Cardamonin Promotes the Apoptosis and Chemotherapy Sensitivity to Gemcitabine of Pancreatic Cancer Through Modulating the FOXO3a-FOXM1 Axis

Huapeng Sun1,#, Na Zhang2,#, Yiqiang Jin3★, and Haisheng Xu3

Abstract
Cardamonin (CAR), a flavone existing in the Alpinia plant, has been found to modulate multiple biological activities, including antioxidant, anti-inflammatory, and anti-tumor effects. Nevertheless, the influence of CAR on pancreatic cancer (PC) is less understood. Here, we conducted in vitro and in vivo experiments to explore the functions of CAR on PC cells’ proliferation, apoptosis and chemosensitivity to gemcitabine (GEM). The growth of PC cells (including PANC-1 and SW1990) was evaluated by the cell counting kit-8 assay, colony formation assay and xenograft tumor experiment. Besides, the apoptosis was determined by flow cytometry and western blot (WB). Moreover, the FOXO3a-FOXM1 pathway expression was tested by reverse transcription-polymerase chain reaction and WB. Our data suggested that CAR restrained cell proliferation, growth and expedited apoptosis both in vitro and in vivo. Moreover, CAR sensitized PC cells to GEM. Mechanistically, CAR heightened FOXO3a while repressed FOXM1. Further loss-of-function assays revealed that down-regulating FOXO3a markedly dampened the anti-tumor effect induced by CAR and accelerated the FOXM1 expression. Our data confirmed that CAR exerted an anti-tumor function in PC dependently by modulating the FOXO3a-FOXM1 axis.

Keywords
pancreatic cancer, cardamonin, FOXO3a, chemotherapy

Introduction
Cancer is 1 of the most severe public health issues worldwide, and pancreatic cancer (PC) is among the most malignant cancers, with a 5 year survival rate of about 4%.1 Clinically, early PC shows no signs and is difficult to diagnose. However, as the disease progresses, the tumor cells rapidly invade the surrounding tissues and organs and develop into a fatal malignancy.2 Clinically, PC is treated by surgical excision, supplemented by chemotherapy, radiotherapy, and other local therapies.3 Unfortunately, the outcomes of PC surgery are not satisfactory due to the high incidence of complications and unresectable primary lesions.4 Meanwhile, the biggest challenge in PC chemotherapy is its resistance to various standard chemotherapeutic drugs, such as gemcitabine (GEM) and adriamycin.5 Hence, we sought to delay PC’s malignant progression of and reduce its chemotherapy resistance from a biomolecular perspective.

1Department of General Surgery, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangyang, Hubei, China
2Department of Pathology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangyang, Hubei, China
3Department of Oncology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, Xiangyang, Hubei, China

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#Contributed equally.

Corresponding Authors:
Yiqiang Jin, Department of Oncology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, No. 136 Jingzhou Street, Xiangcheng District, Xiangyang, Hubei 441021, China.
Email: jjljt47@163.com

Haisheng Xu, Department of Oncology, Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science, No. 136 Jingzhou Street, Xiangcheng District, Xiangyang, Hubei 441021, China.
Email: shenjiaba09@163.com

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Cardamonin (CAR) (molecular formula: C_{16}H_{14}O_{4}; CAS No, 19309-14-9), a flavonoid found in Alpinia plants, has antioxidant, anti-inflammatory, and anti-tumor effects. It has been found that CAR dramatically hampers the proliferation and migration of human melanoma M14 cells and induces apoptosis. Additionally, CAR dampens the proliferation of the mTOR inhibitor-induced HeLa-resistant cervical cancer cells and MCF-7-resistant breast cancer cells, reduces mTOR phosphorylation and raptor protein levels, and improves cellular drug resistance. More importantly, Hossan MS et al stated that Novel Semi-Synthetic Cu (II)-CAR complex (19) exerts significant anti-tumor activity in triple-negative breast cancer and PC by choking the AKT signaling. Meanwhile, a new plant flavonoid, Alpinetin, impedes PC cells' proliferation by modulating the expression of the Bcl2 family and XIAP. Those studies suggest that CAR, which is also a flavonoid, may restrain PC. However, the exact mechanisms and effects of CAR are unclear.

