Sequential administration of pemetrexed and cisplatin reprograms tumor immune microenvironment and potentiates PD-1/PD-L1 treatment in a lung cancer model

Jinxiang Yu,[1] Qianyun Zhang ,[1] Jie Li,[2] Zhaohui Si,[3] Yuanyuan Guo,[4] Xin Xu,[5] Kanjin Wu[1]

[1]Department of Pulmonary and Critical Care Medicine (PCCM) Ward II, Cangzhou Central Hospital, Cangzhou, People's Republic of China
[2]Department of Pharmacy, Cangzhou Central Hospital, Cangzhou, People's Republic of China
[3]Department of Laboratory, Cangzhou Central Hospital, Cangzhou, People's Republic of China
[4]Department of Child Rehabilitation, Cangzhou Central Hospital, Cangzhou, People's Republic of China
[5]Department of Traditional Chinese Medicine 1, Cangzhou Central Hospital, Cangzhou, People's Republic of China

Correspondence to
Dr Qianyun Zhang, Department of Pulmonary and Critical Care Medicine (PCCM) Ward II, Cangzhou Central Hospital, Cangzhou, China;
zhangqianyun001@163.com

Accepted 5 November 2021
Published Online First 6 December 2021

To cite: Yu J, Zhang Q, Li J, *et al*. *J Investig Med* 2022;**70**:792–799.

ABSTRACT

This article aimed to investigate the effects of the administration method of pemetrexed and cisplatin on the efficacy and safety of treating non-small cell lung cancer (NSCLC) and the intrinsic molecular mechanism. Subcutaneous injection of A549 cells into BALB/C nude mice was used to explore the efficacy of different administration methods of pemetrexed and cisplatin in vivo. Immunogenic cell death (ICD) was evaluated by ATP secretion, ecto-CALR expression, and high mobility group protein 1 release. Western blot, qRT-PCR, and immunohistochemical staining were applied to detect the expression of apoptosis, cell cycle, and stimulator of interferon genes (STING) pathway-related markers. Immune microenvironment was evaluated by secretion of cytokines, infiltration of $CD8^+$ T cells, and expression of programmed death molecular ligand-1 (PD-L1). Sequential treatment with pemetrexed and cisplatin inhibited A549 cell-driven tumor formation in nude mice and regulated the expression of apoptosis and cell cycle-related genes. STING pathway and ICD were further activated by sequential treatment with pemetrexed and cisplatin. This sequential administration method increased the levels of interferon β, tumor necrosis factor α, interleukin 12, and C-X-C motif chemokine ligand 10, enhanced the infiltration of $CD8^+$ T cells, and upregulated the expression of PD-L1. Sequential administration of pemetrexed and cisplatin in the treatment of mouse NSCLC model may have a better effect than combination of drugs, providing theoretical basis and potential guidance for clinical medication.

Significance of this study

What is already known about this subject?

- Lung cancer is one of the most common malignant tumors worldwide.
- According to Cancer Statistics (2021), lung cancer is the leading cause of tumor death in both male and female patients.

What are the new findings?

- Sequential treatment with pemetrexed and cisplatin inhibited A549 cell-driven tumor formation in nude mice and regulated the expression of apoptosis and cell cycle-related genes.
- The stimulator of interferon gene pathway and immunogenic cell death were further activated by sequential treatment with pemetrexed and cisplatin.

How might these results change the focus of research or clinical practice?

- Sequential administration of pemetrexed and cisplatin in the treatment of non-small cell lung cancer in a mouse model may have a better effect than combination drugs, providing theoretical basis and potential guidance for clinical medication.

INTRODUCTION

Lung cancer is one of the most common malignant tumors worldwide. According to Cancer Statistics (2021), lung cancer is the leading cause of tumor death in both male and female patients.[1] Lung cancer is divided into non-small cell lung cancer (NSCLC) and small cell lung cancer, of which NSCLC accounts for about 85% of the total population.[2] NSCLC is highly malignant and likely to metastasize and relapse, which are important factors affecting treatment and prognosis.[3] It is particularly important to formulate a reasonable individualized treatment plan for patients with lung cancer to improve quality of life and reduce mortality.[4]

Pemetrexed is a multitarget folate antagonist that inhibits tumor growth by interfering with folate metabolism and DNA synthesis.[5] Its targets are the three enzymes required for purine and pyrimidine synthesis, including thymidylate synthetase, glycinamide ribonucleotide formyltransferase (GARFT), and dihydrofolate reductase (DHFR), which are involved in the biosynthesis of thymidine and purine nucleotides.[6] Compared with many chemotherapy drugs, the side effects of pemetrexed are mild, mainly manifested in the decrease of white cell count, diarrhea, rash, and mucositis.[7] Moreover, with the adoption of some preventive and therapeutic measures, such as the use of vitamins, folic acid, and hormones, the incidence and severity

of many side effects have decreased significantly, making the clinical use of pemetrexed safer.[8] Currently, for NSCLC, the most commonly used first-line chemotherapy is pemetrexed combined with cisplatin or carboplatin.[9] It has been reported that the sequential administration of pemetrexed and cisplatin has a better inhibitory effect on tumor cell proliferation in vitro than the simultaneous administration of the two.[10] However, it is not clear whether such a combination of drugs has a better tumor suppressive effect in mice. This article used a mouse model to explore the effects of the administration method on the efficacy and safety and its intrinsic molecular mechanism.

METHODS

Cell culture

Lung cancer cell line A549 was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, California, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% Antibiotic-Antimycotic (100X) (#15240062; Gibco). Cells were kept in constant temperature incubator of 37°C and 5% CO_2.

Intervention and grouping

Mice were divided into six groups: control group (normal saline group), pemetrexed single-use group, cisplatin single-use group, pemetrexed and cisplatin combination group, schedule 1 group (sequential administration of pemetrexed for 24 hours followed by cisplatin for 24 hours), and schedule 2 group (sequential administration of cisplatin for 24 hours followed by pemetrexed for 24 hours). The dose of pemetrexed was 100 mg/kg[11] and the dose of cisplatin was 2 mg/kg.[12] The administration was started when the tumor size was about 100 mm^3. The combination group was given the two drugs at the same time. Cisplatin (also called DDP) was obtained from West China Hospital Pharmacy and dissolved in double-distilled water (ddH_2O). Pemetrexed was also dissolved in ddH_2O.

