

# Cuproptosis-Related Gene CDKN2A as a Molecular Diagnostic Target in Gastric Carcinoma Based on Transcriptomic Data

Guo Chen<sup>1</sup>, Wenli Zhang<sup>1</sup>, Di Wei<sup>2</sup>, Zeng Li<sup>3</sup>, Jun Lu<sup>2</sup>, Wenying Liu<sup>4</sup>, Lei Wang<sup>5,\*</sup> and Zifan Lu<sup>1,\*</sup>

<sup>1</sup>Translational Medicine Center, Shanxi Provincial People's Hospital, Xi'an, 710068, China

<sup>2</sup>Department of Urology, Xijing Hospital, The Fourth Military Medical University, Xi'an, 710032, China

<sup>3</sup>Department of Gastrointestinal Surgery, Xijing Hospital, The Fourth Military Medical University, Xi'an, 710032, China

<sup>4</sup>Department of Cardiovascular Medicine, Xijing Hospital, The Fourth Military Medical University, Xi'an, 710032, China

<sup>5</sup>Department of Gastrointestinal Surgery, General Hospital of Ningxia Medical University, Ningxia, 750002, China

**\*Corresponding Authors:** Zifan Lu, Translational Medicine Center, Shanxi Provincial People's Hospital, Xi'an, 710068, China, E-mail: pharmluzifan@163.com

Lei wang, Department of Gastrointestinal Surgery, General Hospital of Ningxia Medical University, Ningxia, 750002, China, E-mail: ohney@163.com

**Citation:** Guo Chen, Wenli Zhang, Di Wei, Zeng Li, Jun Lu et al. (2024) Cuproptosis-Related Gene CDKN2A as a Molecular Diagnostic Target in Gastric Carcinoma Based on Transcriptomic Data, Technolock Mol and Cell Biochem 2: 1-12

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## Abstract

Gastric Carcinoma (GC) ranks as the third leading cause of cancer-related mortality globally. Therefore, it is crucial to identify more effective therapeutic targets. Cuproptosis is a newly discovered programmed cell death dependent on overload copper-induced mitochondrial respiration dysregulation. We speculated this regulatory cell death (RCD) mechanism might serve as a potential prognostic predictors and therapy for GC patients. Our findings indicated that the expression levels of 11 genes, namely FDX1, LIPT1, DLD, DLAT, PDHA1, MTF1, GLS, CDKN2A, SLC31A1, and ATP7B, were significantly increased in GC tissues compared to normal tissues ( $P < 0.05$ ). Three genes (CDKN2A, GLS, and MTF1) have predictive value for the prognosis. Patients with lower CRG (Cancer-related Gene) scores exhibited increased immune cell infiltration and expression of immune checkpoint markers. Via molecular docking, saquinavir, folic acid, and growth hormone (GH)-releasing peptides (GHRP) had the best binding affinity with CDKN2A, GLS, and MTF1 protein. CCK8, invasion and migration assay demonstrated that saquinavir could inhibit the proliferation, invasion, and migration of gastric carcinoma cells in vitro. Animal experiment showed that saquinavir treated group had smaller volume and weight tumors. The findings from our study underscore the crucial role of cuproptosis in modulating the advancement, patient outcomes, infiltration of immune cells, and the effectiveness of immunotherapy. CDKN2A as the potential target for gastric carcinoma showed the anticancer effect in vitro and vivo.

**Keywords:** Cuproptosis; Gastric Carcinoma (GC); Tumor Microenvironment; Biomarkers; Prognostic Analysis

**Abbreviations:** ACTH, adrenocorticotrophic hormone; CRGs, cuproptosis-related genes; CRG, Cancer-related Gene; CRH,

corticotropin-releasing hormone; Cu, Copper; DALYs, Disability-Adjusted Life Years; FP, free-progression survival; GC, Gastric Carcinoma; GHRP, growth hormone (GH)-releasing peptides; PFI, progression-free interval; PPS, post-progression survival; RCD, regulatory cell death; OS, overall survival

## Introduction

According to recent reports, worldwide, GC which was responsible worldwide for one in 13 deaths, was the fifth most common cancer in 2020 [1], and was the third leading cause of cancer-related death [2]. GC is a heterogeneous disease, and the lack of GC screening techniques hampers its early diagnosis and leads to poor five-year survival rates [3]. Notably, the prognosis of GC has not improved in recent years [1].