FOXM3a belongs to the Forkhead transcription factor family, and it contributes to diversified cancers such as breast cancer, colon cancer, and bladder cancer. FOXO3a regulates multiple cell life cycles, such as tumor cell proliferation, apoptosis and oxidative stress, and chemoresistance by phosphorylating p-FOXO3a. For example, Rab escort protein 1 interacts with FOXO3a to block its nuclear translocalization, thus abating the FOXO3a-mediated colon cancer cell apoptosis. Also, Hu JQ et al found that Histone deacetylase sirtuin 6 (SIRT6) acts with FOXO3a to accelerate its ubiquitylation, thus co-regulating adriamycin-induced liver cancer cell apoptosis and improving the chemoresistance. The PI3K/AKT/mTOR pathway has been reported to have significant value as a classical carcinogenic pathway in various cancers, including PC. Besides, CAR hinders the proliferation and metastasis of non-small cell lung cancer and esophageal cancer by choking the PI3K/AKT/mTOR pathway. Similarly, CAR restrains the malignant progression of gastric cancer by inactivating STAT3 through attenuation of LncRNA PVT1. Also, Jin J et al claimed that CAR curbed the PI3K/AKT/mTOR/FOXO3a axis. This article brings a new insight for delaying the malignant progression of PC and improving its sensitivity to chemotherapeutic agents from the perspective of drug mechanisms.

**Material and Methods**

**Animals**

Sixteen 4–6-week-old BALB/c nude mice (20-24 g of body weight) (half male and half female) were bought from the Animal Research Centre of Wuhan University and housed in micro-isolation cages in an SPF-rated laboratory animal room, provided with adequate air, food, and water. All experimental procedures conformed to the guidelines for the use and care of animals established by the Institute and were approved by the Ethics Committee of Xiangyang Central Hospital for the Protection of Animals.

**Xenograft Tumor Experiment**

The tumorigenesis experiment was performed in 4–6-week-old athymic BALB/c nude mice. PANC-1 and SW1990 cells (2 × 10⁶) at the logarithmic growth stage were injected into the left abdominal wall of mice with .1 mL PBS, respectively. The injection was implemented with a 1 mL-syringe 30 times. Two weeks after the injection, the mice were randomly divided into 4 groups (2 female and 2 male nude mice per group), that is, the sham group, the CAR group. The tumor weight of mice was determined every 7 days after the tumorigenesis, and the tumor size was measured with precision vernier calipers. The nude mice were killed by cervical dislocation. The tumor volume (V) is equal to (L × W²)/2 (L represented the tumor length; and W represented the tumor width).

**Cell Culture**

PC cell lines (PANC-1 and SW1990) were bought from the Cell Center of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI1640 comprising 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, CA, USA) in an incubator at 37°C with 5% CO₂. The RPMI1640 medium and PBS were provided by Thermo Fisher Scientific (MA, USA). During the logarithmic growth phase, .25% trypsin (Thermo Fisher HyClone, Utah, USA) was employed for cell trypsinization and sub-culture.
Cell Transfection

PANC-1 and SW1990 cells at the logarithmic growth stage were trypsinized, sub-cultured, and grown in 6-well plates (5 × 10^6/well). After the cell growth was stabilized, the small interfering RNA targeting FOXO3a (si-FOXO3a#1; si-FOXO3a#2; si-FOXO3a#3) and the corresponding negative control fragments (si-NC) were transfected into PANC-1 and SW1990 cells using Lipofectamine® 3000 (Invitrogen, Carlsbad, California, USA). Cells were incubated at 37°C with 5% CO2 for 24 hours. Next, the cells were cultured with fresh culture medium for 24 hours.

Cell Treatment

CAR (Article No. HY-N0279) and GEM (Article No. HY-17026) were purchased from Medchemexpress (SH, USA). PANC-1 and SW1990 cells were treated with GEM (0, .0625, .125, .25, .5, 1, and 2 μg/mL) for 24 hours. The treated cells were then harvested. In addition, PANC-1 and SW1990 cells were treated with CAR at doses of 5, 10, and 20 μM for 24 hours, and the treated cells were collected. The growth and apoptosis of the treated cells were monitored.

Cell Counting Kit-8 Assay

Cell viability was determined with the CCK-8 assay. The stably transfected PANC-1 and SW1990 cells were incubated in 96-well plates (1 × 10^3 cells/well) and cultured for 24 hours. Next, 10 μL Cell Counting Kit-8 (CCK8) reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well as per the manufacturer’s instructions. The cells were incubated at 37°C for 1 hour, and the optical density (OD) value at 450 nm was observed on a spectrophotometer (Bio-Rad, CA, USA). Then the OD value was measured at 24, 48, and 72 hours.

