hypoxia in tumor tissue was measured by immunofluorescence assay. Compared with PBS-treatment groups, the tumor tissues of mice treated with Z-TNFα showed a remarkable reduction in HIF-1α expression (areas: 24.7 ± 3.1% vs 3.52 ± 0.7%, Figure 6B). It indicated that Z-TNFα significantly reduced tumor hypoxia, which can be attributed to the normalization of tumor vessel function. These results demonstrated that Z-TNFα could relieve tumor hypoxia and might be used as an ideal radiosensitizer to increase the anti-tumor effect of radiotherapy.

3.6 | Ζ-ΤΝΦα μπροστα στην αντιτυμορ εφέφετο το ΡΤ

Considering that Z-TNFα-induced vessel normalization reduced tumor hypoxia, we proceeded to examine the combined antitumor impact of Z-TNFα and RT. In order to identify the tumor suppression caused by the Z-TNFα mediated sensitization of tumors to RT in vivo, mice with LLC tumor xenografts (around 70 mm³) were randomly divided into four groups (n = 4): (1) PBS, (2) Z-TNFα, (3) RT, and (4) Z-TNFα+RT. As shown in Figure 7A, either PBS-treatment or Z-TNFα did not exhibit any tumor suppression effect. Despite receiving RT (12Gy), the mice did not exhibit significant suppression of tumor growth in comparison to the PBS group (Figure 7A). Nevertheless, there was a notable disparity in the rates at which tumors grew between the Z-TNFα+RT and RT groups. The slowest growth rate of tumors was observed in mice treated with combination of Z-TNFα and RT (Figure 7A). Accordingly, the average tumor weight of Z-TNFα+RT group was only 0.15 ± 0.05 g at the end of the experiment, which was much lighter than those in PBS (0.86 ± 0.17 g), Z-TNFα (0.71 ± 0.03 g) or RT (0.60 ± 0.04 g) group (Figure 7B-C). To further verify enhanced anti-tumor activity of Z-TNFα and RT, tumor tissues were also analyzed by TUNEL assays. As shown in Figure 7D, large number of apoptotic cells (green) were obviously observed in tumor tissue treated with Z-TNFα and RT. These above results demonstrated that Z-TNFα induced fantastic sensitization to RT.

3.7 | Short-term safety evaluation

In short-term safety evaluation assay of treatment, it was observed that all mice remained alive and their body weights showed an increase over time in all groups during the experiment (Figure 8A). As shown in Figure8B, the levels of serum ALT, AST, UREA, CREA, UA and CK in all groups were within the normal range. Moreover, there were no evident histopathological abnormalities detected in the major organs (including the brain, heart, liver, spleen, lung, and kidney) of all the mice that received treatment (Figure 8C). These results indicate that the novel radiosensitizer of Z-TNFα demonstrates favorable safety in a short time.

4 | CONCLUSIONS

Radiotherapy resistance seriously affects the application of radiotherapy in the treatment of lung cancer. Therefore, it is urgent to explore effective radiosensitizing drugs for lung cancer radiotherapy. In this study, the fusion protein Z-TNFα was prepared, and Z-TNFα showed good tumor-targeting ability to LLC tumor in vivo. Z-TNFα normalized tumor vessel and significantly alleviate tumor hypoxia, thus enhancing the anti-tumor effect of radiotherapy. Moreover, Z-TNFα exhibited no evident systemic toxicity throughout the course of the treatment. In summary, Z-TNFα has the potential to be developed as a radiosensitizer for tumor radiotherapy.

AUTHOR CONTRIBUTION

QF, ZG and YL designed and proposed the study. XT, JC, LJ, KZ, FW and YL performed the experiments. QF, ZZ, RY, BT, DY and QW investigated and analyzed the data. QF and XT drafted the manuscript and all authors approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

FUNDING INFORMATION
The sponsor or funding organization had no role in the design or conduct of this research.

DATA AVAILABILITY STATEMENT
The collected and analyzed datasets during this study are available from the corresponding author on reasonable request.

References


**Figures:**

![Diagram A](image1.png) **[A]** TNFα and Z-TNFα expression in LLC cells.

![Diagram B](image2.png) **[B]** PDGFβR expression in LLC cells.

