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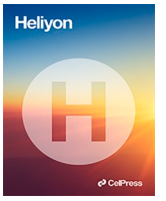

Research article

# Allicin affects immunoreactivity of osteosarcoma cells through lncRNA CBR3-AS1

Wenpeng Xie [a,1], Fengjun Ma [b,1], Luming Dou [c], Wenjie Chang [d], Daotong Yuan [d], Zhimeng Zhang [d], Yongkui Zhang [a,*]

[a] Department of Orthopedics, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, Shandong, 250014, China
[b] Department of Science and Technology,Shandong University of Traditional Chinese Medicine, Jinan, Shandong, 250355, China
[c] Department of Bone Traumatology,Yantai Penglai Traditional Chinese Medicine Hospital, Yantai, Shandong, 265600, China
[d] First Clinical Medical College,Shandong University of Traditional Chinese Medicine, Jinan, Shandong, 250355, China

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ABSTRACT

*Objective:* To analyze the effect of allicin on the immunoreactivity of osteosarcoma (OS) cells and further explore whether its mechanism is related to the long non-coding Ribonucleic Acid (lncRNA) CBR3-AS1/miR-145-5p/GRP78 axis, so as to provide clinical evidence.
*Methods:* The human OS cell line Saos-2 was treated with allicin at 25, 50, and 100 μmol/L, respectively, to observe changes in cell biological behaviors. Subsequently, CBR3-AS1 abnormal expression vectors were constructed and transfected into Saos-2 to discuss their influence on OS. Furthermore, the regulatory relationship between allicin and the CBR3-AS1/miR-145-5p/GRP78 axis was validated by rescue experiments. Finally, a nude mice tumorigenesis experiment was carried out to analyze the effects of allicin and CBR3-AS1/miR-145-5p/GRP78 axis on the growth of living tumors. Alterations in T-lymphocyte subsets were also detected to assess the effect of allicin on OS immunoreactivity.
*Results:* With the increase of allicin concentration, Saos-2 activity decreased and apoptosis increased ($P < 0.05$). In addition, the expression of CBR3-AS1 and GRP78 decreased after allicin intervention, while miR-145-5p increased ($P < 0.05$). Silencing CBR3-AS1 led to reduced Saos-2 activity, enhanced apoptosis, and activated mitophagy and endoplasmic reticulum stress ($P < 0.05$). In the rescue experiment, the effect of CBR3-AS1 on OS cells was reversed by silencing miR-145-5p, while the impact of miR-145-5p was reversed by GRP78. Finally, the tumorigenesis experiment in nude mice confirmed the regulatory effects of allicin and CBR3-AS1/miR-145-5p/GRP78 on tumor growth in vivo. Meanwhile, it was seen that allicin activated $CD4^{+}CD8^{+}$ in OS mice, confirming that allicin has the effect of activating OS immunoreactivity.
*Conclusions:* Allicin activates OS immunoreactivity and induces apoptosis through the CBR3-AS1/miR-145-5p/GRP78 molecular axis.

## 1. Introduction

Osteosarcoma (OS), a malignancy in which the tumor cells directly form bone-like tissue or bone, with high malignancy and rapid

* Corresponding author.
*E-mail address:* 71000356@sdutcm.edu.cn (Y. Zhang).
[1] These authors have contributed equally to this work and share first authorship.

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development, can be divided into central, surface, and intracortical OS according to its occurrence site [1]. According to statistics, the global incidence of OS was about 4–5 per million in 2021, and it is also the most common orthopedic tumor disease at present [2]. Meanwhile, OS is highly metastatic, with approximately 80–90 % of OS patients eventually dying because of systemic metastases [3]. Investigation shows that the 5-year survival of OS patients is only 5–20 %, and its mortality rate is among the best among all malignancies [4]. Therefore, the research on the treatment of OS is of great significance to ensure the life safety of patients [5].

Given the increasing prevalence of chemotherapy resistance in tumor cells, it is advocated in clinical practice to reverse cell resistance through intervention with natural compounds [6]. Among them, allicin (diallyl thiosulfinate) is an organic sulfur compound extracted from the bulbs (garlic heads) of Allium in the Alliaceae family, which is also present in onion and other Alliaceae plants [7]. In recent years, allicin has been found to inhibit the proliferation of gastric, prostate and lung cancers as well as other malignant tumor cells in vitro, and it has been confirmed that its mechanism is mainly to induce apoptosis by increasing the immunological activity of cells [8]. Recently, a study has also confirmed that the use of allicin may help reverse the chemotherapy resistance of OS cells [9], but the specific clinical efficacy and mechanism of action need further research.

The long non-coding Ribonucleic Acid (lncRNA) CBR3-AS1 (hereafter referred to as CBR3-AS1) has received extensive attention in the pathogenic studies of OS [10,11]. In a study on the antibacterial activity of allicin, researchers suggested that CBR3-AS1 may be one of the most critical pathways of action [12]. Therefore, we speculate that the effect of allicin on OS may also be related to the CBR3-AS1 signal transduction pathway and in this way further regulate the immunoreactivity of OS cells. To further explore more comprehensive molecular pathways of action, we conducted a preliminary analysis of its potential downstream target genes through online data, where miR-145-5p and GRP78 caught our attention. In the study of Xie L et al., they showed that CBR3-AS1 promoted the pathological progression of colorectal cancer by sponging miR-145-5p [13]. In OS, the role of miR-145-5p has been repeatedly verified [14,15]. GRP78, on the other hand, is an extremely important target protein in OS, and Gram enhances apoptosis in OS cells by inhibiting CHOP ubiquitination in OS [16,17].

In this study, we will explore the influence of allicin on OS and further analyze whether its mechanism is related to CBR3-AS1, laying a credible foundation for the future clinical application of allicin and more effectively ensuring the prognosis and health of OS patients.

## 2. Materials and methods

### 2.1. Cell data

Saos-2, a human OS cell line purchased from BeNa Culture Collection, was cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (containing 100 U/mL penicillin and 100 mg/L streptomycin) at 37 °C and 5 % $CO_2$, with the culture solution renewed every 2 days.

