

Effects and mechanism of Lanthanum Citrate on the proliferation and apoptosis of hepatocellular carcinoma cell line SMMC-7721

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ABSTRACT

Background/Aims: To investigate the effect and the possible mechanism of lanthanum citrate on the proliferation and apoptosis of human hepatocellular carcinoma (HCC) cell line SMMC-7721 through the Hedgehog (Hh) signaling pathway.

Materials and Methods: Different concentrations of lanthanum citrate and KAAD-cyclopamine (the Hh signaling pathway representative inhibitor) were used to treat SMMC-7721 cells. Cell proliferation was detected using Methylthiazolyl-diphenyl-tetrazolium bromide (MTT) assays. Cell apoptosis was detected using flow cytometry analysis of Annexin V-FITC/propidium iodide (PI). The protein expressions of regulatory genes, such as cell cycle protein D1 (CyclinD1), cyclin-dependent kinase inhibitor 1 (p21), cysteinyl aspartate specific proteinase 3 (Caspase-3), B-cell lymphoma-2 (Bcl-2), glioma-associated oncogene homolog 1 (Gli1), and sonic hedgehog (Shh) were quantified using Western blot assays. The mRNA expressions of Gli1 and Shh were tested using quantitative real-time polymerase chain reaction (qRT-PCR) assays and the protein expressions of Gli1 and Shh were determined using immunofluorescence assays.

Results: The Annexin V-FITC and PI double staining results revealed that the 0.1 mM lanthanum citrate group and the 15 μ M KAAD-cyclopamine group had both increased the apoptosis rate of SMMC-7721 cells. Both lanthanum citrate and KAAD-cyclopamine down-regulated the protein expressions of CyclinD1, Bcl-2, Gli1, and Shh and upregulated the protein expressions of p21 and Caspase-3. Additionally, the immunofluorescence results revealed that the protein expressions of Gli1 and Shh were significantly decreased in both the lanthanum citrate group and the KAAD-cyclopamine group compared to the control group.

Conclusion: Lanthanum citrate inhibits proliferation and promotes apoptosis in HCC SMMC-7721 cells by suppressing the Hh signaling pathway.

Keywords: Hepatocellular carcinoma, the hedgehog signaling pathway, lanthanum citrate, proliferation, apoptosis

INTRODUCTION

The incidence of primary hepatocellular carcinoma (PHC) is markedly higher in China, accounting for more than half of the PHC cases in the world (1). PHC has a high degree of malignancy. There are no obvious early or mid-term symptoms and PHC is prone to intrahepatic and distant metastasis. Thus, early diagnosis is difficult and the best chance for treatment is often missed. At present, surgical resection is the first choice for PHC treatment but post-operative recurrence and metastasis rates are often high and the overall treatment effect is not satisfactory (2).

The occurrence and development of hepatocellular carcinoma (HCC) involve multi-gene participation and a multi-stage, multi-step development process using multiple signaling pathways (3). Recent studies have reported that the Hedgehog (Hh) signaling pathway is ab-

normally activated during tumor development in gastric, colonic, pancreatic, ovarian, breast, and liver cancers (4). As the key components of Hh signaling pathway, sonic hedgehog (Shh) is a binding ligand by initiating this pathway, and glioma-associated oncogene homolog 1 (Gli1) is a family of zinc finger transcription factor that functions as a downstream protein in the Hh signaling pathway. Remarkably, recent study has concluded that hyperactivation of Gli1 and Shh are sufficient to trigger uncontrolled progression of cancer features such as proliferation, migration, and invasion (5). However, the role and mechanism of the Hh signaling pathway in the occurrence, development, invasion, and metastasis of HCC are largely unknown. Rare earth elements (REEs) have the potential for broad application in the field of clinical treatment. REEs have been successfully applied to treat conditions such as burns, skin diseases, thrombo-

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sis, and even cancer. This novel cancer therapy has rapidly attracted interest and inspired many researchers to study the effects of REEs on the malignancy of tumor growth and development. There is conclusive evidence that several REEs compounds have significant inhibitory effects on human hepatoma cells [6]. Lanthanum is a representative REEs and the lanthanum citrate compound is commonly used in cellular drug experiments. Studies have reported that lanthanum citrate displays an anti-HCC effect due to its strong inhibitory influence on cell growth. Lanthanum citrate will transmit signals through the signal transduction pathway even if it does not enter the cells (7). However, the specific molecular mechanism of lanthanum citrate in the treatment of liver cancer is still unclear. Therefore, in this study, we investigate the effect of lanthanum citrate on the proliferation and apoptosis of HCC SMMC-7721 cells as well as the possible mechanisms for these actions. We aim to provide new information and a theoretical basis for the diagnosis and treatment of primary liver cancer.