Xenograft model

To evaluate tumorigenesis and development in vivo, we performed a xenograft assay through subcutaneous injection into BALB/C nude mice. The A549 cells were cultured to the logarithmic growth phase. After trypsinizing the cells, the cells were washed by cold phosphate-buffered saline (PBS) to remove the serum from the cells. The cells were counted and resuspended in PBS to a concentration of 2.5×10^7/mL on ice. A 1 mL syringe was used to gently aspirate the required amount of cell suspension to bounce away air bubbles. The needle of the syringe was placed upwards and inserted into the breast fat pad. Then 200 µL (5×10^6 cells/piece) cell mixture were injected into the mice. The mice were observed every 4 days. The diameter of the subcutaneous tumor was recorded. Before the tumor grew to 1 cm in diameter, the mice were killed by carbon dioxide inhalation asphyxiation method. The tumor was peeled off and washed with PBS. Tumors were neatly arranged on white paper and photographed. Tumors were weighed and the weight was recorded.

Immunohistochemical staining

The dryer was turned on in advance. When the temperature rose to 65°C, the slices were placed on the upper surface of the machine for 30 min to melt the paraffin. Xylene was used for dewaxing. Different concentrations were used to hydrate the slices. Sodium citrate buffer (10 mM, pH=6) was used to restore the antigen and 3% hydrogen peroxide was used to seal the sections. Phosphate-buffered saline with Tween 20 (PBST) was used to clean the sections. Dilution buffer (Zhongshan Jinqiao Company) was used to dilute the primary antibody in a certain proportion, 150 µL of horse radish peroxidase-conjugated secondary antibody (Dako) was used to incubate sections at room temperature, and 1 mL 3,3'-diaminobenzidine (DAB) Substrate Buffer and DAB Concentrate were mixed in a ratio of 1:50. Distilled water was used to stop dyeing. Hematoxylin was used for nuclear staining. A serial pathology section scanner was used to scan the sections.

Western blot

Western blot was performed as standard method. The following antibodies were used: PD-L1 (programmed death molecular ligand-1; E1L3N) XP Rabbit mAb #13684, Phospho-TBK1/NAK (Ser172) (D52C2) XP Rabbit mAb #5483, TBK1/NAK (D1B4) Rabbit mAb #3504, Phospho-IRF-3 (Ser396) (D6O1M) Rabbit mAb #29047, IRF-3 (D83B9) Rabbit mAb #4302, Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb, NF-κB p65 (D14E12) XP Rabbit mAb, and GAPDH (D16H11) XP Rabbit mAb #5174, purchased from Cell Signaling Technology.

Immunogenic cell death detection

ENLITEN ATP Assay System (FF2000; Promega, Leiden, The Netherlands) was applied to estimate ATP release. VICTOR plate reader (PerkinElmer) was used to measure the bioluminescent signal.

Ecto-calreticulin surface exposure (CALR) expression was detected by staining and flow cytometry. Following different treatments, 5% normal goat serum (G9023; Sigma-Aldrich) was used to block A549 cells. Ecto-CALR was then stained by Alexa Fluor 488-conjugated anti-CALR (1:100, ab196158; Abcam) for 1 hour. Non-permeabilized cells were defined by propidium iodide and annexin V. Rabbit IgG (1:100, 199091; Abcam) was used as the isotype control. According to the manufacturer's instructions, high mobility group protein 1 (HMGB1) release was detected by ELISA kit (ST51011; IBL).

RNA extraction and qRT-PCR analysis

Total RNA was extracted by RNeasy Mini Kit (74104; Qiagen) and transcribed to complementary DNA (cDNA) by FastKing RT Kit (With gDNase) (KR116; TIANGEN). quantitative reverse transcription (qRT)-PCR analysis was performed by FastKing One Step RT-qPCR Kit (SYBR Green) purchased from TIANGEN (FP313). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression of the target gene to be tested. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene.

Statistical analysis

All data are presented as mean±SD. One-way or two-way analysis of variance with appropriate post-hoc test was performed to compare data between the groups.

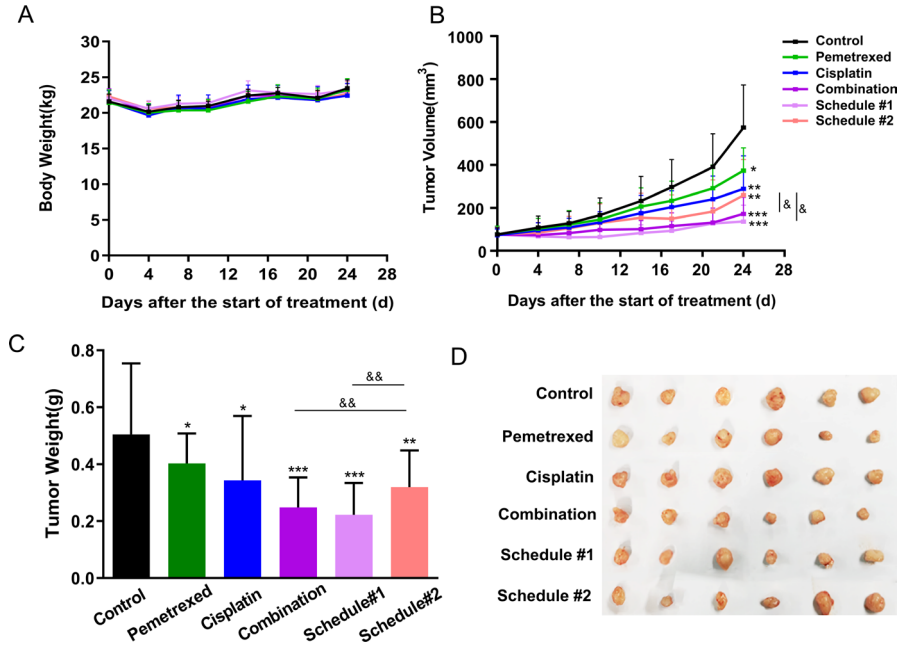

Figure 1 Effects of pemetrexed and cisplatin, alone and in combination or in sequence, on subcutaneous A549 tumor growth. (A) Alteration of weight in each group. (B) Tumor diameters for each mouse. (C–D) Tumors were weighed and photographed after the mice were sacrificed. Data are presented as mean±SD, n=6 for each group. $^{\&}$P<0.05, $^{\&\&}$P<0.01. *P<0.05, **P<0.01, ***P<0.001.