### 2.2. Allicin intervention

After dissolving an appropriate amount of allicin (Fufeng Sinuote Biotech Co., Ltd.) in DMSO, DMEM solution was used to prepare allicin solutions with concentrations of 25, 50, and 100 μmol/L and to achieve DMSO with a final volume fraction of 0.1 %, which was then added to Saos-2 culture medium, labeled as groups A (25 μmol/L), B (50 μmol/L), and C (100 μmol/L) groups, respectively. In addition, the conventionally cultured Saos-2 was set as a normal control group.

### 2.3. Cell viability assay

Cells in each group were seeded into 6-well plates (500 cells per well) for continuous culture, with the culture medium changed every 2 days. Forty-eight hours later, they were immobilized with 4 % paraformaldehyde and stained with 1 % crystal violet, both for 30 min. After drying, the cells were photographed and the number of clones formed was counted.

### 2.4. Detection of cell invasion ability

Cells are seeded at $1 \times 10^5$/well onto a fibronectin-coated polycarbonate membrane insert of the Transwell apparatus (pre-coated with 24 mg/mL of Matrigel, Sigma-Aldrich, Shanghai, China). After 48 h, the number of Giemsa-stained cells adhering to the inferior chamber was calculated by randomly selecting 5 of the predetermined areas under a microscope.

### 2.5. Determination of cell migration ability

Cells were inoculated into a 6-well plate at $5 \times 10^5$/mL. The old culture medium was discarded when the cells reached 80 %–90 % confluence, and then scratches were made perpendicular to the bottom of the plate with a clean micropipette tip. The samples were collected 48 h later for observation and photographing under an inverted microscope. Cell migration rate = (0 h scratch width - scratch width after incubation)/0 h scratch width × 100 %.

### 2.6. Apoptosis rate and cycle assays

The floating and adherent cells were collected, immobilized with 70 % ice ethanol, washed with PBS, and centrifuged. They were then added with RNase A (1 mg/mL) and prodium iodide (PI; 50 μg/mL, MEC, USA) for 30 min of reaction and staining. Finally, cells were collected for flow cytometry and analysis of cell cycle distribution by Modfit software.

### 2.7. Examination of mitochondrial damage

Cells were inoculated into 6-well plates at $5 \times 10^5$/mL for 24 h of culture, and JC-1 dye solution (Thermo Fisher Scientific, USA) was added at 250 μL per well. After 20 min, the cells were observed and photographed under the fluorescence microscope.

### 2.8. Quantification of CBR3-AS1 and miR-145-5p expression

The total RNA isolated by TRIzol (Thermo Fisher Scientific, USA) was reverse transcribed into complementary DNA (cDNA) using a reverse transcription kit (Thermo Fisher Scientific, USA). QRT-PCR analysis was carried out with SYBR Green PCR Premix HS Taq (Thermo Fisher Scientific, USA). CBR3-AS1 and miR-145-5p expression relative to β-actin and U6 were analyzed with $2^{-\Delta\Delta Ct}$ (Table 1), the primer sequences were designed by Nanjing Kingsley Biotechnology Co.

### 2.9. Protein expression detection

Total protein was extracted using a cell lysis buffer. Equal amounts of protein were denatured, transferred to a polyvinylidene fluoride (PVDF, Abcam, USA) via 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Abcam, USA), and incubated with GRP78, LC3-II, Beclin1, CHOP, PERK, and eIF2α primary antibodies (1:1,000, Abcam, USA). A second antibody (1:2,000, Abcam, USA) was added the next day for incubation. Protein gray values were analyzed by Quantity One Software after development with an Enhanced Chemiluminescence (ECL) Kit (Abcam, USA).

### 2.10. Influence of CBR3-AS1/miR-145-5p/GRP78 on OS

Logarithmic-growth-phase Saos-2 cells were inoculated in a 6-well plate after adjusting the cell density to $4 \times 10^5$ cells/mL. Shanghai Hanbio Biotechnology Co. Ltd. was commissioned to design and construct CBR3-AS1 overexpressing lentiviral vector (CBR3-AS1-ov) and its empty vector (CBR3-AS1-ov-nc), as well as CBR3-AS1 expression-silencing lentiviral vector (CBR3-AS1-si) and its empty vector (CBR3-AS1-si-nc). The above vectors were then transfected into Saos-2, respectively, following the Lipofectamine™ 3000 reagent manuals (Thermo Fisher Scientific, USA). Subsequently, cloning, invasion, migration, and apoptosis assays were performed according to the above methods to analyze the impact of CBR3-AS1 on OS.

### 2.11. Rescue experiments

Shanghai Hanbio Biotechnology Co. Ltd. was entrusted to design miR-145-5p expression-silencing sequence (miR-145-5p-si) and GRP78 expression-silencing lentivirus vector (GRP78-si). The cells were intervened as follows [1]: Allicin (the concentration of 100 μmol/L with the most significant intervention effect was selected) intervention + co-transfection of CBR3-AS1-si + miR-145-5p-si + GRP78-si [2]. co-transfection of CBR3-AS1-si + miR-145-5p-si + GRP78-si [3]. co-transfection of CBR3-AS1-si + miR-145-5p-si [4]. co-transfection of miR-145-5p-si + GRP78-si [5]. transfection with miR-145-5p-si alone [6]. transfection with GRP78-si alone [7]. control group with normal culture. Subsequently, cloning, invasion, migration, apoptosis, and other tests were performed according to the above methods for rescue experiments.

### 2.12. Animal data

We purchased 45 SPF healthy BALB/c nude mice, aged 4 weeks and weighing 18–20g, from Cyagen Biosciences (Suzhou) Inc. (Animal Use License Number SCXK (Su) 2022-0016). The animals were kept under controlled temperature (22–25 °C), humidity (40–60 %), and a 12:12h light-dark cycle, and were allowed to eat and drink freely. Animal experiments were approved by the Institutional Animal Care and Use Committee and Animal Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine (approval number 2021-05).

