Identification and verification of the role of crucial genes through which methionine restriction inhibits the progression of colon cancer cells

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Abstract. Studies have shown that methionine restriction (MR) can inhibit tumor progression, but its mechanism in colon cancer is unknown. Through DESeq2 and Edge analysis of the GSE72131 and GSE103602 datasets, 649 co-upregulated and 532 co-downregulated genes affected by MR were identified, respectively. Enrichment analysis showed that these genes were closely associated with tumor progression. Combined with the differentially expressed genes of colon cancer in The Cancer Genome Atlas database, MR affected 330 dysregulated genes in colon cancer. On this basis, a transcriptional regulatory and competing endogenous RNA network was established and F transcription factor 1 and microRNA 17-92a-1 Cluster Host Gene were identified as a key transcription factor and long non-coding RNA, respectively. In addition, four genes (FA Complementation Group I, Holliday Junction Recognition Protein, Karyopherin Subunit Alpha 2 and Kinesin Family Member 15) were identified by analyzing the relationship between dysregulated genes and overall survival. Finally, western blotting, reverse transcription-quantitative PCR, Transwell and other *in vitro* experiments verified that MR inhibits HCT116 colon cancer cell proliferation, metastasis and invasion, induces apoptosis and downregulates 6 hub genes. Collectively, the present study identified potential targets for MR to inhibit colon cancer progression and contributed to the clinical application of MR.

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Introduction

Colon cancer is a common type of gastrointestinal tumor. According to statistics, its incidence ranks third among all tumors and its mortality rates second (1). The specific symptoms, occurrence and development of colon cancer are not obvious and its detection is challenging, which results in patients being diagnosed at the advanced stages (2). The development and progression of colon cancer is a multi-step process, in which accumulated genetic changes can serve an important role. Although considerable progress has been made in the surgery, chemotherapy, radiotherapy and targeted drugs used for colon cancer, there has been no improvement in the overall survival of patients with colon cancer (3). Therefore, identifying new ways for the prevention and treatment of colon cancer is necessary.

There are significant differences in the metabolism between tumor and normal cells and there is great potential for anti-tumor therapy to use these differences to advantage (4). Graziosi *et al* (5) show that gastric tumor cells cannot grow in methionine-deprived media and normal cells can grow normally under methionine-deprived conditions. This may be due to the increase in protein synthesis and transmethylation in tumor cells. The requirement for methionine far exceeds that of normal cells (6). At the same time, due to the low activity of methionine synthase in tumor cells, they cannot use homocysteine to synthesize sufficient endogenous methionine, as can normal cells (5,6). A study by Epner *et al* (7) that the restriction of methionine provided in the intestines for an average of 17 weeks is safe and feasible for patients with advanced metastatic cancer. The short-term methionine restriction (MR) obtained through total parenteral nutrition can improve the efficacy of 5-fluorouracil in patients with advanced gastric cancer (8). These finding suggested that restricting the access of colon cancer cells to methionine is a promising treatment approach.

The Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) and The Cancer Genome Atlas databases (version 23.0; https://portal.gdc.cancer.gov) were used in the present study to comprehensively analyze the effect of MR on the expression of colon cancer genes and preliminarily explore

its anti-tumor mechanism. It was also confirmed that the MR ability inhibited the proliferation, metastasis and invasion of colon cancer HCT116 cells and promoted apoptosis. Finally, a variety of statistical and bioinformatics methods were used to identify and verify the key genes that affect the progression of colon cancer by MR.

Materials and methods

Data collection and processing. The GSE72131 and GSE103602 datasets, which included data on the treatment of HCT116 colon cancer cells with MR, were downloaded from the GEO database. The acquired dataset was analyzed using R 3.6.1 software (https://www.r-project.org/) and 'DESeq2' (version 1.36.0; https://github.com/mikelove/DESeq2) and 'Edge' R (version 2.28.0; https://github.com/jdstorey/edge) packages were used to analyze the two datasets separately and |Log2Fold change|>1 and false discovery rate <0.05 were used as the screening conditions to identify the differentially expressed genes. A Venn diagram was used to obtain the common methionine starvation differentially expressed genes in the two datasets. The colon cancer IlluminaHiSeq dataset was downloaded from UCSC Xena (https://xena.ucsc.edu), matching colon cancer samples were extracted and the 'Limma' R package (9) was used to identify genes that are differentially expressed in colon cancer. The Venn diagram was used to analyze the effect of MR treatment on the differentially expressed genes in colon cancer. The original 'CEL' files of the GSE41445 dataset (containing three human colon cancer cell lines: HT116, HT29 and SW480) and the GSE29316 dataset (containing human colonic tissue cell line CCD-18Co) were downloaded from the GEO database. The expression data of the four cell lines were extracted from the 'CEL' original file by running the 'RMA' function using R3.6.1 software and the 'Combat' function was used to perform background correction and remove batch effects. The expression of vital genes in these cells was extracted and Student's t-test was used for differential analysis of CCD-18Co and HT116, HT29 and SW480, respectively.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. The GO resource provides a computational representation of current scientific knowledge on the functions of genes from a number of different organisms, from humans to bacteria. The ontology is usually composed of a set of categories (or terms or concepts), between which there is a relationship. It describes current understanding of the biological field from three aspects (GO domains): Molecular Function (MF), Cellular Component (CC) and Biological Process (BP). The KEGG is used to understand the advanced functions of biological systems (such as cells, organisms and ecosystems) from molecular-level information (especially large-scale molecular datasets generated through genome sequencing and other high-throughput sequencing). To understand the molecular mechanism and related biological signaling changes in colon cancer cells after MR, the 'clusterprofiler' R package (version 4.4.1; https://doi.org/10.1016/j.xinn.2021.100141)was used to perform GO and KEGG functional enrichment analysis on MR-affected differentially expressed genes.

