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Kaempferol Triggers Ferroptosis of Gastric Cancer Cells by the P53/SLC7A11/GPX4 Pathway Based on PCR Array and *In Vitro* Experiments

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Abstract **Objective:** This work seeks to elucidate the molecular mechanism by which kaempferol inhibits the growth of Gastric Cancer (GC) cells via the ferroptosis pathway. **Methods:** The CCK8 detection was used to assess the viability of GC cells treated with kaempferol and oxaliplatin, both individually and in combination, to evaluate potential synergistic effects, while the EDU experiment was employed to determine the impact of kaempferol on DNA synthesis. The PCR array of cell death pathways was used to screen ferroptosis related genes in GC cells intervened by kaempferol and the levels of key markers were quantified with specific assay kits. Mitochondrial morphological alterations were also discovered using the transmission electron microscope. The protein expression levels of NQO1, p53, SLC7A11 and GPX4 in GC cells were analyzed by Western blot experiment following kaempferol treatment. **Results:** Kaempferol concentration dependently reduced the viability and DNA synthesis of GC cells, with IC50 values of 92.75 μM in HGC27 and 69.74 μM in MKN45 cells. When combined with oxaliplatin, with a Loewe synergy score of 17.621 for HGC27 cells and 13.931 for MKN45 cells, showing a synergistic effect. The PCR array detection indicated that following kaempferol intervention, P53 expression was increased, while NQO1, SLC7A11 and GPX4 expressions were downregulated. Meanwhile, kaempferol markedly decreased GSH levels while elevating MDA, Fe^{2+} and ROS levels in GC cells. The results of the Western blot experiments corroborated the PCR array findings, demonstrating that kaempferol induced ferroptosis in GC cells by modulating the P53/SLC7A11/GPX4 pathway. **Conclusion:** Kaempferol could promote ferroptosis in GC cells through the P53/SLC7A11/GPX4 signaling pathway, even for act as a sensitizer agent when combing with oxaliplatin for the treatment of GC.

Key Words Kaempferol, Gastric Cancer, Ferroptosis, P53/SLC7A11/GPX4 Axis

INTRODUCTION

Gastric Cancer (GC) is a malignant neoplasm with high morbidity and mortality inside the digestive system. Newly diagnosed GC cases are predominantly seen in Asia and South America and helicobacter pylori infection is one of the main pathogenic factors [1,2]. Helicobacter pylori secrete chemicals associated with pathogenesis, thereby establishing a persistent state of infection. This prolonged infection will result in chronic inflammation. Following several years of progression, it may ultimately develop into GC [3]. Prompt diagnosis, regular monitoring and timely intervention can dramatically decrease GC mortality while significantly enhancing patient survival duration and quality of life. In recent years, traditional Chinese medicine and its extracts have emerged as significant therapeutic agents for tumor treatment, characterized by low toxicity, great efficacy and the absence of drug resistance.

The advancement of contemporary medical technologies has increasingly rendered the study of traditional Chinese medicine monomers a focal point of interest. The monomers are active compounds derived from traditional Chinese medicine herbs or natural products, possessing a distinct chemical structure and pharmacological properties, thereby offering a better elucidated mechanism and a more dependable scientific foundation for the antitumor investigations of traditional Chinese medicine. Kaempferol is a flavonoid component that naturally occurs in tea as well as several common vegetables and fruits [4]. Kaempferol has been extensively investigated within the medical community due to its diverse biological activities,

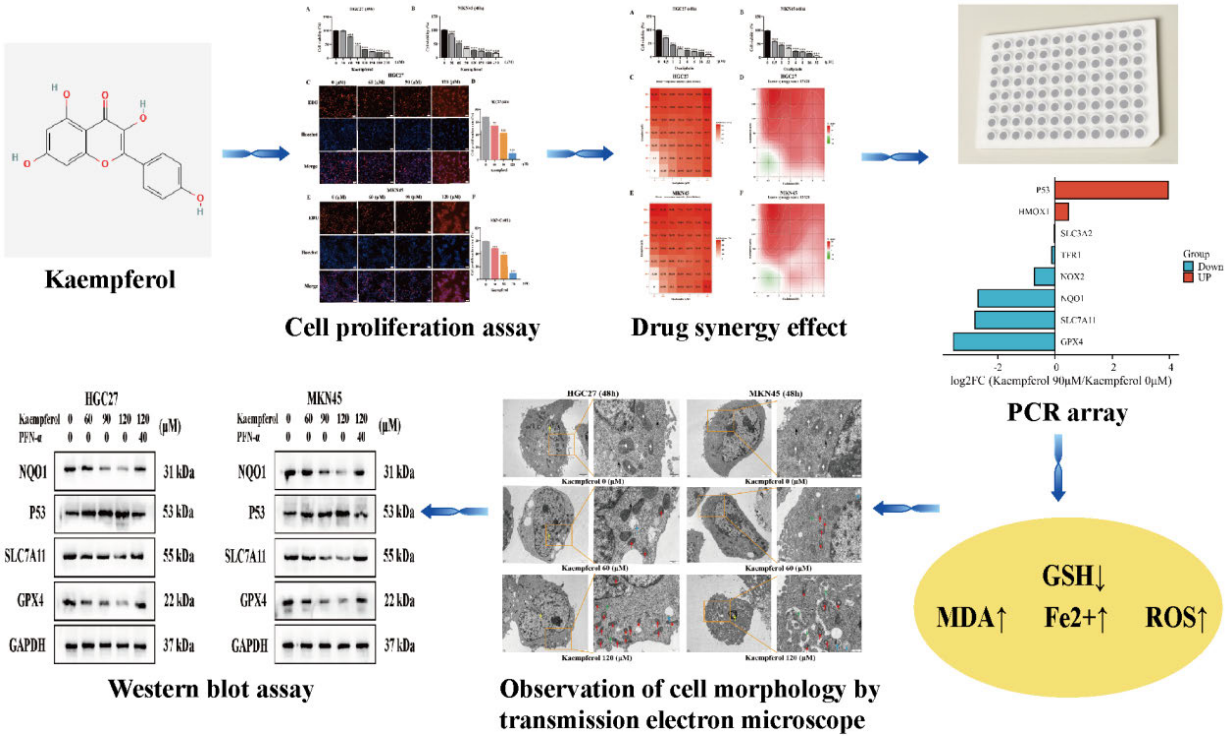

Figure 1: Experimental Workflow Illustrating the Anticancer Effects of Kaempferol