Despite the significant progress in our understanding of the molecular causes of GC, the exact mechanism underlying its development remain unknown. A deeper understanding of novel gene candidates is crucial for better understanding the molecular mechanism of pathogenesis, which would improve patient survival [3]. Therefore, there is an urgent need to identify more convincing and suitable biomarkers for GC.

Copper (Cu) is an essential cofactor for all organisms. However, it becomes toxic if its concentration exceeds a threshold that is maintained by evolutionarily conserved homeostatic mechanism [4]. Tsvetkov et al. [5] recently identified a novel Cu-induced cell death pathway known as cuproptosis, which was distinct from all other known mechanisms of RCD, including apoptosis, ferroptosis, pyroptosis, and necroptosis [6]. The investigators found that Cu-induced cell death is mediated by an ancient mechanism, namely protein lipoylation. Previous research has established a link between Cu homeostasis and various types of cancer [7-10]. Despite this, there remains the gap in our understanding of the connection between the recently described process of cellular proptosis and the development of GC, as well as its influence on the tumor immune microenvironment and response to immunotherapy. Consequently, it is essential to investigate the biological and pathological activities related to cuproptosis, clarify the mechanisms by which cuproptosis affects GC progression, and identify potential targets for its diagnosis and effective treatment. Such insights are vital for the early detection, diagnosis, and management of GC. In our present study, we intended to comprehensively investigate the molecular alterations and clinical relevance of cuproptosis-related genes (CRGs) in GC through accessing and analysing a public health database. Next, we explored the anticancer effects of CRGs as potential targets both in vivo and in vitro. Our analysis highlights the importance of CRGs in GC development and lays the foundation for the therapeutic application of cuproptosis regulators in the treatment of GC.

## Materials and Methods

### Data Preprocessing

RNA sequencing data of 443 patients diagnosed with STAD were extracted from TCGA dataset, available at <https://portal.gdc.cancer.gov/projects/TCGA-STAD>. Normal tissue samples were procured from the GTEx data portal at <https://www.gtexportal.org/home/datasets>. To further confirm the expression levels of CRGs, we downloaded and utilized datasets GSE19826 and GSE29272 from the Gene Expression Omnibus database, accessible at <https://www.ncbi.nlm.nih.gov/geo/>.

The protein expression level of CRGs in tumors compared to normal tissue was obtained from the Human Protein Atlas database (<https://www.proteinatlas.org/>).

The web-based resource at <http://ualcan.path.uab.edu/analysis.html>, known for its extensive capabilities, was employed to evaluate the expression patterns and prognostic significance of DEGs. A Student's t-test was utilized to calculate the corresponding p-values.

We used the cBioPortal ([www.cbioportal.org](http://www.cbioportal.org)), a comprehensive web resource, can visualize and analyze multidimensional can-

cer genomics data, to analyze the mutation and CNV data of all CRGs in 478 total GC samples (TCGA, PanCancer Atlas).

### Functional Enrichment Analysis of CRGs

To functionally annotate CRGs identified by the aforementioned comparison groups, annotation and visualization of GO terms was used by GO enrichment analysis and metaspice. The overlaps between differently expressed gene lists of GO terms were performed by enrichment analysis circle diagram. The DEGs were then introduced into the FunRich (functional enrichment analysis tool) (<http://www.funrich.org/>) for KEGG pathway analysis.

To further elucidate the functions of the potential targets, we conducted a functional enrichment analysis. This involved examining the GO annotations for the targets to identify their biological processes and also enriching for KEGG pathways. For visual representation, boxplots were created using the ggplot2 package in R software.

### Genemania

GENEMANIA (<http://genemania.org/search/>) was used to construct a gene-gene interaction network for DEGs to evaluate the functions of these genes.

### Kaplan–Meier Plotter Database Analysis

The Kaplan–Meier Plotter database (<http://kmplot.com/analysis/>), a comprehensive online platform offering survival analysis of 54,675 genes in 21 various tumor types. We executed analyses for overall survival (OS), free-progression survival (FPS) and post-progression survival (PPS) of CRGs in GC.

### CRGs Mutations and Prognosis

cBioPortal (<https://www.cbioportal.org>) can be used to explore, visualize, and analyze multidimensional cancer genome data [30].

