Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.144

RESEARCH ARTICLE

FOXM1 Inhibits SUV39H1 to Regulate the CSE/H2S Pathway in Promoting Ferroptosis of Gastric Cancer Cells

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ARTICLE HISTORY (24-674)

Received: October 24, 2024
Revised: December 17, 2024
Accepted: December 20, 2024
Published online: January 20, 2025

Key words:
Cell
Expression
Ferroptosis
FOXM1
Gastric cancer
Proliferation
SUV39H1

ABSTRACT

The role of FOXM1 in ferroptosis remains largely unknown. We aimed to explore the role of forkhead box M1 (FOXM1) suppression in promoting the ferroptosis of gastric cancer cells. Groups were set for SGC-7901 cells, including si-FOXM1 group, si-FOXM1+Fer-1 group, si-NC group, si-SUV39H1 group, si-SUV39H1+Fer-1 group, si-FOXM1+pcDNA3.1+SUV39H1 group, si-FOXM1+pcDNA3.1+ SUV39H1+Fer-1 group, and control group. CCK-8 method was employed to determine the proliferation of cells. ELISA was conducted to measure the iron content in cells. In comparison with the control group, the cell survival rate, hydrogen sulfide (H_2S) yield and protein expressions of SUV39H1, cystathionine γ-lyase (CSE), glutathione peroxidase 4 (GPX4) and solute carrier family 7 member 11 (SLC7A11) declined significantly in the si-FOXM1 group, while the relative fluorescence intensity of reactive oxygen species (ROS) and the relative iron content increased ($P<0.05$). Si-FOXM1+pcDNA3.1+SUV39H1 and si-FOXM1+pcDNA3.1+ SUV39H1+Fer-1 groups, especially the latter group, had significantly higher cell survival rate, H_2S yield and protein expressions of SUV39H1, CSE, GPX4 and SLC7A11, and significantly lower relative fluorescence intensity of ROS and relative iron content than those of the si-FOXM1 group ($P<0.05$). FOXM1 suppression is capable of acting on gastric cancer cells through proliferation inhibition combined with ferroptosis promotion.

To Cite This Article: Li Z, Hou W, Wang J, Han X, Zhang L, Tariq M and Wang X, 2025. FOXM1 inhibits SUV39H1 to regulate the CSE/H2S pathway in promoting ferroptosis of gastric cancer cells. Pak Vet J, 45(1): 360-366. http://dx.doi.org/10.29261/pakvetj/2024.144

INTRODUCTION

Malignancies, especially gastric ones, have been a great threat both for humans and domestic animals with the major share in canines (Avital *et al*., 2019; Abrams *et al*., 2019; Ohmi *et al*., 2021). Gastric cancer in canine and human is discussed together in most studies because of similarities in many aspects (Hugen *et al*., 2017; Araújo *et al*., 2022). Both in humans and animals, in the early stages of gastric cancer (GC), clinical signs are from mild to absent, and the most common signs are anorexia, vomiting, lethargy and weight loss (Withrow, 2013; Hugen *et al*., 2017; Avital *et al*., 2019). The Lauren's criteria for humans have successfully been adapted to canine gastric cancer (Araújo *et al.*, 2022). GC has high prevalence in humans as compared to animals (Bray and Neiger, 2011; Seim-Wikse, 2013), but this prevalence estimate may be misleading because most of the animal owners do not pursue completely in the diagnostic steps of GC (Seim-Wikse *et al*., 2013; Ohmi *et al*., 2021). Patients subjected to early GC can have a survival rate exceeding 95% within 5 years after excision (Röcken, 2023). Due to the insidious onset, however, most GC patients have been in late stage when diagnosed, and they are prone to postoperative metastasis, resulting in a survival rate of lower than 30% within 5 years. Therefore, it is necessary to search for a new treatment method for GC.

Being a transcriptional factor, Forkhead box M1 (FOXM1) is a Fox family member involved in various physiological processes ranging from the modulation of cell proliferation, differentiation, and apoptosis to the maintenance of stem cell pluripotency (Sher *et al.,* 2022). It has been verified that FOXM1 has high expressions in colon cancer, liver cancer, and GC, and plays crucial roles in the angiogenesis, metastasis, and infiltration of tumors (Kopanja *et al.,* 2022). Moreover, experiments on FOXM1

in animal models have shown it to cause delay in the aging process, thereby increasing the life expectancy (Zhang *et al.,* 2023). However, there are few reports on the role of FOXM1 in ferroptosis.

