Up-regulation of E-cadherin by the combination of Methionine restriction and HDAC2 intervention for inhibiting gastric carcinoma metastasis

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<th>Journal:</th>
<th>Acta Biochimica et Biophysica Sinica</th>
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<td>Manuscript ID</td>
<td>ABBS-2023-160.R4</td>
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<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>24-Sep-2023</td>
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| Complete List of Authors: | Li, Yi-Fan  
Liu, Chen-Xi  
Xin, Lin; The Second Affiliated Hospital of Nanchang University,  
Liu, Chuan  
Cao, Jia-Qing  
Yue, Zhen- Qi  
Sheng, Jie  
Yuan, Yi-Wu  
Zhou, Qi  
Liu, Zhi-Yang |
| Keywords: | Methionine restriction, HDAC2, gastric cancer, metastasis |
Original Article

Upregulation of E-cadherin by the combination of methionine restriction and HDAC2 intervention for inhibiting gastric carcinoma metastasis

Yifan Li1,†, Chenxi Liu2,†, Lin Xin1,*, Chuan Liu1, Jiaqing Cao1, Zhenqi Yue1, Jie Sheng1, Yiwu Yuan1, Qi Zhou1, and Zhiyang Liu1

1 Department of General Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China, and 2 Excellent Ophthalmology Class 221, School of Ophthalmology & Optometry, Nanchang University, Nanchang 330006, China.

†These authors contributed equally to this work and should be regarded as co-first authors.

*Correspondence address. Tel: +86-791-86312173; E-mail: lin_xin2006@163.com

Running title: Synergetic MR and HDAC2 intervention in GC metastasis

Received: 03-Apr-2023
Accepted: 27-Jul-2023

Abstract

Invasion and metastasis are the leading causes of death in individuals with malignant tumors, including gastric cancer. In this study, we aimed to explore the effect and related mechanisms of methionine restriction (MR) on gastric carcinoma metastasis. In the MR cell model, gastric carcinoma cells were cultured in MR medium, and in the animal model, BALB/c nude rodents were administered a methionine-free diet after receiving injections of MKN45 cells into the caudal vein. Transwell assays were used to detect cell invasion and migration. Chromatin immunoprecipitation was performed to investigate the levels of H3K9me2, H3K27Ac, or H3K27me3 on the E-cadherin promoter. MR inhibited gastric carcinoma cell migration, invasion, and lung metastasis. MR increased E-cadherin while reducing H3K27me3 levels on the E-cadherin promoter. E-cadherin expression in gastric carcinoma cells was adversely regulated by HDAC2. Overexpressing HDAC2 reduced H3K27Ac levels on the E-cadherin promoter, while interfering with HDAC2 increased H3K27Ac levels. HDAC2 interference under MR conditions further upregulated E-cadherin expression and inhibited gastric carcinoma cell migration, invasion, and lung metastasis. MR combined with HDAC2 interference promoted E-cadherin expression by mediating the methylation and acetylation of E-cadherin, thus inhibiting the invasion, migration, and lung metastasis of gastric...
carcinoma cells. Our study provides a new theoretical basis for the inhibitory effect of MR in gastric cancer.

**Keywords:** methionine restriction, HDAC2, gastric cancer, metastasis

**Introduction**

Gastric cancer is one of the most prevalent cancers in China and even the world, with 1 million new cases every year. Gastric cancer is the fourth most common cancer in men and the seventh most common in women, and it is most prevalent in individuals over 55 years old [1]. The pathogenesis of gastric cancer is unknown and may be related to a variety of factors, such as smoking, age, long-term *Helicobacter pylori* infection, environmental factors, lifestyle, diet type, genetics, and economic level [2]. Early gastric cancer is typically asymptomatic or has very modest signs, making detection challenging. When the clinical symptoms appear, the lesions have already progressed, which suggests that the tumor cells have metastasized. Metastasis is a complex challenge for cancer treatment.

