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Curcumin enhances drug sensitivity of gemcitabine-resistant lung cancer cells and inhibits metastasis

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Received November 18, 2020, accepted December 16, 2020

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Pharmazie 76:538-543 (2021) *doi: 10.1691/ph.2021.0927*

This study aimed to investigate the effects of curcumin (Cur) on the proliferation, migration, and invasion of gemcitabine (GEM) resistant lung cancer A549 cells (A549/GEM), and the potential mechanism. After treating with GEM, individually or combined with Cur, the inhibition, migration, and invasion of A549/GEM were tested by the CCK8, transwell, and cell wound healing assays, respectively. QRT-PCR and Western blot were used to detect mRNA and protein markers. Finally, the therapeutic effects of GEM, individually or combined with Cur, were verified in nude mice. The results indicated that the combined application of Cur and GEM can improve the sensitivity of A549/GEM to the GEM. Compared with the GEM, GEM plus Cur significantly decreased the migration and invasion of A549/GEM cells. The expression levels of *MMP9*, *Vimentin,* and *N-cadherin* were significantly decreased, while the *E-cadherin* expression was increased. *In vivo* experiments showed a better therapeutic effect of GEM combined with Cur than that of GEM alone, and the combination therapy did not cause more toxicity to animals. In summary, Cur reversed GEM resistance and inhibited the EMT process in A549/GEM cells. GEM, combined with Cur, is safe and more effective in the treatment of non-small cell lung cancer.

1. Introduction

Currently, lung cancer is the leading cause of cancer-related death in humans. The survival rate of the patients was improved after surgical resection and multiple chemotherapy regimens (Hirsch et al. 2017). However, metastasis of lung cancer and resistance to chemotherapy are still the two major factors leading to poor survival in lung cancer patients. These patients have a poor prognosis, with a 5-year survival rate of only 18% (Álvaro and Sonia 2019). To improve the sensitivity of chemotherapy, developing new drug adjuvants that are safe and effective is the focus of researchers.

Curcumin (Cur) is a natural yellow pigment existing in the roots of turmeric, a perennial plant, and is widely used as a colorant for spices and food (Zendehdel et al. 2019). In traditional medicine, Cur has been used to treat a variety of diseases, including rheumatism, liver disease, cough, sinusitis, anorexia, and biliary tract diseases (Bharat and Ajaikumar 2009). In recent years, more and more studies have shown that Cur has an inhibitory effect on the occurrence, development, and metastasis of a variety of cancers. Cur is also a key research object of combination chemotherapy (Cheng et al. 2018; Ghasemi et al. 2019). However, there are relatively few studies on Cur in combination with other chemotherapy drugs for non-small cell lung cancer. Here, this study investigated the use of Cur in combination with gemcitabine, a chemotherapy drug for advanced lung cancer, in the treatment of non-small cell lung cancer, indicating that Cur can improve the sensitivity of lung cancer to chemotherapy as a drug adjuvant.

2. Investigations and results

2.1. Inhibition of proliferation of A549 and A549/GEM cells by GEM

A549 and A549/GEM cells were treated with 0, 30, 60, 90, 120, and 150 μmol/L of GEM for 48 h, respectively. The CCK8 assay found that the proliferation of A549 and A549/GEM cells was inhibited by GEM in a concentration-dependent way (Fig. 1A). The IC_{50} values of A549 and A549/GEM cells were calculated to be 73.84 μmol /L and 143.4 μmol /L, respectively. This indicated that the inhibitory effect of GEM on the proliferation of A549 cells was significantly higher than that of A549/GEM cells. A549/GEM cells were less sensitive to GEM.

2.2. Inhibitory effect of Cur on the proliferation of A549/GEM cells

Cur is a polyphenolic compound derived from the root of turmeric, a traditional Chinese medicine (Fig. 1B). Many studies have shown that Cur can improve the sensitivity of drug-resistant cells to chemotherapy. A549/GEM cells were treated with 0, 3, 6, 9, 12, and 15 μmol /L of Cur for 48 h, respectively. CCK8 results showed that Cur had a significant inhibitory effect on A549/GEM cells in a dose-dependent manner and the IC_{50} is 11.78 μmol /L (Fig. 1C). To avoid the influence of Cur on cell proliferation inhibition, we applied IC_{10} (3 μmol /L) at the later stage, that is, the concentration with an inhibition rate of no more than 10% was applied to conduct the combined application on cells.

