ORIGINAL RESEARCH

Phospholysine Phosphohistidine Inorganic Pyrophosphate Phosphatase Regulates Oxidative Stress Response to Affect the Progression of Gastric Cancer

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ABSTRACT

Background • Phospholysine phosphohistidine inorganic pyrophosphate phosphatase (LHPP) has been reported to have anti-carcinogenic effects in gastric cancer, but the specific mechanisms by which LHPP influences GC remain unclear. This study aims to investigate the effect and mechanism of LHPP on GC.

Methods • In the in vivo experiments, we constructed a GC mouse model to investigate the impact of LHPP on tumor growth and the expression of related proteins in mice. In the in vitro experiments using human GC cells, we established LHPP overexpression and knockdown cell lines to study the potential mechanisms of LHPP in the progression of GC. We also explored the influence of ROS on the function of LHPP in GC by culturing cells under low glucose and H_2O_2 conditions.

Results • In vivo experiments, comparing the tumor development of mice, it was found that LHPP inhibited tumor formation in vivo. Compared with the NC group, it was found that overexpression of LHPP led to a decrease in the expression levels of ROS-related proteins and the protein expression levels of p-Src, p-ERK, and MMP-9 after LHPP overexpression. In vitro experiments, it was found that LHPP overexpression inhibited the migration and invasion of GC cells. However, this regulatory effect of LHPP on GC cells was suppressed when ROS levels increased.

Conclusion • The regulation of oxidative stress response by LHPP is an important mechanism in the development of GC. LHPP inhibits the development of GC by inhibiting the Src-ERK pathway and MMPs. Our study provides a reliable working basis for future in-depth research. (*Altern Ther Health Med.* 2024;30(12):450-455).

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INTRODUCTION

Gastric cancer (GC) remains one of the leading causes of cancer death, and GC is the third most common tumor-related disease worldwide.[1] Although treatment strategies have improved in recent years, the effectiveness of treatment remains limited. This suggests that there are still gaps in our current GC treatment. Therefore, finding effective targeted treatment strategies to delay the progression of GC is of great significance for improving patients' survival rate and quality of life. In this context, our study focused on exploring how LHPP can be applied to address the GC treatment gap.

Oxidative stress response refers to the process in which the redox equilibrium in cells is broken, producing excessive active oxides, which causes damage to cell structure and function. It has been found that oxidative stress exists in GC tissues. ROS levels are often significantly increased during the development and progression of tumors and are involved in regulating the activities of several signaling pathways, including the Src-ERK pathway.[4] Previous studies have shown that the Src-ERK (Proto-oncogene tyrosine-protein kinase SRC-Extracellular regulated MAP kinase) pathway plays a key role in a variety of cancers, such as GC,[5] lung cancer,[6] pancreatic cancer,[7] rectal cancer[8] and so on. As a membrane-binding switch molecule connecting many important signal pathways inside and outside the cell, Src kinase can regulate a variety of signal transduction pathways related to tumorigenesis and development after being activated by various signals on the surface of the cell membrane. An ERK signaling pathway involves key biological processes such as cell proliferation and transcriptional regulation. Therefore, it is clinically important to investigate the inhibition of the ROS/Src-ERK pathway to intervene in the development of GC. LHPP (phospholysine phosphohistidine inorganic pyrophosphate phosphatase) is a histidine phosphatase protein, which as a new tumor suppressor has been shown to play an important cancer inhibitory role in cancerous tissues, such as hepatocellular carcinoma,[9] cervical

carcinoma,[10] and colorectal carcinoma.[11] Role. Recent studies have shown that LHPP can inhibit gastric cancer progression through the PI3K/AKT/mTOR signaling pathway. The present study attempted to further reveal the biological function of LHPP protein in GC development.

Therefore, this study aimed to explore the therapeutic potential of LHPP on GC and further clarify its mechanism of action in regulating oxidative stress response and the Src-ERK pathway. In this study, the anti-cancer effects of LHPP were verified through in vivo and in vitro experiments, and the regulatory effects of LHPP on oxidative stress and the Src-ERK pathway were investigated. The results are expected to provide insight into the mechanism of action of LHPP in GC and reveal key molecules that regulate oxidative stress response and the Src-ERK pathway. This will provide a theoretical basis for developing new targeted therapy strategies and provide a new direction for the individualized treatment of GC. Ultimately, we hope that this study will provide GC patients with more effective treatment options to delay cancer progression and further improve their survival rate and quality of life.