MATERIALS AND METHODS

Cells, reagents, and antibodies

The HCC SMMC-7721 cell line was provided by the Scientific Research Center of the First Affiliated Hospital of Gannan Medical University. The reagents used include Dulbecco's modified Eagle's medium (DMEM; BI, 0014136, USA), 10% fetal bovine serum (FBS; AusGeneX, FBSSA00418-2, Australia), penicillin-streptomycin (BI, 1634678, USA), Phosphate-Buffered Saline (PBS; BI, 0033016, USA), Methylthiazolyldiphenyl-tetrazolium bromide (MTT) Cell Proliferation and Cytotoxicity Assay Kit (Solarbio, M1020, Beijing, China), Lanthanum oxide (La_2O_3 , 325.81 g/mol, Shanpu, 1312-81-8, Shanghai, China), KAAD-cyclopamine (Aladdin, 4449-51-8, E1528101, Shanghai, China), Annexin V-FITC Apoptosis Detection Kit (Beyotime, C1062L, Shanghai, China), BCA Protein Assay Kit (Solarbio, PC0020, Beijing, China), FastQuant RT Kit (with gDNase; TIANGEN, KR106, Beijing, China), and SuperReal Premix Plus (with SYBR Green; TIANGEN, FP205, Beijing, China). The source of primary antibodies were as follows: anti-CyclinD1 (1:200, Abcam, ab16663, USA), anti-p21 (1:1000, Abcam, ab218311, USA), anti-Caspase-3 (1:500, Abcam, ab13847, USA), anti-Bcl-2 (1:500, Solarbio, K106396P, Beijing, China), anti-Gli1 (1:500, Solarbio, A11793R, 20160914, Beijing, China), and anti-Shh (1:500, Solarbio, A11455R, 20160914, Beijing, China). In addition, the following secondary antibodies were used: anti-mouse IgG peroxidase labeled antibodies (1:10000, Sigma, A4416, USA), and anti-rabbit IgG per-

oxidase labeled antibodies (1:5000, Sigma, A6154, USA). The present study was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University and the written informed consent was not necessary for this manuscript.

Preparation of the lanthanum citrate compound

Lanthanum oxide (purity >99.99%) was dried at 105-110°C for 2 h. It was dissolved with 1:1 HCl to form lanthanum chloride and evaporated to dryness after accurate weighing. The lanthanum chloride was then dissolved completely with citric acid solution (2.5 mol/L) and the pH was adjusted to 7.0 using NaOH solution. Finally, the volume was determined by ddH₂O and the lanthanum citrate was filtered, sterilized, and reserved at 4°C. The required concentration was achieved through dilution with serum-free DMEM media before using.

Cell cultures

The SMMC-7721 cells were cultured at 37°C and 5% CO₂. The median lethal dose (IC₅₀) of drugs to cells was calculated according to the MTT assays and then directed to the lanthanum citrate group and the KAAD-cyclopamine group. Therefore, the experiment was divided into three groups: the blank control group received only PBS without drugs, the experimental group received 0.1 mM lanthanum citrate, and the Hh signaling pathway inhibitor group received 15 µM KAAD-cyclopamine.

MTT assays

An MTT assay was performed to determine the effect of lanthanum citrate and KAAD-cyclopamine on cell proliferation. Both assays were performed in 96-well plates, with eight concentration gradients and five secondary wells. The concentrations of lanthanum citrate were 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mM. The concentrations of KAAD-cyclopamine were 0, 0.5, 1, 5, 10, 15, and 20 µM. The blank control consisted of medium containing no cells. The assays were performed following the MTT Cell Proliferation and Cytotoxicity Assay Kit manufacturer's instructions. The SMMC-7721 cells were treated with the aforementioned drug concentrations for 48 h, then approximately 20 µL of MTT reagent (Solarbio, 298-93-1, Beijing, China) was added to each well. The 96-well plates were constantly stimulated for 4h and subsequently, a blue precipitate appeared at the bottom. Next, 150 µL of dimethyl sulfoxide (DMSO; Solarbio, 67-68-5, Beijing, China) was added to each well before the culture fluid was suctioned and the absorbance of cells at 490 nm was measured using an enzyme-labeled instrument (Thermo, Varioskan Lux, USA).