**Table 1**
Sequence of primers.

| | Forward primer (3′-5′) | Reverse primer (3′-5′) |
|---|---|---|
| CBR3-AS1 | AGTAGTTGCTTGTCCTAT | AAGTCAGTAAGTCCTAAGT |
| β-actin | CTGGGACGACATGGAGAAAA | AAGGAAGGCTGGAAGAGTGC |
| miR-145-5p | TGTCCAGTTTTCCCAGGAATC | CTCAACTGGTGTCGTGGAGTC |
| U6 | CCT GCTTCGGCAGCACAT | AACGCTTC ACGAATTTGCGT |

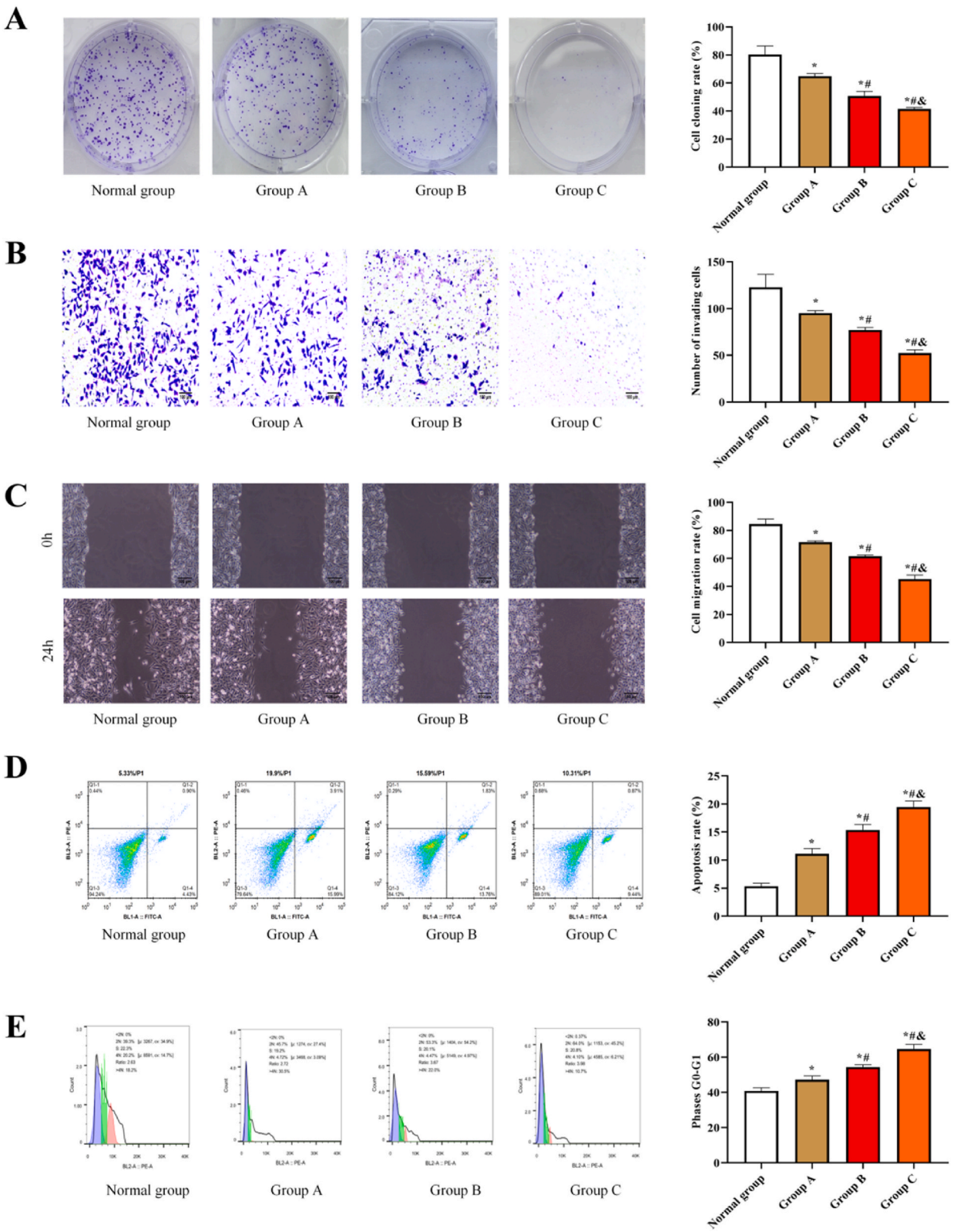


**Fig. 1.** Influence of allicin on biological behavior of OS cells. (A) Effect of allicin on clonogenic capacity of OS cells. (B) Effect of allicin on OS cell invasion ability. (C) Effect of allicin on migration ability of OS cells. (D) Effect of allicin on OS cell apoptosis. (E) Effect of allicin on OS cell cycle. *, #, & indicate $P < 0.05$ with normal group, group A, group B.

### 2.13. Tumorigenesis experiment in nude mice

35 mice were selected and randomly divided into 7 groups (n = 5), which were injected subcutaneously with 500 μL of the 7 groups of cells in the rescue experiments into the right armpit of mice using a micro syringe after resuspension and cell density adjusting ($1 \times 10^7$ cells/mL). On the 14th day of modeling, mice were anesthetized by intraperitoneal injection of 8 μL/g lidocaine and then killed by neck dissection, and the whole tumor tissue was removed. The long and short diameters of the tumor were measured with a vernier caliper, and the volume and weight was calculated.

### 2.14. T lymphocyte subpopulation assay

The control group cells were inoculated into the remaining 10 mice, which were randomly divided into 2 groups after 14 d. One group was intervened by tail vein injection of allicin, and the other group was injected with an equal amount of saline. Subsequently, all mice were executed, and 1 mL of blood from the abdominal aorta of each group was placed in an appropriate amount of erythrocyte lysate, incubated in an ice-water bath for 30 min and then centrifuged to obtain peripheral blood T lymphocytes. The peripheral blood T-lymphocytes were obtained by centrifugation. 500 μLT of lymphocytes were mixed with FITC-*anti*-mCD8 and PE-*anti*-mCD4 antibodies (Abcam, USA), and incubated for 35 min, avoiding light, and then the precipitate was washed by centrifugation (1000 r/min), and then resuspended and mixed with PBS.