### Analysis of Correlation with Immune Infiltration

Tumor Immune Estimation Resource (TIMER <http://timer.comp-genomics.org/>) is a data source for comprehensive analysis of tumor-infiltrating immune cells. The Immune infiltration cells include Neutrophils, Macrophages, B cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and Dendritic cells, etc. Which can effectively predict the prognosis of patients. We have studied the correlation between CRGs expression and these immune infiltrating cells by employing TIMER.

### RNA Extraction and Reverse Transcription-Quantitative PCR (RT-qPCR)

Total RNA was extracted with Trizol reagent (Invitrogen, USA), and then reverse transcription was performed using the HiScript II Q RT SuperMix kit for qPCR (Vazyme, R223) according to the manufacturer's instructions. qPCR performed using the ChamQ SYBR qPCR Master Mix kit (Vazyme, Q311) in accordance with the manufacturer's instructions. The primers for all PCR primers, and their internal reference sequences were designed using Primer 5. The thermocycling protocol consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. All amplifications and detections were performed using a real-time PCR machine (Roche, LightCycler<sup>®</sup>96). The expression level of each target gene was determined using  $\beta$ -actin as the normalization control. Relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method [7]. Every experiment was repeated at least three times independently. The following primers were used:

CDKN2A Forward, 5'-3': GATCCAGGTGGGTAGAAGGTC and reverse 5'-3': CCCCTGCAAACCTTCGTCCT,

GLS forward 5'-3' AGGGTCTGTTACCTAGCTTGG and reverse 5'-3': ACGTTCGCAATCCTGTAGATTT,

MTF1 forward 5'-3': CACAGTCCAGACAACAACATCA and reverse 5'-3': GCACCAGTCCGTTTTTATCCAC,

$\beta$ -Actin forward 5'-3': CATGTACGTTGCTATCCAGGC, and reverse 5'-3': CTCCTTAATGTCACGCACGAT.

### Western Blotting

Total protein was extracted using RIPA Lysis Buffer (Beyotime, Guangzhou, China), and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, USA) according to the manufacturer's instructions. Then, a western blot assay was performed as previously described. The primary antibodies used are listed in The following antibodies were used: CDKN2A (1:1000; Abways, CY8312) GLS (1:1000; Aways, CY5719), MTF1(1:1000; Proteintech,25383-1-AP) and  $\beta$ -actin (1:4000, Proteintech,20536-1-AP). Subsequently, the membranes were immunoblotted with secondary antibody (1:10000, Proteintech, SA00001-2). The protein expression was visualized by enhanced chemiluminescence (Millipore, USA). Images were captured using a ChemiDoc XRS imaging system (Bio-Rad, USA), and Quantity One image software was used for the densitometry analysis of each band;  $\beta$ -actin was used as an internal loading control.

### Molecular Docking

In NCGC Pharmaceutical Collection database, 7929 FDA-approved drugs were selected for drug screening. Download the crystal structures numbered 7OZT (CDKN2A), 3voz (GLS), AF-Q14872-F1-model (MTF1) from the PDB database (<https://www.rcsb.org/>). Using Discovery Studio 2019 software to prepare for molecular docking. To remove the water molecules and protein structure heteroatomic processing, as well as proton and hydrogenation atomic, finally using DS software for protein binding sites. The Discovery Studio Libdock program was used for molecular docking. Libdock is a fast molecular docking tool for screening of large libraries of compounds. A higher score means a closer interaction between molecules and proteins, the scores were sorted according to Libdock score. The top 20 Libdock score compounds were selected for the next step of research.

First, the top 20 ligands required for molecular docking were prepared by AutoDock Vina software (<http://vina.scripps.edu/>). For the target protein, the crystal structure of the target protein was obtained from the PDB database (<https://www.rcsb.org/>), including removal of hydrogenation, modified amino acids, optimization of energy, adjust the force field parameters, download ligands (<https://pubchem.ncbi.nlm.nih.gov/>) which meet low energy conformation of ligand structure. Finally, the target structure was docked with the active component structure. Using PyRx software (<https://pyrx.sourceforge.io/>) internal vina (<https://pyrx.sourceforge.io/>) for docking, its Affinity (kcal/mol) value that represents a combination of the combination of ability. The lower the combining ability, the more stable the ligand binds to the receptor.