Invasion and metastasis, which are the major causes of death in patients with malignant tumors, have been shown to be the main characteristics of malignant tumors [3, 4]. A crucial biological process known as epithelial-mesenchymal transition (EMT) allows epithelial-derived malignant tumor cells to migrate and invade. The absence of epithelial cell markers such as E-cadherin is one of the hallmarks of EMT [5]. High methylation of the E-cadherin promoter has been demonstrated in a variety of tumors [6, 7], including gastric cancer [8, 9], and its decreased expression promotes tumor invasion and distant metastasis [10], suggesting that downregulating E-cadherin promoter methylation and promoting its expression is an effective way to inhibit EMT and metastasis of gastric carcinoma cells.

Methionine (Met) is an essential amino acid that can be converted by methyltransferase into S-adenosine methionine (SAM), a methyl donor, to produce a substrate for methylation [11]. Methionine restriction (MR) can downregulate SAM levels [12]. The reduction of SAM can reduce the methylation level of histone H3 [13]. L-methionine-γ-lyase (METase) has the ability to precisely lyse Met in and out of cells and thus can be used as a genetic way to restrict Met [14]. Our previous studies have revealed that the physiological characteristics of gastric carcinoma cells, including autophagy and stem cell property regulation, can be regulated by Met deletion or exogenous addition of METase to regulate the sensitivity of gastric carcinoma cells to chemotherapy drugs [15-17]. Nevertheless, how MR affects the migration and invasion of gastric carcinoma cells remains unclear. Therefore, we speculate that MR may promote E-cadherin promoter expression by reducing its histone methylation level, thus inhibiting EMT and metastasis of gastric carcinoma cells.

In gastric carcinoma cells, high levels of E-cadherin promoter methylation were found to be accompanied by low levels of acetylation, according to a study [18], suggesting that the epigenetic modification of E-cadherin can be carried out from both methylation and acetylation. Through a literature review, we found that histone
deacetylase HDAC2 may be another intervention target for regulating E-cadherin [19]. We speculate that HDAC2 may promote the progression of gastric cancer by mediating the deacetylation of E-cadherin histone and reducing its expression.

In this study, we intend to explore the effects of MR combined with HDAC2 intervention on gastric carcinoma cell metastasis and the related mechanisms.

**Materials and Methods**

**Cell culture**

MKN45 and AGS human gastric carcinoma cells (Procell, Wuhan, CHN) were cultured in complete medium (CM) or methionine restriction (MR) medium (R7513; Sigma Aldrich, St Louis, USA) supplemented with penicillin/streptomycin (Gibco, Grand Island, USA) at 37°C and 5% CO₂.

**Transwell assay**

Transwell chambers (Corning, New York, USA) coated with or without Matrigel (BD Biosciences, CA, USA) were used to conduct invasion and migration experiments, respectively. The Matrigel was removed from –20°C 12 h in advance and placed in a 4°C refrigerator overnight. After dilution, Matrigel was evenly applied on the surface of the polycarbonate film in the upper chamber and placed at 37°C for 1 h to polymerize into the gel. The cells (2 × 10⁵) cultured in CM or MR medium were added to the upper chamber. The lower chamber was filled with RPMI-1640 medium (Gibco) containing 10% fetal calf serum (Gibco). The culture system was incubated in an incubator for 24 h, the upper layer of the medium was removed, and the Transwell membrane was completely immersed in methanol after washing with phosphate-buffered saline (PBS) and fixed at room temperature for half an hour. Subsequently, the methanol was discarded, and the chambers were stained with crystal violet (Zhongshan Bio, Beijing, CHN) for 15 min. Finally, the stained chambers were placed under a microscope (Leica, Wetzlar, Germany), and 5 fields were randomly selected to count the number of penetrating cells in the lower layer.

**Animal experiment**

Six-week-old BALB/c nude mice were housed in Nanchang University’s Laboratory Animal Science Center. All experiments were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Nanchang University. MKN45 cells were injected into BALB/c nude rodents via the caudal vein (2 × 10⁶ cells). After injection, mice were divided into the Met⁺ group (n = 6) and the Met⁻ group (n = 6). Mice in the Met⁺ group were given a normal diet, while mice in the Met⁻ group were given a methionine-free diet. Eight weeks later, the mice were sacrificed, and lung tissue was collected.