Reverse Transcription-Polymerase Chain Reaction

PANC-1 and SW1990 at the logarithmic growth stage were made into the single-cell suspension and inoculated in a sterilized cell culture bottle for 24 hours. After being synchronized with RPMI1640 containing .5% fetal calf serum for 24 hours, the cells were treated with different factors for 0 (the control group), 24, 48, and 72 hours, respectively. Cells in each group were collected at different time points, and the total cellular RNA was extracted with the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) and stored in RNase-free water at −70°C. The concentration and purity of RNA were determined by ultraviolet spectrophotometry. RNA samples were reverse-transcribed into cDNA by MMLV reverse transcriptase and stored at −20°C for later use. Using cDNA as a template, reverse transcription-polymerase chain reaction was performed with FOXO3, FOXM1, and GAPDH primers (Sangon Biotech, Shanghai, China) and SYBR Green qPCR Master Mix (MedChemExpress, NJ, USA). Results: \( \Delta \Delta CT = \Delta CT_{test\ sample} - \Delta CT_{control\ sample} \) represented the gene expression of the samples to be tested relative to the corresponding gene expression in the control sample. The specific primer sequence is shown in Table 1.

Western Blot

After cell treatment, the culture medium was discarded. Then, the protein lysates (Roche) were added, and the total proteins were separated. Afterward, 50 μg total protein was sampled in 12% polyacrylamide gel for 100 V electrophoresis for 2 hours and then transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked at room temperature with 5% skimmed milk for 1 hour, the membranes were washed with TBST 3 times for 10 min each. The membranes were incubated overnight at 4°C with primary antibodies (1:1000; Abcam, MA, USA) of Bax (ab32503), Bcl2 (ab218123), Caspase3 (ab13847), PI3K (ab32089), mTOR (ab134903), FOXO3a (ab23683), and β-actin (ab115777). After the membranes were rinsed with TBST, they were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit IgG (concentration: 1:300) at room temperature for 1 hour. After that, the membranes were washed with TBST 3 times for 10 min each. Finally, the western blot (WB) special reagent (Invitrogen) was adopted for color development, and Image J was employed to analyze the gray intensity of each protein.

Flow Cytometry

The AnnexinV/7-AAD Apoptosis Detection Kit (Southern Biotechnology, Birmingham, Al, USA) was applied to determine the PANC-1 and SW1990 apoptosis. The treated cells were trypsinized, centrifuged, and washed twice with cold PBS. Then, they were resuspended in the binding buffer, with a final concentration of 1 × 10^6 cells/mL. Next, the cells were

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Table 1. The Specific Primer Sequence.

| The Target | Forward (5' -3') | Reverse (5' -3') |
|------------|------------------|------------------|
| FOXO3      | TAAAAGGAGCTGGTTGGGGAG | CCAGCCTGTACCTTCAGTA |
| FOXM1      | AGCCCCAGTCCATCAGAACTC | CCCAAACACGCTATGATGCC |
| GAPDH      | TGGTTGAGCACAGGGTACTT | CCAAGGAGTAAGACCCCCTGG |

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double-stained with AnnexinV and 7-AAD and incubated in darkness at room temperature for 15 min. Finally, we employed Flowoxietry (BD Biosciences, SanJose, CA, USA) to analyze the stained cells by FACS Calibur Flow Cytometer (BD Biosciences, SanJose, CA, USA). The experiment was carried out in triplicate.

**Colony Formation Assay**

PANC-1 and SW1990 cells were inoculated into 6-well plates (1000 cells/well). They were then cultured in RPMI-1640 medium comprising 10% FBS and 1% penicillin/streptomycin for 2 weeks. Afterward, the cells were immobilized with formaldehyde for 10 min and dyed with .5% crystal violet for 10 minutes. After being rinsed with PBS buffer 3 times, the stained colonies were imaged and counted with an optical microscope. Each experiment was repeated 3 times.

**Immunoprecipitation**

PANC-1 and SW1990 cells were lysed with radiomuno-precipitation (RIPA) lysis buffer (Beyotime Institute of
Biotechnology, Shanghai, China) containing fresh protease inhibitors and PMSF. After incubation with the anti-FOXM1 antibody (BD Pharmingen) at 4°C, the cells were further incubated with the protein G Plus/Protein A agarose suspension (Merck) at 4°C for 4 hours. Next, the cells were washed 3 times with cold cleavage buffer and lysed with SDS cleavage buffer at 95°C for 10 minutes. Subsequently, 10% SDS-PAGE gel was applied for WB analysis. The non-specificity was reduced by adding 5% skim milk to TBST to block the nitrocellulose membranes. After being cleared with TBST 3 times for 10 minutes each, the membranes were incubated with the primary antibody overnight at 4°C, maintained with the secondary antibody for 1 hour at room temperature, and then washed with the primary antibody. Binding antibodies were gauged with the chemiluminescent photosensitive HRP kit (Pierce, Rockford, IL, USA).