Ferroptosis is a novel cell death mode depending on iron and peroxidation of lipids. This mechanism has been observed not only in humans but also in other animals (Conrad *et al.,* 2018). Reactive oxygen species (ROS) accumulates under the catalysis of unsaturated fatty acids from cell membranes by divalent iron or lipoxygenase, thus inducing lipid peroxidation and ultimately leading to cell death. Increasingly more genes with antitumor activity have been found, which can resist tumor growth and spread by ferroptosis (Li *et al.,* 2021a). SUV39H1 is the first identified human histone lysine methyltransferase that can specifically catalyze H3K9 methylation to produce H3K9me3, recruit heterochromatin protein 1, and suppress gene transcription by binding to multiple transcriptional repressors. SUV39H1 is highly expressed in liver cancer, GC and colon cancer, and facilitates the occurrence and the development of cancers (Tsai *et al.,* 2022). Furthermore, it also inhibits the angiogenesis in mice (Niu *et al.,* 2023). However, whether SUV39H1 is implicated in the mechanism of ferroptosis needs further investigation. Hydrogen sulfide (H_2S) exists in the human body and participates in many pathophysiological processes, which is considered a new type of gas signal molecule with important biological functions (Duan *et al.,* 2022). It is mainly derived from cystathionine γ-lyase (CSE) which can generate endogenous H_2S by catalyzing L-cysteine (L-Cys). Both H_2S and CSE can mediate the pathophysiological processes of bladder carcinoma, and breast carcinoma, and many other tumors (Panza *et al.,* 2022), but their roles in GC are rarely reported.

Therefore, the roles and the mechanisms of action of FOXM1 in affecting SUV39H1, CSE/H_2S and ferroptosis in GC cells were explored in the present study, aiming to provide a valuable experimental basis for future treatment.

MATERIALS AND METHODS

Apparatus, reagents and cell lines: GC cell line (SGC-7901) was purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (China). FOXM1 small-interfering RNA (si-FOXM1), si-negative control (NC), together with SUV39H1 small-interfering RNA (si-SUV39H1) were bought from Shanghai GenePharm Co., Ltd. (China). pcDNA3.1-SUV39H1 plasmid was obtained from Invitrogen (USA). Iron inhibitor ferrostatin-1 (Fer-1, purity≥95%) was provided by Sigma (USA). Antibodies against glutathione peroxidase 4 (GPX4), solute carrier family 7 member 11 (SLC7A11) and SUV39H1 were supplied by Cell Signaling (USA). ROS assay kit was purchased from Shanghai Beyotime Biotechnology Co., Ltd. (China). Cell counting kit-8 (CCK-8) assay kit was bought from Sichuan Maccura Biotechnology Co., Ltd. (China). Forma311CO2 incubator and StepOnePlus PCR system were obtained from Thermo Firsher Scientific (USA). Fluorescence microscope was provided by Nikon (Japan).

Cell culture: SGC-7901 cells were cultured in an incubator with 5% CO2 at 37°C in the presence of DMEM containing penicillin (100 U/mL), fetal bovine serum (10%) and streptomycin (100 μg/mL), and the medium was replaced with fresh one every 2-3 days. Upon reaching 80% confluence, the cells were passaged, and later assays were carried out on those in the exponential phase of growth.

Cell transfection and grouping: After the adjustment of cell density to 1×105/well, a 6-well plate was employed to seed SGC-7901 cells in the logarithmic exponential phase of growth, followed by transfection according to the manufacturer's manual for Lipofectamine 2000 transfection kit (Thermo Fisher Scientific, USA): si-NC group (transfection of si-NC plasmids into SGC-7901 cells), si-FOXM1 group (SGC-7901 cells undergoing si-FOXM1 plasmid transfection), si-FOXM1+Fer-1 group (transfection of SGC-7901 cells with si-FOXM1 plasmids and culture in medium containing 20 μmol/L Fer-1), si-SUV39H1 group (si-SUV39H1 plasmids transfected into SGC-7901 cells), si-SUV39H1+Fer-1 group (transfection of si-SUV39H1 plasmids into SGC-7901 cells and culture in medium containing 20 μmol/L Fer-1), si-FOXM1+pcDNA3.1+SUV39H1 group (si-SUV39H1 plasmids and pcDNA3.1+SUV39H1 plasmids simultaneously transfected into SGC-7901 cells), si-FOXM1+pcDNA3.1+SUV39H1+Fer-1 group (simultaneous transfection of SGC-7901 cells with si-SUV39H1 plasmids and pcDNA3.1+SUV39H1 plasmids and cultured in medium containing 20 μmol/L Fer-1), and control group (SGC-7901 cells not transfected). The cells were cultured for 48h and then used for later assays.