MATERIALS AND METHODS

Animals

All animal experiments were conducted by institutional guidelines and approved by the hospital Laboratory Animal Center. SPF-grade 4-5 week old male BALB/c nude mice were purchased from Henan Skbex Biology Co., Ltd. for a subcutaneous transplantation experiment. After one week of adaptive feeding in a specific pathogen-free environment (Mice are housed in an animal house in an SPF-grade environment with tightly sterilized drinking water and feed bedding, unrestricted diet, 12h light/12h black), 32 healthy nude mice were randomly selected and divided into four groups with 8 mice in each group. Tumor cells (1×10^7 cells/mice, in 200 μl PBS) were injected subcutaneously into the lateral ventral region of mice. Every 7 days, the tumor volume was measured to calculate the size by the formula: $0.5 \times \text{length} \times \text{width}$.[2] The mice tumor was isolated and measured 4 weeks later. The experimental protocols were approved by Ethics Committee of Handan Central Hospital. Animal experiments were carried out according to the IACUC guidelines, and are reported in accordance with ARRIVE guidelines.

Cell culture

Human gastric cancer cell lines AGS and MKN-45 were purchased from Procell Life Science & Technology Co., Ltd. and routinely cultured in RPMI-1640+10% FBS medium. (Cells were cultured at 37°C in a humidified environment containing 5% CO_2.) The experiment was carried out with cells in good condition in logarithmic growth stage.

Cell transfection

Acquisition of LHPP-mimic(For overexpression of LHPP): It was cloned into pCDH-puro-EGFP vector plasmid by LHPP. LHPP forward primer: 5'-ATG CCG TGG GGC AAC GGC TG-3', and reverse primer: 5'-TCA CTT GTC GGC GTG CTG CAG C-3'. Acquisition of LHPP-inhibitor(For knocking down LHPP): The sequence of LHPP shRNA is 5'-CTA CAT GAA GGC GCT TGA GTA-3'. Insert the designed LHPP shRNA into pMDL, pRev, and pVSVG packaging vectors. The vector was transfected into 293T cells, and the filter filtered and harvested the virus. Then, the cells were transfected for 72 hours for the next experiment.

Western blot

The total protein was extracted from mouse tissues or cells and the protein concentration was detected by a BCA kit (abcam, ab102536). An equal amount of protein (20-30 μg) was separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride membrane. After sealing with 4% milk, the membrane was incubated with primary antibody NOX2(1:1000), NOX4(1:1000), p-Src(1:1000), p-ERK(1:1000), MMP9(1:1000), GAPDH (1:1000) at 5°C overnight and then with secondary antibody (1:5000), after which an enhanced chemiluminescence(ECL) was performed using an ECL kit (Thermo Fisher Scientific Inc)

Cell scratch assay

To assess cell migration, we performed the cell scratch assay. The cells (1×10^5 cells/mL) were inoculated in six-well plates and cultured to a more than 95% confluent degree. Scrape the wound with the tip of a 10μl plastic pipet. After washing with PBS 3 times, the cells were cultured with the serum-free medium. The images were taken with the phase contrast microscope at 0 h and 48 h respectively. The scratch spacing was measured with Image J software.