RESULTS

Sequential treatment with pemetrexed and cisplatin inhibits potent tumor growth

We first tested the tumor-inhibiting effects of various administration methods in the process of A549 cell-driven tumor formation in nude mice. The mice were divided into six groups: control group (normal saline group), pemetrexed single-use group, cisplatin single-use group, pemetrexed and cisplatin combination group, schedule 1 group (sequential administration of pemetrexed followed by cisplatin), and schedule 2 group (sequential administration of cisplatin followed by pemetrexed). We detected changes in the weight of mice and changes in tumor volume. We found that the various medications did not affect the weight of the mice (figure 1A). From the perspective of tumor volume and weight, the efficacy of the combined drug and sequential drug group was significantly better than that of the control and the two-drug single-use group (figure 1B–D). At the same time, the efficacy of the combined drug and schedule 1 group was similar and both were better than the schedule 2 group (figure 1B–D).

Sequential treatment with pemetrexed and cisplatin affects the expression of apoptosis and cell cycle-related genes

We further detected the changes in apoptosis and cell cycle-related genes in tumors derived from nude mice (table 1). The experimental results showed that the messenger RNA (mRNA) levels of apoptosis-related genes Bax and Bcl-2 in the combination and sequential administration groups were significantly higher than those in the control group and the single-drug group, and the schedule 1 group showed stronger expression of Bax and Bcl-2. In addition, we found that the mRNA levels of cell cycle-related tumor suppressor genes p53, p21, and p27 in the combination and sequential administration groups were significantly higher than those in the control group and the single-drug group, and the schedule 1 group showed stronger expression of p21. Furthermore, we found that the mRNA levels of cell cycle-related genes Cdc2, Cdk2, Cdk7, and cyclin D1 in the combination and sequential administration groups were significantly lower than those in the control group and the single-drug group. They were further downregulated in the schedule 1 group, suggesting that the cell cycle was more significantly inhibited. At the same time, we used immunohistochemical (IHC) staining to further detect the expression levels of cleaved caspase 3 and Ki-67 (figure 2A and B). Ki-67 protein is a convenient and reproducible biomarker of cell proliferation. We found that the expression levels of Ki-67 in the combination and sequential administration groups were significantly lower than those in the control group and the single-drug group. They were further downregulated in the combination group. In addition, we found that expression of cleaved caspase 3 in the combination and sequential administration groups was significantly higher than those in the control group and the single-drug group. The efficacy of the combination group was similar to that of the schedule 1 group and was better than the schedule 2 group in increasing the level of cleaved caspase 3. These results indicate that the sequential administration of pemetrexed and cisplatin (schedule 1) is similar to the combination group in terms of inducing apoptosis and inhibiting tumor cell proliferation and may perform better in terms of certain test indicators.

Sequential treatment with pemetrexed and cisplatin induces activation of STING pathway in vivo and in vitro

It has been reported that cisplatin can activate the stimulator of interferon gene (STING) pathway and induce PD-L1

Table 1 Effects of pemetrexed and cisplatin, alone and in sequence or in combination, on apoptosis and cell cycle-related gene expression

Gene name		Control	Pemetrexed	Cisplatin	Combination	Schedule 1	Schedule 2
Apoptosis-related genes							
Bax	Fold change	–	0.81±0.31	1.23±0.55	17.99±8.09**	53.35±18.64**†	21.17±10.35**
	ΔCT	11.47±1.66	12.61±1.91	10.26±1.64	10.91±1.73	9.48±0.47	8.79±0.54
Bcl-2	Fold change	–	0.21±0.10	–1.49±0.97	–5.54±1.05**	–11.26±7.17**†	–4.54±1.04**
	ΔCT	10.21±0.62	10.13±0.81	11.39±0.61	12.14±1.26	13.39±1.03	10.24±0.75
Cell cycle-related genes							
p53	Fold change	–	0.89±0.46	2.77±1.32	5.88±4.57**	6.24±4.12**	2.80±1.75**
	ΔCT	8.18±1.31	6.92±1.21	7.05±1.33	8.15±1.02	5.86±0.94	6.79±1.53
p21	Fold change	–	3.85±1.52*	4.93±1.65**	86.07±23.15**	97.76±25.31**	62.65±31.86**
	ΔCT	6.27±0.45	5.22±0.34	5.79±0.36	6.53±1.29	2.37±0.27	5.08±1.77
p27	Fold change	–	0.35±0.16	2.08±1.41**	6.59±3.79**	6.15±3.27**	4.31±3.43
	ΔCT	6.84±0.32	6.22±1.64	5.51±1.21	5.79±0.71	5.34±0.89	5.75±0.87
Cdc2	Fold change	–	–1.85±0.28	0.18±0.44	–4.26±1.45**	–6.89±1.88**†	–3.02±1.25
	ΔCT	12.70±1.81	10.83±1.84	13.82±1.92	15.54±2.72	14.09±2.14	12.30±1.67
Cdk2	Fold change	–	–1.85±0.43	–0.82±0.27	–6.52±1.45**	–9.55±1.65**	–5.23±1.52**
	ΔCT	7.41±1.16	8.27±1.34	6.73±1.27	6.90±1.18	11.16±1.09	8.18±1.01
Cdk7	Fold change	–	–0.38±0.21	0.65±0.33	–9.26±2.24**	–10.03±5.61**	–7.34±0.42**
	ΔCT	6.38±0.53	6.39±0.75	8.14±1.04	9.14±0.55	10.13±1.07	7.69±0.88
Cyclin D1	Fold change	–	–4.72±0.17*	–6.39±0.36*	–36.78±22.36**	–56.54±20.88**†	–17.41±10.16**
	ΔCT	7.17±0.68	7.74±0.79	7.37±1.21	10.91±0.98	10.07±0.52	8.41±0.61

Quantitative real-time RT-PCR analysis showing fold change and ΔCT values in gene expression.