including antioxidant, antibacterial, anticancer and neuroprotective effects, among others [5-8]. Kaempferol has been shown to activate the NRF2/SLC7A11/GPX4 signaling pathway, augment antioxidant capacity, prevent lipid peroxidation accumulation in neurons subjected to oxygen-glucose deprivation/reperfusion and subsequently suppress ferroptosis [9]. Kaempferol may mitigate liver injury and inflammatory responses in mice with acetaminophen-induced liver damage, as well as improve hepatic iron overload and oxidative stress in these mice. Kaempferol stimulates the NRF2 pathway and enhances the expression of GPX4 in murine liver and human normal hepatocytes and it inhibits acetaminophen-induced ferroptosis [10]. Moreover, kaempferol offers hepatoprotective benefits against oxidative stress caused by arachidonic acid iron and carbon tetrachloride therapy [11]. Flap transplantation is the principal technique for wound repair and kaempferol may enhance flap viability and mitigate ischemia-reperfusion injury by activating the SIRT1-mediated HMGB1/TLR4/NF-κB and NRF2/SLC7A11/GPX4 signaling pathways [12]. These findings demonstrated that kaempferol is intricately associated with the regulation of ferroptosis. Nevertheless, ferroptosis is an atypical cell death mode driven by iron-dependent phospholipid peroxidation, which has been found in recent years and is closely related to the regulatory growth of a variety of tumor cells [13]. Ferroptosis is crucial to the onset, advancement, treatment and prognosis of GC and has attracted more and more attention from researchers [14]. However, no research on whether kaempferol could regulate the ferroptosis pathway in GC cells were reported and even no research on their sensitivity to platinum-based drugs remains scarce. Therefore, it would be valuable for expanding the application prospects of kaempferol in GC combination therapies (Figure 1).

Our previous research demonstrated that kaempferol can impede the migration and invasion of GC cells via the AKT/GSK3B signaling pathway, as evidenced by network pharmacology and *in vitro* experiments [15]. Nonetheless, no study has yet clarified whether kaempferol induces ferroptosis in GC cells via the P53/SLC7A11/GPX4 pathway. This study initially screened the genes related to kaempferol regulating ferroptosis in GC cells by PCR array and subsequently detected the ferroptosis-related indicators to prove the potential regulatory relationship and specific molecular mechanism between kaempferol and ferroptosis in GC to provide a theoretical basis and scientific basis for the application of kaempferol in the treatment of GC.

METHODS

Cell Culture and Reagents

The HGC27 and MKN45 cell lines were acquired from Wuhan Zishan Biotechnology Co., Ltd. The complete medium employed for cell culture comprises 10% fetal bovine serum (Servicebio; Cat No: G8002), 1% penicillin-streptomycin liquid (Servicebio; Cat No: G4003) and RPMI-1640 medium (Servicebio; Cat No: G4535). Cells were cultured in an incubator at 37°C with 5% CO_2 and the medium was replaced every 2-3 days. Kaempferol (Cat No: SK8030) was purchased by Beijing Solarbio Science and Technology Co., Ltd. Oxaliplatin (Cat No: HY-17371) and PFN-α (Cat No: HY-123076) were purchased from

MedChemExpress. The required antibodies for the experiment include NQO1 (1:2000; Immunoway; Cat No: YM8039), P53 (1:20000; Proteintech; Cat No: 10442-1-AP), SLC7A11 (1:700; Zenbio; Cat No: R26116), GPX4 (1:1000; Affinity; Cat No: DF6701), GAPDH (1:5000; Immunoway; Cat No: YM3029), anti-rabbit (1:10000; Immunoway; Cat No: RS0002) and anti-mouse (1:10000; Immunoway; Cat No: RS0001).

CCK8 Detection

The GC cells in the logarithmic growth phase were seeded in 96 well plates at a density of 5×10^3 cells per well and were intervened with complete media containing varying concentrations of kaempferol, oxaliplatin and PFN-α (P53 inhibitor). The 10 μL CCK8 (NCM; Cat No: C6005) solution and 90 μL RPMI-1640 medium were dispensed into each well and incubated at 37°C in the dark for 1-4 hours prior to measuring the absorbance at 450 nm. Furthermore, the synergy effect experiments of kaempferol and oxaliplatin were categorized into the kaempferol group, the oxaliplatin group, the kaempferol combined with oxaliplatin group and the SynergyFinder online platform (https://synergyfinder.fimm.fi) was utilized to determine the synergy score for both drugs [16]. The website indicates that the synergy score of the medications exceeds 10, signifying a synergistic impact among them. The experiments were performed in triplicate (n = 3).

EDU experiment

The GC cells in the logarithmic growth phase were distributed in a 6-well plate and intervened with different concentrations of kaempferol for 48 hours at 90% cell density. According to the manufacturer's instructions, the impact of kaempferol on DNA synthesis in GC cells was assessed by BeyoClick™ EDU Cell Proliferation Kit with Alexa Fluor 555 (Beyotime; Cat No: C0075S). Finally, the inverted fluorescent microscope was employed to capture images and Image J software was utilized for cell enumeration. The experiments were performed in triplicate (n = 3).

PCR Array of Cell Death Pathways Analysis

After the HGC27 cells were treated with kaempferol for 48 hours, the cell samples were harvested to explore the death pathways between kaempferol-treated group and control group using WcGene death screening PCR array. The cell death pathways includes apoptosis, necroptosis, autophagy, ferroptosis and so on. The WcGene death screening PCR array can screen out the mechansim that can explain the cell phenomenon by detecting the key genes regulating different death modes. The experiment procudures as follows: First, the total RNA of cells was extracted with the MolPure® Cell/Tissue Total RNA Kit (Yeasen; Cat No: 19221ES50) according to the manufacturer's instructions and cDNA was synthesized with the Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (Yeasen; Cat No: 11141ES60) reverse transcription. Then cDNA and Hieff® qPCR SYBR® Green Master Mix (No Rox) (Yeasen; Cat No: 11201ES08) were mixed and added to the death screening PCR array (WcGene; Cat No: WC-MRNA0358-H) for quantitative PCR. In the end, the data were calculated to analyze the differentially expressed genes.

Detection of Ferroptosis-Related Indicators

The GC cells subjected to 48 hours of kaempferol intervention were collected into centrifuge tubes. The experiments were performed according to the manufacturer instructions of the GSH Content Assay Kit (Solarbio; Cat No: BC1175), MDA Content Assay Kit (Solarbio; Cat No: BC0025) and Fe^{2+} Content Assay Kit (Solarbio; Cat No: BC5415) and the content of each indicator was tested in the microplate reader. In addition, the ROS levels in GC cells following kaempferol intervention were measured using the ROS Assay Kit (Beyotime; Cat No: S0033S) and assessed via flow cytometry, with analysis conducted using CytExpert software. The experiments were performed in triplicate (n = 3).

Observation of Cell Morphology by Transmission Electron Microscope

When kaempferol interfered with GC cells for 48 hours, the cultured medium was changed and 0.25% trypsin digestion solutions (Servicebio; Cat No: G4012) were added to digest the cells. The digestion process was terminated with a complete medium, followed by centrifugation, after which the supernatant was discarded. The 2.5% glutaraldehyde (EM grade) (Solarbio; Cat No: P1126) was added to the centrifuge tube, the cell mass was gently lifted and suspended in the solution and subsequently observed and photographed utilizing the transmission electron microscope.