2.3. Inhibition of proliferation of A549 and A549/GEM cells by GEM combined with Cur

To study the influence of GEM combined with Cur on the proliferation of A549 and A549/GEM cells, we combined 3 μmol /L of Cur with different concentrations of GEM (0, 25, 50, 75, 100, 125 μmol /L) to treat A549 and A549/GEM cells for 48 h. CCK8 assay showed that GEM combined with Cur increased the proliferation inhibition of A549 and A549/GEM cells in a GEM dose-dependent manner. The IC_{50} values of A549 and A549/GEM cells were 58.2 μmol /L and 98.72 μmol /L, respectively (Fig. 1D). In particular,

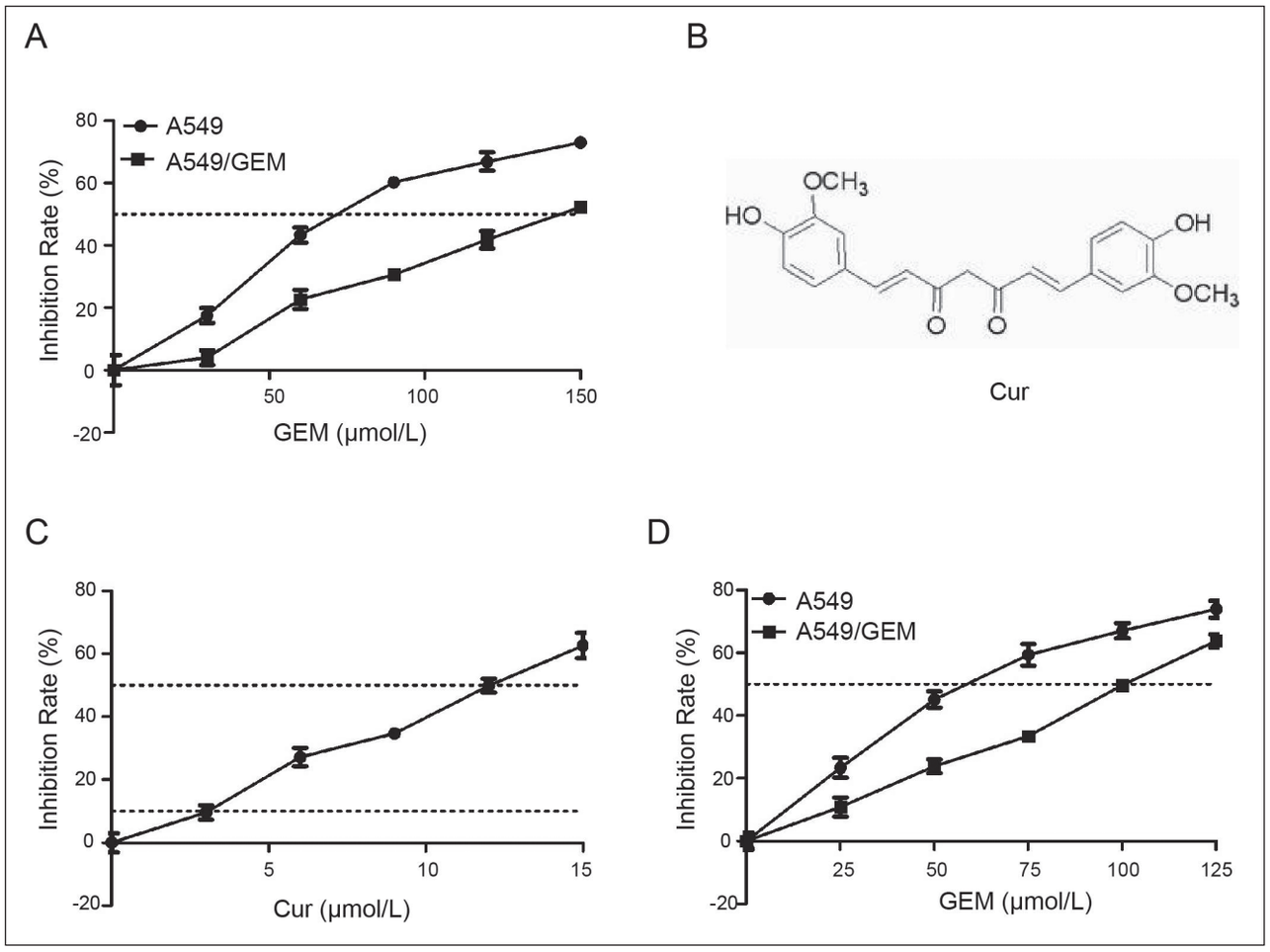

Fig. 1: Effect of GEM combined with Cur on the proliferation of A549 and A549/GEM cells. (A) The cell inhibition rates were determined by the CCK8 assay. A549 and A549/GEM cells were treated with different doses (0, 30, 60, 120 and 150 μmol/L) of GEM for 48 h. (B) The molecular structure of Cur. (C) The inhibition rate of Cur on A549/GEM cells was determined by the CCK8 assay. A549/GEM cells were treated with various doses (0, 3, 6, 9, 12, 15 μmol/L) of Cur for 48 h. (D) The inhibition rate of Cur combined with different doses (0, 25, 50, 75, 100 and 125 μmol/L) of GEM on A549 and A549/GEM cells for 48 h.