MKN45 cells transfected with lentivirus-sh-HDAC2 or lentivirus-sh-NC (RiboBio, Guangzhou, CHN) were injected into BALB/c nude mice via the caudal vein (2 × 10⁶ cells). After injection, mice were split into four groups: Met⁺+sh-NC, Met⁺+sh-HDAC2, Met⁻+sh-NC, and Met⁻+sh-HDAC2. Mice in the Met⁺ group were given a normal diet, while mice in the Met⁻ group were given a methionine-free diet. Eight weeks later, the mice were sacrificed, and lung tissue was collected. The target sequence of shRNA HDAC2 (TRCN0000004823; Sigma) is: 5’-GCAAATACTATGCTGTCAATT-3’.
control is a meaningless sequence attached to the HDAC2 shRNA that does not target the human genome.

**Hematoxylin-eosin (HE) staining**

After cervical dislocation in mice, the lung tissue was removed and immediately frozen in liquid nitrogen. Subsequently, the tissue was cut into slim slices and fixed with 4% paraformaldehyde (Sinopharm, Shanghai, China). After being washed, slices were stained with hematoxylin for 20 min (NobleRyder, Beijing, China), differentiated with differentiation solution (NobleRyder) for 30 s, and stained with eosin for 2 min (NobleRyder). Following xylene transparency, segments were sealed with neutral resin and observed under the microscope. The nucleus is stained blue-purple and the cytoplasm is stained pink.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated from AGS and MKN45 cells using TRIzol (Thermo Fisher Scientific, Waltham, USA). Then, the RNA was reverse transcribed into cDNA using SuperScript™ IV reverse transcriptase (Thermo Fisher Scientific). The obtained cDNA template and PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) were applied for the qPCR, which was centrifuged and added to an ABI 7500 RT-PCR system (Applied Biosystems, Carlsbad, USA). The primers are shown as follows: E-cadherin forward 5′-GGATGTGCTGGATGTGAATG-3′ and reverse 5′-AGCAAGAGCAGCAGAATCAG-3′; HDAC2 forward 5′-AGACTGCAGTTGCCCTTGAT-3′ and reverse 5′-TGCGCAAATTTTCAAACAAA-3′; GAPDH forward 5′-GATGAGATTGGCATGGCTTT-3′ and reverse 5′-CACCTTCACCGTTCCAGTTT-3′. GAPDH was used as a control. Levels of HDAC2 and E-cadherin were examined using the 2$^{-\Delta\Delta C_t}$ method.

**Immunofluorescence**

AGS and MKN45 cells were fixed with 4% paraformaldehyde for 10 min and washed with cold PBS. Cells were then treated with sodium citrate buffers for antigen repair, followed by permeabilization with 0.1% Triton X-100. After that, the cells were blocked with 1% bovine serum albumin (BSA) and incubated with an anti-E-cadherin antibody (1:500; ab40772; Abcam, Cambridge, USA) in a wet box overnight away from light. Following incubation with the secondary antibody (1:500; ab150075; Abcam) for 2 h away from light, cells were sealed with an antifade mounting medium (Beyotime, Shanghai, CHN), and the images were observed and photographed by a confocal fluorescence microscope (Leica).