Construction of transcription regulation and competitive endogenous (ce) RNA network. In order to understand the possible mechanism of the effect MR has on differentially expressed genes in colon cancer, transcriptional regulation and ceRNA network construction were performed on the obtained genes. For the transcriptional regulatory network, DAVID 6.8 (https://david.ncifcrf.gov/) was used to enrich the potential transcription factors of all genes and the transcription factors with a Bonferroni value of <0.05 were considered as meaningful (10). For the ceRNA network, miRcode (http://mircode.org/) was used to compare the obtained genes, in order to obtain the ceRNA network and identify key long non-coding (lnc)RNAs and microRNAs (11). The two networks are visualized using Cytoscape 3.6.1 (https://cytoscape.org/).

Protein interaction network (PPI) construction and screening of independent prognostic candidate genes. The STRING database (version 11.5; https://string-db.org/) contains known protein interactions. To identify core gene networks from differentially expressed genes in colon cancer affected by MR, they were uploaded to the STRING database for online retrieval of protein interactions using default settings. These protein interaction networks were visualized using Cytoscape 3.6.1 and the network analysis tool ranked genes from the inside to the outside by degree value. The molecular complex detection (MCODE) tool was used to conduct a comprehensive analysis of the entire PPI network. The following conditions were set as follows: Degree cutoff=2, node score cutoff=0.2, K-core=2 and max depth=100. The modules were filtered with k>5 as a key module. For genes in key modules, least absolute shrinkage and selection operator (Lasso) and multivariate Cox regression analysis identified gastric cancer-independent prognostic genes while removing collinearity to avoid overfitting.

Cell culture and processing. Human HCT116, HT29 and SW480 cell lines containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 IU/ml penicillin (Beijing Solarbio Science & Technology Co., Ltd.) were cultured in DMEM (Boster Biological Technology). Methionine-restricted cells were cultured in methionine-free (MET-) DMEM (Boster Biological Technology). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C.

Cell proliferation assay. Evaluation of colon cancer cell proliferation through 5-ethynyl-2'-deoxyuridine (EdU) cell proliferation detection. First, staining was performed according to the instructions of the EDU commercial kit (US Everbright Inc.). Cells were incubated with EdU for 4 h at 37˚C and fixed with 4% paraformaldehyde for 15 min at room temperature, cells were treated with 100 μl of YF®594 Azide (US Everbright Inc.) for 30 min at room temperature. Cells in each well were then stained for DNA with 100 μl of DAPI for 30 min at room temperature. Subsequently, under a fluorescence microscope (magnification, x50; Olympus Corporation), three different fields of view were randomly selected for counting analysis.

Apoptosis detection through TUNEL staining. DNA double-strand breaks in apoptotic cells or gaps in one strand

Table I. Primer sequence used in the present study.

Gene ID	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
β-actin	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
E2F1	GGACCTGGAAACTGACCATCAG	CAGTGAGGTCTCATAGCGTGAC
MIR17HG	GGGCCTCCGGTCGTAGTAA	AACAGGTTTCCCTCCGTCGC
FANCI	CCACCTTTGGTCTATCAGCTTC	CAACATCCAATAGCTCGTCACC
HJURP	CAGATGGGGTGGACAACAC	TCTTCCATCCTGTAAGACGTG
KPNA2	CTGTTGGCTCTCCTTGCAGTTC	GCAGGATTCTTGTTGCGGCAAAG
KIF15	AGGAATCTGTATTCGCAACTGTG	ACTTCGTGGGATTACTCCTCTC

result in a series of 3'-OH ends, which are formed by deoxyribonucleotide and biotin under the action of deoxyribonucleotide terminal transferase. Derivatives are labeled to the 3' end of DNA for the detection of apoptotic cells. Based on this principle, TUNEL staining kit (US Everbright Inc.) was used to stain the treated cells. The fixed and permeabilized cells were treated with deoxyribonucleotide terminal transferase at 37˚C for 30 min. subsequently with YF®594 TUNEL reaction in the dark for 60 min. Cells in each well were then stained for DNA with 100 µl of DAPI for 30 min at room temperature. Subsequently, under a fluorescence microscope (magnification, x50; Olympus Corporation), three different fields of view were randomly selected for counting analysis.

Cell migration and invasion experiments. The wound-healing assay measures cell migration. When the cells were close to 95% confluence, 200 µl pipette tips were used to scrape the monolayer vertically. After washing away the cell debris with PBS, an inverted microscope (magnification, x50; Olympus Corporation) was used to analyze the wound gap size at 0, 24 and 48 h after the injury to evaluate the migration ability.

To determine the vertical migration of cells, the cells were suspended in different serum-free media and 2×10^5 cells were seeded in the chamber of a 8.0 µm Transwell® plate (Corning, Inc.). For the cell invasion assay, the upper chamber was pre-coated with Matrigel® (BD Biosciences) for 2 h at 37˚C and the remaining steps were the same as the vertical migration assay. The lower chamber was supplemented with 800 µl serum-containing medium and cultured for 72 h. At room temperature, the cells that invaded into the lower chamber were fixed with methanol for 15 min, stained with 0.1% (w/v) crystal violet (room temperature) for 20 min and analyzed using an inverted microscope (magnification, x50; Olympus Corporation).