the IC_{50} (98.72 μmol /L) of A549/GEM drug-resistant cells was significantly lower than the IC_{50} (143.4 μmol /L) when using GEM alone, indicating that the combined application of Cur and GEM can improve the sensitivity of A549/GEM resistant cells to GEM.

2.4. Effects of Cur combined with GEM on the migration and invasion abilities of A549 and A549/GEM cells

Recurrence and metastasis are the two main causes of death in lung cancer patients. Therefore, we used Cur combined with GEM to treat A549 and A549/GEM cells to study the effect of the combination on the migration and invasion of lung cancer cells. The experiment was divided into four groups: A549 cells treated with GEM (73.84 μmol/L, termed as A549-GEM), A549 cells treated with GEM (58.2 μmol/L) plus Cur (3 μmol/L, termed as A549-GEM+Cur), A549/GEM cells treated with GEM (143.4 μmol/L, termed as A549/GEM-GEM) and A549/GEM cells treated with the combination of GEM (98.72 μmol/L) and Cur (3 μmol/L, termed as A549/GEM-GEM+Cur). The working concentration of GEM in each group was half of the inhibitory concentration. Transwell assay and cell wound healing assay showed that compared with groups treated only with GEM, the cell migration and invasion ability of GEM+Cur groups were significantly reduced (P<0.05) (Figs. 2 and 3), suggesting that the migration and invasion ability of A549 and A549/GEM resistant cells was reduced by the combined application of GEM and Cur.

2.5. Effects of GEM combined with Cur on metastasis-related gene expression of A549 and A549/GEM cells

Total RNA and total protein were collected from four groups of cells as previously described. The expression of metastasis-related genes and proteins was detected by QRT-PCR and Western blotting. The results showed that compared with the GEM groups, the mRNA and protein levels of MMP9, vimentin and N-cadherin in the GEM+Cur groups were downregulated, while the mRNA and protein levels of E-cadherin were upregulated (P<0.05) (Fig. 4).

2.6. In vivo experiments verified the efficacy of GEM combined with Cur in treating non-small cell lung cancer

To compare the efficacy of GEM, individually or combined with Cur, in the treatment of lung cancer, the *in vivo* tumor formation assay was performed. Nude mice injected with A549/GEM resistant cells were treated with either GEM or GEM plus Cur by intraperitoneal injection. The mice were weighed and injected every 3 days. After one month of treatment, mice were sacrificed and dissected. Lung tissues were taken for HE staining. The results showed that in the group treated by GEM plus Cur, the number of lung tumor lesions was significantly less than in the GEM treatment group (P<0.05) (Fig. 5A and B), indicating a better curative effect of GEM combined with Cur to the lung cancer. There was