Data shown are mean±SD for tumors extracted from five different animals.
*P<0.01, **P<0.001 compared with the control group.
†P<0.05 compared with the combination treatment groups.
RT, reverse transcription; ΔCT, Ct(target gene)-Ct(internal control).

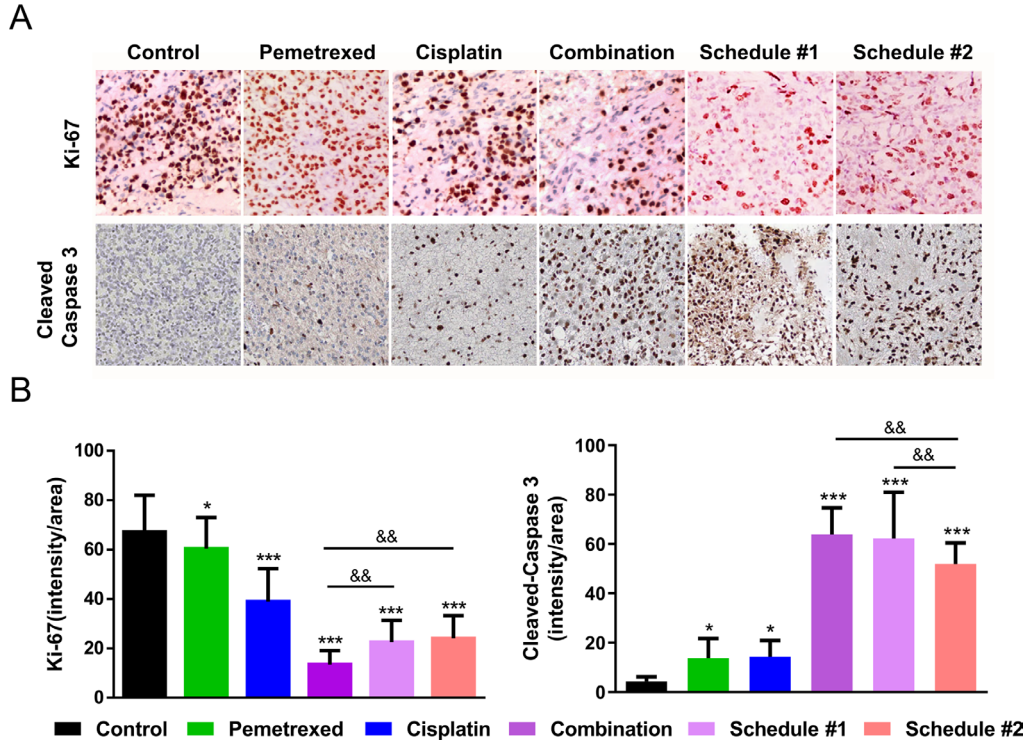

Figure 2 Effects of pemetrexed and cisplatin, alone and in combination or in sequence, on cell proliferation and apoptosis in subcutaneous A549 tumor. Immunohistochemical staining of tumor sections for Ki-67 (proliferation), cleaved caspase 3 (apoptosis) (A) and the corresponding quantification of staining intensity (B) using ImageJ software. Data are presented as mean±SD, n=6 for each group. $^{\&}$P<0.05, $^{\&\&}$P<0.01. *P<0.05, **P<0.01, ***P<0.001.

expression in a variety of preclinical NSCLC models.[13] Therefore, we explored whether the synergistic effect exhibited by pemetrexed and cisplatin in combination or sequential administration is caused by greater activation of the STING pathway at the molecular level. The STING pathway includes the classic STING-TANK-binding kinase 1 (TBK1)-interferon regulatory factor 3 (IRF3) pathway and the non-classic STING-ataxia telangiectasia mutated (ATM)-interferon-γ-inducible factor 16 (IFI16)-nuclear factor κB (NF-κB) pathway.[14] Therefore, we detected the key proteins and their phosphorylation levels in these two pathways in tumor tissues obtained from nude mice (figure 3A). We found that pemetrexed or cisplatin alone can cause activation of these two pathways, as evidenced by increased levels of Phospho-TBK1 (S172), Phospho-IRF3 (S396), and Phospho p65 (S536), and combined and sequential administration groups further increased the expression levels of the three markers. The activation level of STING pathway in the combination medication group and the schedule 1 group was higher than that in the schedule 2 group. It is worth noting that Phospho-IRF3 (S396)/total-IRF3 was significantly increased in the schedule 1 group, which suggests that this sequential medication may be able to further activate the classic STING pathway. Further validation of our results in A549 cells in vitro (figure 3B) showed that the combination of pemetrexed and cisplatin and the order of administration according to schedule 1 may further activate the STING pathway.

Sequential treatment with pemetrexed and cisplatin further triggers immunogenic cell death in vitro

Pemetrexed could induce the occurrence of immunogenic cell death (ICD), and the activation of STING pathway could also cause ICD. Therefore, we further studied the effect of different administration methods of pemetrexed and cisplatin on ICD. This experiment was performed in vitro with A549 cells. By detecting the hallmarks in the supernatant, including ATP, ecto-calreticulin, and HMGB1, we found that the combination group was not significantly different from the pemetrexed single-use group in inducing ICD and even had the opposite effect in the schedule 2 group. In the schedule 1 group, ICD has improved significantly (figure 4A–C). This indicates that the sequential administration of pemetrexed and cisplatin may be able to induce the occurrence of ICD to a greater extent.