### 2.15. Statistical methods

All tests in this study were repeated 3 times, and the results were expressed as (¯χ±s). Multi-group comparisons were made using repeated measures analysis of variance and =]Bonferroni's test. Independent samples *t*-test was used for comparison between groups. Statistical analysis was performed using SPSS 25.0 software (IBM, USA), differences with p-values <0.05 were considered significant.

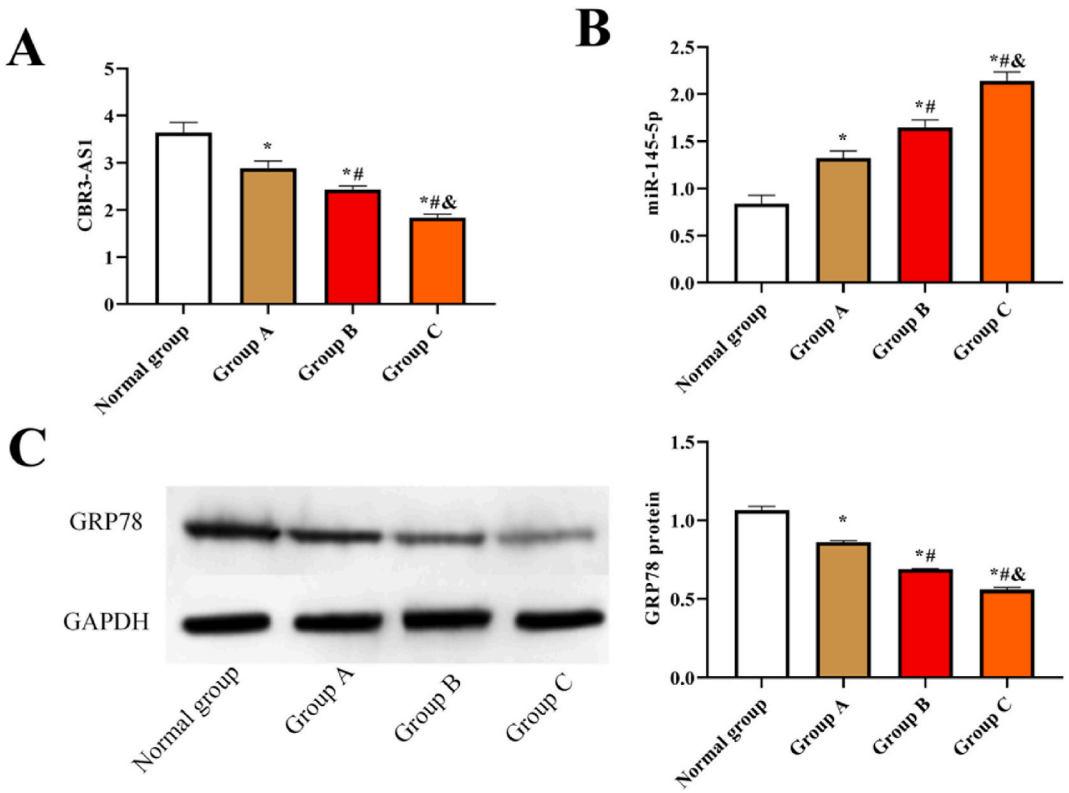


**Fig. 2.** Influence of allicin on CBR3-AS1/miR-145-5p/GRP78 expression in OS cells. (A) Effect of allicin on CBR3-AS1 expression in OS cells (by PCR). (B) Effect of allicin on miR-145-5p expression in OS cells (by PCR). (C) Effect of allicin on GRP78 expression in OS cells (by Western blot, the complete protein imprinting map is in Supplementary Fig. S1). *, #, & indicate P < 0.05 with normal group, group A, group B.