CCK-8 assay of cell proliferation: The 96-well plate was used for inoculation of SGC-7901 cells (1.5×104/mL in each well), followed by culture at 37°C in the presence of 5% CO2 for 48h. Then every well was supplemented with CCK-8 solution (100μL), followed by 1.5h of incubation under the same conditions as above. At last, a microplate reader was applied to measure the optical density of each well at 450nm.

Detection of iron content in cells: SGC-7901 cells were collected with PBS, digested by trypsin, and homogenized by ultrasonication at 4°C, and the iron content in cells was detected according to the kit instructions.

Detection of ROS levels by dihydroethidium (DHE) fluorescence staining: A serum-free medium was used for the dilution of SGC-7901 cells, which were added with DCFH-DA (diluted at 1:1000), reaching a final probe concentration of 10 μmol/L. Following 20min of incubation by the incubator under 5% CO2 and shaking every 5min, the cells were washed and photographed under a fluorescence microscope. Finally, the fluorescence intensity was calculated using ImageJ software (NIH, USA).

Detection of H2S yield in cells by sensitive sulfur electrode assay: SGC-7901 cells were homogenized for the quantification of proteins via the Bradford method. Then the homogenate was transferred to a reaction flask, and added with reaction solutions (2 mmol/L pyridoxal phosphate, 100 mmol/L phosphate buffer, and 10 mmol/L L-Cys) and then 0.5mL of sodium hydroxide. The conical flask was filled with nitrogen for 20s and sealed, followed by 90min of incubation at 37°C using a water bath.

Subsequently, 60-min incubation of the cells supplemented with 500μL of 50% trichloroacetic acid was conducted again at 37°C. Finally, the reaction was terminated, and the liquid in the central well was harvested to measure the H2S level by sensitive sulfur electrode assay. The H2S yield (nmol·mg-1·min-1) was calculated based on the homogenate protein level and the reaction time, i.e. H2S yield=(H2S level/homogenate protein level)/reaction time.

Detection of protein expressions of SUV39H1, CSE, GPX4 and SLC7A11 by Western blotting: Subsequent to lysis with RIPA buffer, the concentration of total protein in SGC-7901 cells was measured by the BCA method. The protein samples at the final concentration of 2 μg/mL were boiled in hot water for 10min and stored in a -20°C refrigerator. Then loading buffer was thoroughly blended with the protein samples (50μg) for denaturation by boiling water. After SDS-PAGE separation, a PVDF membrane was used for protein sample transfer, blocked for 1h by 5% skim milk, and washed with 1 mL/L TBST, prior to 4°C primary antibody (1:1000 diluted) incubation overnight. The next day, after TBST was washed away, secondary antibodies (1:1000 diluted) were added to the membrane for 2h of room-temperature culture. Finally, ECL reagent was added to the cells for exposure and development, and Image Lab software was applied to analyze the bands for gray values.

Statistical analysis: Statistical analyses were accomplished by GraphPad Prism 8.0 software. The normality of data was studied using the Shapiro-Wilk test, and all data were found to be normally distributed. Mean ± standard deviation ($\bar{x} \pm s$) was utilized to express the measurement data. Comparisons among multiple groups were conducted through one-way repeated measures ANOVA, and pairwise comparison between groups was performed using the LSD-t test. $P<0.05$ denoted a difference of statistical significance.

RESULTS

si-FOXM1 inhibited GC cell proliferation and SUV39H1 protein expression and promoted ferroptosis: The differences in cell survival rate, relative fluorescence intensity of ROS, relative iron content, H2S yield and protein expressions of SUV39H1, CSE, GPX4 and SLC7A11 between si-NC and control groups were not statistically significant ($P>0.05$). In comparison with the control group, the cell survival rate, H2S yield and protein expressions of SUV39H1, CSE, GPX4 and SLC7A11 declined significantly, but the relative fluorescence intensity of ROS and the relative iron content exhibited significant increases in the si-FOXM1 group ($P<0.05$). The si-FOXM1+Fer-1 group had significantly higher cell survival rate, H2S yield and protein expressions of SUV39H1, CSE, GPX4 and SLC7A11, in addition to lower relative fluorescence intensity of ROS and relative iron content than those of the si-FOXM1 group ($P<0.05$) (Fig. 1).