### Cell Proliferation Capacity

The cell proliferation capacity was evaluated using the CCK-8 assay. The NCI-N87 and MKN-45 cells were diluted to  $5 \times 10^3$  per well before being plated into a 96-well plate, The cells were incubated for 24h at 37°C in an atmosphere comprising 5% CO<sub>2</sub>. various concentrations of saquinavir, folic acid, GHRP were added and cultivated with cells for an additional 48 h. The CCK-8 reaction solution was added according to the instructions, and the OD at 450 nm (denoted as A450) was measured. Each experimental condition was replicated three times to ensure reliable results. All experiments were performed with mycoplasma-free cells.

### Transwell Migration and Invasion Assays

The transwell chamber (Millipore, USA) was used for migration and invasion assays. To evaluate cell invasion, the chamber was pre-coated with Matrigel (Corning NY, USA). Matrigel was diluted in pre-cooled culture medium according to the manufacturer's instructions. Then, cells ( $2.5 \times 10^4$  cells) were seeded in the upper chamber without serum, saquinavir was added into the upper chamber, while medium with 10% serum was added to the lower chamber for 48 h, then fixed with 4% paraformaldehyde.

hyde for 10 min and stained with crystal violet at room temperature. The migration or invasion cells were photographed by a light microscope (Leica, Germany). Five random fields (400×) were selected and the number of migration or invasion cells were counted. All samples were conducted with three repeats. The migration assay is the same with invasion assay excepting no matrigel was used.

### Xenograft Transplantation in Vivo

Specific-pathogen-free (SPF) 4-week-old male BALB/c-nu mice were purchased from Beijing Vital River Laboratory Animal Technology with SPF-grade rearing environment. Animals were housed in a 12/12 h of light/dark cycle at 22°C with 45-55% humidity and adaptively provided with free access to water and food. The animal studies were approved (approval no. IACUC-20241372) by the Laboratory Animal Welfare and Ethics Committee of the Forth Military Medical University (Xi'an, China). Cells of the NCI-N87 cells were adjusted to  $5 \times 10^6$  and suspended in 200  $\mu$ L PBS, followed by inoculation under the dorsal skin of the nude mice. The tumor size was recorded every three days, and the tumor volume was calculated according to the formula  $V=0.5 \times a \times b^2$ . The experimental group was administered saquinavir via intraperitoneal injection, at a dose of 600 mg/kg, in accordance with previous research for 2 weeks. The control group was administered PBS. All animals were sacrificed after 21 days, the tumors excised and weighed. The tumor tissue, the heart, liver, spleen, lung, and kidney were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5  $\mu$ m thick paraffin sections. There were 6 mice in the intraperitoneal group and 7 mice in the saquinavir group. In the saquinavir group, 1 tumor was not successfully implanted. During the experiment, the mice were also euthanized if there was a tumor larger than 2 cm. We observe the nude mice every day to ensure adequate food and water, as well as the breeding environment was up to standard. We used a small animal anesthesia machine for experimental operation. Inhalation anesthesia was administered with isoflurane which the induction concentration was 2% to 2.5%, and the maintained concentration was 1% to 2.5%. Each nude mouse was kept in a single cage. The maximum tumor volume should not exceed 2000  $\text{mm}^3$ , and each tumor diameter should not exceed 2 cm. The euthanasia is chosen to apply the carbon dioxide asphyxiation. The volume of carbon dioxide in the box was increased by 30% per minute. When we observe that the nude mouse does not breathe for 5 minutes, we can confirm that it is dead.

### Statistical Analysis

The relationships between variables were assessed using either Spearman or Pearson correlation tests. To compare differences in gene expression between adjacent normal and tumor samples, the Wilcoxon test was employed. Survival data were analyzed using Kaplan-Meier estimations and univariate Cox proportional hazards regression models. R software (Version 4.1.2) was utilized for the statistical analysis of bioinformatics outcomes (with statistical significance set at  $P < 0.05$ ).