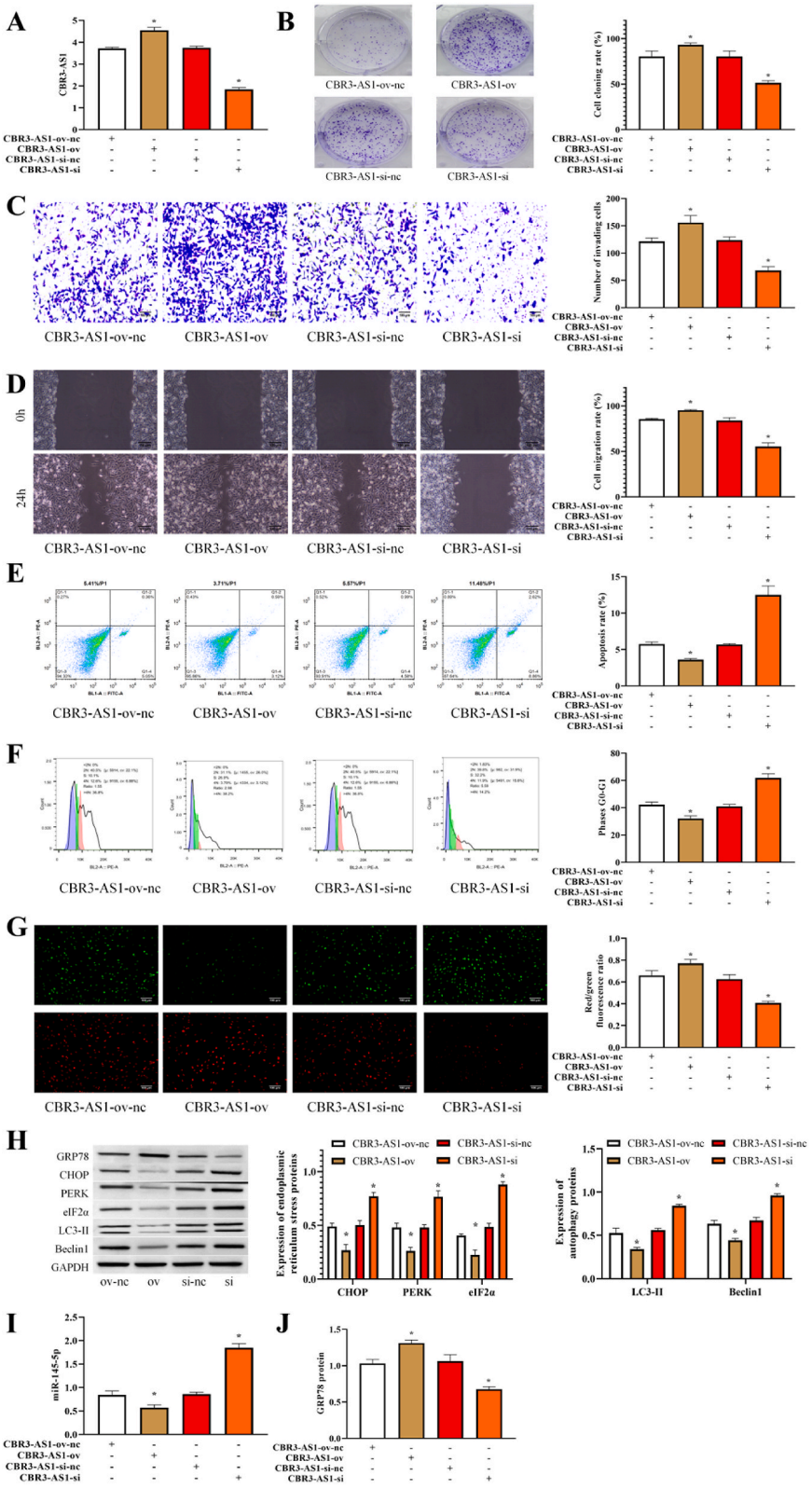


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**Fig. 3.** Effect of CBR3-AS1 on biological behavior of OS cells. (A) Expression of CBR3-AS1 after transfection of the aberrant expression vector. (B) Effect of CBR3-AS1 on clonogenic capacity of OS cells. (C) Effect of CBR3-AS1 on OS cell invasion ability. (D) Effect of CBR3-AS1 on migration ability of OS cells. (E) Effect of CBR3-AS1 on OS cell apoptosis. (F) Effect of CBR3-AS1 on OS cell cycle. (G) Effect of CBR3-AS1 on mitochondrial damage in OS cells. (H) Effects of CBR3-AS1 on endoplasmic reticulum stress and autophagy in OS cells (the complete protein imprinting map is in Supplementary Fig. S2). (I) Effect of CBR3-AS1 on miR-145-5p expression in OS cells. (J) Effect of CBR3-AS1 on GRP78 expression in OS cells. * indicate $P < 0.05$ with corresponding empty carriers.

## 3. Results

### 3.1. Influence of allicin on biological behavior of OS cells

The results of cell cloning experiments showed that the cloning rate of allicin-treated OS cells reduced significantly compared with normal control group, especially in group C ($P < 0.05$, Fig. 1A). Second, in Transwell assay, the number of invading cells was found to be lower in groups A, B, and C than in normal control group ($P < 0.05$, Fig. 1B). The migration rate was the highest in the normal control group and the lowest in group C ($P < 0.05$, Fig. 1C). According to flow cytometry analysis, groups A, B, and C had a lower apoptosis rate (Fig. 1D) and an obviously prolonged G0-G1 phase (Fig. 1E) than the normal control group ($P < 0.05$), with the highest apoptosis rate found in group C ($P < 0.05$).

### 3.2. Influence of allicin on CBR3-AS1/miR-145-5p/GRP78 expression in OS cells

First of all, the CBR3-AS1 expression detection results revealed lower CBR3-AS1 expression in groups A, B, and C compared with normal control group, with the lowest found in group C ($P < 0.05$, Fig. 2A). However, miR-145-5p was higher in groups A, B, and C than in the normal group, and was highest in group C ($P < 0.05$, Fig. 2B). Similarly, GRP78 protein expression was also lower in groups A, B, and C than in normal control group, with group C presenting the lowest level ($P < 0.05$, Fig. 2C).

### 3.3. Effect of CBR3-AS1 on biological behavior of OS cells

After transfection with CBR3-AS1 abnormal expression vectors, CBR3-AS1 expression was detected to verify the transfection success rate. CBR3-AS1 expression was found to be higher in CBR3-AS1-ov group compared with CBR3-AS1-ov-nc group, while that of CBR3-AS1-si was lower versus CBR3-AS1-si-nc group ($P < 0.05$, Fig. 3A), confirming the success of transfection. According to the cell biological behavior test, the cell cloning rate, number of invading cells, and migration rate of CBR3-AS1-ov group were higher compared with CBR3-AS1-ov-nc group, while the cell apoptosis rate was lower and the G0-G1 phase was shorter ($P < 0.05$); the reverse was true for CBR3-AS1-si group ($P < 0.05$, Fig. 3B–F). Finally, based on the results of fluorescence staining of mitochondrial damage, the JC-1 red/green fluorescence ratio was higher in CBR3-AS1-ov group versus CBR3-AS1-ov-nc group, while that of CBR3-AS1-si group was lower compared with CBR3-AS1-si-nc group ($P < 0.05$, Fig. 3G). Further detection of autophagy- and endoplasmic reticulum stress (ERS)-associated proteins showed that LC3-II, Beclin1, CHOP, PERK, and eIF2α protein levels were all decreased in CBR3-AS1-ov group, but increased in CBR3-AS1-si group ($P < 0.05$, Fig. 3H). In addition, miR-145-5p decreased and GRP78 protein expression increased in CBR3-AS1-ov group; while the reverse was true for CBR3-AS1-si group ($P < 0.05$, Fig. 3I and J).