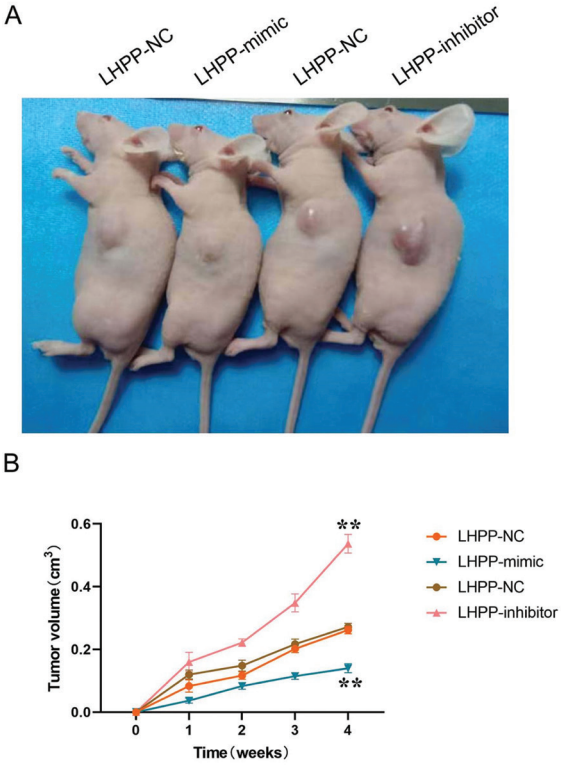
Cell invasion assay

Differently treated cells were suspended (1×10^5 cells/mL) and inoculated with serum-free medium (200μl) into a 24-well Transwell migration chamber (Corning, USA) with an 8μm pore membrane. Matrigel Matrix gel(corning 356234) was added to the top of the Transwell chamber with an 8μm pore membrane at the bottom. On the second day, it was stained with 0.1% crystal violet, fixed with 4% paraformaldehyde, and detected under the microscope. The invading cells were imaged using the microscope, and the number of cells was counted in five independent fields of view. The statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc.). The results were averaged over three independent experiments.

Statistical analysis

The experimental results were repeated three times and expressed as mean ± standard error. All statistical data were calculated using GraphPad Prism 6 software. One-way analysis of variance (ANOVA) was performed comparison test to examine the differences between multiple groups. $P < .05$ was considered statistically significant.

Figure 1. LHPP significantly inhibited the development of GC. A: Representative diagram of isolated tumors of mice in each group. B: Tumor growth curves of each group.



RESULTS

Effect of LHPP on Tumor Growth

To investigate the effect of LHPP on tumor progression in vivo, the HEK 293T cells transfected with LHPP-mimic and LHPP-inhibitor were injected into nude mice. Statistical results showed that LHPP significantly inhibited GC tumor development, the LHPP could inhibit the growth of gastric cancer in mice after the mean tumor volume of the LHPP-mimic group was compared with that of the NC group (P < .05, Figure 1), and inhibiting LHPP could promote the growth of tumors in mice after the mean tumor volume of LHPP-inhibitor group was compared with that of NC group (P < .05, Figure 1).

Changes in Protein Expression

To detect the effects of LHPP on NOX2, NOX4 and p-Src and p-ERK protein expression in mice, we performed Western blot experiments. Western blot results indicated that LHPP regulates the intracellular ROS/Src-ERK signaling pathway. In the LHPP overexpression group, the expression of NOX2 and NOX4 protein was significantly decreased (*P < .05, Figure 2A, B), the expression of p-Src and p-ERK were significantly decreased (P < .001, Figure 2A, B), and the expression of MMP-9 was significantly decreased (P < .05, Figure2A, B). The expression of LHPP was down-regulated in GC tissues, the expression level of ROS-related proteins NOX2 and NOX4 were enhanced (P < .05, Figure 2A, B), p-Src and p-ERK protein expressions were enhanced (P < .05, Figure 2A, B), and MMP-9 protein expression level was enhanced (P < .001, Figure 2A, B).

Figure 2. Expression levels of NOX2, NOX4, p-Src and p-ERK proteins in tumor tissues. A: Protein expression of NOX2, NOX4, and p-Src, p-ERK in tumor tissues of mice. B: Statistical results of NOX2, NOX4, and p-Src, p-ERK protein expression in tumor tissues of mice. (*P < .05, LHPP-mimic vs NC. **P < .001, LHPP-mimic vs NC. ***P < .05, LHPP-mimic vs NC. #P < .05, LHPP-inhibitor vs NC. ##P < .05, LHPP-inhibitor vs NC. ###P < .001, LHPP-inhibitor vs NC.)