Western Blot Analysis of Ferroptosis

When kaempferol interfered with GC cells for 48 hours, the culture medium was discard to collect cell samples, add RIPA buffer (high) (Solarbio; Cat No: R0010) and PMSF (Solarbio; Cat No: P0100) into the cell culture flask and incubate on ice for 30 minutes. The cells were scraped off with a cell scraper and the lysates together with cell fragments were transferred to the EP tube for centrifugation. The protein concentration in the supernatant was measured utilizing the BCA Protein Concentration kit (Solarbio; Cat No: PC0020) and the residual supernatant was boiled with SDS-PAGE loading buffer, 5× (with DTT) (Solarbio; Cat No: P1040) for 10 minutes and thereafter stored at -20°C. The protein was added to the SDS-PAGE gel, followed by electrophoresis, membrane transfer and incubation with primary and secondary antibodies. The membrane was subsequently positioned on the gel imager and subjected to exposure utilizing the Super ECL Detection Reagent Kit (Yeasen, Cat No: 36208ES60). Ultimately, the Image J software is employed to assess the gray value of the target strip. The experiments were performed in triplicate (n = 3).

Statistical Analysis

The experimental data were analyzed using GraphPad Prism 9.3 software. Initially, the normality test was

performed on the data among the multiple groups. When the data followed a normal distribution and exhibited homogeneous variance, the One-way ANOVA was employed. In cases where the variance was heterogeneous, the Welch ANOVA was applied. Conversely, if the data did not adhere to a normal distribution, the Kruskal-Wall's was utilized. The p-value less than 0.05 was considered statistically significant.

RESULTS

Kaempferol Impeded the Proliferation of GC Cells

The CCK8 experiment was employed to investigate the impact of kaempferol on the growth of GC cells by assessing cell viability. The CCK8 experiment results indicated that after 48 hours of kaempferol intervening with GC cells, the IC_{50} for HGC27 cells was 92.75 μM and for the IC_{50} of MKN45 cells was 69.74 μM, with a substantial decrease in cell viability observed as drug concentration increased ($p<0.05$) (Figure 2a-b).

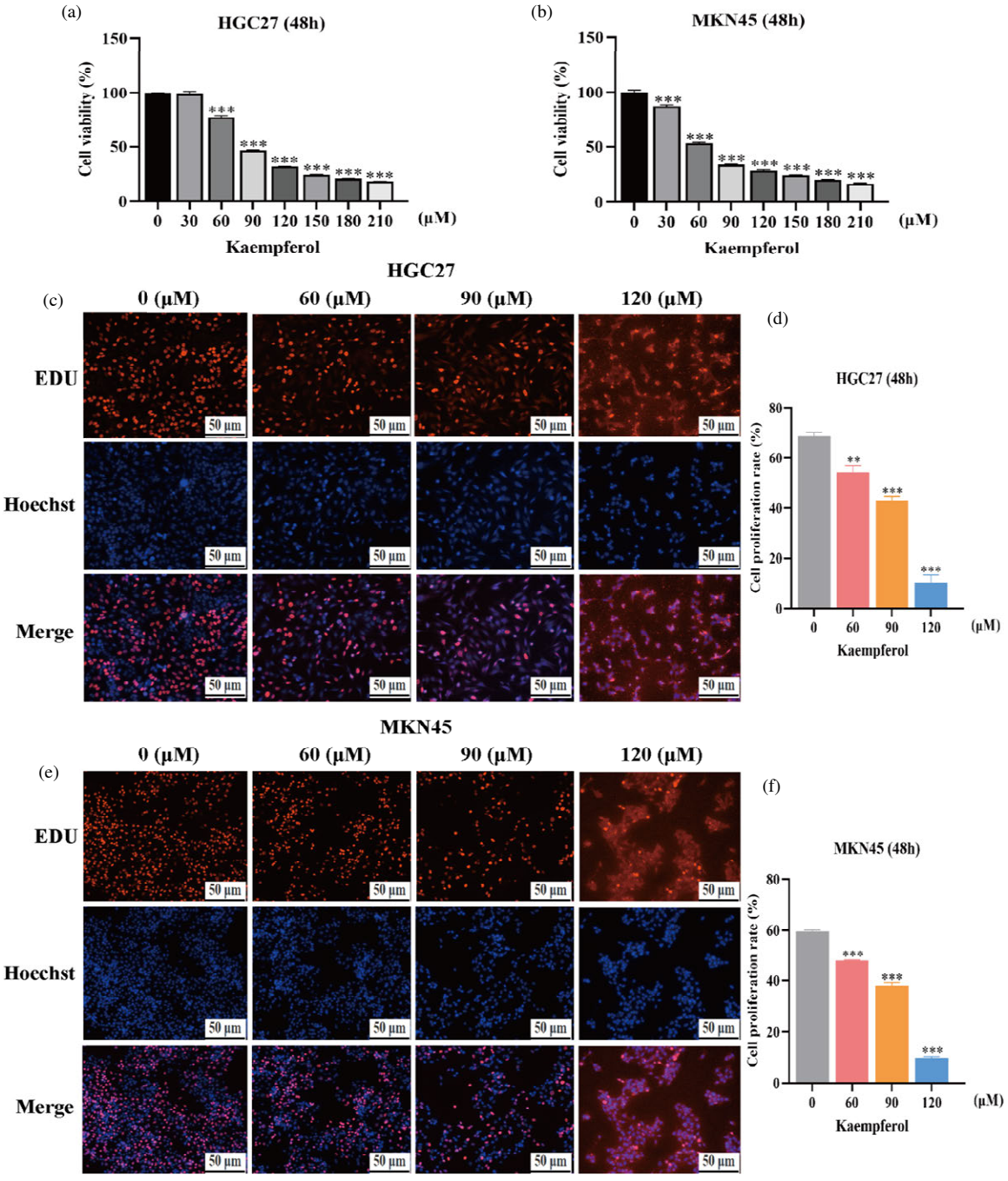

Figure 2(a-f): Kaempferol Suppresses the Proliferation of GC Cells, (a-b) The CCK8 Experiment was Employed to Assess the Cell Viability of GC Cells Following Kaempferol Intervention and (c-f) The EDU Experiment was Employed to Assess the Impact of Kaempferol on the DNA Synthesis Capacity of GC Cells