### 3.4. Rescue experimental results

After intervention and co-transfection, the cell clone rate, number of invading cells, migration rate, apoptosis rate, and cell cycle change of CBR3-AS1-si + miR-145-5p-si and miR-145-5p-si + GRP78-si groups were not significantly different from those of the control group ($P > 0.05$). However, the cell cloning rate, number of invading cells, and migration rate of allicin++CBR3-AS1-si + miR-145-5p-si + GRP78-si and GRP78-si groups were lower compared with control group, especially in allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group; while the apoptosis rate and G0-G1 phase was higher, with a more significant increase in allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group ($P < 0.05$). miR-145-5p-si group showed higher cell clone rate, number of invading cells, and migration rate than control group, as well as a lower apoptosis rate and a shorter G0-G1 phase ($P < 0.05$, Fig. 4A–E). In JC-1 fluorescence staining, the JC-1 red/green fluorescence ratios in descending order in the seven groups of cells were miR-145-5p-si, control, CBR3-AS1-si + miR-145-5p-si, miR-145-5p-si + GRP78-si, CBR3-AS1-si + miR-145-5p-si + GRP78-si, GRP78-si, and allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group (Fig. 4F). Finally, protein detection revealed that LC3-II, Beclin1, CHOP, PERK, and eIF2α protein levels were the lowest in miR-145-5p-si group, and the highest in Allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group ($P < 0.05$); while no marked difference was identified among CBR3-AS1-si + miR-145-5p-si, miR-145-5p-si + GRP78-si, and control groups; nor was there any notable difference between CBR3-AS1-si + miR-145-5p-si + GRP78-si and GRP78-si groups ($P > 0.05$), whose LC3-II, Beclin1, CHOP, PERK, and eIF2α protein levels were higher than the control group, but lower than allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group ($P < 0.05$, Fig. 4G).

### 3.5. Live tumor experimental results

Finally, the results of tumor culture in vivo also showed that the volume and weight of subcutaneous tumor in miR-145-5p-si group

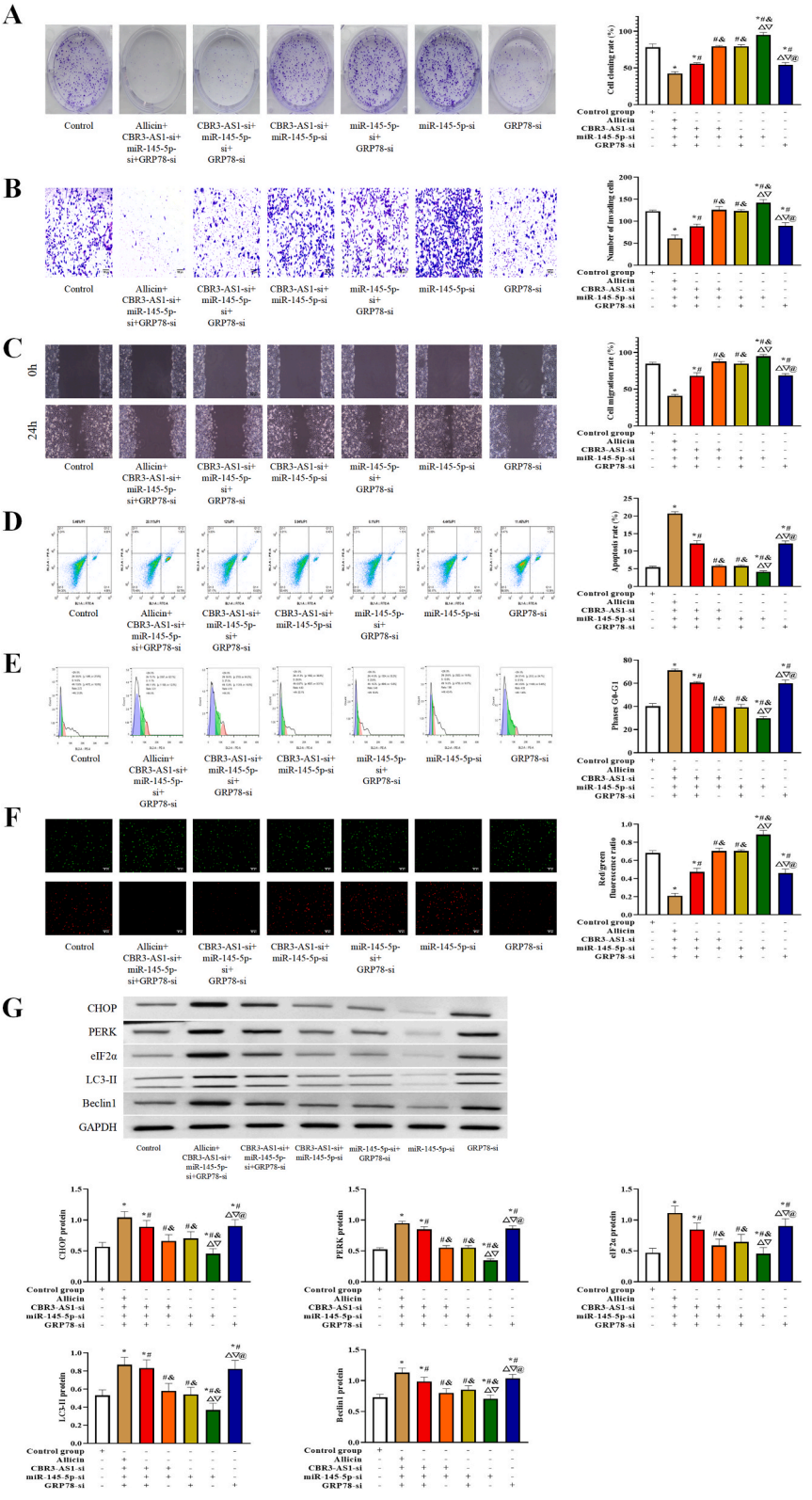


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**Fig. 4.** Rescue experimental results. (A) Comparison of cell clonogenicity. (B) Comparison of invasive cell counts. (C) Comparison of cell migration rates. (D) Comparison of cell apoptosis rates. (E) Comparison of cell cycle. (F) Comparison of mitochondrial damage. (G) Comparison of endoplasmic reticulum stress and autophagy proteins (the complete protein imprinting map is in Supplementary Fig. S3). *, #, &, △, ▽, @ indicate $P < 0.05$ with control group, Allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group, CBR3-AS1-si + miR-145-5p-si + GRP78-si group, CBR3-AS1-si + miR-145-5p-si group, miR-145-5p-si + GRP78-si group, miR-145-5p-si group.