Cell apoptosis detection

We used flow cytometry analysis to detect the apoptosis rate. Trypsin-EDTA solution (0.25% with phenol red, Solarbio, T1320, Beijing, China) was used to digest the SMMC-7721 cells. The cells were cultured for 24 h and then divided into the blank control group, 0.1 mM Lanthanum Citrate group, and 15 μ M KAAD-cyclopamine group. The cells were incubated in the treatments for 48 h before the apoptosis detection was performed. The manufacturer's protocol of the Annexin V-FITC Apoptosis Detection Kit was followed. In brief, the cells were collected from each group and incubated with a mixture of 195 μ L Annexin V-FITC binding buffer and 5 μ L FITC-labeled Annexin V for 10 min at room temperature and then added 10 μ L propidium iodide (PI) in the dark for 20 min at 4°C. The cells were then analyzed using a flow cytometer (BD Bioscience, BD FACSCalibur, USA).

Western blot protein expression analysis

A Western blot was used to analyze the expressions of CyclinD1, p21, Caspase-3, Bcl-2, Gli1, and Shh proteins. The total proteins in each treatment group were extracted with lysis buffer (Solarbio, 9016-45-9, Beijing, China) and phenylmethylsulfonyl fluoride (PMSF; Solarbio, 329-98-6, Beijing, China). The steps were followed according to the manufacturer's instructions. Protein concentrations in each group were determined using a BCA Protein Assay Kit and stored at -78°C. The proteins were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with the primary antibodies overnight at 4°C and then incubated with the secondary antibodies for 1 h at room temperature. Exposure and photographing were performed on the gel imaging analysis system (GE, Amersham Imager 600, USA) to conduct data analysis and processing.

Detection of mRNA expressions of Gli1 and Shh using qRT-PCR

Trizol reagent (Invitrogen, 15596018, USA) was used to extract total RNA from cells in each treatment group for RNA concentration and purity testing. cDNA was synthesized from the total RNA using the FastQuant RT Kit. qRT-PCR was performed using an ABI 7500 Real-time PCR system (Thermo, 7500 RT-PCR, USA) with SuperReal Premix Plus. ImageJ software was used for gray value analysis. GAPDH was used as an internal control. The following primers were present:

Gli1-F: 5'-CCTGGATCGGATAGGTGGTC-3'
 Gli1-R: 5'-GGCTCTTGAACCTCTGGACT-3'
 Shh-F: 5'-CGGGCAGATAGGAAGGTGAT-3'

Shh-R: 5'-GGGCCATAATGAACCACGTC-3'
 GAPDH-F: AGAAGGCTGGGGCTCATTTG-3'
 GAPDH-R: AGGGGCCATCCACAGTCTTC-3'

Immunofluorescence staining protein expression analysis

Immunofluorescence staining was used to determine the protein expressions of Gli1 and Shh in SMMC-7721 cells. The SMMC-7721 cells were treated with drug-free medium, 0.1 mM lanthanum citrate or 15 μ M KAAD-cyclopamine for 24 h. They were fixed in 4% paraformaldehyde/PBS for 15 min and then washed three times with PBS containing 0.1% Triton X-100 (PBST) for 15 min at room temperature. Following blocking in PBS containing 5% BSA (Blocking buffer) for 30 min, the cells were then incubated with the primary antibodies, anti-Gli1 (1:500; diluted with Blocking buffer) and anti-Shh (1:500; diluted with Blocking buffer) for 1 h. The cells were washed three times with PBST and then incubated with Alexa Fluor 594 (Goat anti-rabbit IgG, 1:1000, Abcam, ab150088, USA; diluted with Blocking buffer) for 1 h. The cells were washed three more times with PBST and then incubated with DAPI (1 μ g/ml, Abcam, ab228549, USA) and detected with a Confocal Laser Scanning Microscope (Carl Zeiss LSM 880).

Statistical analysis

SPSS v.18.0 software was used for the statistical analysis. The measurement data were expressed as the mean \pm standard deviation (SD). A comparison between the data was performed using chi-square (χ^2) and student's t-test. $p < 0.05$ was considered to indicate a significant difference between values.

RESULTS

Lanthanum citrate and KAAD-cyclopamine effects on SMMC-7721 cell proliferation

The concentrations of lanthanum citrate in the range of 0.001-1 mM inhibited the proliferation of SMMC-7721 cells. Higher concentrations of lanthanum citrate resulted in increased inhibition of proliferation of SMMC-7721 cells. The inhibitory effect was also enhanced with longer treatment time. One-way analysis of variance indicated that compared with the control group, the proliferation rate of the cells in both treatment groups was significantly lowered. When the lanthanum citrate concentration was 0.1 mM, the cell viability value (43.73 ± 3.53 , Figure 1a, $p < 0.05$) was similar to the IC_{50} after treatment with drugs for 48 h, which significantly contrasted with the control group. For this reason, we chose the 0.1 mM concentration as the subsequent experimental concentration of lanthanum citrate. When the KAAD-cyclopamine con-