Sequential treatment with pemetrexed and cisplatin reprograms the tumor immune microenvironment

Both STING activation and ICD affect the immune microenvironment. We further tested the effects of different administration methods of pemetrexed and cisplatin on the tumor immune microenvironment. We found that the tumors in the schedule 1 group expressed higher levels of interferon β (coding by IFNB1; figure 5A), C-X-C motif chemokine ligand 10 (CXCL10, T cell chemokines; figure 5B), and proinflammatory cytokines including tumor necrosis factor α (TNF-α) (figure 5C) and interleukin 12 (IL-12) (figure 5D). Correspondingly, we found higher $CD8^+$ T cell infiltration in tumor cells in the schedule 1 group (figure 5E). In clinical practice, pemetrexed and cisplatin are usually combined with programmed death-1 (PD-1)/PD-L1 antibody. Therefore, we further detected the effects of different administration methods of pemetrexed and cisplatin on PD-L1 expression. Our results reveal that the schedule 1 group expressed higher levels of PD-L1 (figure 5F), indicating that it is more likely to promote the therapeutic effect of PD-1/PD-L1 antibodies.

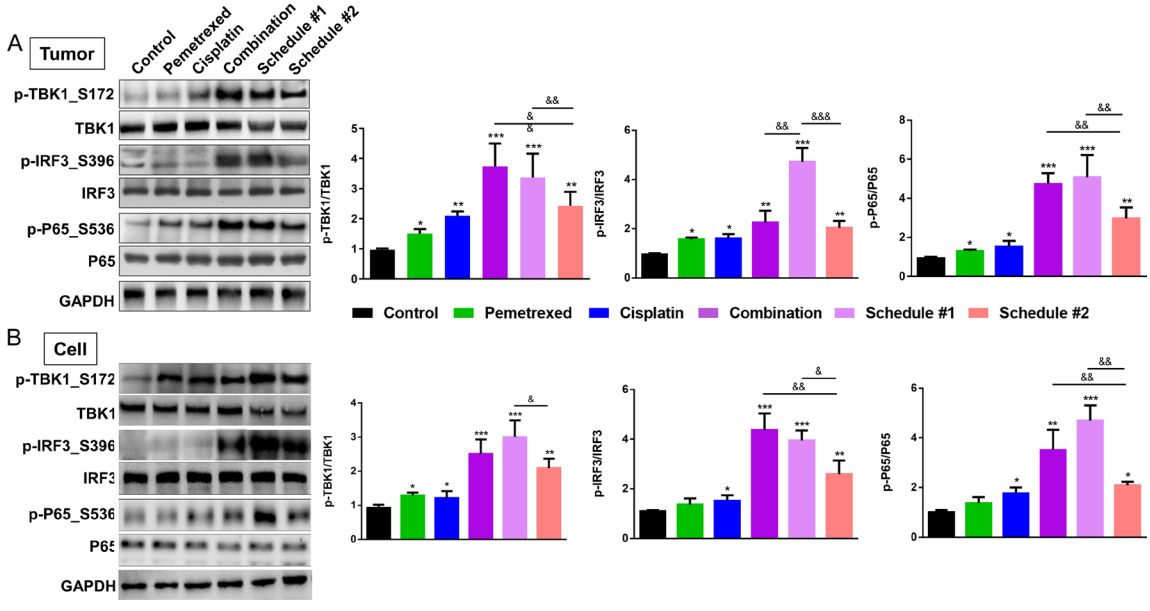

Figure 3 Effects of pemetrexed and cisplatin, alone and in combination or in sequence, on STING pathway. Western blot and quantification analysis to evaluate total and Phospho p65 (S536), total and Phospho-IRF3 (S396), and total and Phospho-TBK1 (S172) in A549 tumors (A) and A549 cells (B) treated as indicated. Data are presented as mean±SD, n=6 for each group. $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001. *P<0.05, **P<0.01, ***P<0.001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRF3, interferon regulatory factor 3; TBK1, TANK-binding kinase 1; STING, stimulator of interferon genes.