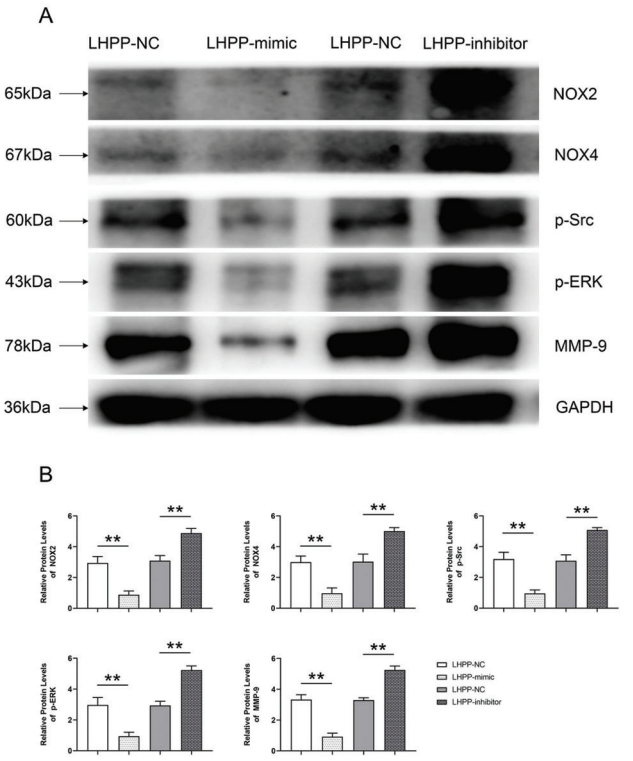


Figure 3. Effect of LHPP on 48h migration of AGS/MKN-45 cells. Representative images of cell scratch assay of AGS and MKN-45 cells transfected with LHPP-mimic or LHPP-inhibitor. The bar chart on the right shows the relative ratio of cell scratches.

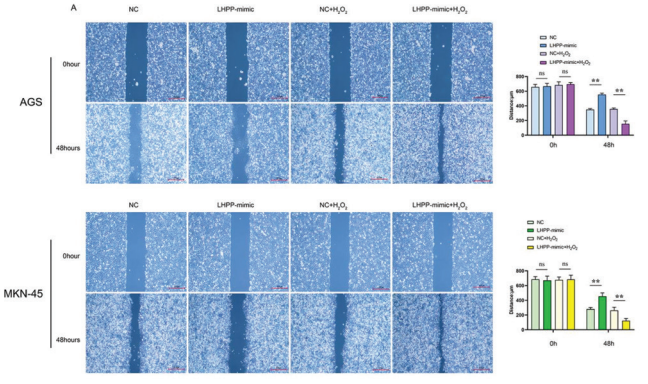


Figure 4. LHPP inhibited the invasion of GC cells. Representative images of AGS and MKN-45 cell invasion assay transfected with LHPP-mimic or LHPP-inhibitor. The statistical graph on the right shows Transwell counting the number of cells passing through the membrane by crystal violet staining. (*P < .05, AGS-LHPP-mimic vs NC. *P < .05, MKN-45-LHPP-mimic vs NC.)(**P < .05, AGS-LHPP-mimic vs NC. **P < .05, MKN-45-LHPP-mimic vs NC.)

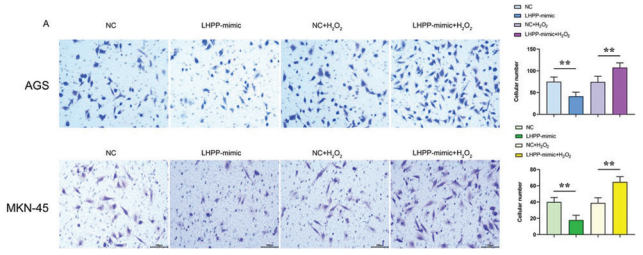


Figure 5. H_2O_2 reversed the regulation of GC cell proteins by LHPP. A: H_2O_2 reversed the regulation of GC cell proteins by LHPP. (*P < .05, LHPP-mimic vs NC. **P < .001, LHPP-mimic vs NC. ***P < .05, LHPP-mimic vs NC.) B: H_2O_2 reversed the migration effects of LHPP on GC cells. C: H_2O_2 reversed the invasion effects of LHPP on GC cells.

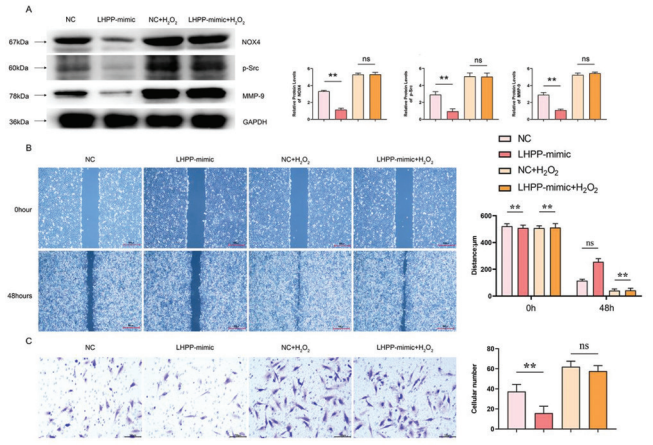
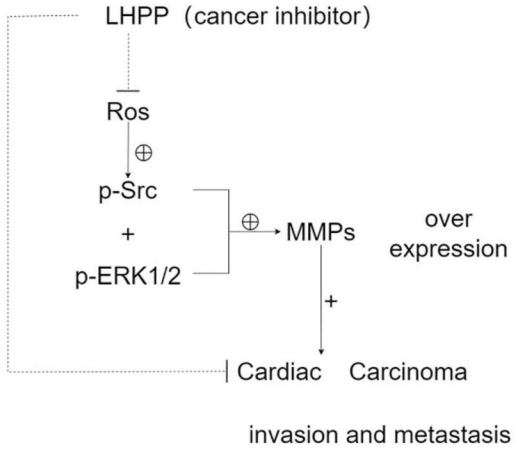