## Results

### CRGs that Exhibit Varied Expression Levels in Gastric Tissue Compared to Normal Biopsies

As mentioned earlier, a set of 12 genes (FDX1, LIAS, LIPT1, DLD, DLAT, PDHA1, PDHB, MTF1, GLS, CDKN2A, SLC31A1, and ATP7B) were identified as being linked to the process of cuproptosis [5]. To verify the role of these genes associated with cuproptosis in GC, we analyzed and compared the expression profiles of the 12 CRGs in both tumor and normal tissue samples, as obtained from the TCGA and GTEx databases. Our analysis included 470 samples of gastric tissue and 1809 samples of normal tissue. As a result, we observed that the expression levels of all 12 genes varied significantly between gastric and normal biopsies (Fig. 1A, B; Supplementary Table 1). Among these, the expression of 11 genes (FDX1, LIPT1, DLD, DLAT, PDHA1, MTF1, GLS, CDKN2A, SLC31A1 and ATP7B) was increased, whereas the expression of one gene (LIAS) remained unchanged in STAD samples (Figure 1A). Furthermore, we examined the relationships between the expression levels of various genes and discovered several strong correlations (Figure 1B). For example, a high positive correlation was observed between the expression of DLD and DLAT ( $r = 0.631$ ,  $P < 0.001$ ) (Figure 1C). Subsequently, we conducted a thorough examination of the molecu-

lar properties of the CRGs using the TCGA Pan Cancer Atlas. The results showed that the top five genes—CDKN2A, ATP7B, LIPT1, GLS and DLAT were altered in 17%, 6%, 5%, 5%, 4%, and 4% of the queried GC samples, respectively (Figure 1D). Additionally, the alteration frequency of tubular pathological type showed higher than other types (Figure 1E).

### Functional Enrichment and Protein–Protein Interaction Analysis of Crgs

To demonstrate the biological functions of CRGs, relevant pathways were analyzed by GO and KEGG databases. The biological processes of the 10 CRGs mainly involved in the GO analysis were iron-sulfur cluster binding, transition metal ion transmembrane transporter activity, oxidoreductase activity, acting on the aldehyde or oxo group pf donors, NAD or NADP as acceptor, oxidoreductase complex, mitochondrial matrix, citrate metabolic process, tricarboxylic acid cycle, acetyl-CoA biosynthetic process and acetyl-CoA biosynthetic process from pyruvate (Figure 2A).

Additionally, in the KEGG pathway enrichment analysis, the 5 CRGs were largely related to Carbon metabolism, Glycolysis/Gluconeogenesis, Pyruvate metabolism, Citrate cycle (TCA cycle) and Central carbon metabolism in cancer (Figure 2B). An analysis of PPIs was conducted to study the relationships among CRGs, and it was found that DLD, DLAT, PDHA1, and PDHB emerged as central or hub genes (Supplementary Figure 1).

### Differential Expression of CRGs in Different Pathologic Stages and Histological Grades of GC

Moreover, we used the TCGA database to analyze the differential expression of CRGs in different pathologic stages and histological grades of GC. The expression of CDKN2A differed significantly between stage2, stage3 and stage 4 and normal tissues respectively (Figure 3A), GLS differed significantly between stage2, stage3, stage 4 and normal tissues (Figure 3B), MTF1 revealed no significantly differed among the different stage groups (Figure 3C). Furthermore, the expression of CDKN2A differed significantly between grade2, grade3 and normal tissues respectively (Figure 3D), GLS differed significantly between grade1, grade2, grade3 and normal tissues respectively (Figure 3E) while that of MTF1 differed no significantly among the different grade groups (Figure 3F). Within the HPA database, our investigation revealed that with the exception of MTF1, CDKN2A and GLS protein expression levels were notably elevated in GC tissues in comparison to the normal tissues (Figure 3G). Similarly, we confirmed the mRNA and protein levels of these three genes in the GC cell lines. CDKN2A and MTF1 mRNA and protein levels were overexpressed in the both cancer cell lines compared to the GES-1 Cell (Figure 3H–I). However, Fig. 3H showed that there was no difference in the mRNA expression level of GLS between GES-1 and MKN-45 cells.

### Predictive Value of CRGs for GC Diagnosis and Prognosis

We then evaluated the prognostic value of CRGs expression by Kaplan–Meier plotter. The results revealed that lower mRNA level of MTF1, LGS and CDKN2A correlated with preferable OS, FPS and PPS in GC samples, respectively (Figure 4A–I). The data suggested these three CRGs to be the potential biomarkers for predicting gastric cancer prognosis.

