Article

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Carbamazepine Inhibits Lung Cancer Metastasis by Suppressing Chemokine Receptor 4 Expression

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Background: Lung cancer is one of the leading causes of cancer-related deaths worldwide, with treatment failure resulting from metastasis. C-X-C chemokine receptor type 4 (*CXCR4*) plays a crucial role in tumor cell migration and metastasis. Recent studies have suggested that the commonly used antiepileptic drug, carbamazepine (CBZ), may impede tumor metastasis; however, its specific mechanism remains unclear.

Methods: In this study, we evaluated the effects of CBZ on the migration and invasion of lung cancer cells through *in vitro* cell cultures and *in vivo* animal models. The regulatory effect of CBZ on *CXCR4* expression was analyzed using western blot and reverse transcription-quantitative polymerase chain reaction techniques. To further validate whether CBZ's anti-metastatic effect is mediated through *CXCR4*, we used the *CXCR4* agonist NUCC-390 and overexpression of the *CXCR4* gene in lung cancer cell lines.

Results: The results demonstrated that CBZ significantly inhibited the migration and invasion of lung cancer cells (*$p < 0.001$). In animal experiments, CBZ treatment significantly reduced the extent of metastasis in the lungs (*$p < 0.01$). Moreover, CBZ downregulated the expression of *CXCR4* (*$p < 0.001$). When NUCC-390 was used or *CXCR4* was overexpressed, the anticancer effect of CBZ was reversed, indicating the anti-metastatic effect of CBZ is closely associated with its inhibition of *CXCR4* expression.

Conclusion: This study reveals, for the first time, a novel mechanism by which CBZ inhibits lung cancer metastasis through the suppression of *CXCR4* expression. These findings offer a new avenue for the treatment of lung cancer using CBZ as a potential agent against lung cancer metastasis. Further research is warranted to explore the clinical potential of CBZ and to offer more treatment options for lung cancer patients.

Keywords: lung cancer; carbamazepine; *CXCR4*; metastasis; tumor cell migration

Introduction

Lung cancer ranks among the leading causes of cancer-related deaths worldwide [1,2]. Despite recent advances in early diagnostic and therapeutic approaches, the long-term survival rate of lung cancer patients remains unfavorable, primarily due to the high metastatic potential of the disease [3]. Tumor metastasis is a complex process involving various interactions between tumor cells and the host environment, with the expression of C-X-C chemokine receptor type 4 (*CXCR4*) closely associated with the invasiveness and metastatic potential of tumor cells [4,5]. Previous studies have reported that *CXCR4* is a key regulatory factor in the migration and metastasis of various tumor cells [6,7]. However, there is an urgent need to effectively inhibit *CXCR4*-mediated lung cancer metastasis [8,9].

In recent years, numerous studies have explored the role of *CXCR4* in the development of lung cancer [5,10]. For instance, Liu *et al*. (2022) [11] highlighted that the binding of *CXCR4* to its ligand C-X-C motif chemokine 12 (*CXCL12*) activates multiple signaling pathways, promoting the survival of lung cancer cells. Zhang *et al*. (2015) [12] reported that *CXCR4* overexpression is negatively correlated with patient prognosis. Furthermore, some preclinical studies have explored small molecule inhibitors targeting *CXCR4* to potentially impede lung cancer progression [13–15]. However, the clinical application of these small molecule inhibitors is limited by their pharmacokinetics and toxicity profiles. Therefore, uncovering new potential applications for existing drugs capable of inhibiting *CXCR4* function is a promising avenue for investigation.

While previous research has revealed the role of *CXCR4* in lung cancer metastasis, there is relatively limited research on how known drugs can inhibit *CXCR4*. Carbamazepine (CBZ), as a widely used antiepileptic drug, has been found to inhibit histone H4 deacetylation in the liver tumor cell line HepG2 through a high-throughput gene expression screening [16]. Since this discovery, different investigators have explored the potential therapeutic effects of CBZ in tumorigenesis, progression, invasion,

and metastasis. In colon cancer, for instance, CBZ was found to have concentration-dependent cytotoxicity on HT-29 cells, inducing apoptosis through caspase 3 expression [17]. In breast cancer, CBZ could promote Her-2 degradation and inhibit tumor proliferation by synergizing with trastuzumab or geldanamycin [18]. Another study has also shown that activation of *CXCR4* promotes tumor proliferation and cancer metastasis through the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) and mammalian target of rapamycin (mTOR) signaling pathways in various cancer types [19]. Currently, there is a lack of research on the impact of CBZ on *CXCR4* and its mechanism of action in lung cancer metastasis. Therefore, exploring whether CBZ can inhibit lung cancer metastasis by affecting *CXCR4* is of great significance for expanding lung cancer treatment strategies.

This study aims to investigate the impact of CBZ on *CXCR4* and its role in inhibiting lung cancer metastasis. We evaluated the effect of CBZ on *CXCR4* expression in lung cancer cells through *in vitro* cell experiments and studied its inhibitory effect on lung cancer metastasis using *in vitro* and *in vivo* models. Additionally, we explored the potential mechanism by which CBZ inhibits *CXCR4*-mediated signaling pathways. The significance of this research lies in the potential to provide a new strategy for lung cancer treatment by utilizing the existing drug CBZ to inhibit lung cancer metastasis, which may not only benefit clinical treatment but also provide a new direction for drug repurposing in lung cancer research.

Materials and Methods

Cell Culture

H1299 (CRL-5803, ATCC, Manassas, VA, USA) and A549 cells (CCL-185, ATCC, Manassas, VA, USA) were cultured in F-12 medium (11765054, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (26170043, ThermoFisher Scientific, Waltham, MA, USA) and 100 U/mL penicillin-streptomycin solution (15140122, Thermo Fisher Scientific, Waltham, MA, USA) in a Forma Steri-Cycle 160i CR CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 3 days. Cell culture medium was collected, and the polymerase chain reaction mycoplasma test kit (13100-01, HuaAn Biotech, Hangzhou, China) was used to verify that H1299 and A549 cells were free of mycoplasma. H1299 and A549 cell lines were authenticated by Short tandem repeat (STR) profiling. For drug intervention experiments, CBZ was added at 10 µM or 20 µM. CBZ was purchased from Sigma-Aldrich (298-46-4, St. Louis, MO, USA). The selective small molecule *CXCR4* receptor agonist NUCC-390 (10 µM) was added to the culture media of the CBZ treatment group to reverse the inhibition of CBZ on *CXCR4*. NUCC-390 was purchased from MedChemExpress (HY-111793, Monmouth Junction, NJ, USA).