Compared to the kaempferol 0 μM group, **p<0.01, ***p<0.001

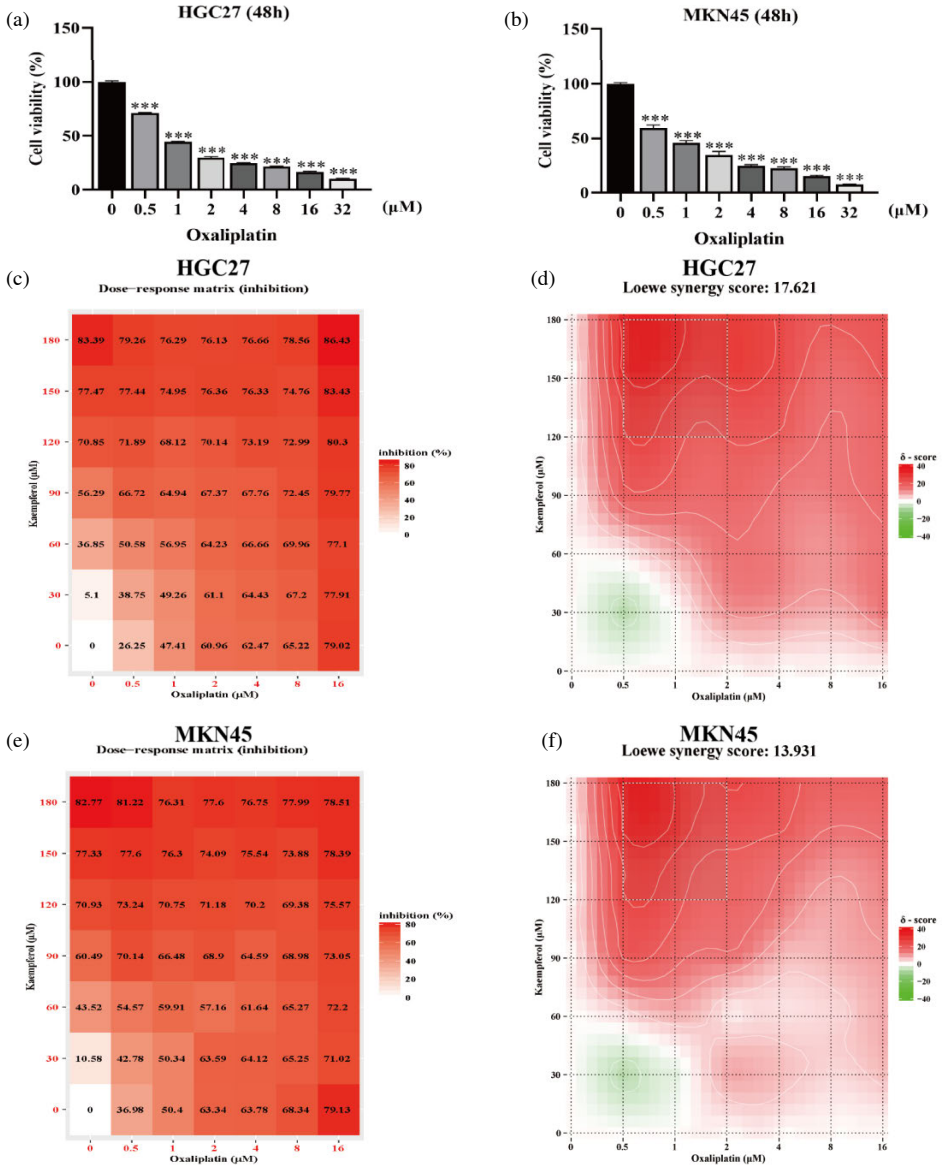

Figure 3(a-f): Kaempferol in Conjunction with Oxaliplatin Exhibits a Synergy Effect on the Suppression of GC cells, (a-b) The CCK8 Experiment was Employed to Assess the Vitality of GC Cells Following Oxaliplatin Intervention, (c, e) The Growth Inhibition Percentage of GC Cells Treated with Kaempferol and Oxaliplatin, Either Alone or in Combination, (d, f) Thermogram Illustrating the Synergy Effect of Kaempferol and Oxaliplatin, Both Alone and in Combination, on GC Cells
Compared to the Oxaliplatin 0 μM group, ***p<0.001

Subsequently, in the EDU experiment, after intervention of gastric cancer cells with different concentrations of kaempferol at 0, 60, 90 and 120 μM, it was found that the cell proliferation rates (100%) of HGC27 cells were 68.77±2.43, 54.50±4.20, 43.01±2.99, 10.43±5.01 and MKN45 cells were 59.63±0.77, 48.12±0.50, 38.03±2.02 and 9.91±0.90, respectively. The results of the EDU experiment showed that kaempferol could significantly limit the DNA synthesis capability of GC cells ($p<0.05$) (Figure 2c-f). In conclusion, kaempferol could inhibit the proliferation of GC in a concentration-dependent manner.

Kaempferol Exhibited a Synergy Effect on GC Cells When Combined with Oxaliplatin

The synergy effect of pharmaceuticals refers to the pharmacological effect produced when two or more drugs are used simultaneously, which is greater than the sum of the pharmacological effects of each drug when used alone. First of all, the drug concentration of oxaliplatin in GC cells was detected using the CCK8 experiment. Subsequently, kaempferol and oxaliplatin were administered individually and in combination to GC cells for 48 hours to investigate the synergy effect of the two agents. The CCK8 experiment results indicated that the IC_{50} value for oxaliplatin in HGC27 cells was 0.97 μM, while in MKN45 cells it was 0.81 μM. Furthermore, oxaliplatin diminished the viability of GC cells in a concentration-dependent manner ($p<0.05$) (Figure 3a-b). The results of CCK8 to detect the synergy effect showed that the Loewe synergy score was 17.621 for HGC27 cells and 13.931 for MKN45 cells (Figure 3c-f). The synergy score of both GC cells exceeded 10, signifying that when kaempferol

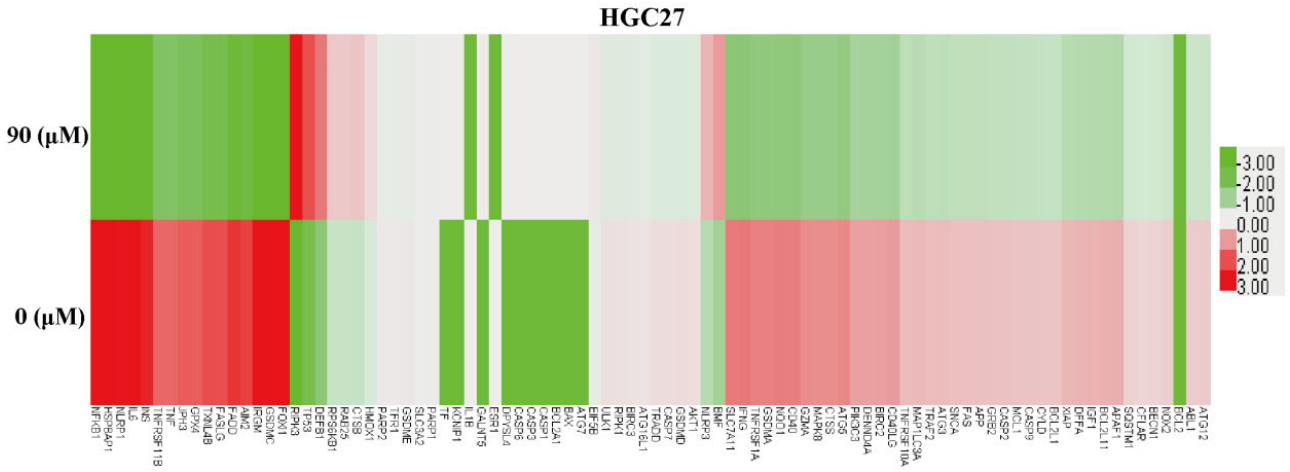

Figure 4: PCR Array Results after 48 hours of Intervention with Kaempferol in HGC27 Cells

was combined with oxaliplatin, the effect of inhibiting GC was stronger than with kaempferol or oxaliplatin alone.