**Immunohistochemistry**

IHC staining was implemented to acquire the expression of FOXO3a and FOXM1 in the tumor tissues. 4-μM-thick paraffin-embedded sections from tumor xenografts were prepared, blocked by 5% fetal goat serum, and respectively incubated with anti-p-FOXO3a (ab23683, Abcam, MA, USA) and anti-FOXM1 (ab207298, Abcam, MA, USA) antibodies. The slides were examined with an optical microscope (Olympus), and the percentage of positive nuclei was quantified using Image J software (NIH, Bethesda, USA).

**Statistical Analysis**

SPSS 16.0 software (SPSS Inc, Chicago, IL, USA) was applied for statistical analysis, and the results were presented as mean±SD (x±s). Student’s t test was used for the mean comparison between the 2 groups, while one-way ANOVA was used for the comparison of multiple means. SNK-q test was employed for the pair-wise comparison of multiple means. P < .05 indicated statistical significance.

**Results**

**CAR Enhanced PC Cell Apoptosis and Reduced Cell Viability**

We treated PANC-1 and SW1990 cells with different doses of CAR (0, 5, 10, 20 μM) for 48 hours to probe the impact of CAR on human PC cell lines. The molecular formula of CAR is shown in Figure 1A. CCK8 and flow cytometry outcomes exhibited a significant decline in PA cell viability (P<.05, Figure 1B) and a facilitation in apoptosis (P<.05, Figure 1C) with increasing concentrations of CAR. As indicated by the cloning assay data, elevated doses of CAR resulted in a significant attenuation in cell proliferation (P<.05, Figure 1D). Meanwhile, WB data revealed that the pro-apoptotic proteins Bax and Caspase3 were up-regulated, and the anti-apoptotic protein Bel2 was down-regulated with the elevation of CAR’s concentrations (P<.05, Figure 1E). These results illustrated
that CAR curbed PC cell proliferation and facilitated apoptosis.

**CAR Facilitated the Chemosensitivity of PC Cells to GEM**

We treated PANC-1 and SW1990 cells with CAR (0, .0625, .125, .25, .5, 1, and 2 μM) with/without GEM (0, .0625, .125, .25, .5, 1, and 2 μg/mL) for 48 hours. As indicated by CCK8 assay results, cell viability declined significantly after GEM treatment. However, compared with the control group, the cell viability of the CAR group was lower (P<.05, Figures 2A and 2B). Additionally, the colony formation assay manifested that PC cell proliferation was distinctly hampered after CAR or GEM treatment (vs the control group). The CAR+GEM group had a less cell colony formation ability compared with GEM group (P<.05, Figures 2C and 2D). These data disclosed that CAR enhanced the chemosensitivity of PC cell to GEM.

**The Effect of CAR on the FOXO3a-FOXM1 Pathway**

We treated PANC-1 and SW1990 cells with CAR (0, 5, 10, 20 μM) for 48 hours to verify the mechanism of CAR in PC. WB results revealed that CAR concentration-dependently hindered the phosphorylation levels of PI3K, AKT, and mTOR in PANC-1 and SW1990 cells (P<.05, Figure 3A). Meanwhile, p-FOXO3a was up-regulated and FOXM1 was down-regulated with the increase of CAR’s doses (P<.05, Figure 3B). Additionally, we conducted the IP experiment with the FOXM1 antibody in PANC-1 and SW1990 cells to explore the interaction between FOXO3a and FOXM1. As a result, FOXO3a bound to FOXM1 (Figure 3C). The above

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**Figure 3.** The effect of CAR on FOXO3a-FOXM1. PANC-1 and SW1990 cell lines were treated with CAR (0 to 20 μM) for 48 hours. (A) WB was carried out to gauge the PI3K/AKT/mTOR expression. (B) The profile of the FOXO3a/FOXM1 pathway was checked by WB. (C) The interaction between FOXO3a and FOXM1 was confirmed by IP. *P < .05, **P < .01, ***P < .001 (vs Control group), N = 3. CAR: cardamonin, WB: western blot.
results hinted that CAR heightened the FOXO3a expression and restrained the FOXM1 level.