Cell Transfection

pCEP4-myc-*CXCR4*-W161Y (Addgene plasmid #98951; RRID: Addgene_98951) was a gift from Dr. Erik Procko from the University of Illinois at Urbana-Champaign, Champaign, IL, USA, and cells were passaged when they reached approximately 80% confluence. Cells were digested with 0.25% trypsin and seeded in 6-well plates. Transfection was performed when the cell density reached approximately 90% on the second day, according to the instructions for Lipofectamine 2000 (Life Technologies, Rockville, MD, USA). A549 cells were transfected with either the *CXCR4* overexpression plasmid (CXCR4 group) or the negative control to *CXCR4* overexpression plasmid (NC group). The culture medium containing normal serum was replaced after 6 hours of transfection, and cells were cultured for 48 or 72 hours before collection for further analysis.

Transwell Assay

Transwell assay was performed using chambers with an 8-µm pore size membrane (SCWP04700, Millipore, Bedford, MA, USA). A549 and H1299 cells, as well as their respective control groups after different treatments, were suspended in labstock culture medium and seeded into the upper chamber at a density of 3.5×10^4 cells per well (Invasion assay requires pre-coating of the upper chamber's membrane with Matrigel) (356234, Life Technologies, Rockville, MD, USA). The lower chamber was filled with 0.6 mL of F-12K medium containing 20% fetal bovine serum. The chambers were then incubated in an incubator for 24 hours. Cells on the upper surface of the membrane were scraped with a cotton swab, and those on the lower surface of the membrane were fixed with 4% paraformaldehyde for 15 minutes. Subsequently, cells were stained with 2.5% crystal violet for 30 minutes. Cell counting was performed by randomly selecting and photographing 3 areas under an inverted microscope (CKX53, Olympus, Tokyo, Japan).

Clonogenic Formation Assay

A549 and NCI-H1299 cells were seeded in 6-well plates (150 cells per well) and cultured in RPMI-1640 medium (11875093, Thermo Fisher Scientific, Waltham, MA, USA). Control, 10 µM CBZ, and 20 µM CBZ groups were set up, with 3 replicates per group. Cells were fixed with 4% paraformaldehyde after 10 days, stained with 0.1% crystal violet for 10 minutes, and observed and counted (>50 cells as one colony) under an optical microscope (CKX53, Olympus, Tokyo, Japan).

Lung Metastasis Animal Model

Thirty-six SPF nude mice (6–8 weeks) were purchased from the Experimental Animal Center (protocol no. SCXK 2021-7846, Jiujiang, China). The mice were housed in a

room with a temperature of 18–26 °C, a relative humidity of 40–70%, a noise level below 85 dB, an ammonia concentration <7.6 mg/m^3, and a ventilation rate of 8–12 times/hour. A549 cells were collected by enzyme digestion and resuspended to a cell density of 2×10^7 cells/mL. In brief, 100 µL of the A549 cell suspension was injected into the tail veins of mice. For *in vivo* experiments on tumor cell metastasis, there were 6 nude mice in each group. Initially, one group received CBZ treatment while another served as the control group to evaluate the inhibitory effects of CBZ on lung tumor metastasis. To further explore the impact of *CXCR4* overexpression on inhibition effects of CBZ in lung tumor metastasis, four experimental groups were designed as follows: control group: nude mice only received the injection of A549 cell; *CXCR4* group: nude mice received the injection of A549 cell and NUCC-390 treatment; CBZ group: nude mice received the injection of A549 cell and CBZ treatment; CBZ+*CXCR4* group: nude mice received the injection of A549 cell, CBZ treatment and NUCC-390 treatment. Every two days, mice in corresponding treatment groups received injections of CBZ (10 mg/kg) and NUCC-390 (3.2 mg/kg) via the tail vein to keep effective serum drug concentration [20]. All thirty-six SPF nude mice were conventionally bred for 21 days. The mice were euthanized using carbon dioxide. The lungs were dissected and fixed in 4% paraformaldehyde (Solarbio, Beijing, China), embedded in paraffin, sectioned, stained with hematoxylin-eosin, and 5 random fields from each sample were selected to count the number of micrometastases under a light microscope using a 10× objective lens (CKX53, Olympus, Tokyo, Japan).

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells using an RNA Rapid Extraction Kit (AM9775, Thermo Fisher Scientific, Wilmington, NC, USA). Subsequently, cDNA was synthesized by reverse transcription, and quantitative real-time polymerase chain reaction (RT-qPCR) was performed using a 20-µL reaction system with SYBR Green PCR Master Mix (4309155, Thermo Fisher Scientific, Wilmington, USA). The reaction system included 10 µL of 2× Ultra SYBR Mix, 0.4 µL of upstream primer, 0.4 µL of downstream primer, 0.8 µL of cDNA, and 8.4 µL of double-distilled water. The reaction conditions were as follows: pre-denaturation at 95 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 1 minute, and extension at 40 °C for 5 minutes. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the internal reference. The primer sequences were designed as follows: *CXCR4* (forward primer: 5′-CAGCAGGTAGCAAAGTGACG-3′ and reverse primer: 5′-GTAGATGGTGGGCAGGAAGA-3′), *GAPDH* (forward primer: 5′-GAAGGTCGGAGTCAACGGATTT-3′ and reverse primer: 5′-ATGGGTGGAATCATATTGGAAC-3′), mouse *CXCR4* (forward primer: 5′-TCAACCTCTACAGCAGCGTTCTCTT-3′ and reverse primer 5′-TGTTGGTGGCGTGGACAAT-3′), mouse *GAPDH* (forward primer: 5′-TTCCGTGTTCCTACCCCCAATG-3′ and reverse primer 5′-TGCCTGCTTCACCACCTTCTT-3′). The relative mRNA expression level was calculated using the $2^{-\Delta\Delta CT}$ method.