si-SUV39H1 inhibited GC cell proliferation and SUV39H1 protein expression and promoted ferroptosis: In comparison to the control group, the si-SUV39H1 group had significant decreases in cell survival rate, H2S yield and protein expressions of SUV39H1, CSE, GPX4 and SLC7A11, as well as significant increases in the relative fluorescence intensity of ROS and relative iron content ($P<0.05$). Furthermore, the si-SUV39H1+Fer-1 group had increased cell survival rate, H2S yield and protein expressions of SUV39H1, CSE, GPX4 and SLC7A11, and decreased relative fluorescence intensity of ROS and relative iron content compared with those of the si-SUV39H1 group ($P<0.05$) (Fig. 2).

si-FOXM1 promoted ferroptosis in GC cells by inhibiting SUV39H1 and regulating CSE/H2S: Compared with the si-FOXM1 group, si-FOXM1+ pcDNA3.1+SUV39H1 and si-FOXM1+pcDNA3.1+ SUV39H1+Fer-1 groups, especially the latter group, had significantly increased cell survival rate, H2S yield and protein expressions of SUV39H1, CSE, GPX4 and SLC7A11 and significantly decreased relative fluorescence intensity of ROS and relative iron content ($P<0.05$) (Fig. 3).

DISCUSSION

As a frequently occurring digestive system malignancy globally, GC poses a serious threat to people's health due to high morbidity and mortality rates. The morbidity and mortality rates of GC rank fifth and third, respectively, in the world, and have shown increasing trends over the recent past years (Chen *et al.,* 2020). It is estimated that by 2030, GC will become a member of the 15 major contributors to the death worldwide. To better understand the pathogenesis and progression of GC, various animal models have been developed, including genetically engineered mice, chemically induced models, and xenograft models (Li *et al.*, 2023; Zhang *et al.*, 2024). GC patients are still experiencing a low 5-year survival rate, despite some recent progress on the therapy (Katai *et al.,* 2018). Therefore, it is of great significance to discover new targets for GC for ameliorating the prognosis.

The Fox family has been reported to participate in tumor occurrence and development by mediating embryonic development, proliferation, differentiation, apoptosis, transformation and tumorigenesis (Wang *et al.,* 2022). FOXM1 is a typical pro-proliferative transcription factor in the Fox family, being closely related to cell proliferation, invasion, metastasis and angiogenesis. It not only contributes to tumor progression, but also activates many factors affecting tumor onset. Guo and Wu (2022) found that FOXM1 regulated NUF2 expression and autophagy via the AKT/PI3K/mTOR signal transduction pathway, thereby mediating the proliferation, migration and invasion of glioma cells. Additionally, Hafez *et al.* (2021) found that down-regulation of FOXM1 made HeLa/DDP (human cervical cancer cell line) more sensitive to cisplatin-based chemotherapy. Moreover, FOXM1 induced the progression of neoplasm in patients with endometrial cancer (Tang *et al.,* 2023). Consistently, we herein found that FOXM1 suppression inhibited GC cell proliferation.

Iron is one of the indispensable elements for maintaining the basic and special functions of cells, but abnormally accumulated Fe^{2+} can directly generate ROS