Western blot analysis. Cells were lysed in RIPA (Beijing Solarbio Science & Technology Co., Ltd.) containing protease inhibitors (Boster Biological Technology) for 20 min on ice. A BCA protein content kit (Beijing Solarbio Science & Technology Co., Ltd.) was used to determine protein concentration. Total protein (40 µg) per well was separated on 10% Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis and transferred onto a PVDF membrane (MilliporeSigma). The membrane was blocked with 5% BSA (CoWin Biosciences) for 1 h at room temperature. The PVDF membrane was soaked with GAPDH (cat. no. 60004-1-Ig; dilution: 1:10,000; ProteinTech Group, Inc.), B-cell lymphoma/leukemia-2 (cat. no. 12789-1-AP; dilution: 1:1,000; ProteinTech Group, Inc.), Bcl-2-associated X (cat. no. 50599- 2-Ig; dilution: 1:1,000; ProteinTech Group, Inc.), Caspase-3 (cat. no. 66470-2-Ig; dilution: 1:1,000; ProteinTech Group, Inc.), N-cadherin (cat. no. ab76057; dilution: 1:1,000; Abcam), E-cadherin (cat. no. ab231303; dilution: 1:1,000; Abcam) and vimentin (cat. no. ab137321; dilution: 1:10,000; Abcam) for 12 h at 4˚C. Following washing with Tris-buffered saline with 0.05% Tween, the membrane was probed with horseradish peroxidase-conjugated goat anti-rabbit (cat. no. BA1070; dilution: 1:5,000; Boster Biological Technology) or goat anti-mouse IgG (cat. no. BM2002; dilution: 1:5,000; Boster Biological Technology) for 1 h at room temperature. The band was detected using Super ECL Plus (US Everbright Inc.). The protein expression results are expressed relative to the GAPDH band density. The expression gray value of every protein was analyzed using Image Lab 5.2.1 (Bio-Rad Laboratories, Inc.).

Expression verification of key genes. The expression of six hub genes was validated following MR in HCT116, HT29 and SW480. Total RNA was extracted from 80-90% confluent cells using TRIzol® Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After the concentration and purity of the RNA were qualified, an RR047a reverse transcription (RT) kit (Takara Bio, Inc.) was used for RT, according to the manufacturer's instructions. β-actin was used as the internal reference gene, and calculate the $2^{-\Delta\Delta Cq}$ value for each hub gene. The calculation formula was as follows: $\Delta\Delta Cq=\Delta Cq_{experimentalgroup}-\Delta Cq_{controlgroup}$, $\Delta Cq=Cq_{targetgene}-Cq_{internalreference}$ (12). The expression level of the target gene was analyzed using an RR820 kit (Takara Bio, Inc.) in the ABI7900-HT system (Thermo Fisher Scientific, Inc.). The qPCR conditions were: Denaturation, 95˚C, 30 sec; annealing, 95˚C, 3 sec; extension, 60˚C, 30 sec; 40 cycles. All primers used were synthesized by Sangon Biotech (Shanghai) Co., Ltd. and the primer sequences are shown in Table I. The above experiments were repeated three times.

Statistical analysis. GraphPad Prism 8.0 (GraphPad Software, Inc.) was used for statistical analysis and the mean ± standard deviation was used to describe the measurement data. The experiments were repeated >3 times and a Student's unpaired t-test was used to compare the two samples. For multiple comparisons, the significance was identified by simple

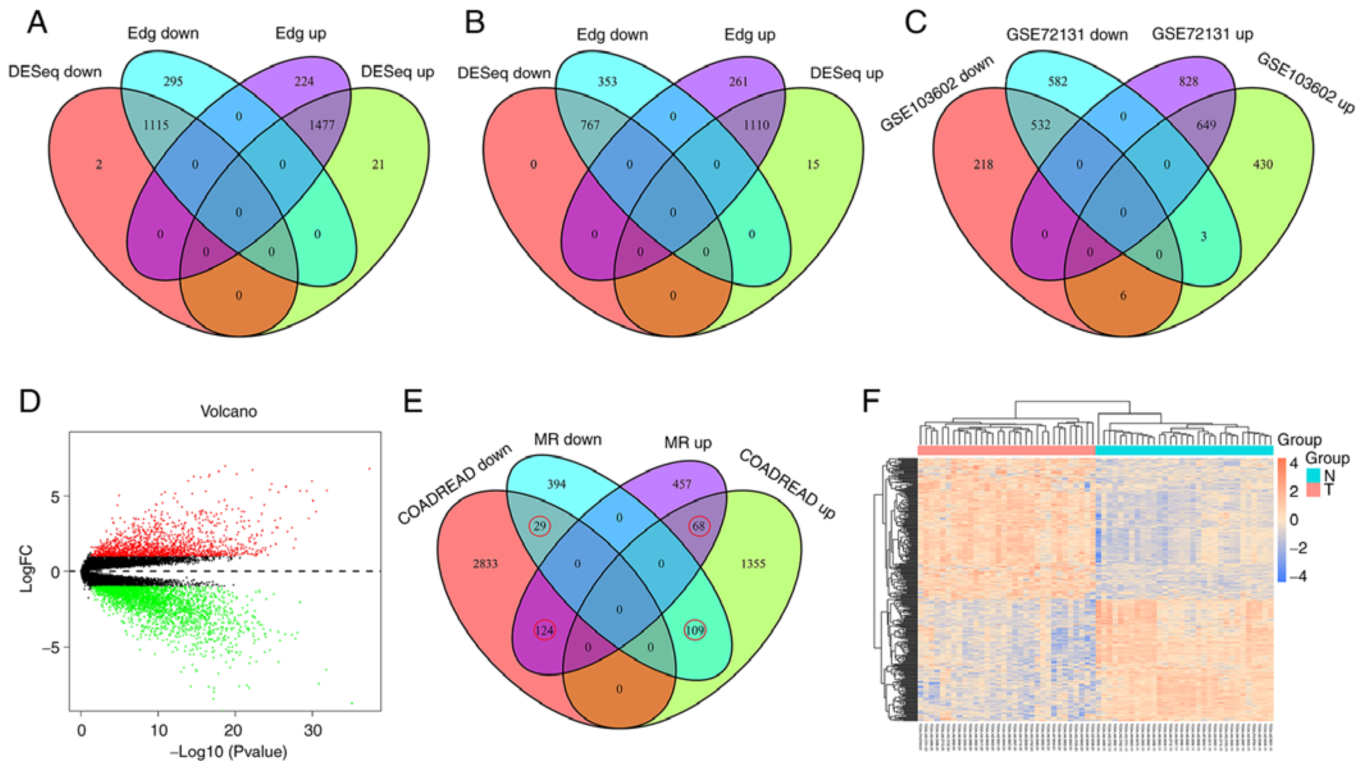

Figure 1. Identifying that methionine restriction affects gene expression in colon cancer. (A) Genes regulated by methionine restriction in GSE72131. (B) Genes regulated by methionine restriction in the GSE103602 dataset. (C) Methionine restriction co-regulated genes in GSE72131 and GSE103602 datasets. (D) Dysregulated genes in colon cancer. (E) Identification of methionine restriction regulating differentially expressed genes in colon cancer. (F) The heat map showed that methionine restriction regulates the expression of differentially expressed genes in colon cancer in matched colon cancer tissues.