Western Blot Analysis

Cells in the logarithmic growth phase were seeded in 6-well plates (1×10^6 cells per well). When the cell confluence reached 90%, cells were washed twice with phosphate-buffered saline. RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) was added, and cells were lysed on ice for 15 minutes. The lysate was centrifuged at 12,000 rpm for 15 minutes at 4 °C, and the supernatant was collected for BCA protein quantification. The proteins were denatured and inactivated at 100 °C for 8 minutes. Subsequently, the proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V for 120 minutes until the molecular weight marker proteins were sufficiently separated. The proteins were then transferred to a polyvinylidene fluoride membrane (IPVH00010, Millipore, Bedford, MA, USA) at 300 mA for 90 minutes. The membrane was blocked with 5% non-fat milk for 2 hours. Rabbit anti-human CXCR4 antibody (diluted 1:2000, ab227767, Abcam, Cambridge, MA, USA) and rabbit anti-human GAPDH antibody (diluted 1:3000, ab181602, Abcam, Cambridge, MA, USA) were added and incubated overnight at 4 °C. The membrane was washed with Tris-buffered saline three times for 6 minutes each, followed by incubation with the secondary antibody, goat anti-rabbit immunoglobulin G (IgG) (32160702, Sigma-Aldrich, St. Louis, MO, USA, diluted 1:4000), at room temperature for 2 hours. The alkaline phosphatase-conjugated goat anti-rabbit secondary antibody was obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (ZB2301, Beijing, China). The membrane was washed with tris-buffered saline and tween 20 (TBST) three times, and the immunoreactive target proteins were visualized with enhanced chemiluminescence reagents in a gel imaging system. ImageJ v1.48 (LOCI, University of Wisconsin, Madison, WI, USA) and Image-ProPlus v7.0 software packages (GraphPad Software, La Jolla, CA, USA) were used to quantify and calculate the intensity of the target proteins. Mean grayscale values were recorded as the raw values of each band. The background signal from each band was subtracted to correct for the non-specific staining. To correct the variations in protein loading or transfer efficiency, the intensity of CXCR4 was normalized to that of GAPDH.

5-Ethynyl-2′-deoxyuridine (EdU) Assay

A549 cells transfected with the *CXCR4* overexpression plasmid or the empty plasmid were seeded in 24-well plates (2×10^4 cells per well) and incubated for 24 hours. Cells were stained using the EdU Cell Proliferation Assay Kit (ST067, Beyotime, Shanghai, China). Three random cell fields were selected and photographed under a fluorescence microscope (BX63, Olympus, Tokyo, Japan) to calculate the average EdU-positive nuclear ratio.

Statistical Analysis

Data were statistically analyzed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Data were presented as mean ± standard deviation. Pairwise comparisons were analyzed using paired *t*-tests. Multiple group comparisons were performed using one-way analysis of variance, and comparisons between two groups were carried out using Fisher's Least Significant Difference Test (LSD-*t* tests). A *p*-value of less than 0.05 was considered statistically significant.

Results

Carbamazepine Inhibits the Migration and Invasion of Lung Cancer Cells

In A549 cells, both 10 µM and 20 µM CBZ significantly inhibited cell migration and invasion ($p < 0.001$), suggesting that CBZ can reduce the metastatic ability of lung cancer cells (Fig. 1A). Similar trends were observed in NCI-H1299 cells, indicating CBZ treatment reduces cell migration, which demonstrates a certain universality of its inhibitory effect (Fig. 1B). For A549 and NCI-H1299 cells, the reduction in the number of colonies after CBZ treatment indicates that CBZ can inhibit the proliferation ability of lung cancer cells (Fig. 1C,D) (($p < 0.001$)).

Carbamazepine Inhibits the Metastasis of Tumor Cells in the Lungs

Hematoxylin-eosin (H&E) staining, a widely employed tissue staining technique, enables the identification of micrometastases in the lungs. In this experiment, we used a nude mouse model, characterized by immunodeficiency, in which A549 cells were transplanted to simulate the growth and metastasis of lung cancer. Representative H&E-stained images show lung tissues from both the treatment group (10 mg/kg CBZ) and the control group, and a comparative analysis of the staining images reveals the presence of micrometastases in the lungs, along with their morphological characteristics. Quantitative assessment of metastatic nodules in the lungs revealed a significant reduction in the number of nodules in the mice treated with CBZ compared to the control group ($p < 0.001$), indicating CBZ effectively inhibits lung metastasis (Fig. 2A,B).

Carbamazepine Affects CXC Chemokine Receptor 4 Expression

RT-qPCR results revealed that CBZ treatment resulted in the downregulation of *CXCR4* gene expression in A549 cells.

Quantitative results showed that the relative mRNA and protein expression level of *CXCR4* in the control group was higher, and the relative expression level of *CXCR4* in the CBZ 10 µM and 20 µM groups was increased with the increase of drug concentration ($p < 0.001$, Fig. 3A,B). RT-qPCR and western blot results showed the downregulation of *CXCR4* gene expression in CBZ-treated NCI-H1299 cells. The control group exhibited the highest *CXCR4* expression, while the CBZ-treated group showed decreased *CXCR4* expression with increasing concentrations, consistent with the results observed in A549 cells ($p < 0.001$, Fig. 3C,D).

CXCR4 Receptor Agonist NUCC-390 Reverses the Anticancer Effect of Carbamazepine

RT-qPCR and western blot analyses were conducted to detect *CXCR4* gene expression. The results showed similar and higher levels of *CXCR4* gene expression in the NC group and the CBZ+NUCC-390 group compared to the CBZ-only treatment group, while the NUCC-390 treatment group had the highest expression of *CXCR4* expression ($p < 0.001$). This indicates that CBZ can inhibit *CXCR4* gene expression, while NUCC-390 can reverse the inhibitory effect of CBZ (Fig. 4A,B). In the NUCC-390 treatment group, the Transwell migration assay showed extensive migration. Similar and higher migration capabilities of lung cancer cells were observed in the NC group and the CBZ+NUCC-390 group compared to the CBZ-only treatment group ($p < 0.001$). This suggests that CBZ significantly inhibits the migration capability of lung cancer cells, while NUCC-390 can restore this migration capability (Fig. 4C). EdU assay results showed the highest lung cancer cell proliferation in the NUCC-390 treatment group ($p < 0.001$), and similar or higher proliferation capabilities in the NC group and the CBZ+NUCC-390 group compared to the CBZ-only treatment group. This indicates that CBZ inhibits the proliferation of lung cancer cells, while NUCC-390 reverses this inhibitory effect (Fig. 4D). The results suggest that CBZ can reduce the migration and proliferation of lung cancer cells by inhibiting the expression of *CXCR4* and that NUCC-390, as a *CXCR4* agonist, can reverse the inhibitory effect of CBZ by promoting *CXCR4* expression.