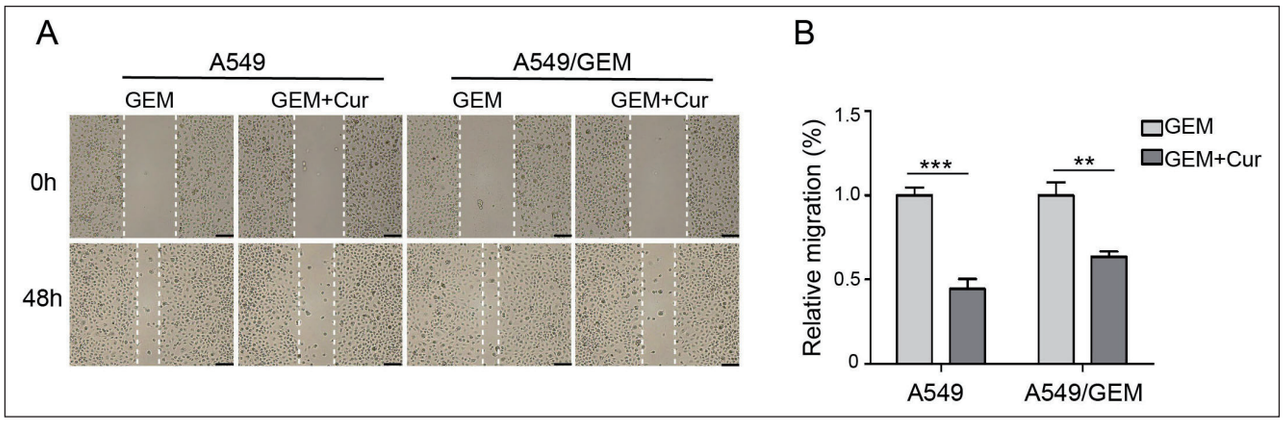

Fig. 2: Effect of GEM combined with Cur on the migration of A549 and A549/GEM cells. (A) and (B) The migration abilities of A549 and A549/GEM cells were analyzed by wound healing assay with IC_{50} of GEM and GEM+Cur for 0 h and 48 h, respectively. Bar=50 μm. $^{**}P<0.01$, $^{***}P<0.001$.