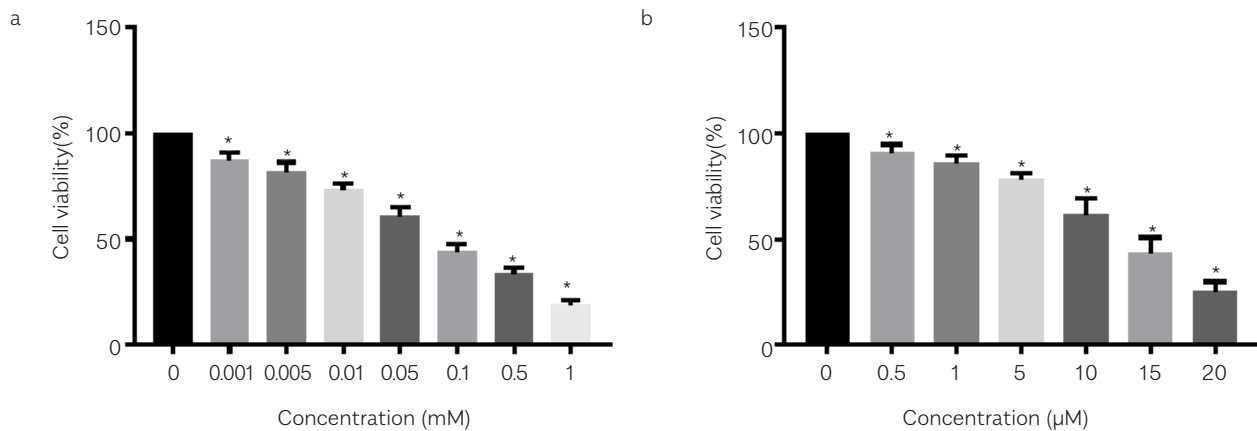


Figure 1. a, b. Both lanthanum citrate and KAAD-cyclopamine decreased cell viability of SMMC-7721 Cells. (a) Effects of different concentrations of lanthanum citrate on the proliferation of SMMC-7721 Cells. * $p < 0.05$, compared with the control group (0 mM). (b) Effects of different concentrations of KAAD-cyclopamine on the proliferation of SMMC-7721 Cells. * $p < 0.05$, compared with the control group (0 μM).

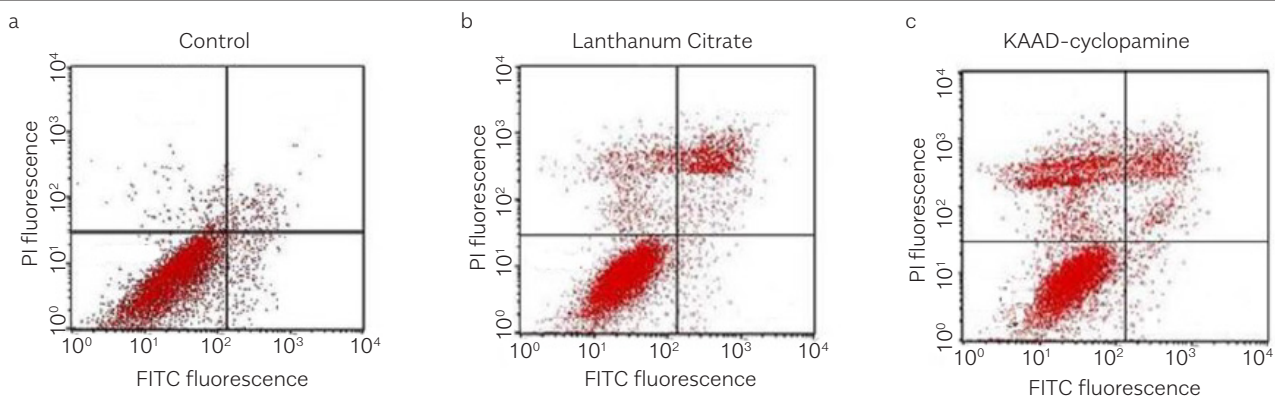


Figure 2. a-c. Effects of different treatments on SMMC-7721 cells apoptosis detected by flow cytometry. (a) Cells were treated with drug-free medium for 48 h and then evaluated using an Annexin V-FITC Apoptosis Detection Kit. (b) Cells were treated with 0.1 mM lanthanum citrate for 48 h and then evaluated using an Annexin V-FITC Apoptosis Detection Kit. (c) Cells were treated with 15 μM KAAD-cyclopamine for 48 h and then evaluated using an Annexin V-FITC Apoptosis Detection Kit. PI, propidium iodide.