Overexpression of CXCR4 Reverses the Anticancer Effect of Carbamazepine

The results indicated that the *CXCR4* group exhibited the highest expression level, indicating overexpression of the *CXCR4* gene. The CBZ-treated group showed the lowest expression of *CXCR4* ($p < 0.001$), demonstrating the ability of CBZ to inhibit the transcription of the *CXCR4*

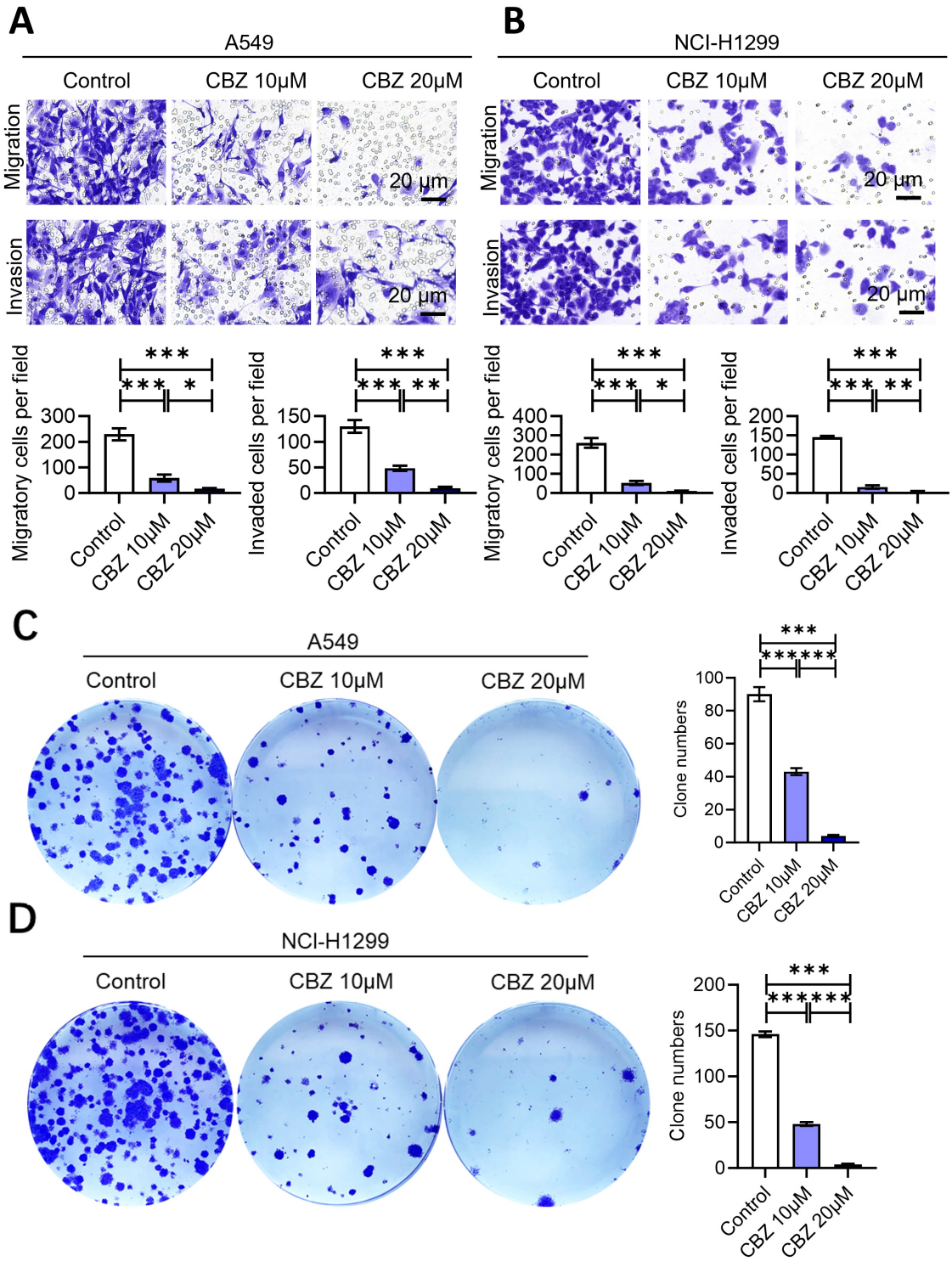

Fig. 1. Inhibition of migration and invasion of lung cancer cells by carbamazepine. (A) Evaluation of cell migration and invasion in A549 cells after treatment with different doses of carbamazepine (CBZ). (B) Evaluation of cell migration and invasion in NCI-H1299 cells after treatment with different doses of CBZ. (C,D) Evaluation of cell colony formation in A549 cells (C) and NCI-H1299 cells (D) after treatment with different doses of CBZ. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n = 3.

gene. The expression levels of *CXCR4* in the NC group and the CBZ+*CXCR4* group were similar, indicating the overexpression of *CXCR4* can reverse the inhibitory effect of CBZ (Fig. 5A,B). Transwell assay results showed that the *CXCR4* group exhibited the strongest migration capability ($p < 0.001$), which resulted from the promotion of

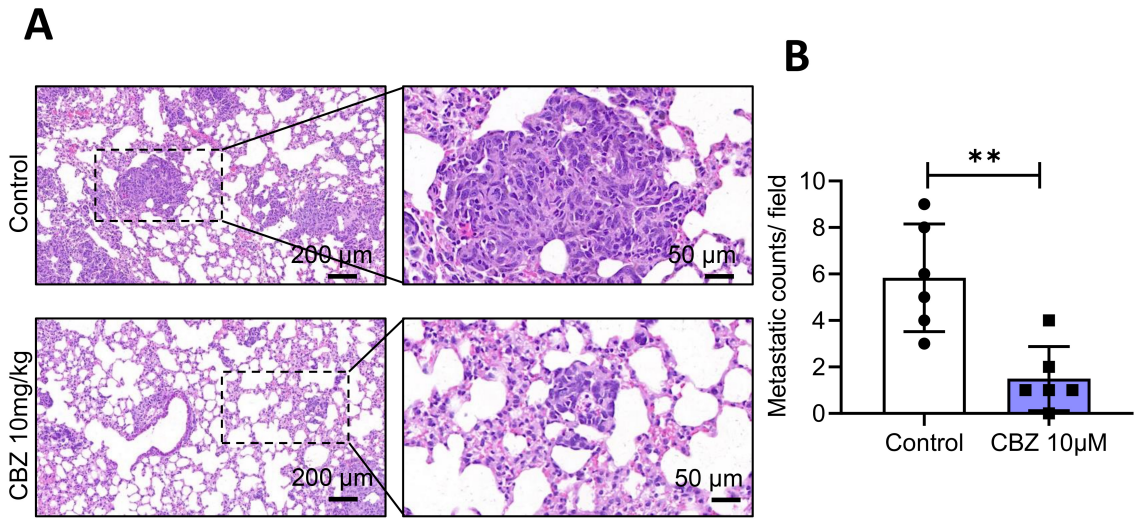

Fig. 2. Inhibition of tumor cell metastasis to the lungs by carbamazepine. (A) Hematoxylin-eosin staining of representative images of lung micrometastases (lung cancer cell A549) in a nude mouse model after carbamazepine (CBZ) treatment. (B) Quantitative analysis of lung metastatic nodules. $^{**}p < 0.01$. n = 6.