**Chromatin immunoprecipitation**

The ChIP kit ab500 was used to examine the combination of the E-cadherin promoter and dimethylated histone H3 at lysine 9 (H3K9me2), trimethylated histone H3 at lysine 27 (H3K27me3), or acetylation of lysine 27 on histone 3 (H3K27Ac). AGS and MKN45 cells were treated with trypsin and incubated at 37°C for 5 min to produce a single-cell suspension (3×10^7 cells). After centrifugation, the supernatant was discarded, and the cells were resuspended in a buffer A/formaldehyde/PBS mixture. The final formaldehyde concentration was approximately 1.1%. Glycine was added to seal the formalin, followed by mixing and centrifugation to precipitate the cells. Then, the cells were lysed, and the DNA was chopped into 200–1000 bp fragments using an ultrasonic
crusher. Add 1× ChIP buffer/PI mixture to the clipped chromatin and swirl for 5 s. The antibody (H3K9me2, H3K27me3, or H3K27ac) was added to the corresponding sample and incubated overnight at 4°C. Afterward, the prepared beads were added and incubated at 4°C for 50 min. qPCR was conducted to measure the levels of the E-cadherin promoter. E-cadherin promoter sequence: sense-5′-GGGCTGGGATTCGAACCCAGTG-3′ and antisense-5′-CCAATCAGCAGCGCGGACCC-3′.

**Cell transfection**

The vectors for HDAC2 overexpression (OE-HDAC2), HDAC1 interference (si-HDAC1), HDAC2 interference (si-HDAC2), and HDAC3 interference (si-HDAC3) were provided by RiboBio (Guangzhou, CHN). AGS or MKN45 cells were seeded in a 24-well plate until 80% confluence and then transfected with OE-HDAC2, si-HDAC1, si-HDAC2, or si-HDAC3 using Lipofectamine® RNAiMAX (Invitrogen, CA, USA) according to the manufacturer’s instructions. After transfection for 48 h, cells were collected for subsequent detection. Si-HDAC1: 5′-CCGGUCAUGUCCAAAGUAA-3′; si-HDAC2: 5′-GCCTCATAGAATCCGCATGdtdt-3′; si-HDAC3: 5′-CGGUGUCCUUCCACAAUA-3′; si-NC: 5′-CAGTCGCGTTTGCGACTGGdtdt-3′.

**Western blot analysis**

AGS and MKN45 cells were lysed with pierce RIPA buffer (Thermo Fisher Scientific) to obtain the protein samples. The cells were blown into a mixture with a pipette gun and then left at 4°C for 45 min. After ultrasonic lysis, the cells were centrifuged at 15000 g for 10 min, the supernatant was taken, and the precipitate was discarded. The protein concentration was then measured using a BCA kit (Thermo Fisher Scientific). Then, electrophoresis and transmembrane operation were applied to the protein samples. Afterward, the membranes were trimmed according to molecular weight and then blocked with 5% BSA and incubated with anti-HDAC2 antibody (1:8000, ab12169; Abcam), anti-E-cadherin antibody (1:5000, ab40772; Abcam), and anti-GAPDH antibody (1:1000, ab8245; Abcam) at 4°C. After 16 h, the membranes were washed with PBS, followed by incubation with the diluted secondary antibody for 2 h. The blots were observed using ECL reagents (Thermo Fisher Scientific) [20].

**Statistical analysis**

Data were analysed using GraphPad Software and expressed as the mean ± SD. The difference between the two groups was evaluated via Student’s t-test, and the difference among multiple groups was evaluated via one-way (or two-way) analysis of variance. P < 0.05 was considered to be statistically significant.

**Results**

*Methionine restriction (MR) prevents gastric carcinoma cell migration, invasion, and in vivo metastasis*

MKN45 and AGS cells were cultured in CM or MR medium for 24 h. Transwell experiments revealed that MR culture reduced migration (Figure 1A) and invasion (Figure 1B) in MKN45 and AGS cells. In addition, apoptosis detection demonstrated that MR treatment for 24 h had no significant influence on gastric carcinoma cell apoptosis, whereas MR treatment for 48 h increased the apoptosis of gastric carcinoma...
cells (Supplementary Figure S1A). BALB/C nude mice were injected with MKN45 cells and then given a normal diet (Met+) or a methionine-free diet (Met−). Lung tissue treated with HE showed that nude rodents in the Met− group had fewer lung metastases than those in the Met+ group (Figure 1C). The data suggested that MR inhibited cell migration, invasion, and lung metastases.