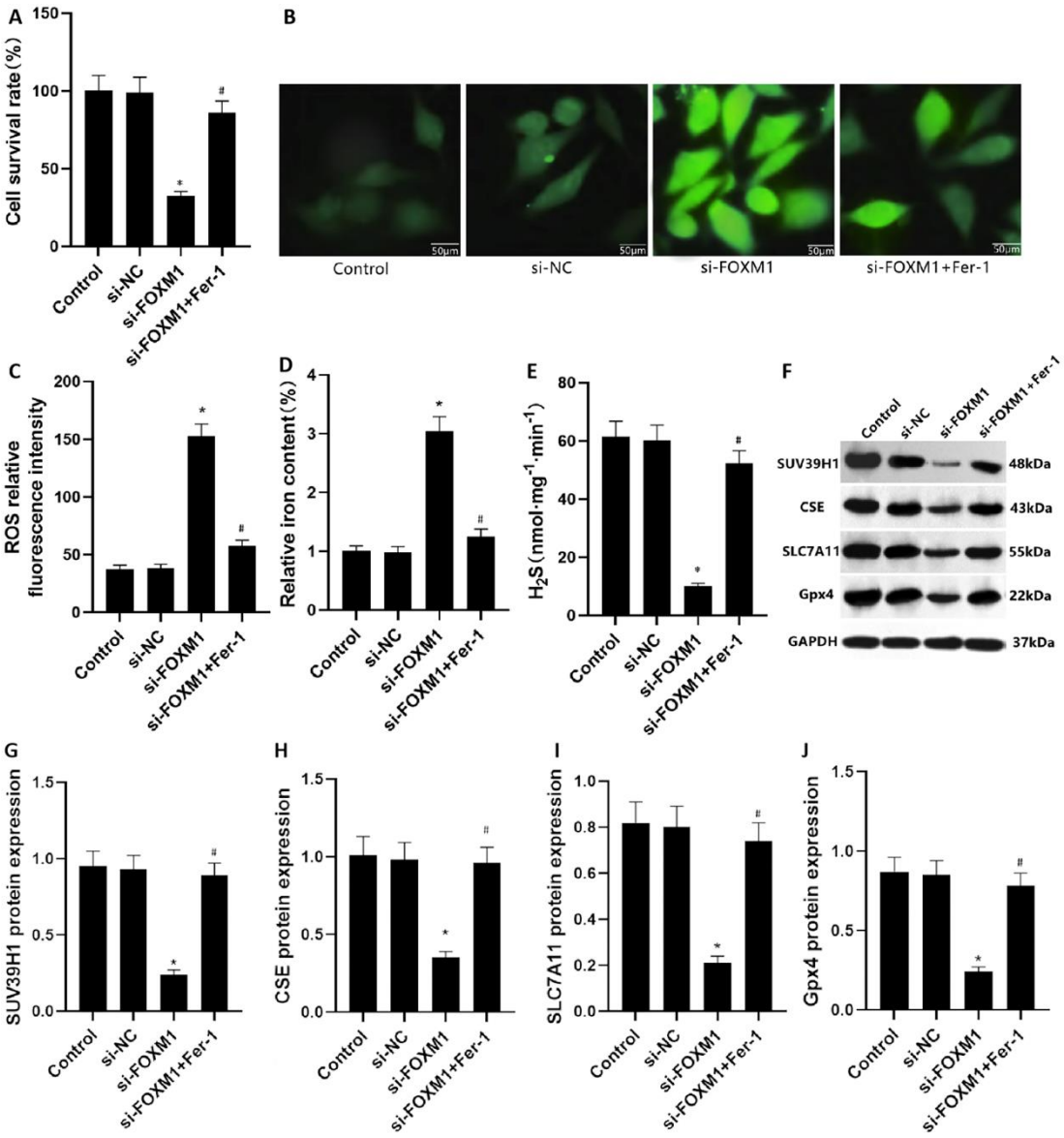

Fig. 1: si-FOXM1 inhibited GC cell proliferation and SUV39H1 protein expression, and promoted ferroptosis. A: Comparison of cell survival rate. B: ROS levels detected by DHE fluorescence staining (×200). C: Comparison of relative fluorescence intensity of ROS. D: Comparison of relative iron content in cells. E: Comparison of H_2S yield in cells. F: Protein expressions of SUV39H1, CSE, GPX4 and SLC7A11 detected by Western blotting. G: Protein expression quantification for SUV39H1 in cells. H: Quantification of cellular CSE at protein level. I: Protein expression quantification for SLC7A11 in cells. J: Quantification of cellular GPX4 at protein level. $^{\#}P<0.05$ *vs.* si-FOXM1 group. $^{*}P<0.05$ *vs.* control group

through the Fenton reaction, resulting in excessive lipid peroxidation, reducing the antioxidant capacity of cells, and inducing ferroptosis (Ni *et al.,* 2021). GPX4 is an antioxidant enzyme able to scavenge lipid peroxides in cells. In the case of GPX4 expression decline or dysfunction, lipid peroxides in cells cannot be effectively eliminated, thus contributing to ferroptosis (Wang *et al.,* 2022). SLC7A11 is a carrier protein involved in glutathione synthesis and maintenance of intracellular redox balance. When the expression of SLC7A11 declines, the glutathione level in cells decreases, then increasing the risk of ferroptosis (Lee and Roh, 2022). In this study, FOXM1 suppression increased iron and ROS content and inhibited the protein expressions of GPX4 and SLC7A11 in GC cells, implying the possible involvement of ferroptosis in GC cell death. Furthermore, the ferroptosis inhibitor Fer-1 attenuated the inhibitory role of FOXM1 suppression in the proliferation of GC cells in addition to the protein expressions of GPX4 and SLC7A11 and its promoting effects on the iron and ROS content, suggesting that FOXM1 suppression facilitated the ferroptosis of GC cells.