Kaempferol Induced Ferroptosis in GC Cells

Inducing ferroptosis in neoplastic cells has emerged as a promising anticancer approach. This work initially identified the mRNA expression of ferroptosis-related indicators using a death screening PCR array and chose genes with |log2FC|>2.5 for subsequent investigation (Figure 4 and 5a). The PCR array findings indicated that kaempferol dramatically downregulated the mRNA expression of NQO1, SLC7A11 and GPX4 in HGC27 cells following 48 hours of intervention. Prior research has demonstrated that P53 has a role in the control of ferroptosis [17]. The PCR array results indicated that P53 was increased following kaempferol intervention in HGC27 cells.

Ferroptosis may induce cell death by the buildup of iron ions, lipid peroxidation and disruption of the antioxidant system, with its mechanism involving alterations in GSH, MDA, Fe^{2+} and ROS levels. This study employed a microplate reader and flow cytometry to assess alterations in indicators associated with ferroptosis. After intervention with kaempferol in GC cells, we measured the levels of GSH in HGC27 cells to be 1.00±0.02 (0 μM), 0.92±0.02 (60 μM), 0.53±0.01 (90 μM) and 0.31±0.02 (120 μM) (Figure 5b); The levels of GSH in MKN45 cells were 1.00±0.01 (0 μM), 0.71±0.02 (60 μM), 0.61±0.01 (90 μM) and 0.47±0.02 (120 μM) (Figure 5c). The levels of MDA in HGC27 cells were 1.00±0.08 (0 μM), 2.44±0.84 (60 μM), 5.21±0.42 (90 μM) and 6.23±0.55 (120 μM) (Figure 5d); The levels of MDA in MKN45 cells were 1.00±0.13 (0 μM), 2.54±0.57 (60 μM), 4.40±0.35 (90 μM) and 5.74±0.65 (120 μM) (Figure 5e); The levels of Fe^{2+} in HGC27 cells were 1.00±0.20 (0 μM), 1.36±0.12 (60 μM), 1.97±0.23 (90 μM) and 4.66±0.14 (120 μM) (Figure 5f); The levels of Fe^{2+} in MKN45 cells were 1.00±0.06 (0 μM), 2.11±0.10 (60 μM), 3.25±0.37 (90 μM) and 5.76±0.61 (120 μM) (Figure 5g); The levels of ROS in HGC27 cells were 1.00±0.07 (0 μM), 1.22±0.01 (60 μM), 1.58±0.01 (90 μM) and 1.62±0.01 (120 μM) (Figure 5h-i); The levels of ROS in MKN45 cells were 1.00±0.08 (0 μM), 1.49±0.02 (60 μM), 1.71±0.02 (90 μM) and 1.86±0.05 (120 μM) (Figure 5j-k). In comparison to the 0 μM group, the GSH levels in the kaempferol intervention group were markedly diminished, whereas the levels of MDA, Fe^{2+} and ROS were significantly elevated ($p<0.05$), suggesting that kaempferol could induce ferroptosis in GC cells by disrupting iron metabolism and compromising the antioxidant system.

Ultrastructural analysis of kaempferol-induced ferroptosis in GC cells

Mitochondria play an important role in ferroptosis processes. This study examined the ultrastructural alterations of GC cells subjected to kaempferol intervention using a transmission electron microscope (Figure 6).

On the one hand, the transmission electron microscope revealed that the nucleus of HGC27 cells exhibited irregularity and unevenness, displaying a distinct binuclear membrane structure without perinuclear space expansion, as indicated by the yellow arrow. In the HGC27 0 μM group, the architecture of the mitochondrial bilayer membrane within the cytoplasm is distinctly observable, with lamellar cristae seen. The cristae are approximately arranged in parallel lamellae, as indicated by the white arrow. The structure of the endoplasmic reticulum is normal without obvious expansion, as indicated by the black arrow. In the HGC27 60 μM group, certain mitochondria in the cytoplasm exhibited atrophy and were smaller, with a reduction or absence of cristae and a rise in the electron density of the mitochondrial membrane, as indicated by the red arrow. The structure of the endoplasmic reticulum is normal without obvious expansion, as indicated by the black arrow. A limited number of autophagic lysosomes are observable in the cytoplasm, characterized by a monolayer membrane structure, with cytoplasmic components having undergone degradation, as indicated by the blue arrow. In the HGC27