### Nomogram Development and Validation for GC

To simplify the use of the prognostic model in clinical practice, we incorporated four clinical and genetic characteristics from patients in the TCGA dataset and utilized the multivariable Cox regression analysis to create the nomogram. We then employed discrimination and calibration techniques for OS, progression-free interval (PFI) and disease-specific survival (DSS) outcomes (Figure 5). A nomogram integrating CRGs expression and independent clinical risk factors (age, pathological, stage, N stage and M stage) was constructed (Figure 5A–C). A worse prognosis was represented by a higher total number of points on the nomogram. Meanwhile, calibration plots were closed to the idea curve (i.e., a 45°line), showed the favorable concordance between the predicted OS, DSS or PFI and the observed OS, DSS, PFI at 1, 3 and 5 years of survival (Figure 5D–F).

### Correlation between Expression of CRGs and Immune Infiltration Levels in GC

It remains unclear if CRGs have an impact on the recruitment of immune cells within the tumor microenvironment, which

could in turn influence the prognosis of GC. TIMER database was used to estimate immune infiltration levels in GC. CDKN2A expression was negatively correlated with CD8+ T cell infiltration ( $r = -0.11$ ,  $p = 3.41e-02$ ), macrophage ( $r = -0.079$ ,  $p = 1.31e-01$ ) and dendritic cell ( $r = -0.112$ ,  $p = 3.08e-02$ ). No correlation was observed with tumor purity, B cell, CD4+ T cell, neutrophil (Figure 6A), GLS expression was positively correlated with tumor purity ( $r = 0.041$ ,  $p = 4.3e-01$ ), B cell ( $r = 0.127$ ,  $p = 1.43e-02$ ), CD4+ T cell ( $r = 0.188$ ,  $p = 2.97e-04$ ), Macrophage ( $r = 0.077$ ,  $p = 1.41e-01$ ). GLS expression was negatively correlated with CD8+ T cell infiltration ( $r = 0.1888$ ,  $p = 2.97e-04$ ) and neutrophil ( $r = -0.049$ ,  $p = 3.46e-01$ ). No correlation was observed with dendritic cell ( $r = 0.022$ ,  $p = 6.77e-01$ ) (Figure 6B). MTF1 expression was positively correlated with B cell ( $r=0.057$ ,  $p = 2.74e-01$ ), CD4+ T cell ( $r = 0.194$ ,  $p = 1.94e-04$ ), macrophage ( $r = 0.128$ ,  $p = 1.34e-02$ ), neutrophil ( $r = 0.134$ ,  $p = 9.61e-03$ ), dendritic cell ( $r = 0.136$ ,  $p = 8.74e-03$ ). MTF1 expression was negatively correlated with tumor purity ( $r = -0.08$ ,  $p = 1.17e-01$ ). No correlation was observed with CD8+ T cell infiltration ( $r = 0.015$ ,  $p = 7.75e-01$ ) (Figure 6C).

## Molecular Docking

In order to confirm if CDKN2A, GLS, MTFI can become a potential therapeutic target for gastric cancer, we employed the molecular docking to mimic the interaction between the drugs and these three proteins. Fast molecular docking screening protein was performed using Libdock score (Supplementary Table 1, 3, 5). Using PyRx software for finally docking and the top 5 binding affinity score compounds were selected (Supplementary Table 2, 4, 6). From 7929 FDA-approved drugs, we found that saquinavir, folic acid, and GHRP were respectively docked into the CDKN2A, GLS, MTFI, which were demonstrated the best binding affinity (Figure 7A-F).

## CDKN2A as the Therapeutic Target for Gastric Cancer was Proved the Good Anticancer Effect

Cells viability decreased as saquinavir concentration increased both in NCI-N87 and MKN-45. When the saquinavir concentration was 20 $\mu$ M, the cells viability was 91 % and 77 % both in NCI-N87 and MKN-45. When the saquinavir concentration was 70 $\mu$ M, the cells viability was 9 % and 21 % both in NCI-N87 and MKN-45. However, in folic acid and GHRP, when the concentration was 70 $\mu$ M, the cells viability was still from 83 % to 91 % (Figure 8A-C). Hence, saquinavir which targeted the CDKN2A was chose for further investigation in our study.

Migration and invasion are the key regulation processes in the progression of gastric cancer. Because of MKN-45 cells was partial suspended growth, we select NCI-N87 cells for migration and invasion assay. Figure 8D, E showed the results of a migration assay for a treatment period of 48 h. Saquinavir inhibited the migration ability of NCI-N87 cells in a minimum effective inhibitory concentration. The invasive inhibition ability of Saquinavir was proved using a Matrigel invasion assay (Figure 8D, F).