Endogenous H_2S has some biological effects on tumor cells, and one of its main mechanisms is to regulate cell proliferation and apoptosis. Endogenous H_2S can contribute to the proliferation of colon cancer and ovarian cancer cells, and decreasing its level can significantly promote apoptosis (Hellmich and Szabo, 2015; Szabo, 2021). Li *et al*. (2014) found that tumor necrosis factor-α promoted the binding of transcription factor SP1 and CSE gene enhancer to elevate CSE protein expression level and thus facilitate H_2S synthesis. Meanwhile, a sulfhydrylation reaction occurs between H_2S and p65 subunit of NF-κB, and then the expression of downstream anti-apoptotic genes is up-regulated, exerting an anti-apoptotic effect. In this study, FOXM1 suppression reduced H_2S and CSE levels in GC cells, suggesting that FOXM1 suppression may promote the ferroptosis of GC cells by inhibiting the CSE/H_2S signaling pathway.

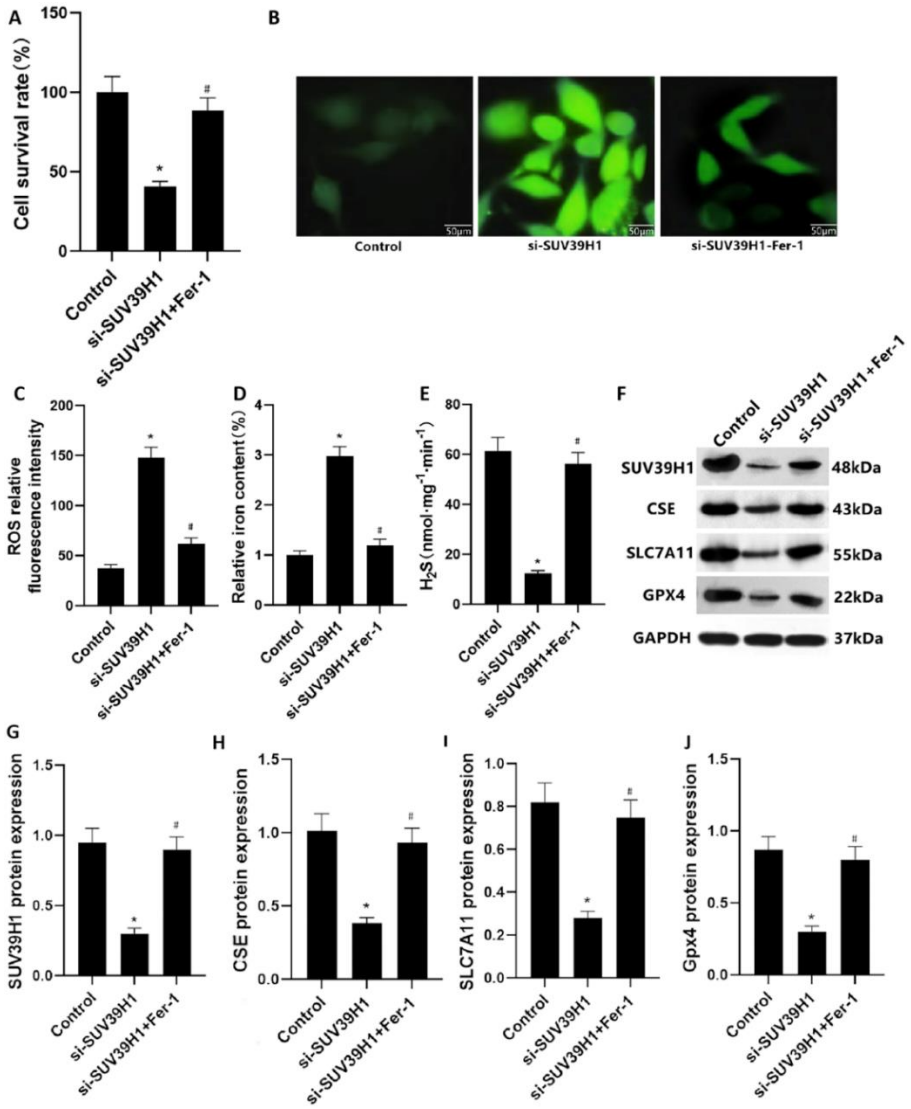

Fig. 2: si-SUV39H1 inhibited GC cell proliferation and SUV39H1 protein expression and promoted ferroptosis. A: Comparison of cell survival rate. B: ROS levels detected by DHE fluorescence staining (×200). C: Comparison of relative fluorescence intensity of ROS. D: Comparison of relative iron content in cells. E: Comparison of H_2S yield in cells. F: Protein expressions of SUV39H1, CSE, GPX4 and SLC7A11 detected by Western blotting. G: SUV39H1 protein expression in cells obtained through quantification. H: Expression of CSE protein in cells by means of quantitative determination. I: Quantification of SLC7A11 in cells at protein level. J: Expression of cellular GPX4 protein *via* quantitative analysis. $^{\#}P<0.05$ *vs*. si-SUV39H1 group. $^{*}P<0.05$ *vs*. control group.