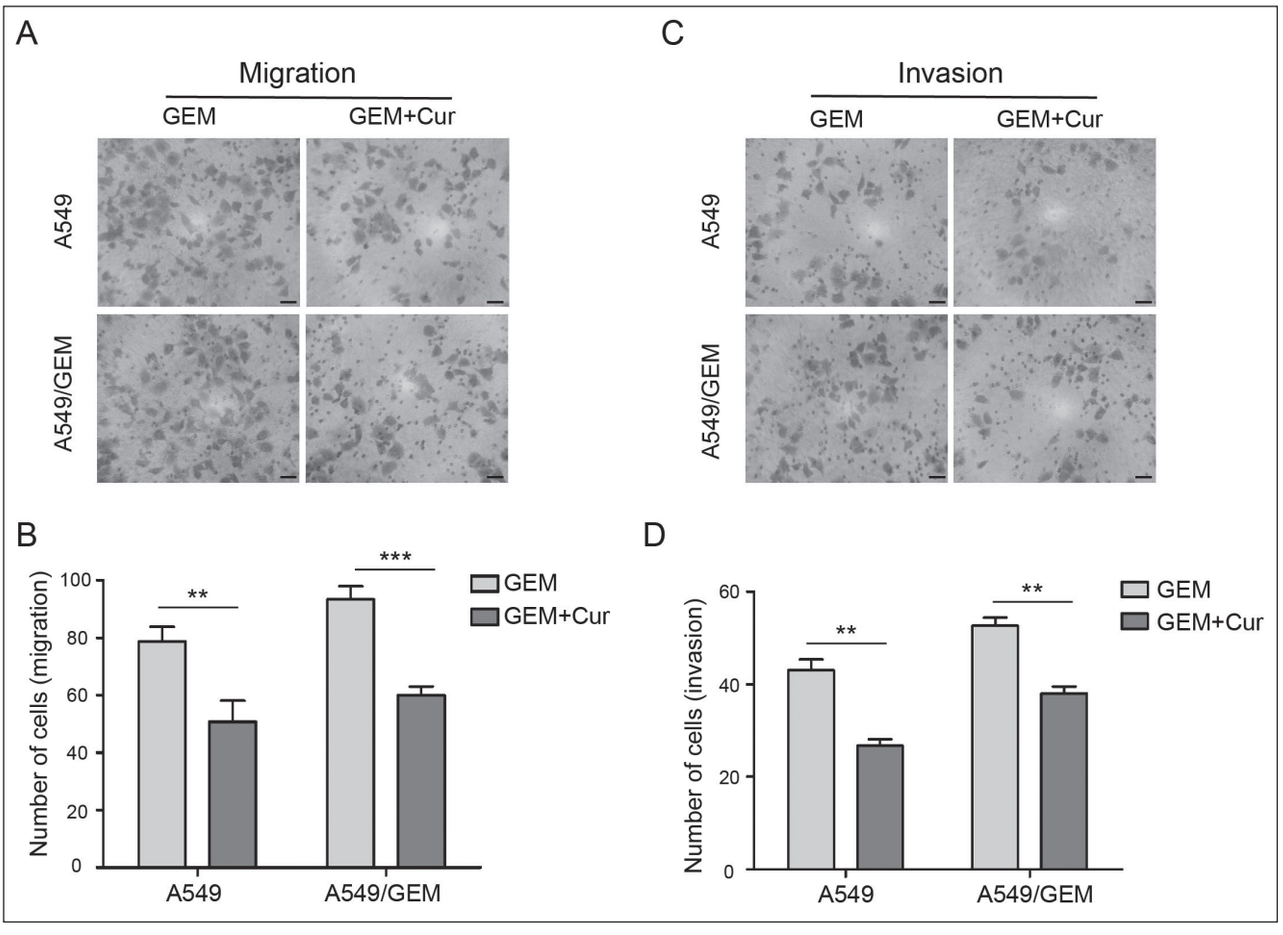

Fig. 3: Effect of GEM combined with Cur on inhibiting migration and invasion of A549 and A549/GEM cells. (A) and (B), The effect of GEM combined with Cur on inhibiting migration of A549 and A549/GEM cells was examined by the transwell assay with the IC_{50} of GEM and GEM+Cur for 48 h. (C) and (D) The effect of GEM combined with Cur on inhibiting invasion of A549 and A549/GEM cells was examined by transwell assay with the IC_{50} of GEM and GEM+Cur for 48 h. The migration and invasion abilities of A549 and A549/GEM cells were quantified by counting the number of stained cells under microscopy. Bar=20 μm. $^{**}P<0.01$, $^{***}P<0.001$.

no significant difference in body weight between the two groups (Fig. 5C), implying that adding Cur to GEM did not cause more toxicity to the mice.

3. Discussion

Lung cancer is a malignant tumor of the respiratory system, which does not lead to obvious symptoms in the early stage and is usually advanced or metastatic when it is diagnosed. In recent years, with the changes in people's lifestyles and the global environment, the incidence and death rate of lung cancer have increased significantly. Lung cancer has become the leading cause of cancer death in China. Lung cancer can be divided into four subgroups: squamous cell carcinoma, adenocarcinoma, large cell lung cancer, and small cell lung cancer, according to the pathological morphology. The first three types are also referred to as non-small cell lung cancer due to their similar biological traits. Chemotherapy is a common means of lung cancer treatment, which can significantly