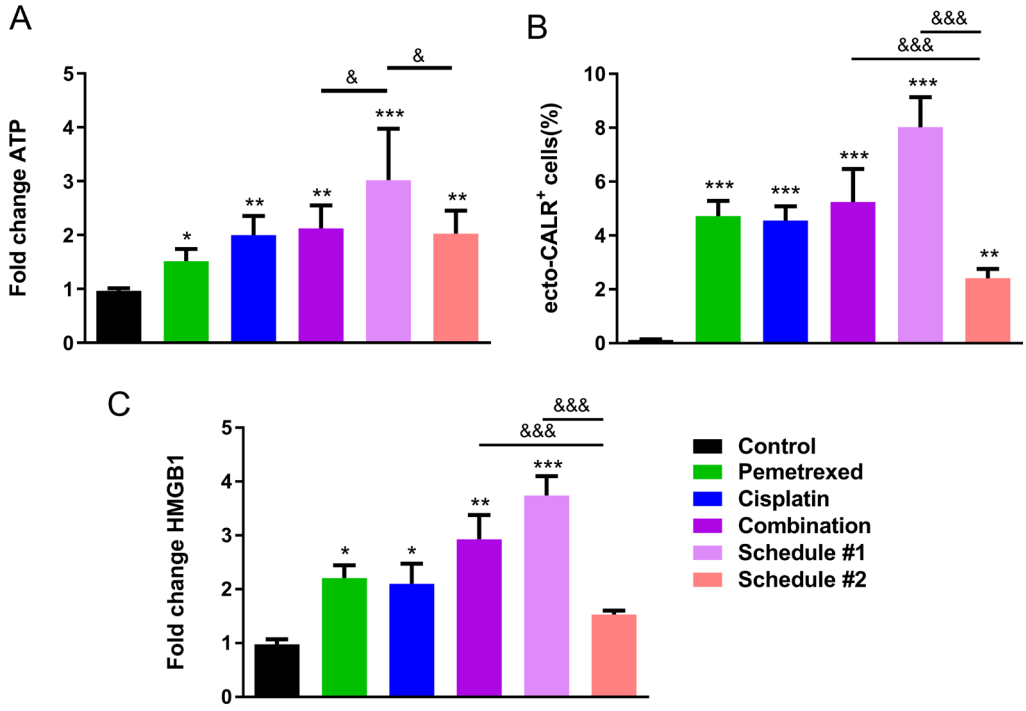

Figure 4 Effects of pemetrexed and cisplatin, alone and in combination or in sequence, on immunogenic cell death in A549 lung cancer cell lines. ATP secretion (A) percentages of ecto-CALR-positive (ecto-CALR$^+$) cells (B) and HMGB1 release (C) in A549 cell lines. Data are presented as mean±SD, n=6 for each group. $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001. *P<0.05, **P<0.01, ***P<0.001. HMGB1, high mobility group protein 1.

DISCUSSION

The treatment methods for lung cancer include surgery, chemotherapy, radiotherapy, molecular targeted therapy, gamma knife therapy, immunotherapy, and traditional Chinese medicine treatment.[15] Surgical treatment and radiotherapy are feasible in early-stage NSCLC.[15] However, due to the insidious incidence of lung cancer and the lack of specificity of symptoms, most patients are already in the advanced stage at the time of diagnosis and have lost the opportunity for surgery, and chemotherapy has become one of the main treatments for advanced NSCLC.[16] Platinum-based combined with third-generation lung cancer chemotherapy drugs are the current standard first-line treatment for advanced NSCLC.[17] However, the current chemotherapy effect for patients with advanced NSCLC is still unsatisfactory. Although increasing the dose intensity can increase the efficacy of chemotherapy, the adverse reactions also increase, making it difficult for most patients to tolerate, especially elderly patients and patients with impaired functions of multiple organs.[18] Therefore, identifying better treatments to improve efficacy and reduce complications is a common concern in oncology.

Maintenance therapy is defined as continuing to receive cytotoxic drugs or targeted drugs until the disease progresses after completing the standard cycle of first-line chemotherapy and reaching disease control.[19] However, the adverse effects of traditional chemotherapy drugs are relatively large. Patients with long-term treatment have greater cumulative toxicity and poorer quality of life, which may affect the efficacy and benefit.[20] In addition, due to poor patient tolerance, the proportion of patients who can receive more than four cycles of treatment is low, which affects the possible survival benefits of continuous treatment and limits the development effect of maintenance therapy.[21] Toxicity and adverse reactions caused by first-line platinum-containing drugs are usually caused by platinum drugs.[22] The results of this study show that the sequential administration of pemetrexed and cisplatin (schedule 1) is similar to the combination group in terms of inducing apoptosis and inhibiting tumor cell proliferation and may perform better in terms of certain test indicators. At the same time, the adverse reactions and toxicity of the sequential administration of the two drugs may be lower than that of the combination of the two drugs. These are worthy of further discussion.

The STING signaling pathway can identify abnormal cytoplasmic double-stranded DNA and mediate the innate immune response.[23] When unstimulated, the STING protein is mainly located on the endoplasmic reticulum, with a few located on the mitochondria.[24] When abnormal double-stranded DNA appears in the cytoplasm, the conformation of STING protein changes and then STING transfers to the perinuclear region to recruit and phosphorylate TBK1.[24 25] After STING and TBK1 are further activated by phosphorylation at specific sites, the complex rapidly transfers from the endoplasmic reticulum to the Golgi apparatus and further phosphorylates the transcription factors IRF3 and NF-κB.[26] These transcription factors then translocate to the nucleus, turn on the transcription of innate immune genes, and regulate the transcription of type I interferon or other proinflammatory genes (such as IL-6 and TNF-α) and the release of various downstream inflammatory factors.[27] Therefore, tumor cells are often accompanied by damage to the STING signaling pathway and mutations and deletions