cell migration by the overexpression of *CXCR4*. CBZ significantly inhibited the migration capability of lung cancer cells ($p < 0.001$), while the migration capabilities of the NC group and the CBZ+*CXCR4* group were similar, indicating the overexpression of *CXCR4* can reverse the inhibitory effect of CBZ (Fig. 5C). EdU, a marker of cell proliferation, is used to assess the proliferative activity of cells by measuring their ability to synthesize DNA. The results showed that the *CXCR4* group exhibited the strongest proliferation capability ($p < 0.001$), as the overexpression of *CXCR4* promoted cell proliferation. The cell proliferation capability was lowest in the CBZ group, indicating the inhibition of lung cancer cell proliferation by CBZ. The similar proliferation capabilities of the NC group and the CBZ+*CXCR4* group suggested that the overexpression of *CXCR4* can reverse the inhibitory effect of CBZ (Fig. 5D). The results indicate that CBZ inhibits the migration and proliferation of lung cancer cells by suppressing the expression of *CXCR4*. However, when *CXCR4* is overexpressed, the inhibitory effect of CBZ is reversed, demonstrating the crucial role of *CXCR4* in the migration and proliferation of lung cancer cells.

Carbamazepine Inhibits Tumor Cell Metastasis to the Lungs, and Overexpression of CXCR4 Reverses the Inhibitory Effect of Carbamazepine

The H&E staining of lung micrometastases revealed the characteristic morphological features of lung cancer cell metastasis in nude mouse models subjected to different treatments. Microscopic observation of lung tissues stained with H&E provided visual evidence of the presence and extent of tumor cell metastasis in the lungs. To evaluate the protein expression level of CXCR4 during treatment, western blotting of *CXCR4* expression was performed using mouse lung metastatic nodule tissues. The overexpression of *CXCR4* enhanced the migration capability of lung cancer cells, resulting in an increased number of lung metastatic nodules and a higher protein expression of CXCR4 compared to the NC group ($p < 0.001$). This indicated the promoting role of *CXCR4* in lung cancer metastasis. After treatment with CBZ, the number of lung metastatic nodules and the expression of CXCR4 protein decreased ($p < 0.001$), suggesting that CBZ can inhibit the metastasis of lung cancer cells, possibly through the suppression of *CXCR4* expression or function. In the context of CBZ treatment, the overexpression of *CXCR4* by NUCC-390 resulted in an increased number of lung metastatic nodules ($p < 0.001$) and *CXCR4* expression similar to that of the NC group, indicating the overexpression of *CXCR4* reversed the inhibitory effect of CBZ, further confirming the critical role of *CXCR4* in the inhibition of lung cancer metastasis by CBZ (Fig. 6A–C). These results demonstrate that CBZ effectively inhibits *CXCR4* expression, thereby reducing the metastasis of lung cancer cells. When *CXCR4* is overexpressed by the agonist NUCC-390, the inhibitory effect of CBZ is reversed, indicating the crucial role of *CXCR4* in lung cancer metastasis, and suggesting CBZ may exert its anti-tumor metastatic effect by inhibiting *CXCR4*.

Discussion

This study aimed to investigate the impact of CBZ on lung cancer metastasis and its potential mechanism of action. Our experimental results demonstrated that CBZ significantly inhibited the migration and invasion of lung cancer cells and suppressed the metastasis of tumor cells