Methionine restriction (MR) reduces H3K27me3 levels on the E-cadherin promoter

E-cadherin has been reported to be downregulated in many malignancies, including gastric cancer [21]. MR culture upregulated the mRNA levels of E-cadherin in MKN45 and AGS cells (Figure 2A). Immunofluorescence staining also showed that MR treatment increased the level of E-cadherin protein in AGS and MKN45 cells (Figure 2B). The methylation level of CpG islands on the E-cadherin promoter region was then detected, and the findings revealed that MR had no appreciable impact on methylation (data not shown). H3K9me2 and H3K27me3 play a role in controlling E-cadherin expression in gastric carcinoma cells [22]. Therefore, we further explored how MR treatment affected H3K9me2 and H3K27me3 levels on the E-cadherin promoter. MKN45 and AGS cells were cultured under MR conditions for 0, 6, 24, and 48 h, followed by ChIP experiments. The findings revealed that H3K27me3 levels on the E-cadherin promoter were significantly reduced at 6 h following MR culture and remained at a low level for the duration of this study, namely, 48 h, but there was no significant change in H3K9me2 (Figure 2C,D). These results indicated that MR led to a decrease in H3K27me3 levels on the E-cadherin promoter.

HDAC2 regulates the acetylation of the E-cadherin promoter

Considering the increased expression of HDAC2 in gastric cancer and its regulatory effect on E-cadherin histone deacetylation, we next explored the regulatory effect of HDAC2 on E-cadherin in gastric carcinoma cells. MKN45 and AGS cells were transfected with OE-HDAC2 or si-HDAC2. qRT–PCR and western blot assays revealed that highly expressed HDAC2 inhibited E-cadherin expression in MKN45 and AGS cells, while interference with HDAC2 increased E-cadherin expression (Figure 3A-C). Moreover, interference with HDAC1 and HDAC3 had no significant effect on E-cadherin (CDH1) expression (Supplementary Figure S2). We also detected apoptosis after interference with HDAC2, and the results showed that HDAC2 intervention had no significant effect on the apoptosis of gastric carcinoma cells (Supplementary Figure S1B). A ChIP experiment was performed to confirm that HDAC2 modulates the acetylation of the E-cadherin promoter, and the H3K27ac antibody was employed for immunoprecipitation. The findings revealed that the H3K27ac level on the E-cadherin promoter decreased after overexpression of HDAC2, while H3K27ac levels on the E-cadherin promoter increased after interference with HDAC2 (Figure 3D). The above data indicated that HDAC2 might inhibit E-cadherin transcription by inhibiting E-cadherin promoter acetylation.

HDAC2 expression intervention combined with methionine restriction (MR) upregulates E-cadherin expression

We further investigated the effect of HDAC2 on E-cadherin in gastric carcinoma cells under MR conditions. The qRT–PCR (Figure 4A,B) and western blot (Figure 4C) experiments indicated that MR increased the expression of E-cadherin in MKN45
and AGS cells but had no discernible impact on the expression of HDAC2; interference with HDAC2 increased the level of E-cadherin, and interference with HDAC2 further increased the level of E-cadherin under MR conditions. Immunofluorescence staining also showed that interference with HDAC2 further increased the level of E-cadherin under MR conditions (Figure 4D). The amount of H3K27me3 on the E-cadherin promoter was detected by ChIP assay, and the findings revealed that MR decreased this level but that interference with HDAC2 had no appreciable impact on it (Figure 4E). We also used a ChIP assay to detect the H3K27ac level on the E-cadherin promoter. The results showed that the H3K27ac level of the E-cadherin promoter was unaffected by MR, but it increased significantly after interference with HDAC2 (Figure 4F). Results indicated that MR reduced the level of H3K27me3 on the E-cadherin promoter, while interfering with HDAC2 increased the level of H3K27ac on the E-cadherin promoter, and jointly promoted E-cadherin transcription.