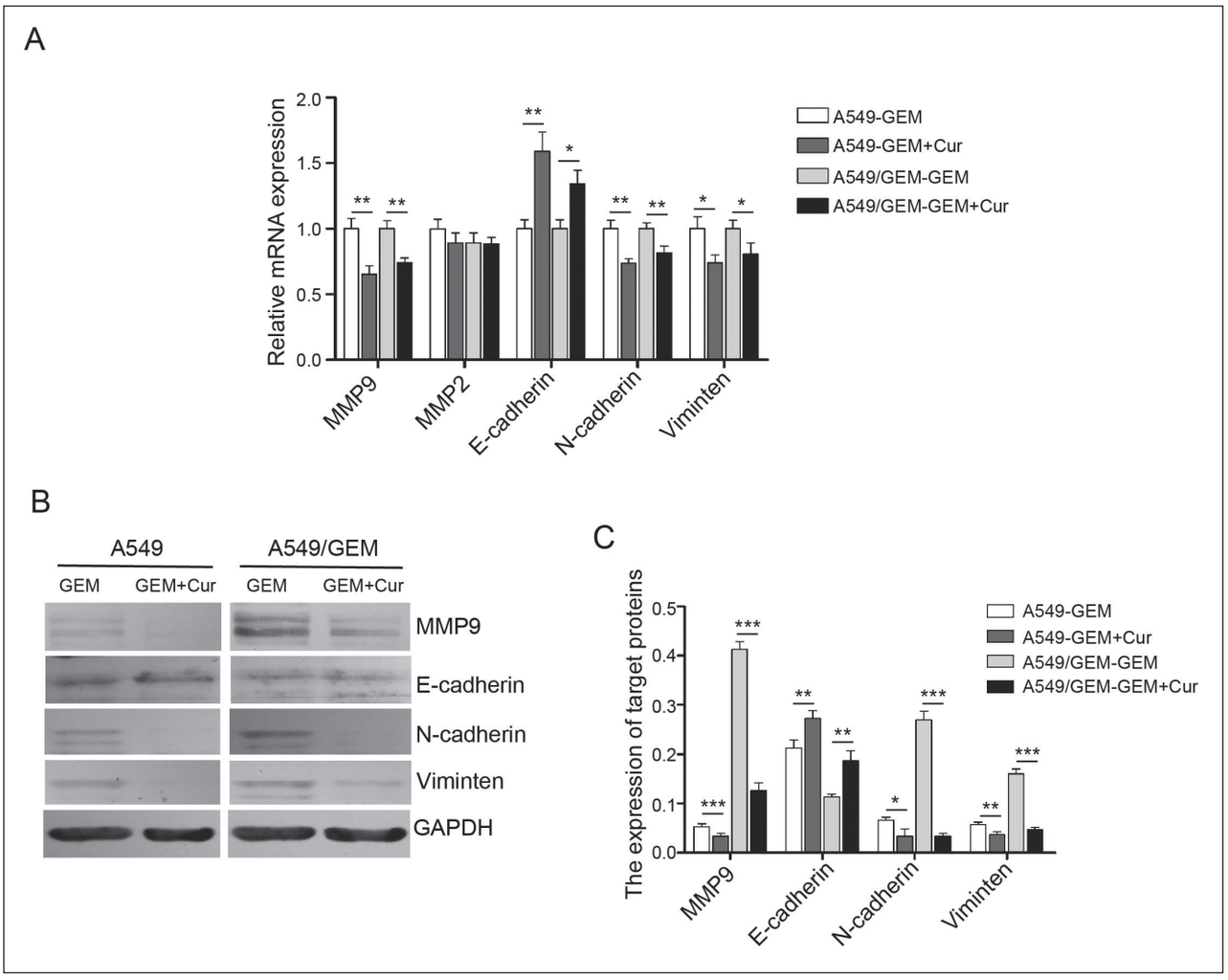

Fig. 4: Effect of GEM combined with Cur on the metastasis-associated genes of A549 and A549/GEM cells. (A) The changes of metastasis-associated genes after GEM or GEM+Cur treatment were detected by QRT-PCR. (B) and (C) MMP9, Vimentin, E-cadherin, and N-cadherin were detected by Western blot after GEM or GEM+Cur treatment. *P<0.05, **P<0.01, ***P<0.001.

improve the survival rate of patients. However, with the application of chemotherapy drugs, the drug resistance of lung cancer gradually emerged. The insensitivity to the drugs promoted the development of tumors, which also leads to the spreading of the tumor cells to new locations through infiltration, lymph nodes, blood, and other ways to form new lesions. Therefore, the discovery of new chemotherapy drugs and adjuvants are very important.

The effect of traditional Chinese medicine on tumors has been widely studied. Cur is the main component of the traditional Chinese medicine Curcuma. Previous studies have shown that Cur has anti-inflammatory (Lee et al. 2019), antioxidant (Chang et al. 2019), antiviral (Li et al. 2019), anti-cancer (Elmansi et al. 2017), and other pharmacological effects. In 2015, Lee et al. showed that Cur could not only downregulate the expression of matrix metalloproteinase 2 and 9 (*MMP2* and *MMP9*) but also inhibit the progression and metastasis of oral cancer by reducing the expression of epithelial-mesenchymal transformation (EMT) -related proteins such as Snail, Twist, E-cadherin and P53 (Lee et al. 2015). Yin et al. (2019) also showed that when treating the colorectal cancer HCT116/OXA cell line, a combination of Cur with oxaliplatin could inhibit the expression of p-p65 and bcl-2, as well as the EMT process through the TGF-β/Smad2/3 signaling pathway, thereby significantly reducing the resistance of colorectal cancer HCT116/OXA cell line to the oxaliplatin. Besides, researchers found that Cur made cells sensitive to the 5-FU, thereby inhibiting tumor growth in xenotransplantation and theoretical models (Toden et al. 2015). Although Cur has been reported to increase the sensitivity of cancer cells to chemotherapeutic agents, the exact molecular mechanism of this action is not yet fully understood. Zhang et al. (2018) discussed the mechanism of sensitization. They found that Cur inhibited the proliferation of drug-resistant cells and enhanced apoptosis. The combination of Cur and irinotecan could upregulate E-cadherin expression and downregulate the expression of vimentin and N-cadherin, suggesting that EMT plays an important role in irinotecan resistance in colon cancer cells. Cur can reverse the resistance of colon cancer cells to irinotecan by reversing the EMT process.

EMT is a process of transition from epithelial cells to mesenchymal cells in a specific environment and is the main cause of tumor invasion and metastasis (Ombrato and Malanchi 2014). In this study, we found that A549/GEM drug-resistant cells were sensitive to the combination of Cur and GEM. This combined application can improve the inhibition rate of A549/GEM cells, upregulate the E-cadherin expression, and downregulate the expression of MMP9, vimentin and N-cadherin by inhibiting the EMT process, which reduces the migration and invasion ability of lung cancer cells. Furthermore, the *in vivo* assay showed that the efficacy of GEM combined with Cur was significantly better than GEM alone, and this combination did not cause higher toxicity to animals.