one-way ANOVA followed by a Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Gene expression affected by MR. The effects of MR on gene expression were identified in the GSE72131 and GSE103602 datasets using the 'Edge' and 'DESeq' packages. In the GSE72131 dataset, 1,477 upregulated and 1,115 downregulated genes were identified (Fig. 1A). In the GSE103602 dataset, 1,877 dysregulated genes were identified, including 1,110 upregulated and 767 downregulated genes (Fig. 1B). The intersection of the two datasets identified a total of 649 co-upregulated and 532 co-downregulated genes (Fig. 1C), which were considered to be differentially expressed genes affected by MR. In addition, the dysregulated genes in colon cancer were also analyzed, with a total of 1,532 upregulated and 2,986 downregulated genes identified (Fig. 1D). To identify the effect of methionine starvation on these differentially expressed genes in colon cancer, Venn diagram analysis was performed on the two. Finally, 330 genes were identified (Fig. 1E) and cluster analysis of these genes in paired colon samples was performed using heat maps (Fig. 1F).

GO and KEGG function enrichment analysis. To understand the influence of MR on the molecular mechanism of colon cancer, the obtained MR-regulated genes were enriched and analyzed. For 649 upregulated genes, BP was enriched in extracellular matrix organization, extracellular structure organization, response to nutrient levels and I-κB kinase/NF-κB signaling; CC was mainly enriched in the collagen-containing extracellular matrix, collagen trimer; MF was mainly enriched in extracellular matrix structural constituent, protein tyrosine/threonine phosphatase activity, extracellular matrix structural constituent conferring tensile strength, MAPK tyrosine/serine/threonine phosphatase activity and MAPK phosphatase activity (Fig. 2A; Table SI). KEGG was mainly enriched in cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptors pathway, IL-17 signaling pathway and starch and sucrose metabolism (Fig. 2C). For 532 downregulated genes, BP was enriched in DNA replication, DNA conformation change, DNA-dependent DNA replication and negative regulation of cell cycle process; CC was mainly enriched in chromosome regions and mitochondria; MF was mainly enriched in DNA-dependent ATPase activity, ATPase activity and ubiquitin-like protein ligase binding (Fig. 2B; Table SI). KEGG was mainly enriched in DNA replication, cell cycle, pyrimidine metabolism, drug metabolism-other enzymes, glycine and serine and threonine metabolism (Fig. 2D).

Transcription regulation and ceRNA network. A transcriptional regulatory network was constructed for differentially expressed genes regulated by MR in colon cancer. For the obtained 330 genes, five types of transcription factors [zinc finger protein 423 (ZNF423, ROAZ), E2F Transcription Factor (E2F), Activating Transcription Factor (ATF), TAL1β-ITF2 and Activator Protein 1 (AP1)] and 298 downstream coding genes were identified. It is worth noting that the differential genes identified included E2F family gene