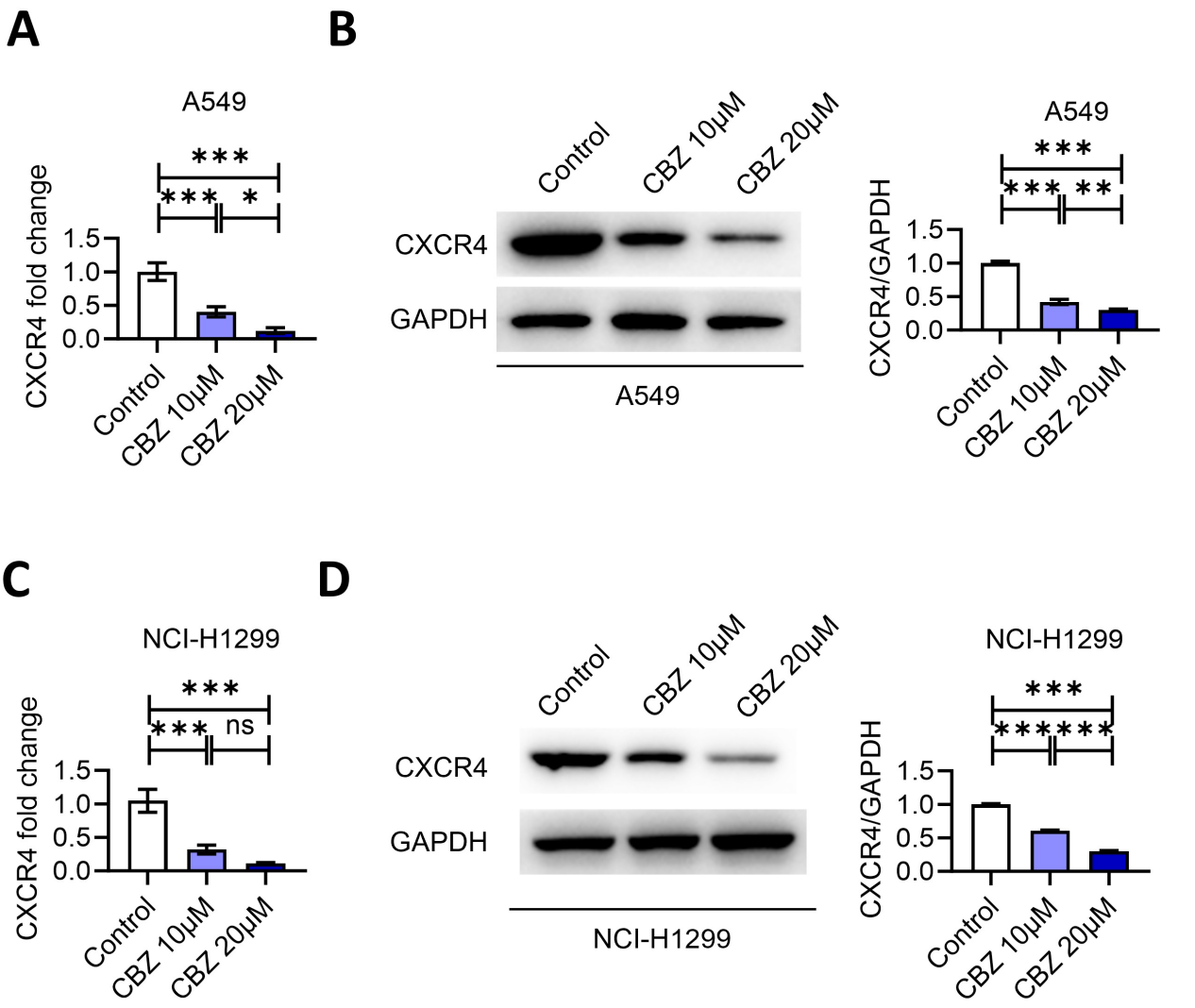

Fig. 3. Effect of carbamazepine on C-X-C chemokine receptor type 4 (*CXCR4*) expression. (A) RT-qPCR results show the inhibition of *CXCR4* gene expression in A549 cells by carbamazepine (CBZ). (B) Immunoblot and quantitative analysis demonstrating downregulation of *CXCR4* expression in A549 cells by CBZ. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. (C) RT-qPCR results show the inhibition of *CXCR4* gene expression in NCI-H1299 cells by CBZ. (D) Immunoblot and quantitative analysis demonstrating downregulation of *CXCR4* expression in NCI-H1299 cells by CBZ. GAPDH was used as a control. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, ns, not significant; RT-qPCR, quantitative real-time polymerase chain reaction. n = 3.

to the lungs. Additionally, we found that CBZ affected the expression of *CXCR4*. Furthermore, both NUCC-390 treatment and *CXCR4* overexpression reversed the antitumor effect of CBZ. These findings support our hypothesis that CBZ inhibits lung cancer metastasis by suppressing *CXCR4*.

CXCR4, a G protein-coupled receptor closely associated with the migration and metastasis of tumor cells, and its ligand CXCL12/stromal cell-derived factor 1 (SDF-1), have been demonstrated to be key factors promoting the migration and metastasis of tumor cells in various cancers [21–24]. In lung cancer, another study collected 244 patients who were clinically diagnosed with T1N0M0 NSCLC. Their results showed the increased expression of *CXCR4* in tumor tissues compared to healthy tissues. Patients who carried positive *CXCR4* expression had a lower five-year disease-free survival and a lower five-year overall survival [25]. Therefore, inhibiting *CXCR4* signaling may provide a new strategy for treating lung cancer.

CBZ, a commonly used antiepileptic drug, primarily exerts its antiepileptic effects by stabilizing the inactivated state of voltage-gated sodium channels [26,27]. However, the non-antiepileptic effects of CBZ remain incompletely understood. Our study revealed the potential role of CBZ in inhibiting lung cancer metastasis, suggesting its potential as a therapeutic agent for lung cancer. The results indicated