were the highest, followed by CBR3-AS1-si + miR-145-5p-si, miR-145-5p-si + GRP78-si, and control group, which showed no statistical difference among them ($P > 0.05$). The subcutaneous tumor volume and weight in CBR3-AS1-si + miR-145-5p-si + GRP78-si group and GRP78-si group were consistent ($P > 0.05$), while those in allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group were the lowest ($P < 0.05$, Fig. 5A). T-lymphocyte subpopulation assays, on the other hand, showed that $CD4^+$ and CD4+/CD8+ were significantly higher and $CD8^+$ was lower in this group of mice compared with the control group after allicin injection ($P < 0.05$,

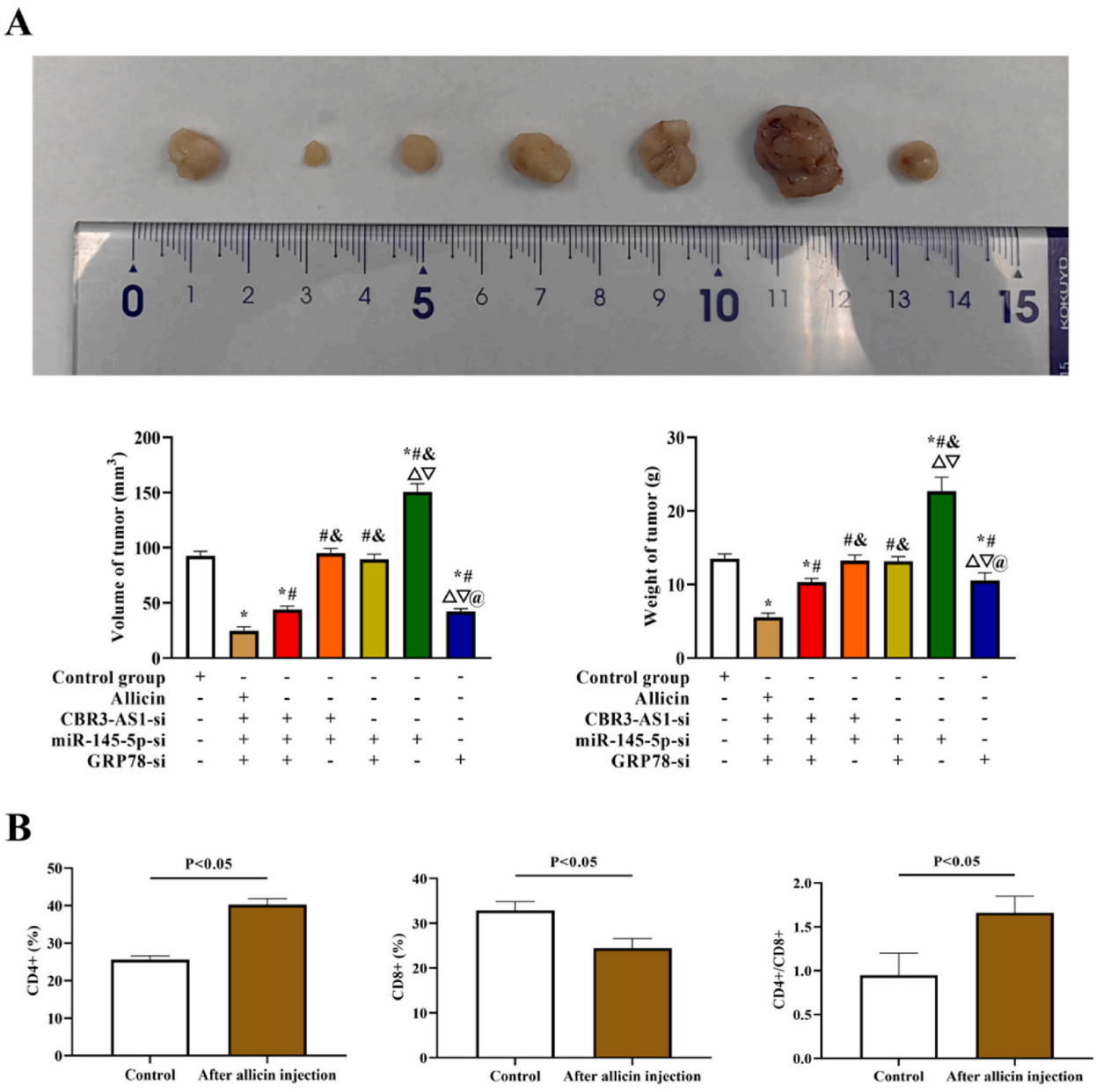


**Fig. 5.** Live tumor experimental results. (A) Allicin affects OS growth through CBR3-AS1/miR-145-5p/GRP78. (B) Effect of allicin on T-lymphocyte subpopulations in OS-loaded mice. *, #, &, △, ▽, @ indicate $P < 0.05$ with Allicin + CBR3-AS1-si + miR-145-5p-si + GRP78-si group, CBR3-AS1-si + miR-145-5p-si + GRP78-si group, CBR3-AS1-si + miR-145-5p-si group, miR-145-5p-si + GRP78-si group, miR-145-5p-si group.

Fig. 5B).

## 4. Discussion

In this study, we found that allicin can inhibit the growth of OS cells and promote its apoptosis, which lays the foundation for future clinical therapeutic applications of allicin. Secondly, the mechanism of action of allicin on OS may be related to CBR3-AS1/miR-145-5p/GRP78, but further studies are still needed to confirm it.