*Interference with HDAC2 inhibits gastric carcinoma cell metastasis under methionine restriction (MR) conditions.*

MKN45 and AGS gastric carcinoma cells transfected with si-HDAC2 (or si-NC) were cultured under CM or MR conditions. Transwell experiments revealed that both MR culture and interference with HDAC2 reduced migration (Figure 5A) and invasion (Figure 5B) in MKN45 and AGS cells and that interference with HDAC2 further inhibited cell migration and invasion. BALB/C nude mice were injected with lentivirus-sh-HDAC2 or lentivirus-sh-NC-transfected MKN45 cells and then given a normal diet (Met⁺) or a methionine-free diet (Met⁻). HE staining of lung tissue showed that the number of lung metastases in the Met⁻ + sh-HDAC2 group was the lowest, indicating that MR and HDAC2 intervention could further inhibit lung metastasis of gastric carcinoma cells (Figure 5C). The results suggested that MR treatment combined with HDAC2 interference could further inhibit the metastasis of gastric carcinoma cells.

**Discussion**

The majority of gastric cancer patients are in the metastatic stage at the time of diagnosis, which dramatically increases the mortality risk [23]. Current treatments can prolong patients' lives but often have many side effects, and some even develop drug resistance [24]. In recent years, dietary intervention to alter cancer progression and treatment outcomes has caused extensive research [25]. Met is an essential amino acid whose metabolism is disrupted in a variety of illnesses. MR has been used as a therapeutic strategy in clinical trials [26]. In our study, MR was found to suppress the migration and invasion of gastric carcinoma cells as well as reduce lung metastasis. Further experiments showed that MR reduced H3K27me3 levels on the E-cadherin promoter. In addition, HDAC2 regulates E-cadherin promoter acetylation. We also found that HDAC2 expression intervention combined with MR promoted E-cadherin transcription and further restrained gastric carcinoma cell metastasis.

Met is an essential amino acid in cell metabolism. As a precursor of SAM, it can affect a variety of regulatory processes, such as RNA, DNA, and histone methylation [26]. MR has been shown in numerous studies to effectively prevent cancer growth and development by regulating their metabolism [24, 27]. MR has been reported to reduce
tumor growth and increase the survival rate of mice [28]. A nutritional deprivation study showed that Met deficiency had the most substantial inhibitory effect on the migration and invasion of breast cancer cells, as well as the ability to significantly inhibit lung metastasis in tumor-bearing mouse models [29]. Another study discovered that Met deficiency or diet restriction can enhance the chemotherapy sensitivity of melanoma and decrease metastasis [30]. In this study, we discovered that MR culture decreased the migration and invasion of gastric carcinoma cells (MKN45 and AGS). Furthermore, in vivo experiments also revealed that nude mice fed a Met-free diet had fewer lung metastases. The data suggested that MR might be utilized to inhibit gastric cancer.

EMT is vital to tumor development and metastasis. The activation of EMT, in which epithelial cells acquire mesenchymal cell characteristics and enhance cell motility and migration, is a critical step in cancer metastasis [4]. Key molecules of EMT, including E-cadherin, are also important targets for tumor therapy. E-cadherin is the most important adhesion molecule in epithelial cells. It has been confirmed in a variety of tumors that E-cadherin levels are downregulated and considered to be a key metastasis inhibitor [31-33]. Changes in promoter methylation and histone modification generally reduce E-cadherin expression [32]. In Met metabolism, Met reacts with ATP to produce SAM, which is the most important methyl donor in the body and participates in the methylation reaction catalyzed by Met transferase. MR significantly reduced SAM levels, and the decreased SAM could further reduce the trimethylation of histone H3 lysine 4 [12, 13]. Additionally, it has been reported that reduced gastric carcinoma cell migration, invasion, and metastasis are related to H3K9me2 and H3K27me3 changes on the E-cadherin promoter. Here, we discovered that MR upregulated the E-cadherin content via qRT-PCR and immunofluorescence staining experiments. In addition, our data indicated that MR could reduce the H3K27me3 level on the E-cadherin promoter, increase the transcription of E-cadherin, and inhibit the metastasis and invasion of gastric carcinoma cells.