**Knocking Down FOXO3a Declined PC Cells’ Chemosensitivity to GEM**

To figure out the function of FOXO3a in GEM chemosensitivity of PC cells, we set up a FOXO3a knockdown cell model (Figure 4A). As a result, we discovered FOXM1 was significantly up-regulated in FOXO3a-down-regulated cells (Figure 4A). PC cells’ viability under was tested. It turned out that down-regulating FOXO3a notably elevated the viability of PC cells (Figures 4B and 4C). Moreover, PC cells with lower level of FOXO3a showed less chemosensitivity to GEM (compared with the si-NC group) (Figures 4D and 4E). Hence, knocking down FOXO3a curbed GEM chemosensitivity of PC cells.

**Down-regulation of FOXO3a Reversed CAR-Mediated Anti-tumor Effects in PC Cells**

To explore the mechanism by which CAR regulated the FOXO3a-FOXM1 axis and affected PC cells, we transfected PANC-1 cells with si-NC or si-FOXO3a and 20 μM CAR for 48 hours, respectively. CCK8 results manifested that by contrast with the control group, the cell viability and colony formation ability of PANC-1 cells were curbed in the CAR+si-NC group (Figures 5A and 5B). However, PANC-1 cells in the CAR+si-FOXO3a group gained accelerated cell viability and colony formation ability (Figures 5A and 5B). The GEM-sensitivity was gauged; the results testified that the CAR+si-FOXO3a group had signally impeded GEM-sensitivity when compared with the CAR+si-NC group (Figure 5C). We then conducted WB to examine the FOXO3a-FOXM1 axis in PANC-1 cells. Interestingly, compared with the control group, FOXO3a was up-
regulated and FOXM1 was down-regulated in the CAR+si-NC group, while down-regulating FOXO3a had the opposite results. In contrast, FOXO3a was down-regulated and FOXM1 was up-regulated in the CAR+si-FOXO3a group compared with that of the CAR+si-NC group (P < .05, Figure 5D). These findings hinted that CAR restrained cell proliferation and viability and induced apoptosis dependently through facilitating FOXO3a and dampening FOXM1.

CAR Attenuated PC Cell Growth In Vivo

PANC-1 and SW1990 cells were subjected to the xenograft tumor experiment in nude mice for further evaluation of the impact of CAR on PC. We found that compared with the sham group, CAR treatment restrained PANC-1 and SW1990 cells’ growth (Figures 6A-6F). The FOXO3a/FOXM1 pathway expression was further examined by WB. As a result, CAR treatment up-regulated FOXO3a and repressed FOXM1 expression (vs the sham group) (Figure 6G). Meanwhile, treatment with CAR notably lowered the phosphorylation levels of PI3K, AKT and mTOR compared to the sham group (Figure 6H). As indicated by IHC outcomes, CAR elevated the FOXO3a expression and hampered FOXM1 profiles compared to the sham group (Figure 6I). Hence, CAR showed anti-tumor effect via modulating the PI3K/AKT/mTOR and FOXO3a/FOXM1 axis.

Discussion

PC is among the common digestive tract malignancies with a high fatality rate. Due to the lack of apparent manifestation in early PC, almost all PC patients are diagnosed with varying degrees of tumor cell metastasis. Drug chemotherapy is an effective treatment method for PC. However, PC is prone to drug resistance. The characteristics of fewer pancreatic blood vessels and more matrices make the chemotherapy drugs unable to be effectively delivered to tumor cells, which greatly weakens the effect of chemotherapy. Meanwhile, chemotherapy drugs such as GEM have poor therapeutic effects on PC. Therefore, it is significant to study the drug mechanism of PC for its clinical treatment. This experiment explores the effect of CAR’s regulating FOXO3a-FOXM1 on PC cell apoptosis and GEM chemosensitivity.