Figure 6. LHPP can delay the progression of GC by regulating the ROS-mediated Src-ERK pathway



LHPP inhibits the spreading of GC cells

Wound healing and Transwell assays were performed to investigate whether LHPP affected GC cell migration. The results indicated that LHPP inhibited cell migration and invasion by regulating the ROS/Src-ERK signaling pathway. The scratch assay results showed that the relative ratio of scratch spacing in the LHPP overexpression cells was greater than that in the NC group at 48 hours (Figure 3). The relative ratio of cell scratch spacing in the LHPP inhibitor group was lower than that in the NC group at 48 h, and LHPP reduced cell migration ability (Figure 3). Transwell assay results showed that the overexpression of LHPP inhibited the invasion of GC cells: The number of cell invasions was significantly reduced in the LHPP overexpression group compared with the NC group (P < .05, P < .05 Figure 4). And the LHPP inhibition group showed the opposite result: The number of cell invasions increased significantly in the LHPP inhibitor group compared with the NC group (P < .05, P < .05 Figure 4).

ROS reversed the regulation of GC cell protein by LHPP

A corrective experiment was conducted to investigate whether LHPP is regulated by oxidative stress. The expression levels of NOX2 and NOX4 proteins in the LHPP overexpression group were significantly decreased (P < .05, Figure 5A), the expression of p-Src and p-ERK was significantly decreased (P < .05, Figure 5A), and the expression of MMP-9 was significantly decreased (P < .05, Figure 5A). There was no difference in the expression of NOX2, NOX4, p-Src, p-ERK and MMP-9 proteins in LHPP overexpression group after 1 μM H_2O_2 stimulation (P < .05, P < .001, Figure 5A)

ROS reversed the inhibition of GC cell spreading by LHPP

To further explore the cell migration and invasion regulated by LHPP through oxidative stress, we went on to do cell scratch and Transwell invasion experiments. The cell scratch results showed that the scratch spacing of LHPP overexpression was larger than that of the NC group at 48 h, and there was no difference between the scratch spacing of the LHPP overexpression group and the NC group after H_2O_2 stimulation (P < .05, Figure 5B). Transwell assay results showed that LHPP overexpression inhibited GC cell invasion: the number of cell invasions was significantly reduced in the LHPP overexpression group compared with the NC group. There was no significant difference in the number of cells between the LHPP overexpression group and the NC group after H_2O_2 stimulation: There was no difference in the number of cell invasions between the LHPP overexpression group and the NC group (P < .05, Figure 5C). The above results suggest that LHPP regulates the Src-ERK signaling pathway through ROS which in turn regulates GC cell migration and invasion.

DISCUSSION

Gastric cardia cancer (GCC) occurs at the top of the stomach, near the junction of the esophagus, and is one of the

leading causes of cancer-related death worldwide.[12] The main finding of our study is that LHPP delays GC progression by modulating the ROS-mediated Src-ERK pathway. In this study, we focused on LHPP. Research suggests that LHPP may be a tumor suppressor in multi-system tumors: Hindupur SK's study demonstrated that LHPP is a new tumor suppressor in hepatocellular carcinoma,[13] Sun W's study demonstrated that LHPP inhibits the development of thyroid cancer by regulating AKT/AMPK/mTOR signaling,[14] and Wang X's study demonstrated that LHPP down-regulates the pyruvate kinase subtype M2 (PKM2) in glioblastoma, thereby inhibiting the growth of glioblastoma.[15] Our results compare the biological functions of LHPP in GC with those in other cancer types, and reveal its specific role in cell proliferation, apoptosis, and migration. This helps to deepen the understanding of the biological characteristics of GC tumors and provides important clues for the development of targeted GC therapeutic strategies.