Song et al. [18] clarified that in gastric carcinoma cells, the methylation level of E-cadherin decreased after increasing E-cadherin acetylation, and E-cadherin transcription was activated. Therefore, we also investigated the acetylation of E-cadherin. HDAC2 has been shown to reduce E-cadherin expression in pancreatic cancer by mediating histone deacetylation [19]. HDAC2 has been reported to be critical for the development of gastric cancer [34]. In our study, HDAC2 was found to negatively regulate E-cadherin expression in gastric carcinoma cells. Moreover, the ChIP assay verified that HDAC2 negatively regulated H3K27ac levels on the E-cadherin promoter, suggesting the regulation of HDAC2 on the acetylation of the E-cadherin promoter. Furthermore, Kim et al. [35] claimed that HDAC2 expression was increased in gastric cancer, and in vitro experiments showed that interference with its expression could inhibit the growth and metastasis of gastric carcinoma cells. Likewise, we found that under the restriction of methionine, interference with HDAC2 further upregulated E-cadherin expression by increasing the H3K27ac level at the E-cadherin promoter, synergistically promoted E-cadherin transcription, and further inhibited cell migration, invasion, and lung metastasis of gastric carcinoma cells.
Conclusively, our present study proved that MR has an inhibitory impact on gastric carcinoma cell invasion, migration, and lung metastasis. By exploring the influencing mechanism, we found that MR may promote the expression of the E-cadherin promoter by downregulating its methylation level. We also found that inhibiting HDAC2 may slow the progression of gastric cancer by increasing the expression of E-cadherin histone by mediating its deacetylation.

Funding
This work was supported by the grants from the Jiangxi Province Key Research and Development Program (No. 20203BBG73056), the Jiangxi Province Academic and Technical Leaders Training Program for Major Disciplines (Leading Talents Program: 20213BCJ22014), the National Natural Science Foundation of China (No. 8216100399), and the Science and Technology Project of Jiangxi Provincial Health Commission (No. 202310030).

Conflict of Interest
All authors declare that they have no conflict of interest.

References


Figure legends

**Figure 1. Effects of methionine restriction (MR) on migration, invasion, and metastasis of gastric carcinoma cells**  Human gastric carcinoma cells MKN45 and AGS were cultured with complete medium (CM) and methionine restriction (MR) medium for 24 h, respectively. N=3. (A,B) Cell migration and invasion were detected by Transwell assay. *P<0.05, **P<0.01 vs CM. MKN45 cells were injected into BALB/c nude mice. Mice were divided into two groups: Met⁺ and Met⁻. N=6. (C) HE staining was performed to observe metastasis in lung tissue, and the number of metastatic lung nodules was counted. Scale bar: 100 μm. **P<0.01 vs Met⁺.

**Figure 2. Effect of methionine restriction (MR) on H3K27me3 levels on the E-cadherin promoter**  Human gastric carcinoma cells MKN45 and AGS were cultured with complete medium (CM) and methionine restriction (MR) medium for 48 h, respectively. N=3. (A) The expression of E-cadherin mRNA was measured detected by qRT-PCR. *P<0.05, **P<0.01 vs CM. (B) Immunofluorescence staining was performed. Scale bar: 25 μm. (C,D) A ChIP assay was performed to measure the levels of H3K27me3 and H3K9me2 on the E-cadherin promoter. *P<0.05, **P<0.01 vs CM.

**Figure 3. Regulation of HDAC2 on the acetylation of the E-cadherin promoter**  MKN45 and AGS human gastric carcinoma cells were transfected with OE-HDAC2 or si-HDAC2. N=3. (A‒C) The expression of E-cadherin and HDAC2 was measured detected by qRT-PCR and western blot. *P<0.05, **P<0.01, ***P<0.001 vs OE-NC or si-NC; #P<0.05, ##P<0.01, ###P<0.001 vs si-NC. (D) The effect of HDAC2 on the acetylation of the E-cadherin promoter was investigated by ChIP assay. *P<0.05 vs OE-NC; #P<0.05 vs si-NC.