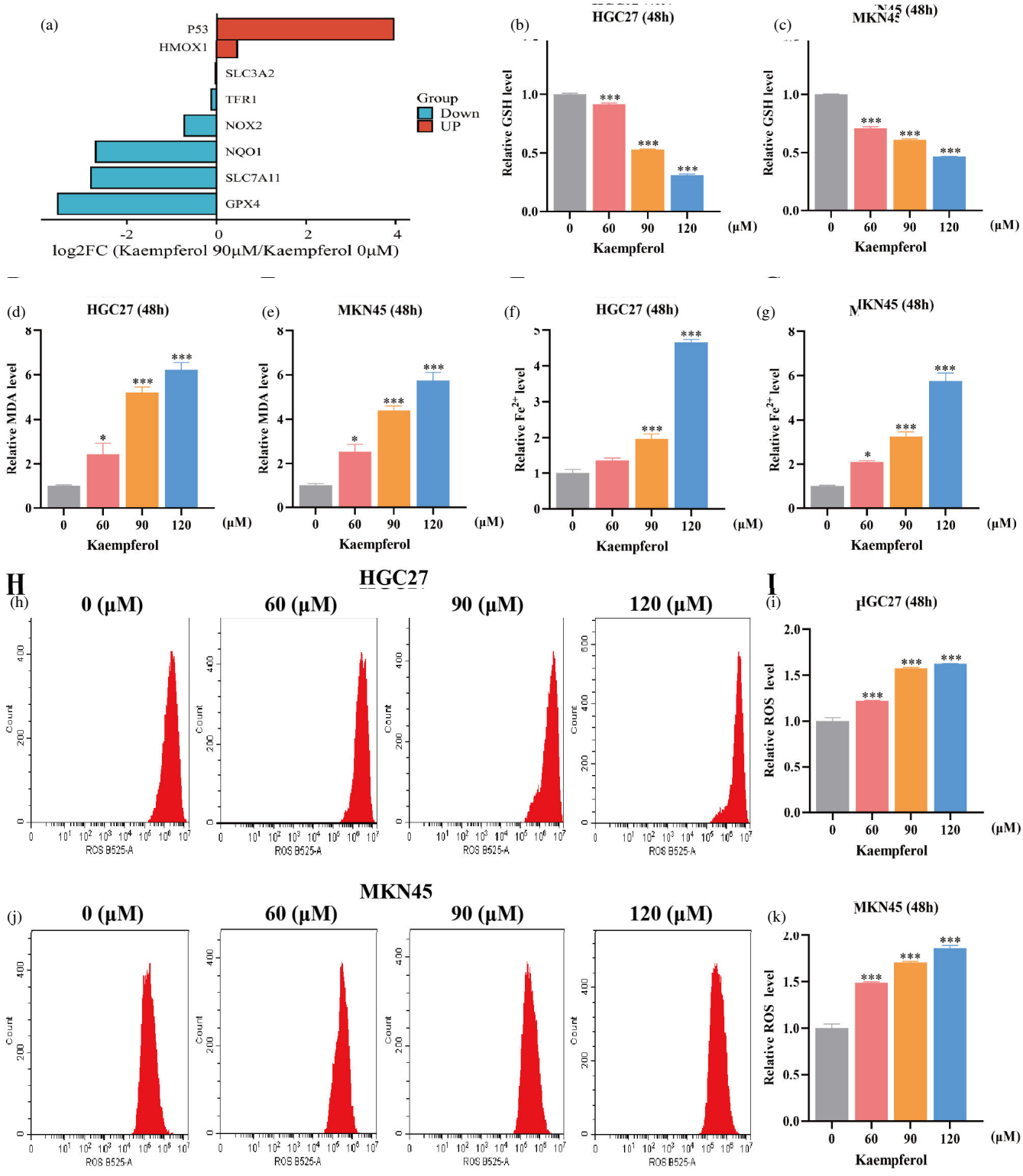

Figure 5(a-k): Kaempferol May Promote Ferroptosis in GC Cells, (a) The Death Screening PCR Array was Employed to Screen Genes Related to Kaempferol Regulating Cell Death Pathway in HGC27 Cells and the Four Ferroptosis Related Genes with |log2FC|>2.5 were Screened for Further Analysis, (b-c) Alterations in GSH Levels Following Kaempferol Intervention in GC Cells, (d-e) Alterations in MDA Levels Following Kaempferol Intervention in GC Cells, (f-g) Alterations in Fe^{2+} Levels Following Kaempferol Intervention in GC Cells and (h-k) Alterations in ROS Levels Following Kaempferol Intervention in GC Cells

Compared to the Kaempferol 0 μM Group, *p<0.05, ***p<0.001)

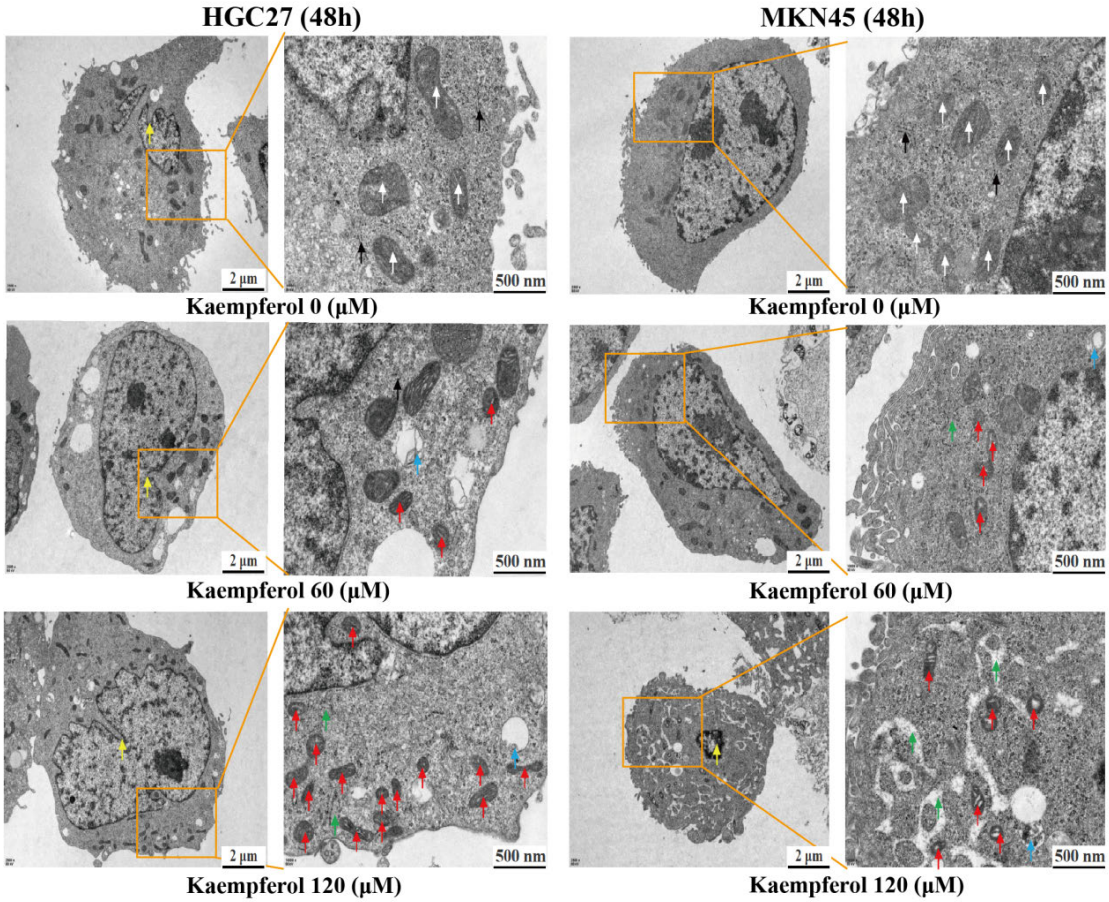

Figure 6: Ultrastructural Alterations in GC Cells Following Kaempferol Intervention

120 μM group, a large number of mitochondria in the cytoplasm became atrophied and smaller, cristae decreased or disappeared and the electron density of the mitochondrial membrane increased, as indicated by the red arrow. The endoplasmic reticulum expands, as indicated by the green arrow. A limited quantity of autophagic lysosomes is observable in the cytoplasm, characterized by a monolayer membrane structure with the cytoplasmic constituents having undergone degradation, as indicated by the blue arrow.

On the other hand, transmission electron microscopy revealed that in the MKN45 0 μM group, the cell nucleus was regular, the shape of the binuclear membrane was clear and the perinuclear space was not dilated. The mitochondrial bilayer membrane structure in the cytoplasm is clear and lamellar cristae can be seen. The cristae are roughly arranged in parallel lamellae, as indicated by the white arrow. The endoplasmic reticulum has a normal structure without significant expansion, as indicated by the black arrow. In the MKN45 60 μM group, the nuclear morphology was regular, the binuclear membrane structure was clear and the perinuclear space was not dilated. Certain mitochondria in the cytoplasm become atrophy and smaller, with a decrease or absence of cristae and an increase in the electron density of the mitochondrial membrane, as indicated by the red arrow. The endoplasmic reticulum expands, as indicated by the green arrow. Several autophagosomes are present in the cytoplasm, characterized by a double-membrane vacuole structure that contains cytoplasmic components, as indicated by the blue arrow. In the MKN45 120 μM group, the nucleus exhibited pyknosis and the chromatin within the nucleus was condensed, as indicated by the yellow arrow. A substantial quantity of mitochondria in the cytoplasm undergo atrophy and are smaller, with a reduction or absence of cristae, while the electron density of the mitochondrial membrane escalates, as indicated by the red arrow. A large number of endoplasmic reticulum expanded significantly, as indicated by the green arrow. A limited quantity of autophagic lysosomes is observable in the cytoplasm, characterized by a monolayer membrane structure with the cytoplasmic constituents having undergone degradation, as indicated by the blue arrow.