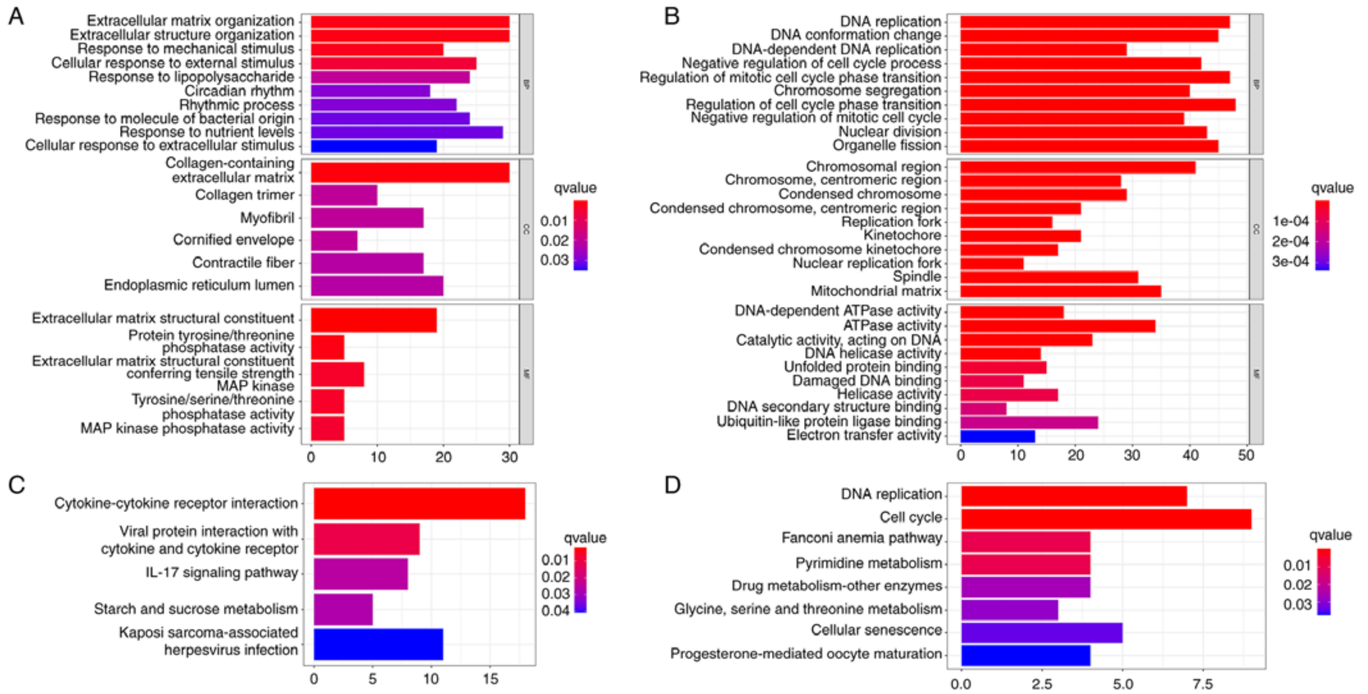

Figure 2. Enrichment analysis of methionine restriction regulatory genes. (A) GO enrichment analysis of upregulated genes. (B) KEGG function enrichment analysis of upregulated genes. (C) GO enrichment analysis of downregulated genes. (D) KEGG function enrichment analysis of downregulated genes. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

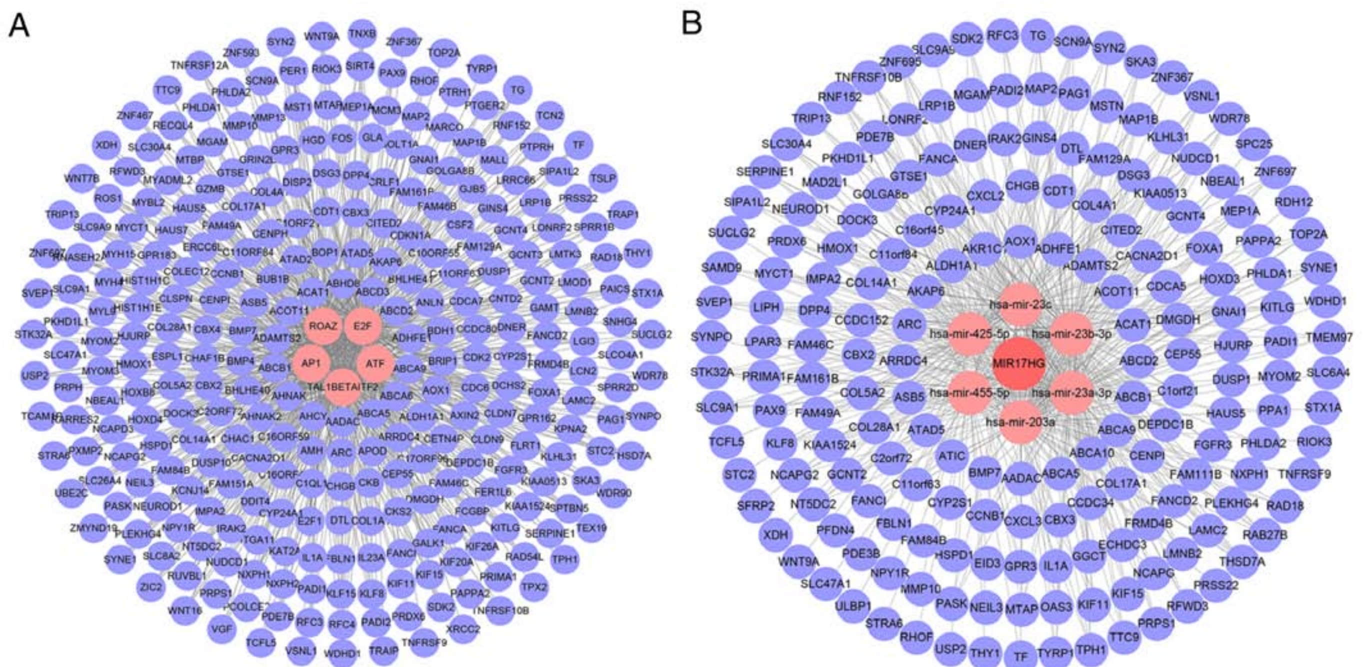

Figure 3. Construction of transcriptional regulation and ceRNA network based on methionine restriction regulation of differentially expressed genes in colon cancer. (A) The transcriptional regulatory network screened out the key transcription factor E2F1. (B) The ceRNA network identified the key lncRNA MIR17HG. E2F1, E2F transcription factor 1; ceRNA, competitive endogenous RNA; lncRNA, long non-coding RNA.

F transcription factor 1 (E2F1; Fig. 3A). In addition, these 330 genes were found to contain two lncRNAs [MiR-17-92a-1 Cluster Host Gene (MIR17HG) and Small Nucleolar RNA Host Gene 4], 325 protein-coding genes and three pseudogenes. On this basis, a ceRNA network was constructed and five miRNAs downstream of MIR17HG were identified (has-mir-23c, has-mir-23b-3p, has-mir-23a-3p, has-mir-203a, has-mir-455- 5p and has-mir-425-5p) and 190 miRNA target genes (Fig. 3B).

Construction of PPI network and identification of independent prognostic genes. A total of 325 encoded proteins were