Table 1. Effects of lanthanum citrate and KAAD-cyclopamine on survival or apoptosis rate of HCC SMMC-7721 cells (mean±SD, %).

| Group | Survival rate | Apoptosis rate |
|--------------------------|---------------|----------------|
| Control group | 0.924±0.024 | 0.043±0.015 |
| 0.1 mM lanthanum citrate | 0.622±0.089* | 0.292±0.039* |
| 15 μM KAAD-cyclopamine | 0.520±0.067* | 0.457±0.061* |

* $p < 0.05$, compared with the control group (phosphate-buffered saline without drugs). HCC: hepatocellular carcinoma; SD: standard deviation.

centration was 15 μM, the cell viability value (43.70 ± 7.09 , Figure 1b, $p < 0.05$) was approximately equal to the IC_{50} after 48 h of treatment. Thereby, we chose this concen-

tration as the subsequent experimental concentration of KAAD-cyclopamine.

Lanthanum citrate and KAAD-cyclopamine effects on SMMC-7721 cell apoptosis

Both the lanthanum citrate and KAAD-cyclopamine treatments promoted apoptosis of hepatoma cells. When compared with the blank control group, the SMMC-7721 cells treated with 0.1 mM lanthanum citrate for 48 h had a significantly higher apoptosis rate (0.292 ± 0.039 , $p < 0.05$). When the cells were treated with 15 μM KAAD-cyclopamine, the apoptosis rate (0.457 ± 0.061 , $p < 0.05$) was also significantly higher than in the control group. See Figure 2 and Table 1 for details.

Lanthanum citrate and KAAD-cyclopamine effects on CyclinD1, p21, Caspase-3, Bcl-2, Gli1, and Shh protein expressions

The results of the Western blot showed that the relative expression levels of CyclinD1, Bcl-2, Gli1, and Shh proteins in the control group, 0.1 mM lanthanum citrate

group, and 15 μ M KAAD-cyclopamine group were shown in Table 2, respectively.

Compared with the control group, the differences were statistically significant (Figure 3, $p < 0.05$). These data indicated that both lanthanum citrate and KAAD-cyclopamine downregulated the protein expressions of CyclinD1, Bcl-2, Gli1, and Shh.

To verify whether lanthanum citrate and KAAD-cyclopamine influenced the protein expressions of Gli1 and Shh in SMMC-7721 cells, we then used the immunofluorescence staining method to determine the protein expressions of Gli1 and Shh within cells. The results showed that the fluorescence intensity of Gli1 in the nucleus was weaker in both the lanthanum citrate group and KAAD-cyclopamine group (Figure 4a). The fluorescence intensity of Shh in the cytoplasm was weaker in both the lanthanum citrate group and KAAD-cyclopamine group (Figure 4b), indicating that both treatments downregulated the protein expressions of Gli1 and Shh.

The relative expression levels of p21 and Caspase-3 proteins in the control group, 0.1 mM lanthanum citrate

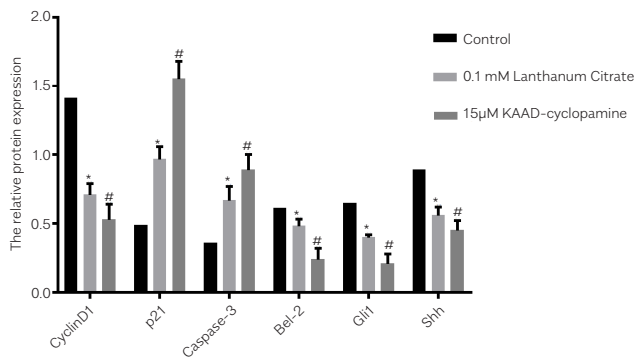


Figure 3. Effects of lanthanum citrate and KAAD-cyclopamine on protein expressions of CyclinD1, p21, Caspase-3, Bcl-2, Gli1, and Shh. * $p < 0.05$, compared with the control group (drug-free cell culture medium) and # $p < 0.05$, compared with the control group (drug-free cell culture medium).