thus facilitate H_2S synthesis. Meanwhile, a sulfhydrylation reaction occurs between H_2S and p65 subunit of NF-κB, and then the expression of downstream anti-apoptotic genes is up-regulated, exerting an anti-apoptotic effect. In this study, FOXM1 suppression reduced H_2S and CSE levels in GC cells, suggesting that FOXM1 suppression may promote the ferroptosis of GC cells by inhibiting the CSE/H_2S signaling pathway.

It is well-documented that gene mutation and epigenetic alterations may play important roles in the pathogenesis of cancer (Chu *et al.,* 2020). Histone modification abnormality can mediate the occurrence and development of diseases by causing the imbalance of epigenetic regulation. However, these epigenetic alterations are reversible, which also provide new therapeutic targets (Chrun *et al.,* 2017). SUV39H1 is an enzyme that can catalyze the methylation of histone H3K9, which participates in heterochromatin formation. It not only mediates the segregation and stability of chromosomes, but also contributes to the occurrence of tumors by affecting tumor suppressor protein Rb (Lu *et al.,* 2020). Akçay *et al*. (2022) found that SUV39H1 had a significantly increased expression in prostate cancer and promoted the stimulated prostate cancer cells to migrate and infiltrate. Besides, Li *et al*. (2021b) reported that SUV39H1 promoted ovarian cancer cell proliferation and had an apparent relationship with patients' poor prognosis. In the present study, FOXM1 suppression inhibited SUV39H1 protein expression in GC cells. Therefore, we postulated that FOXM1 suppression may inhibit SUV39H1 expression to enhance GC cell ferroptosis. Furthermore, SUV39H1 suppression also inhibited the protein expressions of GPX4 and SLC7A11 and increased iron and ROS content. Fer-1 attenuated the promoting effect of SUV39H1 suppression on the ferroptosis of GC cells, suggesting that SUV39H1 suppression can also facilitate the ferroptosis of GC cells. SUV39H1 has been reported to indirectly regulate the CSE/H_2S relationship through interacting with transcription factors. For example, SUV39H1 can bind to transcription factor Sp1 and form a complex in its upstream promoter region, thus regulating the transcription of CSE gene (Chuang *et al.,* 2011). In this study, SUV39H1 suppression reduced H_2S and CSE levels in GC cells, while Fer-1 attenuated such an inhibitory effect. Therefore, we hypothesized that FOXM1 suppression may promote the ferroptosis of GC cells by inhibiting the CSE/H_2S signaling pathway mediated by SUV39H1. To verify this hypothesis, si-FOXM1 plus pcDNA3.1-SUV39H1 was used for the