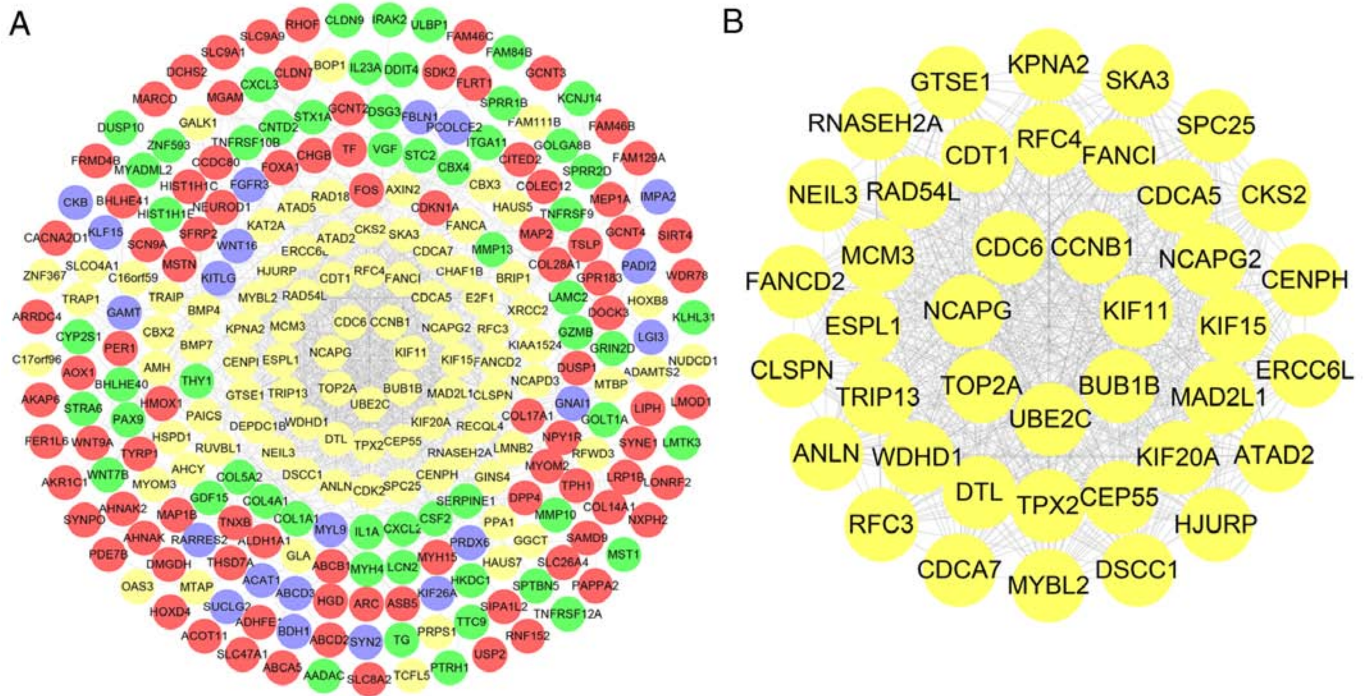

Figure 4. PPI network construction and key network identification. (A) Establishment of PPI network base on methionine restriction regulation of differentially expressed genes in colon cancer. (B) MCODE identified critical networks, k=15.6. Blue nodes represent downregulated genes in colon cancer that were down-regulated again after MR treatment, red nodes represent downregulated genes in colon cancer that are upregulated after MR treatment, yellow nodes represent upregulated genes in colon cancer that are downregulated after MR treatment, green nodes represent upregulated genes in colon cancer that were upregulated again after MR treatment. PPI, protein interaction network; MCODE, molecular complex detection; MR, methionine restriction.

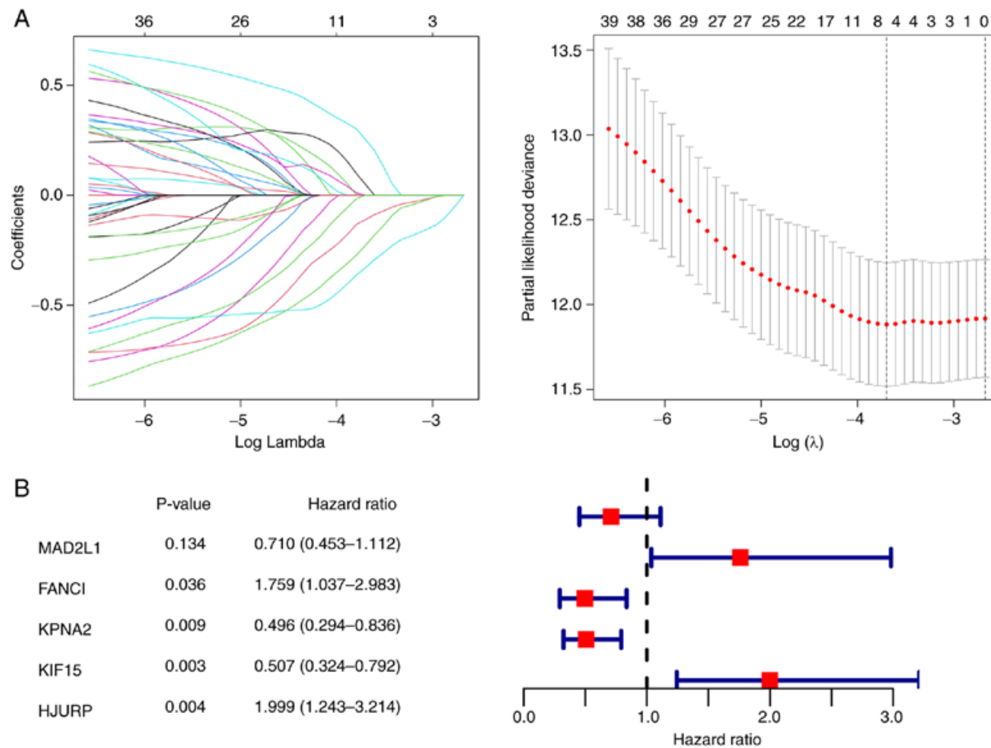

Figure 5. Lasso regression and multivariate Cox regression analysis identify independent prognostic genes from key modules. (A) Lasso regression analysis. (B) Multivariate Cox regression analysis identified four independent prognostic genes.

uploaded to STRING and the independent and unconnected proteins from the overall network were removed to obtain a network of 256 genes and 1,321 edges (Fig. 4A). Using MCODE, a key network with k=15.6 was identified from the network, which contained 41 genes and 675 edges (Fig. 4B). For these 41 candidate genes, Lasso regression analysis