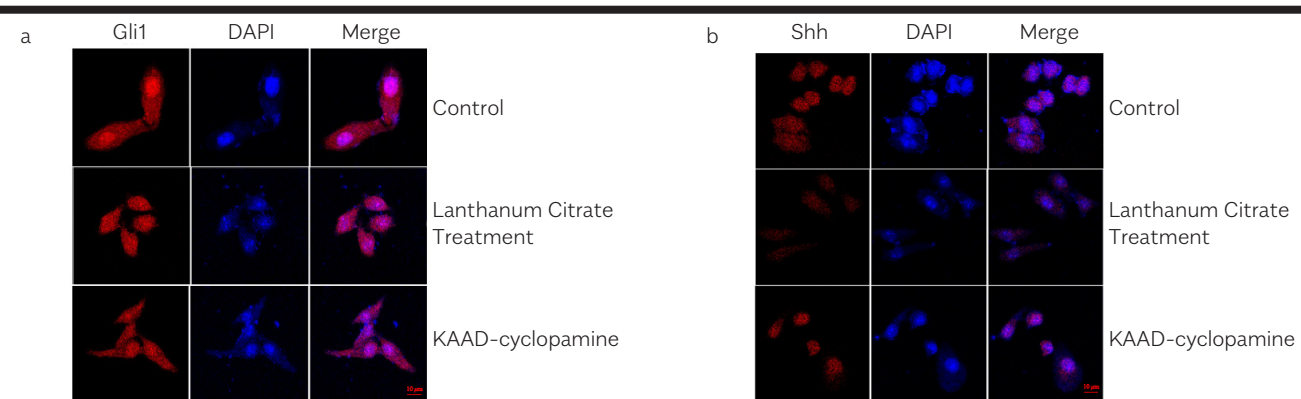


Figure 4. a, b. Immunofluorescence staining of SMMC-7721 cells treated with drug-free medium, 0.1mM lanthanum citrate and 15 μ M KAAD-cyclopamine for 24 h, using antibody Gli1 and Shh. (a) Representative images of Gli1 immunofluorescence staining in SMMC-7721 cells. (b) Representative images of Shh immunofluorescence staining in SMMC-7721 cells. Nuclei were stained with DAPI. Scale bar: 10 μ m.

Table 2. The relative protein expressions of CyclinD1, Bcl-2, Gli1, Shh, p21, and Caspase-3 in HCC SMMC-7721 cells (mean \pm SD).

| Group | CyclinD1 | Bcl-2 | Gli1 | Shh | p21 | Caspase-3 |
|-----------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Control group | 1.41 \pm 0.21 | 0.53 \pm 0.11 | 0.65 \pm 0.13 | 0.89 \pm 0.10 | 0.49 \pm 0.08 | 0.36 \pm 0.05 |
| 0.1 mM lanthanum citrate | 0.71 \pm 0.08* | 0.48 \pm 0.05* | 0.40 \pm 0.02* | 0.56 \pm 0.06* | 0.97 \pm 0.09* | 0.67 \pm 0.10* |
| 15 μ M KAAD-cyclopamine | 0.53 \pm 0.11* | 0.24 \pm 0.08* | 0.21 \pm 0.07* | 0.45 \pm 0.07* | 1.55 \pm 0.13* | 0.89 \pm 0.11* |

* $p < 0.05$, compared with the control group (phosphate-buffered saline without drugs).

HCC: hepatocellular carcinoma; SD: standard deviation.

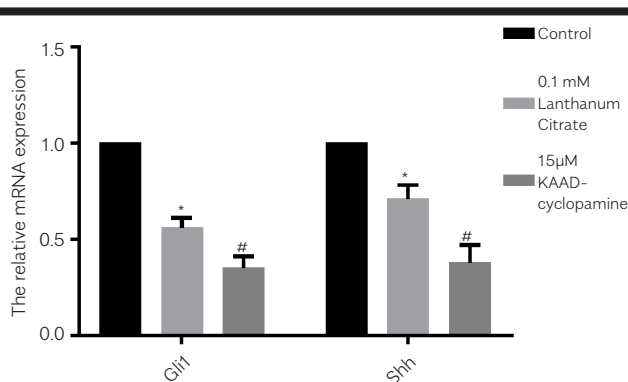


Figure 5. Effects of lanthanum citrate and KAAD-cyclopamine on mRNA expression of Gli1 and Shh. * $p < 0.05$, compared with the control group (drug-free cell culture medium) and # $p < 0.05$, compared with the control group (drug-free cell culture medium).

group, and 15 μ M KAAD-cyclopamine group were also shown in Table 2, respectively. Compared with the control group, the differences were statistically significant (Figure 3, $p < 0.05$). These data demonstrated that both the lanthanum citrate and KAAD-cyclopamine treatments upregulated the protein expressions of p21 and Caspase-3 in comparison to the control.