Although previous studies have validated the inhibitory behavior of allicin on tumor cells [18–20], its effect on OS remains unclear. Therefore, this study first confirmed the influence of allicin on the biological behavior of OS cells. We observed obviously inhibited OS cell proliferation, invasion, and migration, enhanced apoptosis, and cell arrest in the G0-G1 phase under the intervention of allicin, confirming that allicin could effectively inhibit the activity of OS cells and induce their apoptosis, consistent with the previous research results [21]. As we all know, the development of malignant tumors is closely related to the enhancement of cell proliferation capacity and the decrease of cell apoptosis capacity, and three biological processes (i.e., mitochondrial signaling, calcium ion environment, and oxidative stress) are typical events leading to cell apoptosis [22]. During allicin-induced apoptosis, some components of apoptosis pathways have been extensively studied, such as up-regulation of Bax, down-regulation of Bcl-2, release of cytochrome C, and over-activation of Caspase-3/9 [23,24]. In the proteomics study of Luo R et al., it was found that about 50 % of allicin-sensitive proteins in human gastric cancer cell lines were closely related to apoptosis [25]. Therefore, allicin plays an important role in accelerating tumor cell apoptosis. However, its specific mechanism has not been fully validated, and there is still a lack of reliable references and guidance in clinical practice.

As mentioned earlier, CBR3-AS1, as a newly discovered member of the lncRNA family, is located in the antisense region of CBR3 and was first identified for its aberrant expression in breast cancer [26]. With the deepening of research, the role of CBR3-AS1 in OS has attracted increasing attention [27], and the research on the action pathway of allicin also points to the possibility that CBR3-AS1 may be one of the key links [12]. Therefore, we further examined the expression of CBR3-AS1 in OS cells under the intervention of allicin, which showed decreased CBR3-AS1 levels, suggesting that allicin may have an inhibitory effect on CBR 3-AS1 expression. Moreover, after further regulating CBR3-AS1 expression, the cellular biological behavior of OS also changed obviously, that is, the activity was enhanced after CBR3-AS1 was upregulated, and apoptosis was induced after CBR3-AS1 expression was silenced. These results are consistent with previous studies on the pathogenesis of CBR3-AS1 [28], suggesting that silencing CBR3-AS1 is beneficial for OS treatment, and the inhibitory effect of allicin on CBR3-AS1 may be the key regulatory mechanism. On the other hand, ERS and autophagy are known to be typical physiological processes in the apoptotic process [29]. Therefore, we further detected the expression of autophagy- and ERS-associated proteins in cells. Similarly, silencing CBR3-AS1 led to an obvious increase in autophagy- and ERS-associated protein expression; moreover, a greatly reduced red/green fluorescence ratio was indicated by JC-1 fluorescent staining, demonstrating that silencing CBR3-AS1 expression can activate mitophagy and ERS in OS cells, thus inducing apoptosis [30]. Based on the inhibitory effect of allicin on CBR3-AS1 expression mentioned above, we can preliminarily understand its mechanism of action.

Therefore, we also examined miR-145-5p and GRP78 after allicin intervention. As expected, miR-145-5p increased under the intervention of allicin, while GRP78 decreased. Moreover, in the rescue experiments, we observed that silencing miR-145-5p alone promoted OS activity, while silencing GRP78 had a biological effect consistent with CBR3-AS1, consistent with previous research results [31,32]. The absence of significant difference in biological behavior among CBR3-AS1-si + miR-145-5p-si, miR-145-5p-si + GRP78-si, and control groups indicated that the effect of silencing CBR3-AS1 on OS cells was reversed by silencing miR-145-5p, while the effect of miR-145-5p was reversed by GRP78. However, after being treated with allicin, CBR3-AS1, miR-145-5p, and GRP78 simultaneously, OS cells showed more significant mitophagy and ERS and increased apoptosis rate than the control group, fully indicating the signal transduction pathway relationship between the CBR3-AS1/miR-145-5p/GRP78 molecular axis, and that the effect of allicin on OS cells is carried out through the CBR3-AS1/miR-145-5p/GRP78 molecular axis. Finally, through the subcutaneous tumorigenesis experiment, we further learned that the growth regulation effects of allicin and CBR3-AS1/miR-145-5p/GRP78 molecular axis in the growth process of living tumors are consistent with the above cell experiments, which not only shows that allicin has a very high clinical application potential in treating OS in the future, but also verifies the results of the above experiments.

Finally, tumor immunity is the basis of tumor radiotherapy, and the mechanism of the body's immune response to tumors and the escape of tumor cells from the immune effect greatly determines how to investigate new tumor treatment options [33,34]. As mentioned in a previous study, allicin can be used to treat arthritis by modulating immune homeostasis in arthritic mice [35], so we hypothesized that allicin might have the same effect on OS. In the T-lymphocyte subpopulation assay, we observed that $CD4^+$ and CD4+/CD8+ were elevated and $CD8^+$ was decreased in mice injected with allicin, suggesting that allicin has the effect of activating OS immunoreactivity, which verifies the above viewpoints, and is in line with the previous studies on the mechanism of action of allicin [36]. And these results not only fully confirmed the therapeutic potential of allicin in OS, but also laid a better foundation for subsequent related studies.

However, there are still many limitations to be addressed. For example, we need to further confirm the effects of miR-145-5p and GRP78 on OS through independent cell experiments, and validate the targeted regulatory relationship between CBR3-AS1/miR-145-5p/GRP78 molecular axes. At the same time, it is necessary to carry out in vivo experiments to analyze the side effects of allicin. In the future, we will carry out more comprehensive and in-depth research and analysis to address the shortcomings mentioned above.

## 5. Conclusion

Allicin can effectively activate the immunoreactivity of OS cells and induce the apoptosis of OS cells,the mechanism of which is carried out by activating the mitochondrial autophagy and ERS of OS cells through the CBR3-AS1/miR-145-5p/GRP78 molecular axis. In the future, allicin is expected to be a new clinical treatment scheme for OS, thus providing a reliable safety guarantee for the prognosis of patients.

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## Ethics approval statement

The experiment was approved by the Institutional Animal Care and Use Committee and Animal Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine (approval number 2021-05).

## Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## CRediT authorship contribution statement

**Wenpeng Xie:** Writing – review & editing, Writing – original draft. **Fengjun Ma:** Formal analysis. **Luming Dou:** Methodology. **Wenjie Chang:** Investigation. **Daotong Yuan:** Supervision, Software. **Zhimeng Zhang:** Visualization, Validation. **Yongkui Zhang:** Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

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

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Not applicable.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31971.

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