## CDKN2A Inhibitor Saquinavir Demonstrated the Anticancer Effect in Vivo

To investigate the CDKN2A inhibitor saquinavir whether could inhibit the gastric cancer cells in vivo, an NCI-N87 cell GC murine xenograft model was established. The nude mice received saquinavir with intraperitoneal injection for 2 weeks after NCI-N87 cells inoculation. The results showed that the tumors treated by saquinavir was lighter. The weight of tumors in treated groups and control groups were  $0.16 \pm 0.12$  g and  $0.45 \pm 0.13$  g, respectively. The volume of tumors was  $400.9 \pm 340.4$  mm<sup>3</sup> and  $99.19 \pm 158.1$  mm<sup>3</sup> (Figure 9A-C). The immunohistochemistry showed that the expression of CD31, Ki-67, CDKN2A were reduced in treated group (Figure 9D). To explore the acute toxicity of saquinavir, the heart, liver, spleen, lung, and kidney were excised for hematoxylin-eosin staining (Figure 9E). The results indicated that there was no acute injure in these organs.

## Discussion

In summary, although there has been a downward trend, GC continues to be a significant contributor to cancer-related mortality. While early detection through screening is crucial for high-risk groups, addressing the prevalence of *Helicobacter pylori* infection and other established risk factors is essential for GC prevention. Monitoring the incidence rate among younger individu-

als is necessary to determine if this upward trend will persist. It is imperative that research and governmental efforts concentrate on implementing preventive strategies that can alter the prevalence of risk factors, thus providing long-term benefits to public health. It is projected that in 2020, GC will account for 770,000 fatalities and 1.1 million new cancer cases globally. Moreover, by 2040, the number of GC-related deaths is expected to rise to approximately 1.3 million, with roughly 1.8 million new diagnoses [59].

China has the highest incidence, mortality, and Disability-Adjusted Life Years (DALYs) rates for gastric cancer globally [8]. Hence, a thorough comprehension of the genetic context and the tumor microenvironment is crucial for the prevention, therapy, and prognostic assessment of GC. Cuproptosis, a novel form of RCD, is distinct from apoptosis, ferroptosis, and necroptosis, and relies on mitochondrial respiration [5]. In our study, we investigated the prognostic significance of CRGs expression in GC, given its unclear role in tumor development as a form of RCD. Our investigation revealed that 11 of the 12 CRGs exhibited increased expression, whereas 1 gene showed no significant difference in expression between GC and normal tissues. Functional analyses exhibited that pathways related to TCA cycle were enriched, and the CRGs were also proven to be associated with the pathologic stages and histological grades of GC. Using the Kaplan-Meier Plotter database, we found that higher expression of CRGs in GC correlated with reduced OS, DSS, and PFI. No prior studies have explored the relationship between CRG expression and GC progression, suggesting that CRGs may serve as prognostic markers for GC. In this study, the prognostic score was formulated using three CRGs: CDKN2A, GLS, and MTF1. CDKN2A, cyclin-dependent kinase inhibitor 2A, OMIM 600160, is a well-known tumor suppressor gene that encodes p16INK4A and p14ARF proteins, which play a crucial role in cell cycle regulation. The expression of CDKN2A is closely linked to the development of various types of tumors through its involvement in cell cycle control [9-12].

In cancer, the two GLS isozymes exhibit contrasting functions in tumor development. GLS is associated with tumor growth and malignancy, being controlled by the c-MYC oncogene, while GLS2 generally exhibits tumor-suppressive properties and is governed by the p53 protein [13-16]. GLS has been found to be consistently overexpressed in various types of cancers, including breast cancer [17-19], prostate cancer [20], colorectal cancer [21, 22], lung cancer [23]. Rapidly growing malignant cells have elevated mRNA levels and enhanced GLS protein expression [16, 24-26] and GLS enzymatic activity correlates with poor disease outcome in liver, lung, colorectal, breast and brain tumors [16, 23, 27-29]. MTF1, a zinc finger transcription factor, enhances cell survival by activating targets like metallothionein (MT1), MMPs, ZnT-1, and ZIP-1, which are involved in metal binding and zinc regulation [30, 31]. In breast, lung, and cervical cancers, MTF1 expression is increased [32]. MTF1 is elevated in colorectal cancer and linked to copper balance [33]. Our study has multiple advantages. We are the first team to develop a prognostic model on the basis of CRGs in GC.