![Diagram C](image3.png) **[C]** Protein binding in LLC cells.
Figure 1

A

B

C

Cell Viability (%)

Concentration of protein (μM)

Cell Area (%)

PBS
Z-TNFα
RT
Z-TNFα+RT

0.05
0.1
0.25
0.5
1
2
5
10

0
20
40
60
80
100
120

**

n.s.

n.s.

n.s.
Figure 2
Figure 3

Figure 4
Figure 1. Z-TNFζ specifically binds to PDGFRβ-positive PCs. (A) The SDS-PAGE of purified protein. (B) PDGFRβ expression of LLC and PCs cells was detected by flow cytometry. (C) PDGFRβ-dependent cellular binding of Z-TNFζ. In the specific binding test, a total of $2 \times 10^5$ cells were cultured with FAM-Z-TNFζ for one hour and subsequently examined using flow cytometry. For the blocking assay, cells were preincubated with unbound Z-TNFζ for 30 minutes before the incubation with FAM-Z-TNFζ.

Figure 2. Cell cytotoxic and radiosensitization effects of Z-TNFζ in vitro. (A) Cytotoxicity of Z-TNFζ in LLC cells was measured by CCK-8 assay. (B) Radiosensitization effect of Z-TNFζ in vitro was evaluated by cell cloning assay. After different treatments, LLC cells were fixed, stained and taken pictures. (C) Cell areas were analyzed in the cell cloning assay.

Figure 3. Tumor-targeting ability of Z-TNFζ in vivo. (A) The optical imaging of mice bearing LLC tumor xenografts (circle indicated). Three mice with tumors in every group were given an intravenous injection of either Cy7-Z-TNFζ or an equivalent amount of Cy7-TNFζ. They were then scanned at 0, 1, and 2 hours after the injection using the IVIS optical imaging system. (B) Investigation of the distribution of Z-TNFζ in mice with LLC tumor xenografts. At 2 hours after injection, mice were euthanized and major organs/tissues were collected, then scanned using the IVIS optical imaging system. (C) The analysis involved examining the uptake of Z-TNFζ in the organs/tissues. (D) Localization of Z-TNFζ and PDGFRβ+ cells in LLC tumor tissues. FAM-Z-TNFζ was injected intravenously into mice that had LLC tumor grafts. After the passage of 60 min, the tumor grafts were extracted and sliced while being kept frozen, then subjected to staining using an antibody against PDGFRβ (red).

Figure 4. The Z-TNFζ promoted tumor vessel normalization in structure and function. Mice bearing LLC tumor grafts were intravenously injected with Z-TNFζ or PBS every other day for three times. (A) Tumor vessels were shown by CD31 staining. (B) Tumor vessel permeability. Following the treatment, mice received an intravenous injection of FITC-labeled dextran and were then allowed to circulate for 10 min. After perfusing the heart, the tumor grafts were extracted and examined using immunofluorescence analysis.

Figure 5. Z-TNFζ treatment increased the perfusion of tumor vessels. LLC tumor-bearing mice were given intravenous injections of Z-TNFζ or PBS every alternate day for a total of three administrations. Following the treatments, the mice received an intravenous injection of FITC-lectin. Heart-perfusions with PBS and 2% PFA were conducted after approximately 1.5 hours, followed by the collection of tumor grafts for immunofluorescence analysis.

Figure 6. Z-TNFζ treatment relieved tumor hypoxia. (A) Hypoxia in tumor grafts. LLC tumor-bearing mice were given intravenous injections of Z-TNFζ or PBS every alternate day for a total of three administrations. Following the treatment, mice received an intravenous injection of pimonidazole, which was then circulated for a duration of 90 min. The FITC-conjugated anti-Hypoxyprobe-1 antibody was used to stain the tumor tissues (green). (B) HIF-1α expression in tumor grafts. Following the treatment, the tumor grafts were acquired and subjected to staining with the HIF-1α antibody (red). DAPI (blue) was utilized to visualize the cell nuclei.

Figure 7. Z-TNFζ treatment enhances the antitumor effect of RT. (A) Tumor growth curves in different treatment groups. (B) Tumor weights of tumors that were isolated in various groups. (C) Tumor photographs of isolated tumors in different groups. (D) Images of tumor stained with the TUNEL from every group of treatments. DAPI (blue) was used to stain the nuclei.

Figure 8. Short-term safety evaluation. (A) Weights of the bodies were documented daily in various groups receiving different treatments. (B) Biochemical blood markers were used to assess the liver (ALT and AST), kidney (Crea, Urea, and UA) and heart (CK) functions. (C) Representative images of the major organs obtained, stained with H&E.