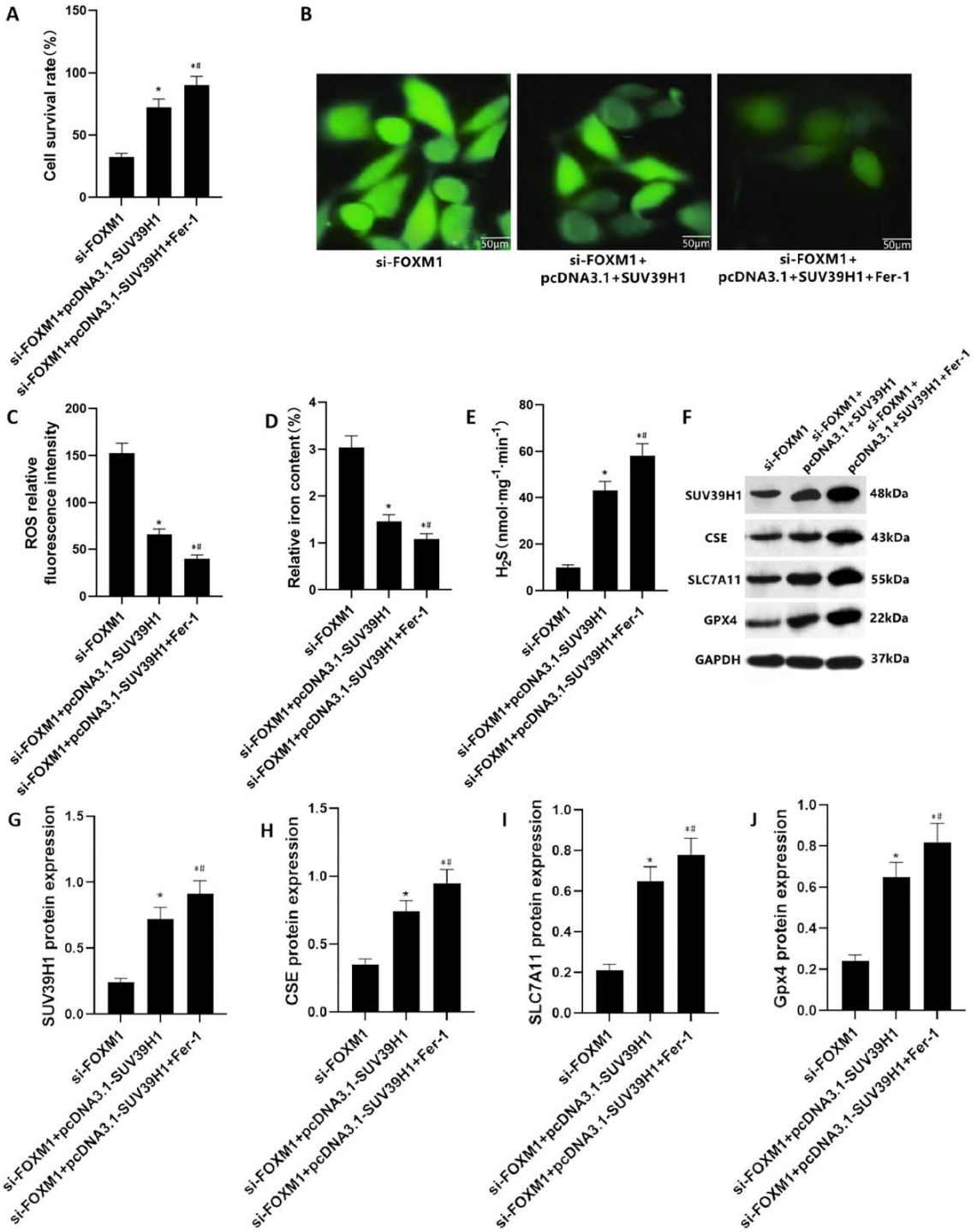

Fig. 3: si-FOXM1 promoted ferroptosis in GC cells by inhibiting SUV39H1 and regulating CSE/H_2S. A: Comparison of cell survival rate. B: ROS levels detected by DHE fluorescence staining (×200). C: Comparison of relative fluorescence intensity of ROS. D: Comparison of relative iron content in cells. E: Comparison of H_2S yield in cells. F: Protein expressions of SUV39H1, CSE, GPX4 and SLC7A11 detected by Western blotting. G: Protein expression of SUV39H1 in cells detected by quantitative assay. H: Protein expression quantification for CSE in cells. I: Quantification of cellular SLC7A11 at protein level. J: GPX4 protein expression in cells obtained through quantitative determination. $^{\#}P<0.05$ *vs.* si-FOXM1+pcDNA3.1+SUV39H1 group. $^{*}P<0.05$ *vs.* si-FOXM1 group.

intervention with GC cells. The results showed that overexpression of SUV39H1 attenuated the promoting effect of FOXM1 suppression on the ferroptosis of GC cells, and Fer-1 further weakened the promoting effect of si-FOXM1 plus pcDNA3.1-SUV39H1 on ferroptosis.

Conclusions: In view of the rapidly increasing rate of malignancies, especially the gastric cancer, investigation for the new and reliable treatment options is direly needed. For this purpose, therapies targeting FOXM1 can be very effective. For testing this hypothesis, the current research was carried out. The results of this study revealed FOXM1's suppression to able to promote the ferroptosis of GC cells while repressing their proliferation by inhibiting SUV39H1 expression and CSE/H2S signaling pathway. Hence, FOXM1 can be a new target for GC therapy. However, further extensive and deeper trials are needed to verify the effectiveness of this approach in treating and preventing GC cases.

Authors contribution: Zhihu Li was responsible for the study design and data analysis. Wenpei Hou and Jia Wang participated in data collection and analysis. Xia Han, Muhammad Tariq and Lianxing Zhang provided technical support and assisted with data interpretation. Xingdong Wang supervised the entire project and provided critical revisions to the manuscript. All authors reviewed and approved the final version of the manuscript for submission.

Funding: This study was financially supported by the Health Industry Plan Management Project of Gansu Province (No. GSWSKY-2019-96).

Conflict of interest: There is no conflict of interest among the authors.

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