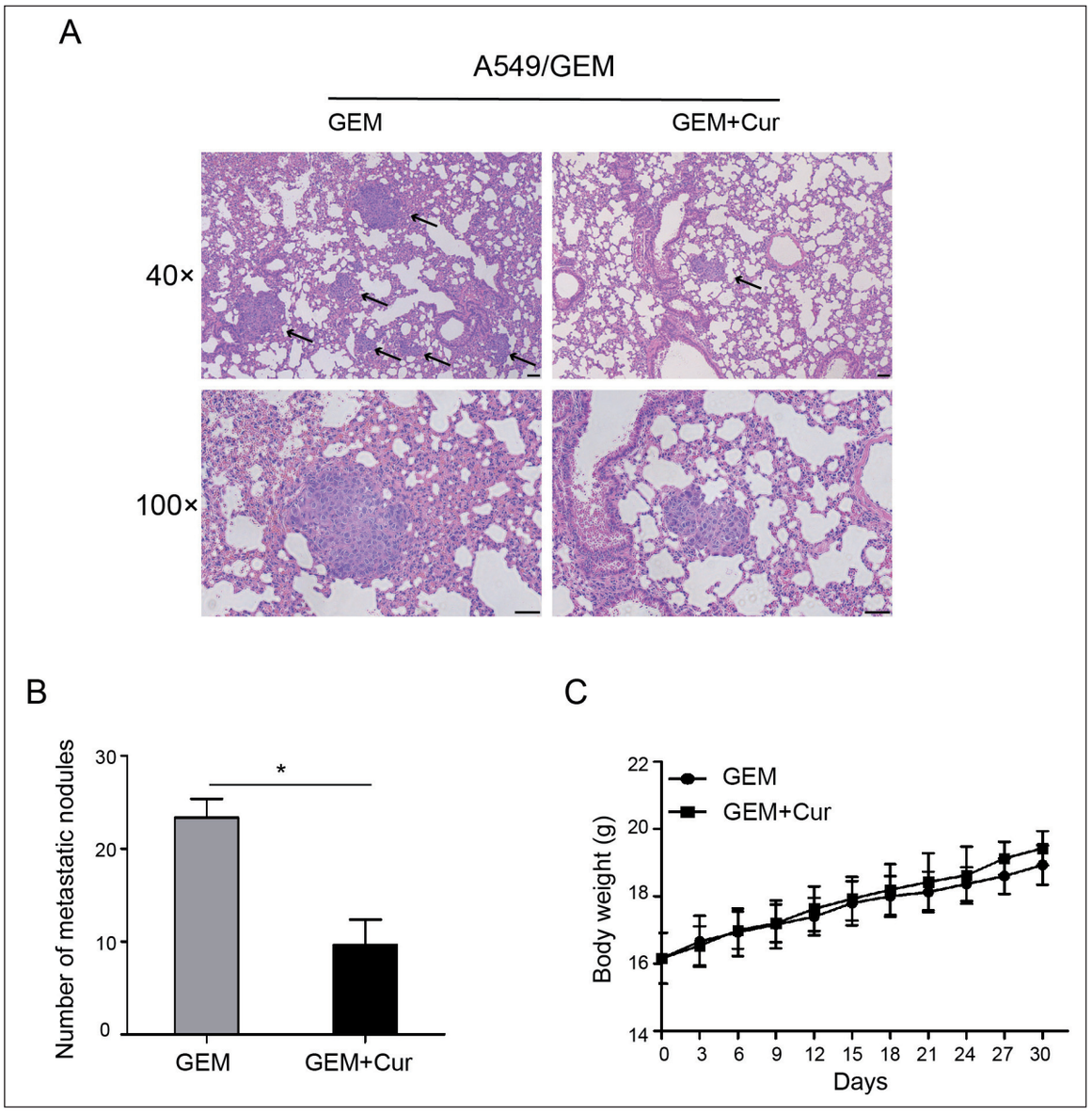

Fig. 5: The efficacy of GEM combined with Cur in treating non-small cell lung cancer *in vivo*. (A) and (B) HE staining of lung tissue sections from the GEM and GEM+Cur treatment groups. The statistical diagram of metastases was shown. (C) The weights of mice were tested every three days. Bar=50 μm. **P*<0.05.

4. Experimental

4.1. Cell culture

Human lung cancer A549 cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. A549 cells were cultured in RPMI-1640 (Gibco) medium with 10% FBS (Gibco) and incubated at 37 °C in a cell incubator of 5% CO_2. GEM concentration was titrated to establish the drug-resistant A549/GEM cell line and 5 μmol /L was applied to maintain the drug resistance. A549/GEM cells were cultured in the same medium as the A549 cells. Cur was dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 medium (final volume fraction of DMSO < 0.01%). Gemcitabine hydrochloride was diluted with RPMI-1640 medium.