We discovered a hidden connection between CRGs levels and immune cell infiltration, with CDKN2A expression showing an inverse relationship with DCs and CD8<sup>+</sup> T cells. GLS expression was negatively correlated with CD8<sup>+</sup> T cell infiltration. It is widely recognized that DCs are the most potent antigen-presenting cells, activating CD8<sup>+</sup> T cells through cross-priming and subsequently triggering antitumor immunity. Presence of CD8<sup>+</sup> T cells in ovarian cancer is associated with prolonged survival [34]. Our findings thus highlight the significance of cuproptosis in the TME and GC immunotherapy, offering new perspectives for immune checkpoint blockade treatment.

Our study showed that CDKN2A, GLS, and MTF1 were the potential targets for GC treatment. Then we used molecular docking to screen the drugs which could connect these proteins. We found that saquinavir demonstrated the ability inhibiting the proliferation, invasion, and migration of GC cells in vitro. In animal experiment, tumors treated by saquinavir were smaller in weight and volume. We also found that in tumors the expression of CDKN2A was reduced. These results indicated that saquinavir, which was the HIV protease inhibitor used in antiretroviral therapy, could also inhibit the GC [35]. The possible mechanism is that saquinavir could inhibit the expression of CDKN2A, which promoting the cuproptosis.

Despite our thorough examination of cuproptosis in GC and identification of potential targets for further investigation into GC



progression, this study has limitations. It is highly feasible that the emergence and progression of tumors are intricately linked not only to the microenvironment but also to the body general response. Tumors generate a variety of substances, including cytokines, immune mediators, neurotransmitters, hypothalamic hormones, and glucocorticoids. These malignancies can manipulate the central neuroendocrine and immune systems, thereby undermining the body's natural defenses against cancer[36]. For example, corticotropin-releasing hormone (CRH) is a peptide consisting of 41 amino acids, which is derived from a larger precursor, the 196-amino acid preprohormone. The principal role of CRH is to stimulate the synthesis of adrenocorticotrophic hormone (ACTH) in the pituitary gland. CRH can also be correlated with the tumorigenesis of some cancers[37], such as gastric cancer[38]. A challenging question is whether CRGs also impact the neuroendocrine mechanism. As our cancer samples were derived from retrospective TCGA and GEO database analyses, more prospective case studies are needed. Further research is essential to uncover the precise molecular mechanisms by which cuproptosis influences gastric cancer advancement.

In conclusion, achieving a comprehensive understanding of tumors, their microenvironments, and the intricate dynamics of neuroimmunology endocrine mechanisms, along with their interrelationships, is essential for developing holistic therapeutic strategies in the future.

## Disclosure

## Funding

This research was funded by grants from the Key R&D Program of Ningxia Province (grant number: 2021BEG03037), the Medical Enhancement Program (grant number: 2020SWAQ06), Incubation fund of Shaanxi Provincial People's Hospital (grant number: 2023YJY-15)

## Competing Interests

The authors declare that they have no competing interests

## Ethics Approval and Consent to Participate

All animal studies were approved by the Laboratory Animal Welfare and Ethics Committee of the fourth military Medical University (No. IACUC-20241372). Written informed consent was obtained from all patients. Registry and the Registration No. of the study/trial: N/A. Animal Studies: N/A

## Acknowledgements

The authors would like to thank the study participants and research staff for their contributions and commitment to this study

## Availability of Data and Materials

The data sets analyzed during the current study are available in the TCGA (<https://portal.gdc.cancer.gov/>), accession numbers TCGA-STAD, STAD-FPKM; GEO repository (<https://www.ncbi.nlm.nih.gov/geo/>), accession numbers GSE19826 and GSE29272

## Authors' Contributions

Conceptualization, GC, LW and ZFL; Data curation, DW, JL; Formal analysis, WYL; Funding acquisition, GC, LW and ZFL; Investigation, ZL; Methodology, GC, WLZ and WYL; Resources, ZL; Software, WLZ; Supervision, GC and LW; Validation, ZL;

Writing – original draft, JL and DW; Writing – review & editing, GC. All authors reviewed the manuscript. All authors read and approved the final manuscript

## Patient Consent for Publication

Not applicable

## Date Availability Statement

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

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