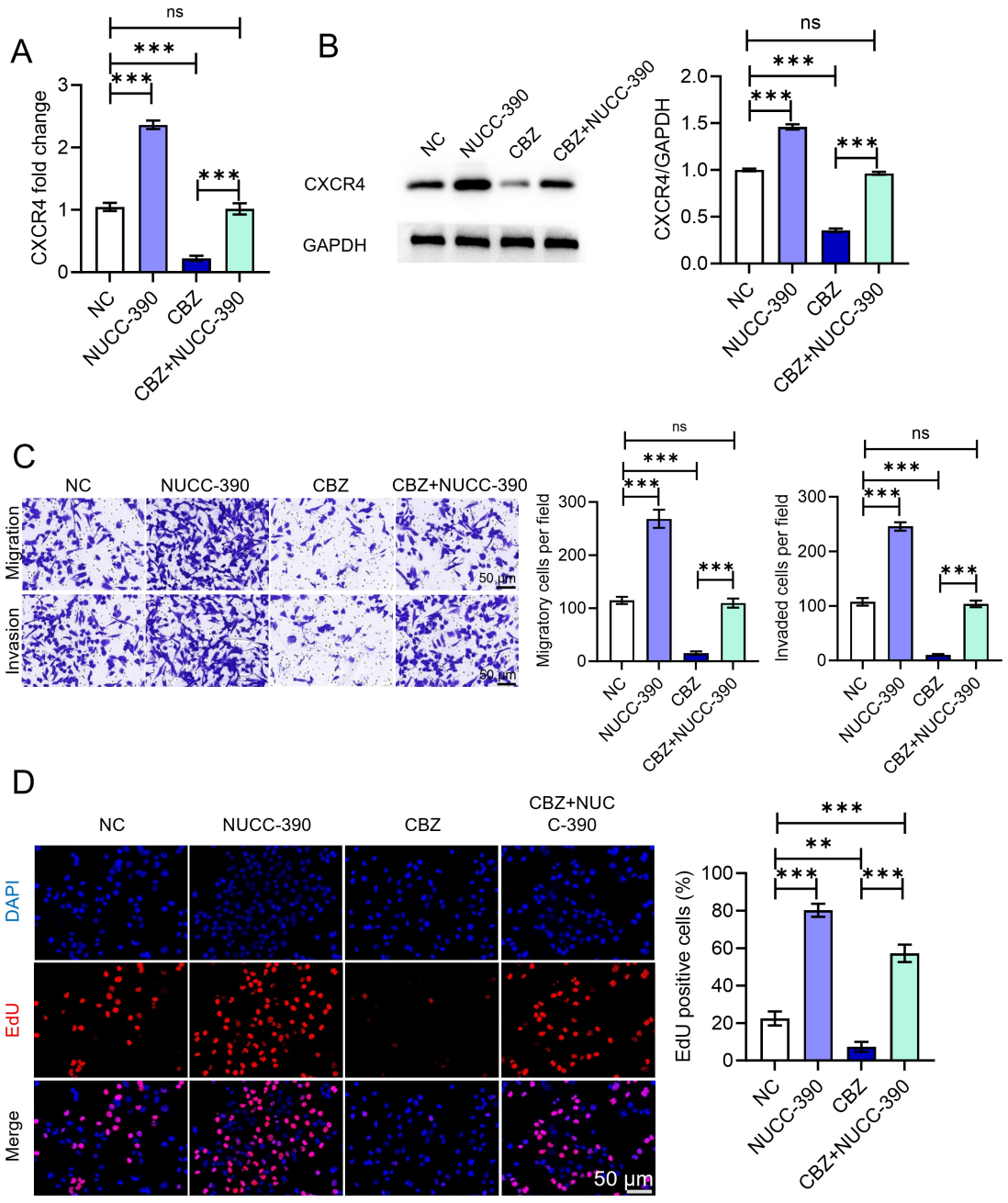

Fig. 4. *CXCR4* receptor agonist NUCC-390 reverses the anticancer effect of carbamazepine. (A) RT-qPCR results showing *CXCR4* gene expression. (B) Immunoblot and quantitative analysis of *CXCR4* expression. GAPDH was used as a control. (C) Transwell assay evaluating the migration of lung cancer cells in different treatment groups *in vitro*. (D) 5-Ethynyl-2′-deoxyuridine (EdU) assay assessing the proliferation capacity of lung cancer cells in different treatment groups. ns, not significant; $^{**}p < 0.01$, $^{***}p < 0.001$. n = 3.

that CBZ significantly inhibits *CXCR4* expression, which may be a key mechanism through which CBZ inhibits the migration and metastasis of lung cancer cells. The central role of *CXCR4* in the action of CBZ was provided by the reversal of the inhibitory effect through NUCC-390 treatment and *CXCR4* overexpression. These results suggest that CBZ may exert its anticancer effect by downregulating the expression of *CXCR4* or inhibiting its signaling pathways.

The key findings of this study are as follows: (1) CBZ significantly inhibits the migration and invasion of lung cancer cells; (2) CBZ downregulates the expression of *CXCR4* in lung cancer cells; and (3) CBZ inhibits the metastatic potential of lung cancer cells by affecting *CXCR4*-related signaling pathways. Previous studies have highlighted the crucial role of *CXCR4* in the migration and invasion of lung cancer cells [28–30]. Our findings align with these studies, further substantiating the potential of

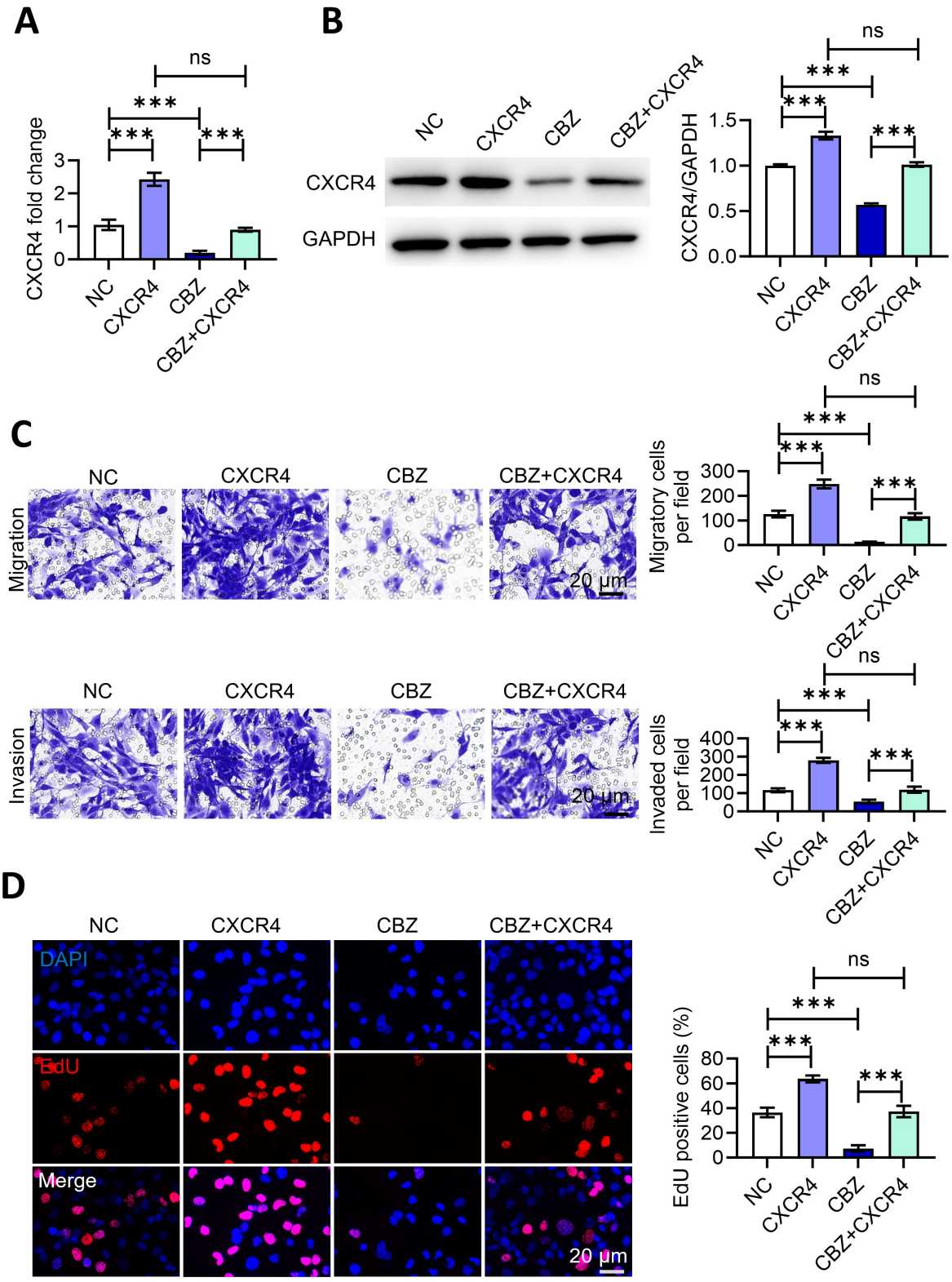

Fig. 5. Overexpression of *CXCR4* reverses the anticancer effect of Carbamazepine. (A) RT-qPCR detects *CXCR4* gene expression. (B) Immunoblot and quantitative analysis of *CXCR4* expression. GAPDH was used as a control. (C) Transwell assay evaluating the migration of lung cancer cells in different treatment groups *in vitro*. (D) EdU assay assessing the proliferation capacity of lung cancer cells in different treatment groups. ns, not significant, ***$p < 0.001$. n = 3.