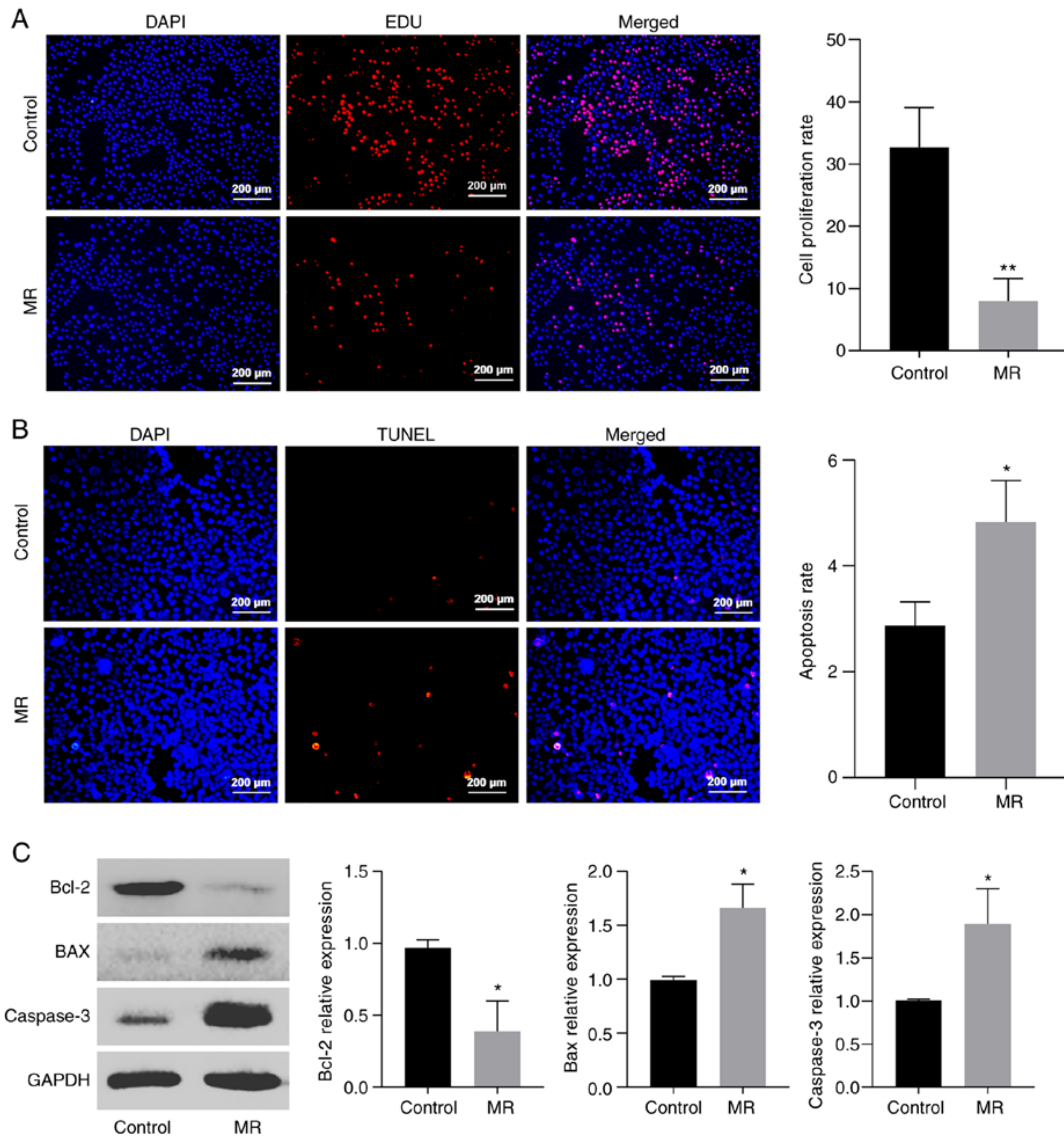

Figure 6. Methionine restriction inhibits the proliferation of colon cancer cells and promotes apoptosis. (A) EDU showed that methionine restriction reduced the number of proliferating colon cancer cells (scale bar=200 μm). (B) Methionine restriction promotes apoptosis by TUNEL staining. (C) Methionine restriction downregulated the expression of Bcl-2 and increased that of Bax and Caspase-3. *P<0.05 and **P<0.01. EdU, 5-ethynyl-2'-deoxyuridine.

was first used to remove collinearity and five prognostic genes [Mitotic arrest deficient 2 like 1, FA complementation group I (FANCI), Karyopherin subunit alpha 2 (KPNA2), Kinesin family member 15 (KIF15) and Holliday junction recognition protein (HJURP)] were identified (Fig. 5A). Furthermore, using multivariate Cox regression analysis (Fig. 5B), four out of these five genes (FANCI, KPNA2, KIF15 and HJURP) were identified as independent prognostic genes (P<0.05).

Effect of MR on the proliferation and apoptosis of colon cancer cells. First, EDU staining was used to analyze the effect of MR on the proliferation of colon cells (Fig. 6A) and the results showed that the cell proliferation ability of the MR group was weaker than that of the control group (P<0.01). The apoptotic rate was also analyzed (Fig. 6B) following MR and it was found to be significantly higher than that of the control group (P<0.05). By analyzing the expression of apoptosis-related proteins (Fig. 6C), the expression of Bax and Caspase-3 was significantly increased, while that of Bcl-2 was decreased, which also confirmed that MR can induce apoptosis (P<0.05).