Matrix metalloproteinase-9 (MMP-9) regulation plays a key role in inflammation response, angiogenesis, wound healing, and differentiation of human embryonic stem cells.[16,17] However, the high expression of MMP-9 may increase tumorigenesis and metastasis.[18,19] At the same time, increased production of reactive oxygen species (ROS) has been detected in various cancers. ROS can activate pro-tumor signaling, enhance cell survival and proliferation, and drive DNA damage and genetic instability.[20] In addition to the respiratory chain, the main source of ROS in cells is NADPH oxidase (NOX). NOX is an important cellular source of ROS production and is involved in NADPH superoxide production under many pathological conditions. The physiological functions of NOX play various roles in cell signaling, gene expression regulation, cell growth, differentiation, and death. ROS activation of the MMP-9 signaling pathway has been identified in various cells.[18,21] The results of this study are consistent with those of previous studies. Through our study, we provide new insights into the specific roles of MMP-9 and ROS in GC and further elucidate the importance of LHPP in regulating these molecular pathways.

In this study, we explored the potential role of LHPP in developing GC. We investigated its mechanism of delaying tumor progression by regulating the ROS-mediated Src-ERK pathway. Through in vitro experiments and in vivo models, further studies have shown that LHPP affects the activity of the Src-ERK pathway by regulating ROS levels, thus affecting the proliferation, migration, and invasion of GC. The results show that the overexpression of LHPP reduces the expression of ROS-related proteins in GC cells, and the overexpression of ROS under H2O2 counteracts the overexpression of LHPP and inhibits the phosphorylation of the Src-ERK signaling pathway. This suggests that LHPP may influence GC progression in a ROS-dependent manner. Our study further revealed that ROS are involved in tumor growth and metastasis through activation of the Src-ERK pathway in GC. Regulation of ROS levels by LHPP may have affected the activation status of the Src-ERK signaling pathway, which in turn affected the proliferation and metastatic ability of tumor cells. This finding emphasizes the important role of ROS-mediated signaling pathways in GC progression and reveals the criticality of LHPP as a regulator. These findings provide new ideas and possibilities for the design and implementation of future GC therapeutic strategies.

In our study, although new insights were gained regarding the important role of LHPP in GC and its Src-ERK pathway mediated through ROS, there are some limitations that require further attention. First, the sample size was relatively limited, which may have restricted the comprehensiveness of our analysis of the association between LHPP and GC development. Second, our study mainly relied on cell lines and animal models, and these model systems may have limitations for simulating the complex pathophysiological processes of human gastric cancer. These limitations may affect the interpretation of our findings, and thus future studies require clinical validation with larger sample sizes and modeling system studies that are more relevant to humans.

In terms of future research directions, we should focus on the following aspects: first, we need to study the interactions of LHPP with other signaling molecules in greater depth, especially with other key factors in the ROS-mediated pathway such as AKT and MAPK. This will help to reveal a more comprehensive mechanism of LHPP's role in tumor development. Second, we should further explore the role of LHPP in regulating the tumor immune microenvironment and tumor stem cells to deepen our understanding of the function and mechanism of LHPP in GC.

Targeting the incorporation of therapeutic paradigms and providing new therapeutic avenues, our findings provide important clues for the development of new therapeutic strategies. For example, drugs targeting the ROS-mediated Src-ERK pathway may become a new option for future GC treatment, whereas targeting LHPP regulation may become a strategy to intervene in the tumor microenvironment and enhance the efficacy of immunotherapy. In clinical practice, we may consider augmenting existing therapies by activating LHPP or inhibiting ROS-mediated pathways, or developing novel targeted drugs against these pathways.

In conclusion, this study reveals the mechanism of LHPP in delaying the progression of GC by regulating the ROS-mediated Src-ERK pathway. This research provides new therapeutic strategies for the treatment of GC and broadens our understanding of tumor development and cancer treatment. However, our results still need further verification and an in-depth study of molecular mechanisms. In the future, we will further explore the specific mechanism of action of LHPP and its interaction with ROS, Src-ERK pathway, and other related signaling molecules, to provide a more in-depth theoretical and experimental basis for diagnosing and treating GC.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest related to this study.

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None

AUTHOR CONTRIBUTIONS

Xuebing Song and Yuchuan Ma designed the experiments and flow of work. Xuebing Song, Yuchuan Ma, Wulin Zhang and Tao Jiaperformed the experiments, statistics and prepared the manuscript. The manuscript was written and reviewed by all the authors.

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