**Figure 4. Effect of interference with HDAC2 combined with methionine restriction (MR) on E-cadherin expression**  MKN45 and AGS human gastric carcinoma cells were transfected with si-HDAC2 or si-NC. N=3. (A,B) The expression of E-cadherin mRNA and HDAC2 mRNA was measured by qRT-PCR. *P<0.05, **P<0.01, ***P<0.001 vs si-NC or MR-. (C) The expression of E-cadherin protein and HDAC2 protein was detected by western blot. *P<0.05, **P<0.01, ***P<0.001 vs si-NC or MR-. (D) E-cadherin expression was detected by immunofluorescence staining. Scale bar: 25 μm. (E,F) A ChIP assay was performed to measure the levels of H3K27me3 and H3K27ac on the E-cadherin promoter. *P<0.05, **P<0.01, ***P<0.001 vs si-NC or MR-. (E,F) A ChIP assay was performed to measure the levels of H3K27me3 and H3K27ac on the E-cadherin promoter. *P<0.05, **P<0.01, ***P<0.001 vs si-NC or MR-. (E,F) A ChIP assay was performed to measure the levels of H3K27me3 and H3K27ac on the E-cadherin promoter. *P<0.05, **P<0.01, ***P<0.001 vs si-NC or MR-. (E,F) A ChIP assay was performed to measure the levels of H3K27me3 and H3K27ac on the E-cadherin promoter. *P<0.05, **P<0.01, ***P<0.001 vs si-NC or MR-.

**Figure 5. Effect of interference with HDAC2 on gastric carcinoma cell metastasis under methionine restriction (MR) conditions**  Human MKN45 and AGS human gastric carcinoma cells were transfected with si-HDAC2 or si-NC. N=3. (A,B) Cell migration and invasion were detected by Transwell assay. *P<0.05, **P<0.01, ***P<0.001 vs si-NC or MR-. MKN45 cells infected with lentivirus-sh-HDAC2 or lentivirus-sh-NC were injected into BALB/c nude mice. Mice were divided into four groups: Met⁺+sh-NC, Met⁺+sh-HDAC2, Met⁺+sh-NC, and Met⁺+sh-HDAC2. N=6. (C) HE staining was performed to observe metastasis in lung tissue, and the number of metastatic lung nodules was counted. Scale bar: 100 μm. *P<0.05, ***P<0.001 vs sh-NC or Met⁺.
171x119mm (300 x 300 DPI)
For Peer Review

148x120mm (300 x 300 DPI)
Supplementary Figure S1. The detection of apoptosis. (A) Apoptosis was detected by flow cytometry after MR treatment for 12, 24, and 48 h. **P<0.01 vs CM. (B) MKN45 cells were transfected with the HDAC2 interference sequence and corresponding negative control, and apoptosis was detected 48 h later by flow cytometry.
Supplementary Figure S2. The detection of CDH1 levels. MKN45 cells were transfected with HDAC1 (A) and HDAC3 (B) interference sequences and corresponding negative controls, and CDH1 expression was detected 48 h later by qRT-PCR.
Invasion and metastasis are primary reasons for death in individuals with gastric cancer. In this study, we found that MR combined with HDAC2 interference promoted E-cadherin expression by mediating the methylation and acetylation of E-cadherin, thus inhibiting invasion, migration, and lung metastasis of gastric carcinoma cells. (1) MR prevents gastric carcinoma cell migration, invasion, and lung metastasis. (2) MR promotes E-cadherin expression by reducing the level of histone methylation on the cadherin promoter. (3) HDAC2 reduces E-cadherin expression by mediating deacetylation of E-cadherin histones.
The diagram illustrates the effects of HDAC2 interference/inhibition on E-cadherin expression and its impact on migration and invasion. 

- **MR**
  - H3K27me3 → Transcription
  - E-cadherin

- **HDAC2 interference/inhibition**
  - H3K27ac → Transcription
  - E-cadherin

- Decreased migration
- Decreased invasion