4.2. CCK8 assay

A549 and A549/GEM resistant cells were seeded to 96-well plates with 5 ×10^3 cells /well and cultured in a 5% CO_2 cell incubator at 37 °C overnight. Cells were divided into the control group and the experimental group. Different concentration gradients of GEM or Cur were set up in the experimental group. 10 μl of CCK8 was added to each well after 48 h of treatment. Then cells were incubated at 37 °C, 5% CO_2 for 2 h. OD_{450} was detected. Cell proliferation inhibition rate (IR) was calculated as the formula: IR (%) = (1 - OD_{450}(experimental group)/OD_{450}(control group)) × 100%.

4.3. Transwell assay

A549 and A549/GEM drug-resistant cells were inoculated into the upper chamber of the transwell at a concentration of 3×10^4 cells /200 μL RPMI-1640. The 500 μL medium containing 20% FBS was added to the lower chamber of the transwell. Both upper and lower chambers were combined with the diluted drug. The culture plate was placed in a cell incubator for 48 h. The membrane penetrating cells were stained with crystal violet and photographs were taken under a 200-fold microscope.

Transwell chambers coated with matrigel were used to detect the invasion ability of the cells. The cells were inoculated at a concentration of 6× 10^4/200 μL RPMI-1640. The following steps are the same as above described.

4.4. QRT-PCR

A549 cells and A549/GEM resistant cells were exposed to GEM or GEM plus Cur for 48 h, respectively. Trizol method was used to extract the total RNA from cells of each group. The relative quantification of the genes in the Table was performed by reverse transcription and real-time quantitative PCR. GAPDH was used as the internal reference. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative transcription of each gene. Primers used are listed in the Table.

Table 1: Primer sequences of QRT-PCR

Target		Primer sequence
MMP2	*F:*	*5'-AGCGAGTGGATGCCGCCTTTAA-3'*
	R:	*5'-CATTCCAGGCATCTGCGATGAG-3'*
MMP9	*F:*	*5'-GCCACTACTGTGCCTTTGAGTC-3'*
	R:	*5'-CCCTCAGAGAATCGCCAGTACT-3'.*
E-cadherin	*F:*	*5'-GCCTCCTGAAAAGAGAGTGGAAG-3'*
	R:	*5'-TGGCAGTGTCTCTCCAAATCCG-3'*
N-cadherin	*F:*	*5'-CCTCCAGAGTTTACTGCCATGAC-3'*
	R:	*5'- GTAGGATCTCCGCCACTGATTC-3'*
Vimentin	*F:*	*5'- AGGCAAAGCAGGAGTCCACTGA-3'*
	R:	*5'- ATCTGGCGTTCCAGGGACTCAT-3'*
GAPDH	*F:*	*5'-GTCTCCTCTGACTTCAACAGCG-3'*
	R:	*5'-ACCACCCTGTTGCTGTAGCCAA-3'*

F: Forward Sequence; R: Reverse Sequence

4.5. Western blot

Antibody against MMP9 (13667T, Cell Signaling Technology, USA) was purchased from CST company. Antibodies against Vimentin (A11423, ABclonal, China), N-cadherin (A3045, ABclonal, China) and E-cadherin (A11509, ABclonal, China) were purchased from ABclonal company (https://abclonal.com.cn/). Proteins were extracted by the mixture of Radio Immunoprecipitation Assay (RIPA) lysate and protease inhibitors. Bio-Rad wet transfer system was used. The rest of the steps follow the routine method. Finally, they were developed with the ECL luminescent kit (Thermo).

4.6. In vivo experiments

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The trial was conducted in accordance with the Declaration of Helsinki. All animal experiments were performed under a project license granted by the ethics committee, in compliance with the national or institutional guidelines for the care and use of animals. Ten male BALB/c nude mice, 5-week-old, were raised in an SPF animal laboratory. A549/GEM resistant cells were collected and resuspended with PBS and injected into the tail veins of nude mice. For each mouse, 1×10^6 cells in 150 μL of PBS were injected. One week after inoculation, nude mice were randomly divided into two groups and given the drug therapy by intraperitoneal injection.

4.7. Statistical analysis

All experimental data were statistically analyzed by the SPSS 19.0 and Graphpad Prism 5 software. The data were represented by ?x±s. ANOVA was used to analyze the data. Statistical graphs were made by Graphpad Prism 5 software. $P<0.05$ was considered statistically significant.

Conflicts of interest: The authors declared no potential conflicts of interest for the research, authorship, and/or publication of this article.

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