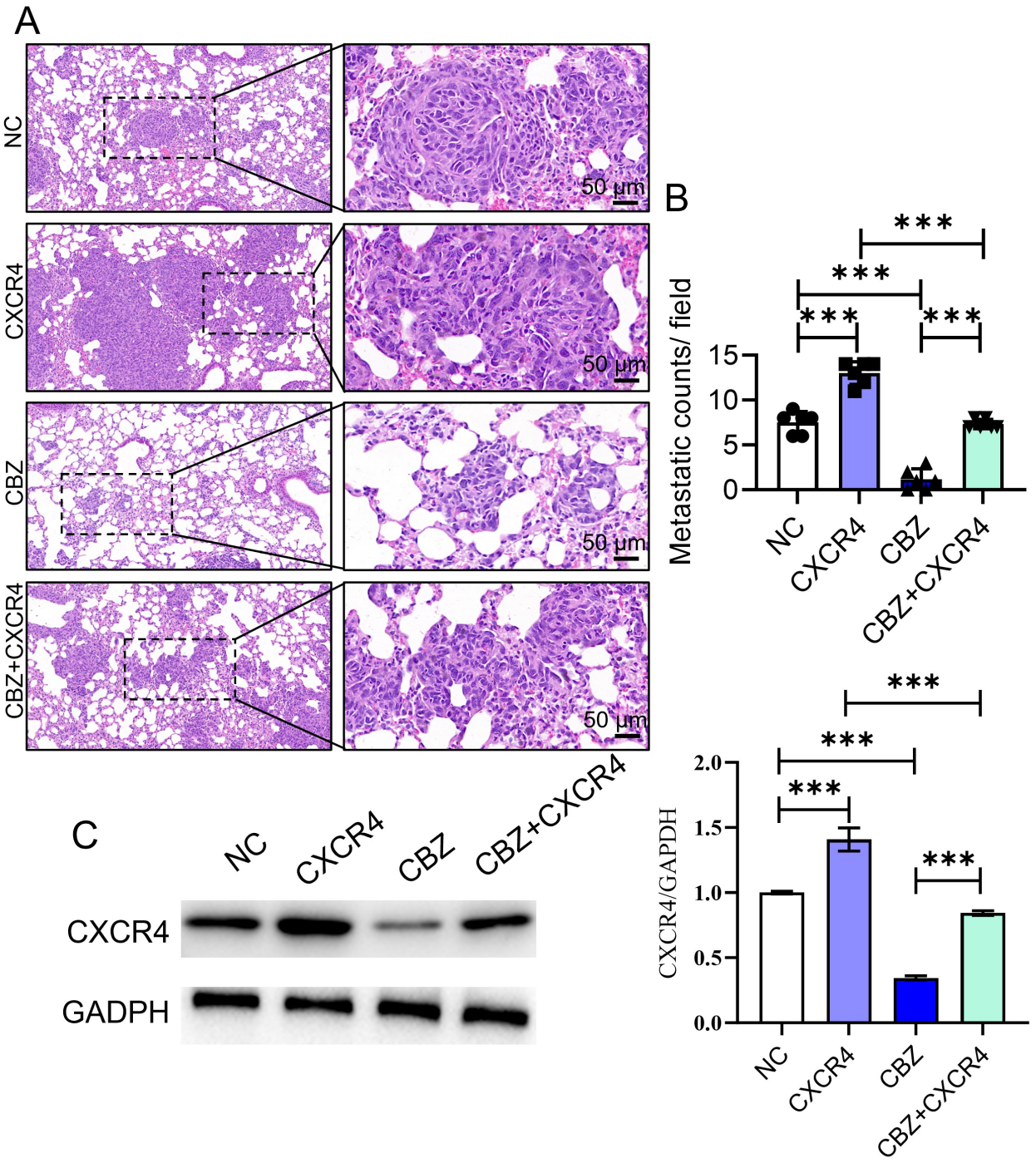

Fig. 6. Inhibition of tumor cell metastasis to the lungs by carbamazepine, while overexpression of *CXCR4* reverses the inhibitory effect of carbamazepine. (A) Hematoxylin-eosin (H&E) staining of representative images of lung metastasis (lung cancer cell A549) in nude mouse models from different treatment groups *in vivo*. (B) Quantitative analysis of lung metastatic nodules. (C) Immunoblot and quantitative analysis of *CXCR4* expression from mouse lung metastatic nodule tissues. GAPDH was used as a control. ***$p < 0.001$. n = 6.

CXCR4 as a promising therapeutic target for lung cancer. Regarding the second key finding, our data corroborate the perspectives of other studies which suggest that drugs can impede tumor progression by affecting the expression of *CXCR4*. Lastly, our third key finding complements previous research, which has demonstrated that interfering with the *CXCR4* signaling pathway can reduce the likelihood of tumor metastasis.

This study has some limitations. First, while our study confirmed the impact of CBZ on *CXCR4* in cell models, further research is warranted to investigate its *in vivo* effects and underlying mechanisms. Second, the impact of CBZ on *CXCR4* may not represent the exclusive antitumor mechanism; therefore, further studies are necessary to explore alternative pathways. Additionally, future studies should evaluate the dose-responsive relationship of CBZ, its long-term safety profile, and its impact on normal lung function.

Conclusion

In conclusion, our study provides preliminary experimental evidence for using CBZ in the treatment of lung cancer. Subsequent research should prioritize validating the antitumor effects of CBZ *in vivo*, elucidating its specific mechanism of action, and evaluating the practicality of its clinical application.

Availability of Data and Materials

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Author Contributions

CZ: Conception, Design, Materials, Data Collection, Analysis, Literature Review, and Drafting. XM: Design, Materials, Analysis, Literature Review, and Writing. ZL: Supervision, Data Collection, Data Analysis, Literature Review, and Writing. DL: Supervision, Materials, Data Collection, Data Analysis, and Writing. CC: Materials, Data Collection, Data Analysis, Writing, and Critical Review. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

This study was reviewed and approved by the Animal Ethics Committee of Yichun University (No. YCU-YXY-2023-187).

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Conflict of Interest

The authors declare no conflict of interest.

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