Upon the intervention of kaempferol in GC cells, mitochondrial atrophy and smaller was seen, diminished or absent cristae and an increase in mitochondrial membrane electron density. These alterations indicated the occurrence of mitochondrial malfunction, a significant ultrastructural characteristic of ferroptosis induced by kaempferol.

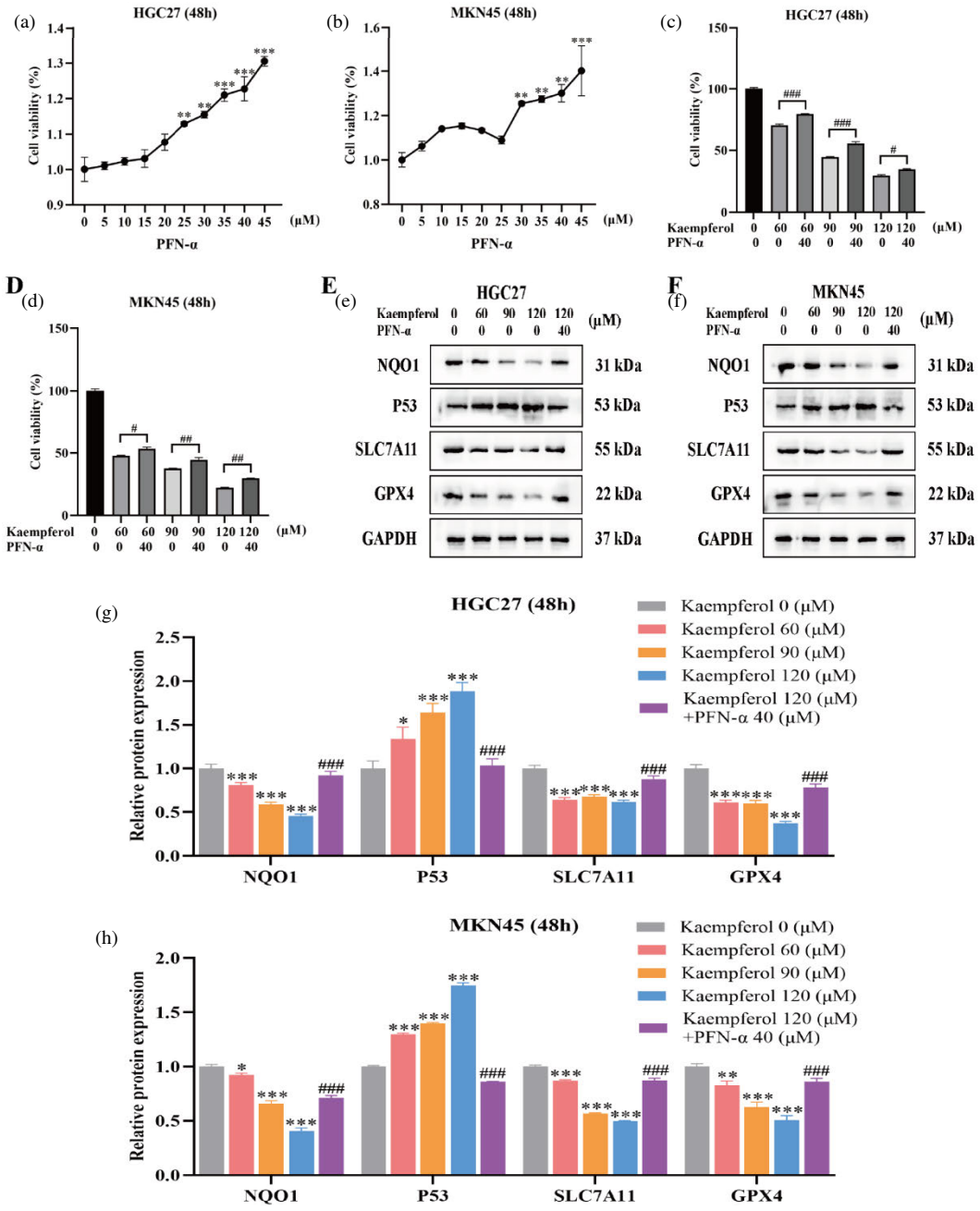

Figure 7(a-h): Kaempferol Modulated Ferroptosis in GC Cells Via the P53/SLC7A11/GPX4 Signaling Pathway, (a-b) The CCK8 Experiment was Employed to Assess the Vitality of GC Cells Following PFN-α Intervention. (Compared to the PFN-α 0 μM group, **p<0.01, ***p<0.001), (c-d) Effects of Kaempferol and PFN-α Alone or in Combination on the Viability of GC Cells, ($^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001), (e-f) The Protein Expression Levels of NQO1, P53, SLC7A11 and GPX4 were Detected by the Western Blot Experiment After the Intervention of Kaempferol and PFN-α in GC Cells
Compared to the PFN-α 0 μM Group, *p<0.05, **p<0.01, ***p<0.001; Compared to the Kaempferol 120 μM Group, $^{\#\#\#}$p<0.001

Kaempferol Modulated P53/SLC7A11/GPX4 Signaling Pathway to Facilitate Ferroptosis

The death screening PCR array indicated that kaempferol might alter the expression levels of ferroptosis-related genes in GC cells. However, P53 is a quintessential tumor suppressor gene and its role in ferroptosis is intricate. Research indicates that wild-type P53 can suppress the expression of SLC7A11, diminish cystine absorption, therefore lower intracellular GSH levels, inhibit the activity of GPX4 and facilitate ferroptosis [18]. This study utilized several doses of PFN-α to intervene with GC cells for 48 hours, with 40 μM chosen for further experimental investigation (p<0.05) (Figure 7a-d). The findings of the Western blot experiment indicated that, in comparison to the 0 μM group, kaempferol substantially upregulated P53 and downregulated the expression levels of NQO1, SLC7A11

and GPX4 proteins. On the other hand, in comparison to the kaempferol 120 μM group, the combination intervention of kaempferol and PFN-α significantly reduced P53 levels while enhancing the expression levels of NQO1, SLC7A11 and GPX4 proteins ($p<0.05$) (Figure 7e-h). The findings indicate that kaempferol may facilitate ferroptosis in GC cells through modulation of the P53/SLC7A11/GPX4 signaling pathway.