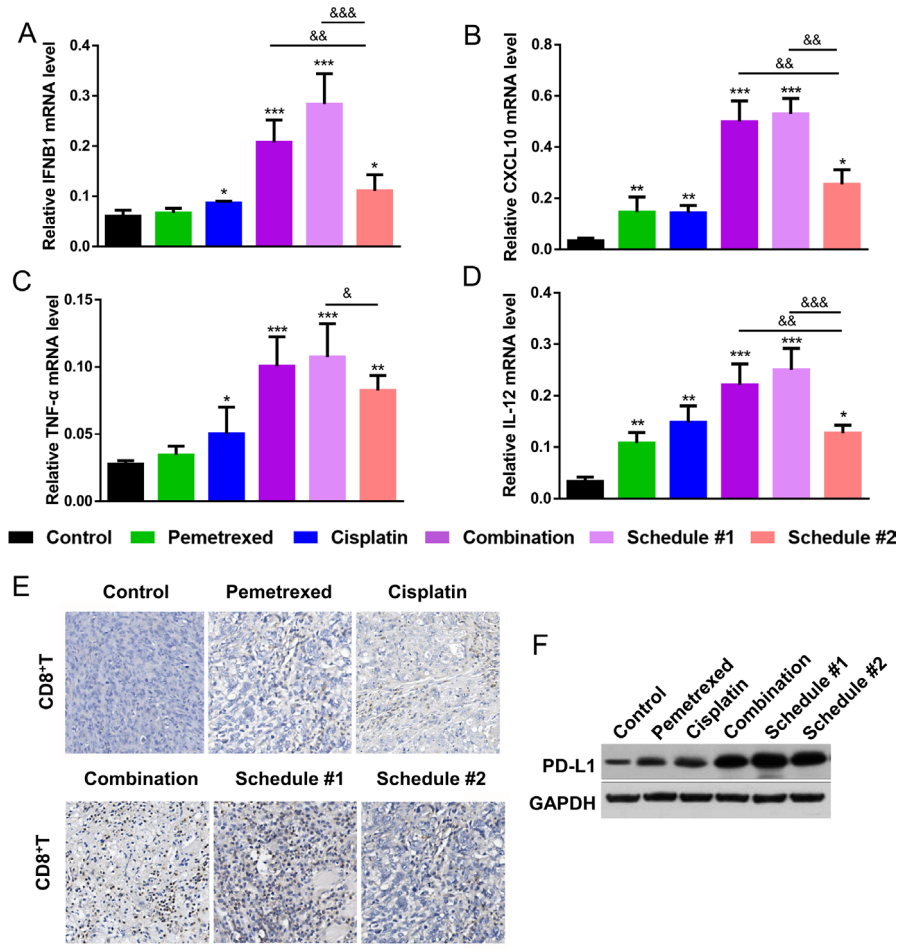

Figure 5 Pemetrexed and cisplatin in sequence generates T cell inflamed tumor microenvironment. qRT-PCR analysis of IFNB1(A), CXCL10 (B), TNF-α (C), and IL-12 (D) gene expression. (E) Immunohistochemical staining of the tumor sections for infiltrated $CD8^+$ T cells. (F) PD-L1 expression in tumors was analyzed by western blot. Data are presented as mean±SD, n=6 for each group. $^{\&}P<0.05$, $^{\&\&}P<0.01$, $^{\&\&\&}P<0.001$. *P<0.05, **P<0.01, ***P<0.001. IFNB1, interferon beta 1; CXCL10, C-X-C motif chemokine ligand 10; IL-12, interleukin 12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT, quantitative reverse transcription; mRNA, messenger RNA; PD-L1, programmed death molecular ligand-1.

of the type I interferon gene to escape DNA detection pathways and survive.[28] The results of this study indicate that the combination of pemetrexed and cisplatin and the order of administration according to schedule 1 can further activate the STING pathway. However, in the schedule 2 group, the activation of the STING pathway was not further improved, and on the contrary there was a significant decrease. Therefore, in the treatment of NSCLC, the order of administration is also a very noteworthy aspect which may affect the final therapeutic effect.

In recent years, it has been discovered that the immune system plays an important role in the occurrence and development of cancer.[29] The immune system can not only suppress the growth of tumor cells and remove tumor cells through immune monitoring, but also screen out cancer cells through escape mechanisms.[29] Usually, the antigen-presenting cells in the body's immune surveillance system recognize tumor surface antigens and present them to cytotoxic T lymphocytes (CTL).[30] The activated CTL eliminates tumor cells by inducing apoptosis.[31] With the development of tumor immune escape mechanism, it is found that the negative immune regulation of some immune checkpoints plays an important role in tumor formation.[32] PD-1 is a relatively mature immunoscreening molecule[33] and an immunosuppressive receptor. PD-L1 and programmed death molecular ligand-2 (PD-L2) are two known PD-1 ligands, both of which are B7 family immunoglobulins.[34] PD-L1 and PD-L2 are expressed on the surface of most cancer cells, which can specifically bind to PD-1 on the surface of T cells, activate the PD-1 signaling pathway, inhibit T cells from killing tumors, and downregulate the body's immune response.[35] Konishi *et al*[36] used IHC staining methods to confirm the relationship between PD-1/PD-L1 signaling pathway and NSCLC for the first time. The results show that all NSCLC specimens express PD-L1, but different samples have different expression levels of PD-L1.[36] The PD-1/PD-L1 monitoring site plays a negative regulatory role in the antitumor immunity of NSCLC. PD-1 inhibitors can block the negative signal transduction pathway of PD-1 and PD-L1, enhance the body's immune response, and open up a new direction for the treatment of advanced lung cancer. In this study, we found that sequential treatment

with pemetrexed and cisplatin increased the levels of interferon β, TNF-α, IL-12, CXCL10, and the infiltration of $CD8^+$ T cells, suggesting that this administration method can regulate the immune microenvironment. Notably, we found that sequential treatment with pemetrexed and cisplatin enhanced the levels of PD-L1 compared with combined treatment, indicating that this order of administration is more likely to promote the therapeutic effect of PD-1/PD-L1 antibodies.

CONCLUSION

In conclusion, our results showed that sequential administration of pemetrexed and cisplatin inhibited tumor formation driven by A549 cells in nude mice. The sequential administration regulated the expression of apoptosis and cell cycle-related genes and further activated STING pathway and ICD. Compared with combination of two drugs, sequential administration of pemetrexed and cisplatin enhanced the infiltration of $CD8^+$ T cells and upregulated the expression of PD-L1, providing theoretical basis and potential guidance for clinical medication.

Contributors Concept or design: JY and QZ. Acquisition of data: JY, QZ, JL, ZS, YG, XX, and KW. Analysis or interpretation of data: JY, QZ, JL, and ZS. Guarantor: QD. Drafting of the manuscript: JY, QZ, JL, ZS, YG, XX, and KW. Critical revision of the manuscript for important intellectual content: all authors. All authors had full access to the data, contributed to the study, approved the final version for publication, and take responsibility for its accuracy and integrity.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The animal studies were reviewed and approved by the ethics committee of Cangzhou Central Hospital (2019/c32).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