CAR has been confirmed to exert an anti-inflammatory, anti-proliferative, and anti-tumor role in various malignant tumors. For example, CAR hampers CRC cell growth, induces cell cycle arrest, and facilitates apoptosis by inactivating NF-κB. In addition, CAR inactivates mTOR and its downstream target ribosome S6 kinase 1 (S6K1), thus abating the proliferation, invasion, and metastasis of Lewis lung cancer (LLC) cells. All the above reports show that CAR has a tumor-suppressive effect in diversified cancers. As a flavonoid, Wogonin impedes PC by hindering ROS-activated autophagy and intensifying apoptosis. Similarly, Fisetin, a natural flavonoid, curbs PC cells’ proliferation by inducing DNA damage through RFXAP/KDM4A-dependent...
histone H3K36 demethylation. Moreover, Guo Y et al claimed that dietary bioflavonoid quercetin repressed PC growth and induced apoptosis by antagonizing SHH and the TGF-β/Smad pathway. These studies suggest that various flavonoids contribute to PC. Based on this, we speculate that CAR, which is also a flavonoid, contributes to PC. It is encouraging to note that CAR curbs PC cells’ proliferation and intensifies apoptosis in this article.

As the report goes, GEM improves the prognosis of advanced PC. However, the resistance of PC cells to GEM severely limits the effectiveness of chemotherapy, increases the treatment costs, and leads to more toxic and side effects. In addition, PC cells’ growth and metastasis are enhanced after drug resistance, which weakens apoptosis and affects epithelial–mesenchymal transition (EMT). Some researches have revealed that LINC 00346 targets miR-118-3p, and overexpressing LINC 00346 facilitates PC cells’ proliferation and colony formation and enhances the chemoresistance of PC cells to GEM. In contrast, knocking down LINC 00346 boosts the therapeutic effect of GEM on PC. Also, Xian G et al discovered that Simvastatin restrains tumor-associated macrophage–mediated chemoresistance of PC cells to GEM by blocking the TGF-β1/GFI-1 axis. The above studies elaborated the resistance of PC cells to GEM from the molecular perspective and the drug mechanism. This research also studied the effect of CAR on the chemoresistance of PC cells from the drug mechanism and revealed that CAR abated the resistance of PC cells to GEM.

From the above studies, we have learned that the resistance of PC mediates the abnormal expression of genes and the activation of key signal pathways. FOXO3a, a tumor suppressor transcription factor, boosts cell cycle arrest, senescence and cell death, regulates tumor cell proliferation and apoptosis, and affects PC cell resistance. FOXO3a is often down-regulated or inactivated in cancer cells, especially in drug-resistant cells. As a crucial downstream target of FOXO3a, FOXM1 interacts with FOXO3a to modulate the growth, proliferation, and apoptosis of various tumor cells, affecting cell drug resistance. Several studies have demonstrated that the expression of p-FOXO3a, FOXO3a, and the downstream target of FOXM1 (SIRT2) is negatively correlated with Lapatinib sensitivity in nasopharyngeal carcinoma cells, and SIRT2 mediates FOXO3a deacetylation and facilitates Lapatinib resistance in the cells. In addition, all-trans-retinoic acid (RA; a retinoid) and bexarotene declined the proliferation of oral squamous cell carcinoma (OSCC)–derived cell lines via heightening FOXO3a and repressing FOXM1. Collectively, those studies confirmed that the FOXO3a-FOXM1 pathway contributes to tumor progression and acts as a promising chemotherapy target. Interestingly, a previous study has disclosed that CAR induced G2/M arrest and apoptosis in breast cancer cell lines by enhancing the expression and nuclear translocation of FOXO3a via upstream c-Jun N-terminal kinase. The effect of the FOXO3a-FOXM1 axis on PC was also confirmed in this experiment, which signified that CAR intensified PC cell apoptosis and dampened GEM resistance by regulating the FOXO3a-FOXM1 axis.

Overall, this research confirmed that CAR effectively hindered tumor cell growth in tumor-bearing mice. In addition, CAR elevated FOXO3a expression and hampered FOXM1, thus inducing PC cells’ apoptosis and improving their chemosensitivity to GEM. The research on CAR brings a new treatment direction for chemotherapy in PC patients. Nevertheless, this experiment also has some shortcomings. For instance, its feasibility has not been verified by clinical
specimens, which will be further studied in the follow-up experiments.

**Author Contributions**
Conceived and designed the experiments: Yiqiang Jin, Haisheng Xu; performed the experiments: Huapeng Sun, Na Zhang; statistical analysis: Huapeng Sun, Na Zhang; wrote the paper: Huapeng Sun, Na Zhang. All authors read and approved the final manuscript.

**Declaration of Conflicting Interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Ethics Approval**
Our study was approved by the Ethics Review Board of Xiangyang Central Hospital, Affiliated Hospital of Hubei University of Arts and Science.

**Data Availability Statement**
The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

**ORCID iD**
Yiqiang Jin https://orcid.org/0000-0001-5231-3250

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