DISCUSSION

Malignant tumor is one of the major diseases threatening human health worldwide. According to the data of the International Agency for Research on Cancer in 2022, there are around 970,000 new cases of GC and almost 660,000 deaths, ranking fifth in the world in terms of morbidity and mortality [19]. Despite a decline in the prevalence of GC in recent decades, the overall prognosis for patients remains pessimistic [20]. Despite the substantial advancements in contemporary medicine in the treatment of GC, conventional treatment methods, including surgery, radiation and chemotherapy, still face several challenges, including drug resistance, high recurrence rate and the impact on the quality of life of patients [21]. Consequently, it is crucial to explore and develop unique and effective tumor treatment options to enhance clinical efficacy and social benefits.

Traditional Chinese medicine, as a significant component of conventional medicine, possesses a lengthy history and rich experience in tumor prevention and therapy. Recent study findings have validated that kaempferol shows significant activity in the realm of antitumor. Kaempferol exerts an antitumor impact by many mechanisms, such as the inhibition of tumor cell proliferation, migration and invasion; the induction of programmed cell death; and the regulation of the cell cycle. These characteristics make kaempferol a new antitumor candidate drug with important development value [22-26]. Kaempferol may effectively impede the proliferation of triple-negative breast cancer cells while simultaneously inducing apoptosis and G2/M phase arrest [27]. Kaempferol may reduce the viability and induce apoptosis of pancreatic cancer cells in a dose-dependent manner [28]. In cholangiocarcinoma, kaempferol could efficiently suppress colony formation, migration and invasion of cholangiocarcinoma cells, as well as induce apoptosis *in vitro*. The *vivo* experiments demonstrated that the volume of subcutaneous xenografts in the kaempferol treatment group was significantly smaller compared to the control group and could inhibit the number and volume of metastases in the lung metastasis model, suggesting that kaempferol may be a promising candidate for cholangiocarcinoma treatment [29]. In this study, we first demonstrated that kaempferol could inhibit the proliferation of GC cells in a dose-dependent manner through CCK8 and EDU experiments. Oxaliplatin is a platinum compound. Its mechanism of action primarily involves disrupting the replication and repair processes of tumor cell DNA, leading to the demise of tumor cells and so exerting an antitumor effect. However, the primary role of drug synergy is to augment therapeutic efficacy, diminish drug dose and adverse effects and postpone the development of drug resistance. The experimental results of this study also indicated that the combination of kaempferol and oxaliplatin exhibits a synergy effect on inhibiting the growth of GC cells. These findings suggest that kaempferol may serve as a chemosensitizing agent for platinum-based therapy in GC. Studies have found that the combination of kaempferol and doxorubicin exhibits enhanced inhibitory effects on the viability, migration, invasion capacity, cell cycle progression, DNA damage response and mitochondrial function of liver cancer cells, compared with kaempferol or doxorubicin alone [30]. Consequently, kaempferol impedes the proliferation, migration and invasion of tumor cells and other biological phenotypes, serving as a crucial mechanism for its antitumor action and a significant criterion for assessing the antitumor efficacy of pharmaceuticals.

Ferroptosis is an iron-dependent new programmed cell death mode, which is different from apoptosis and autophagy. The primary mechanism of ferroptosis is that under the action of ferrous iron or ester oxygenase, it catalyzes the high expression of unsaturated fatty acids on the cell membrane to produce lipid peroxidation, thus inducing cell death [31]. P53, one of the most important tumor suppressor genes, is frequently mutated in human tumors. P53, functioning as a transcription factor, is stabilized and activated in response to various genotoxic and cellular stress signals, such as DNA damage and hypoxia, thereby resulting in cell cycle arrest, senescence and metabolic adaptation [32]. SLC7A11 could inhibit ROS-induced ferroptosis. P53 transcription could suppress SLC7A11 activity, diminish cystine input and hence decrease GSH synthesis and ROS buildup. Nonetheless, GSH may reduce polyunsaturated fatty acids by the catalysis of GPX4, therefore decreasing ferroptosis [33,34]. The level of MDA exhibited a positive correlation with the extent of ferroptosis. During ferroptosis, the buildup of iron ions and the elevation of ROS within cells exacerbate lipid peroxidation, resulting in a substantial increase in MDA levels [35]. This work was first revealed by PCR array high throughput screening technology that kaempferol intervention in GC cells resulted in the upregulation of P53 mRNA expression and the downregulation of NQO1, SLC7A11 and GPX4 mRNA expression. The protein expression levels of the aforementioned indicators were also similar. However, in contrast to the kaempferol 120 μM group, the combination of PFN-α and kaempferol could restore the protein expression levels of P53, NQO1, SLC7A11 and GPX4 observed with kaempferol alone. Because many researchers have done a lot of research on the P53/SLC7A11/GPX4 signaling pathway, this pathway has become a classic pathway for ferroptosis-related research [36-38]. This study subsequently detected ferroptosis-related indicators and revealed that, in comparison to the 0 μM group, kaempferol reduced GSH levels while elevating MDA, Fe^{2+} and ROS levels in GC cells. In neuroblastoma, hippophandine A-C, three kaempferol derivatives, at a

certain dose, can mitigate hydrogen peroxide-induced damage to SH-SY5Y cells, decrease MDA levels and elevate superoxide dismutase, catalase and GSH levels. Mitochondria, a crucial organelle that governs the internal metabolism of tumor cells, always affects the development of tumors. Under the observation of transmission electron microscopy, this study discovered that kaempferol could induce mitochondrial atrophy and become smaller, reduce or disappear cristae and increase mitochondrial membrane electron density in GC cells. These morphological alterations indicate mitochondrial malfunction in ferroptosis. In conclusion, kaempferol may modulate ferroptosis in GC cells through various targets, offering a reference for the creation of novel antitumor agents.

Traditional Chinese medicine monomer has the advantages of high active ingredients, stable structure and few side effects. It demonstrates distinct advantages in the therapy of tumor-mediated ferroptosis but the current research still has some limitations. Currently, research on the mechanisms by which traditional Chinese medicine monomers regulate ferroptosis in tumor therapy is inadequate and it mostly remains in the experimental phase, without clinical validation. Then the intricacy of medication research and development, safety evaluation and the burdensomeness of clinical trials result in a poor conversion rate to clinical application. It is recommended to enhance multiomics research to explore mechanisms, conduct organoid and clinical experiments to validate efficacy and safety, improve evaluation accuracy, facilitate the connection of drug research and development with clinical application, bolster policy support and financial investment and ensure robust backing for the research, development and application of traditional Chinese medicine monomer drugs.

CONCLUSIONS

In conclusion, kaempferol could promote the ferroptosis of GC cells through the P53/SLC7A11/GPX4 signaling pathway *in vitro*. This discovery not only helps to explore the potential application value of kaempferol but also provides novel tactics and methodologies for contemporary cancer treatment. However, currently, we have only conducted in vitro studies and have not yet performed in vivo experimental investigations. Consequently, we were unable to corroborate the findings of the in vitro research with an in vivo tumorigenic investigation. In future research, we will address the limitations of existing studies and conduct in vivo and clinical translation studies to elucidate how kaempferol regulates ferroptosis, thereby providing new insights and directions for GC therapy.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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