ORCID iD

Qianyun Zhang http://orcid.org/0000-0002-1451-6679

REFERENCES

1 Siegel RL, Miller KD, Fuchs HE, *et al*. Cancer statistics, 2021. *CA Cancer J Clin* 2021;71:7–33.
2 Bade BC, Dela Cruz CS. Lung cancer 2020: epidemiology, etiology, and prevention. *Clin Chest Med* 2020;41:1–24.
3 Jonna S, Subramaniam DS. Molecular diagnostics and targeted therapies in non-small cell lung cancer (NSCLC): an update. *Discov Med* 2019;27:167–70.
4 Jones GS, Baldwin DR. Recent advances in the management of lung cancer. *Clin Med* 2018;18:s41–6.
5 Adjei AA. Pharmacology and mechanism of action of pemetrexed. *Clin Lung Cancer* 2004;5:S51–5.
6 Liang J, Lu T, Chen Z, *et al*. Mechanisms of resistance to pemetrexed in non-small cell lung cancer. *Transl Lung Cancer Res* 2019;8:1107–18.
7 Hill J, Vargo C, Smith M, *et al*. Safety of dose-reduced pemetrexed in patients with renal insufficiency. *J Oncol Pharm Pract* 2019;25:1125–9.
8 Dumoulin DW, Visser S, Cornelissen R, *et al*. Renal toxicity from pemetrexed and pembrolizumab in the era of combination therapy in patients with metastatic Nonsquamous cell NSCLC. *J Thorac Oncol* 2020;15:1472–83.
9 Nishio M, Barlesi F, West H, *et al*. Atezolizumab plus chemotherapy for first-line treatment of Nonsquamous NSCLC: results from the randomized phase 3 IMpower132 trial. *J Thorac Oncol* 2021;16:653–64.
10 Kano Y, Akutsu M, Tsunoda S, *et al*. Schedule-dependent interactions between pemetrexed and cisplatin in human carcinoma cell lines in vitro. *Oncol Res* 2006;16:85–95.
11 Kuo W-T, Tu D-G, Chiu L-Y, *et al*. High pemetrexed sensitivity of docetaxel-resistant A549 cells is mediated by TP53 status and downregulated thymidylate synthase. *Oncol Rep* 2017;38:2787–95.
12 Ou W, Ye S, Yang W, *et al*. Enhanced antitumor effect of cisplatin in human NSCLC cells by tumor suppressor LKB1. *Cancer Gene Ther* 2012;19:489–98.
13 Della Corte CM, Sen T, Gay CM, *et al*. STING pathway expression identifies NSCLC with an Immune-Responsive phenotype. *J Thorac Oncol* 2020;15:777–91.
14 Zhou L, Xu Q, Huang L, *et al*. Low-dose carboplatin reprograms tumor immune microenvironment through STING signaling pathway and synergizes with PD-1 inhibitors in lung cancer. *Cancer Lett* 2021;500:163–71.
15 Hoy H, Lynch T, Beck M. Surgical treatment of lung cancer. *Crit Care Nurs Clin North Am* 2019;31:303–13.
16 Collins LG, Haines C, Perkel R, *et al*. Lung cancer: diagnosis and management. *Am Fam Physician* 2007;75:56–63.
17 Rossi A, Di Maio M. Platinum-based chemotherapy in advanced non-small-cell lung cancer: optimal number of treatment cycles. *Expert Rev Anticancer Ther* 2016;16:653–60.
18 Pirker R. Chemotherapy remains a cornerstone in the treatment of nonsmall cell lung cancer. *Curr Opin Oncol* 2020;32:63–7.
19 Campochiaro C, Della-Torre E, Lanzillotta M, *et al*. Long-term efficacy of maintenance therapy with rituximab for IgG4-related disease. *Eur J Intern Med* 2020;74:92–8.
20 Ling C, Evans D, Zhang Y, *et al*. The effects of cognitive behavioural therapy on depression and quality of life in patients with maintenance haemodialysis: a systematic review. *BMC Psychiatry* 2020;20:369.
21 Gupta S, Nag S, Aggarwal S, *et al*. Maintenance therapy for recurrent epithelial ovarian cancer: current therapies and future perspectives - a review. *J Ovarian Res* 2019;12:103.
22 Dehghanpour S, Pourzamani HR, Amin MM, *et al*. Evaluation of toxic effects of platinum-based antineoplastic drugs (cisplatin, carboplatin and oxaliplatin) on green alga Chlorella vulgaris. *Aquat Toxicol* 2020;223:105495.
23 Loo TM, Miyata K, Tanaka Y, *et al*. Cellular senescence and senescence-associated secretory phenotype via the cGAS-STING signaling pathway in cancer. *Cancer Sci* 2020;111:304–11.
24 Wan D, Jiang W, Hao J. Research advances in how the cGAS-STING pathway controls the cellular inflammatory response. *Front Immunol* 2020;11:615.
25 Chen Q, Sun L, Chen ZJ. Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. *Nat Immunol* 2016;17:1142–9.
26 Zhang X, Bai X-C, Chen ZJ. Structures and mechanisms in the cGAS-STING innate immunity pathway. *Immunity* 2020;53:43–53.
27 Jiang M, Chen P, Wang L, *et al*. cGAS-STING, an important pathway in cancer immunotherapy. *J Hematol Oncol* 2020;13:81.
28 Flood BA, Higgs EF, Li S, *et al*. STING pathway agonism as a cancer therapeutic. *Immunol Rev* 2019;290:24–38.
29 Abbott M, Ustoyev Y. Cancer and the immune system: the history and background of immunotherapy. *Semin Oncol Nurs* 2019;35:150923.
30 Leone RD, Powell JD. Metabolism of immune cells in cancer. *Nat Rev Cancer* 2020;20:516–31.
31 Aerts JG, Hegmans JP. Tumor-specific cytotoxic T cells are crucial for efficacy of immunomodulatory antibodies in patients with lung cancer. *Cancer Res* 2013;73:2381–8.
32 Schmid-Bindert G, Jiang T. First-line nivolumab (anti-PD-1) monotherapy in advanced NSCLC: the story of immune checkpoint inhibitors and "the sorcerers apprentice". *Transl Lung Cancer Res* 2015;4:215–6.
33 Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* 2015;27:450–61.
34 Hatam LJ, Devoti JA, Rosenthal DW, *et al*. Immune suppression in premalignant respiratory papillomas: enriched functional CD4+Foxp3+ regulatory T cells and PD-1/PD-L1/L2 expression. *Clin Cancer Res* 2012;18:1925–35.
35 Homet Moreno B, Parisi G, Robert L, *et al*. Anti-PD-1 therapy in melanoma. *Semin Oncol* 2015;42:466–73.
36 Konishi J, Yamazaki K, Azuma M, *et al*. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin Cancer Res* 2004;10:5094–100.