Effects of Lanthanum Citrate and KAAD-cyclopamine on mRNA expressions of Gli1 and Shh

The qRT-PCR results indicated that the relative mRNA expression levels of Gli1 in the 0.1 mM lanthanum citrate group and 15 μ M KAAD-cyclopamine group were 0.56 ± 0.05 and 0.35 ± 0.0 , respectively; the relative mRNA expression levels of Shh were 0.71 ± 0.07 and 0.38 ± 0.09 , respectively. Compared with the control group, the differences were statistically significant (Figure 5, $p < 0.05$). These results implied that both lanthanum citrate and KAAD-cyclopamine downregulated the transcriptional expressions of Gli1 and Shh.

DISCUSSION

It is reported that REEs and their complexes have dose-dependent anti-cancer effects, which may be caused by inhibition of DNA synthesis within tumor cells and expression of related genes, preventing the proliferation of tumor cells (8). Cytological assays also indicate that lanthanum citrate has a dose-dependent effect on tumor cells including HepG2 cells. At a lower concentration, the proliferation of cells is promoted slightly, while at a high concentration there was a promoting effect on the apoptosis of tumor cells (9). Studies have also reported that the same dose of rare earth ion (Ln^{3+} ; 0.1 mM) can in-

hibit the proliferation of rat hepatoma cells but promote the proliferation of primary hepatocytes (10). Therefore, when lanthanum citrate is at a suitable concentration, normal hepatocytes exhibit no or low response, while tumor cells exhibit a greatly increased response that is not caused by cytotoxicity. However, higher concentrations of lanthanum citrate may be toxic to normal and/or abnormal liver cells (11).

Our MTT assays revealed that the antiproliferative effect of lanthanum citrate on the SMMC-7721 cell line was significantly higher than that of the blank control group, indicating that lanthanum citrate has a significant inhibitory influence on the proliferation of hepatoma cells. We detected the IC_{50} of lanthanum citrate on SMMC-7721 cells, providing a suitable drug concentration for subsequent studies. Moreover, we used the Annexin V-FITC and PI double staining method to determine the apoptosis rate of SMMC-7721 cells and the results showed that the apoptosis rate of the lanthanum citrate group was significantly higher than that of the blank control group. This suggests that lanthanum citrate can also promote the apoptosis of hepatoma cells. In addition, the results demonstrated that compared with the control group, lanthanum citrate can significantly downregulate the mRNA and protein expressions of key components of the Hh signaling pathway in HCC cells such as Shh and Gli1. This indicates that lanthanum citrate has inhibitory effects on the Hh signaling pathway in hepatoma cells.

Uncontrolled proliferation of cells is the main cause of malignant tumors. The uncontrolled growth of tumor cells is attributed to the destruction of the cell growth cycle mechanism. CyclinD1 is a kinase that regulates the DNA replication process in the cell cycle. CyclinD1 activates Rb protein by linking with CDK4, then Rb protein is separated from nuclear transcription factor E2F, E2F enters the cell nucleus and promotes transcriptional expression of target genes related to the G1/S phase, prompting cells to pass the G1/S phase of cell check period. The expression of CyclinD1 protein in HCC tissues is significantly higher than that in paraneoplastic and normal tissues. CyclinD1 is one of the target genes in the Hh pathway. The downstream activation molecule Gli1 of the Hh pathway can bind to the promoter of CyclinD1 in the nucleus, promote the transcription of CyclinD1 and upregulate its expression, so that the cells can rapidly proliferate (12). Research conducted by Guodong He et al. (13) on hepatoma specimens confirmed that the Hh pathway activation was accompanied by overexpression of CyclinD1 protein and mRNA. Furthermore, Hailong Tian et al. (14)

reported that after the transfection of siRNA-Gli silencing the Gli1 gene of U251 cells, the expressions of CyclinD1 and Bcl-2 proteins and mRNA were all decreased. They suggested that the effect of the Hh pathway on cell proliferation and apoptosis was achieved through the regulation of Cyclin D1 and Bcl-2 by Gli1. After downregulating the expression of Gli1, the transcription of CyclinD1 was reduced, and the proliferation of cells was inhibited.

The protein p21 is a CDK kinase inhibitor. The role of p21 is to downregulate activity by linking with CDK so that the cells enter the G1 phase for DNA self-inspection and repair. The expression of p21 protein is often accompanied by suppression of cancerous tissues in HCC patients. As a negative regulator of the cell cycle, p21 is of great significance in preventing the rapid passage of tumor cells through the cell checkpoint. Gli1 regulates the expression of p21. Lei Wang et al. (15, 16) demonstrated that when transfection of siRNA-Gli1 interferes with the expression of Gli1, the expression of p21 increases, the expression of CyclinD1 decreases, and the proliferation of KYSE-30 cells decreases. Therefore, they suggest that the effect of Gli1 on cell proliferation may be related to the downregulation of CyclinD1 and the upregulation of p21. Additionally, a study by Wenxia Su et al. (17) demonstrated that silencing Gli1 molecules can inhibit the proliferation of K562 cells. It is suggested that the mechanism of Gli1 promotion of cell proliferation is through the activation of c-myc and promotion of upregulation, while c-myc down-regulates the expression of p21 and promotes cell proliferation. The results of our Western blot also indicated that CyclinD1 protein expression was significantly downregulated in the cells treated with lanthanum citrate, and p21 protein expression was significantly increased. The mechanism of the prominent inhibition of the proliferation of SMMC-7721 cells by lanthanum citrate may be associated with the inhibition of the Hh signaling pathway, the decreased expression of Gli1 mRNA and protein resulting in the decreased expression of CyclinD1 and increased expression of p21 protein expression in the liver cancer cell cycle.

It is generally considered that abnormal apoptosis is one of the main factors for the occurrence of malignant tumors. The detection methods of apoptosis include morphological observation and biochemical indicator detection. In our study, Annexin V-FITC in conjunction with PI staining was used to detect apoptosis. Bcl-2 is a negative regulator of apoptosis and is a target gene of the Hh pathway. The gene sequence that binds to the nucleus of the Gli protein family can be found in the promoter se-

quence of Bcl-2. Previous studies have reported that Bcl-2 contains one Gli1 binding domain and three Gli2 binding domains. Gli1 binds to the Bcl-2 promoter to promote the transcription and expression of Bcl-2 and inhibit apoptosis (18, 19). Bcl-2 can be downregulated by KAAD-cyclopamine by blocking the activation of Gli1 downstream of the Hh pathway and the expression of the Bcl-2 gene, which promotes the apoptosis of SMMC-7721 cells. Wen Jiang et al. (20) reported that cyclopamine can inhibit the transcription of Gli1, downregulate the expression of Bcl-2 in HSQ-89 cells and promote apoptosis. Moreover, Jun Liu et al. (21) indicated that after cyclopamine acted on HSC-6 cells, Gli1 and Bcl-2 could be downregulated to improve cell apoptosis rate.

Caspase-3 protein is an effector apoptosis protein in the Caspases protein family. The low expression of caspase-3 is closely related to the anti-apoptosis of hepatoma cells. Caspase proteins can be divided into primary apoptotic proteins and active apoptotic proteins according to the relevant activation period (22). When an apoptotic protein stimulating factor is present, the original pro-apoptotic plasminogen is activated to produce an enzymatic activity, which further cleaves and activates the downstream effector pro-apoptotic plasmin, thereby degrading a protein necessary for various life activities such as cell proliferation and differentiation.

p53 is a tumor suppressor gene that can induce the apoptosis of mutant cells, which is of great significance in tumor research. Gli1 can be connected with the ubiquitin ligase B-TrCP, while enhancing its stability, and destroying the stability of p53, preventing its aggregation and its induction of apoptosis in cells (23, 24). The activation of p53 can promote the activation of caspase-3 and p21, and caspase-3 can cleave p21 into a 14 kD fragment, which binds to CDK, blocks the cell cycle and DNA replication, and promotes apoptosis of hepatoma cells (25). In our study, the expression of Bcl-2 protein was downregulated and the Caspase-3 protein was overexpressed with the apoptosis of SMMC-7721 cells treated with lanthanum citrate. This result suggests that blocking the Hh signaling pathway can promote the apoptosis of hepatoma cells by inhibiting the expression of Bcl-2 gene and promoting the activation of Caspase-3.

In conclusion, to the best of our knowledge, this was the first time to report that lanthanum citrate downregulated the expression of Gli1 and Shh significantly, indicating that lanthanum citrate exerts anti-proliferation effect in HCC cells via blocking the Hh signaling pathway. In ad-

dition, the findings of this study revealed the potential mechanism of lanthanum citrate which inhibited proliferation and promoted apoptosis of hepatoma cells. However, this study is only for experiments *in vitro*. In the future, we will conduct further experiments with lanthanum citrate using more HCC cell lines and animals *in vivo* to determine their efficacy and safety.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of the First Affiliated Hospital of Gannan Medical University.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – B.C., Z.H.; Design – B.C., Z.H.; Supervision – B.C., Z.H.; Resource – B.C.; Materials – B.L.; Data Collection and/or Processing – Z.H., B.C.; Analysis and/or Interpretation – Z.H., B.C., B.L.; Literature Search – Z.H.; Writing – Z.H.; Critical Reviews – B.C., Z.H.

Conflict of Interest: The authors have no conflicts of interest to declare.

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