Effect of MR on colon cancer cell invasion and migration. The cell scratch test can reflect the horizontal migration ability of colon cancer cells and the results showed that the horizontal migration ability of methionine-restricted cells is lower than that of the control group (P<0.01; Fig. 7A). Similarly, in Transwell experiments, MR can inhibit the vertical migration of colon cells (P<0.05; Fig. 7B). In addition,

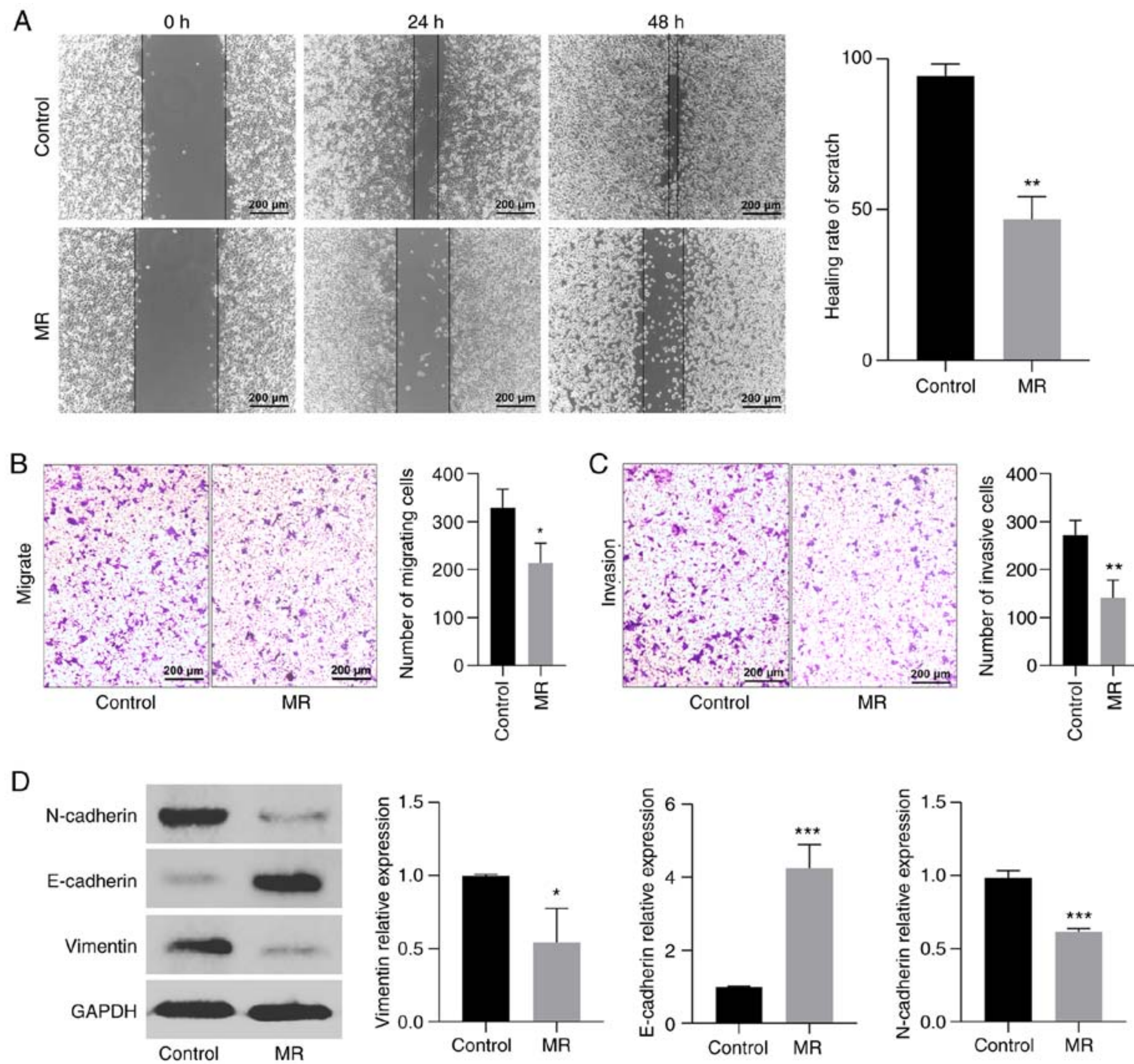

Figure 7. Methionine restriction inhibits the invasion and migration of colon cancer cells. (A) Cell scraping experiments show that methionine restriction inhibits the horizontal migration of colon cancer cells (scale bar=200 μm). (B) Transwell method shows that methionine restriction inhibits the vertical migration of colon cancer cells (scale bar=200 μm). (C) Methionine restriction inhibits the invasiveness of colon cancer cells. (D) Methionine restriction reduces the expression of N-cadherin and vimentin and induces high expression of E-cadherin. *P<0.05, **P<0.01 and ***P<0.001.

invasion experiments showed that MR caused a decrease in invasion ability (P<0.01; Fig. 7C). Finally, the expression of metastasis-related proteins was analyzed (Fig. 7D). Following MR, the expression of N-cadherin and vimentin decreased, while that of E-cadherin increased, suggesting that MR inhibited colon cancer metastasis (P<0.05).

Verification of the expression of key genes affected by MR. In the transcriptional regulatory network, the key transcription factor E2F1 was identified. In the ceRNA network, it was found that MIR17HG was the core lncRNA of the network. By identifying candidate genes obtained from the key network of the PPI network, combined with Lasso regression and multivariate Cox regression, four independent prognostic genes (FANCI, KPNA2, KIF15 and HJURP) were identified. Gene expression changes in HCT116, HT29 and SW480 colon cancer cell lines were compared with those in CCD-18Co normal colon fibroblasts (Fig. 8A). The results showed that the expression of E2F1, FANCI, HJURP, KPNA2 and KIF15, MIR17HG was significantly increased in colon cancer (P<0.01). Next, RT-qPCR was performed to verify the expression of these six genes following MR in HCT116, HT29 and SW480 colon cancer cell lines (Fig. 8B-D). The results showed that the expression of the above six genes was significantly downregulated following MR, which was consistent with our previous analysis in two data sets (P<0.05).

Discussion

Colon cancer usually occurs at the junction of the rectum and the sigmoid colon, mainly in individuals aged 40-50. Modern society has brought significant changes in the diet and living habits of individuals. Obesity, metabolic syndrome, sedentary, physical inactivity and high-calorie diet have lowered the age in which colon cancer occurs (13). Over 30 years ago, Halpern *et al* (14) found that there is a methionine-dependent metabolic phenotype in tumor cells. Studies on colon cancer have shown that methionine supplements can

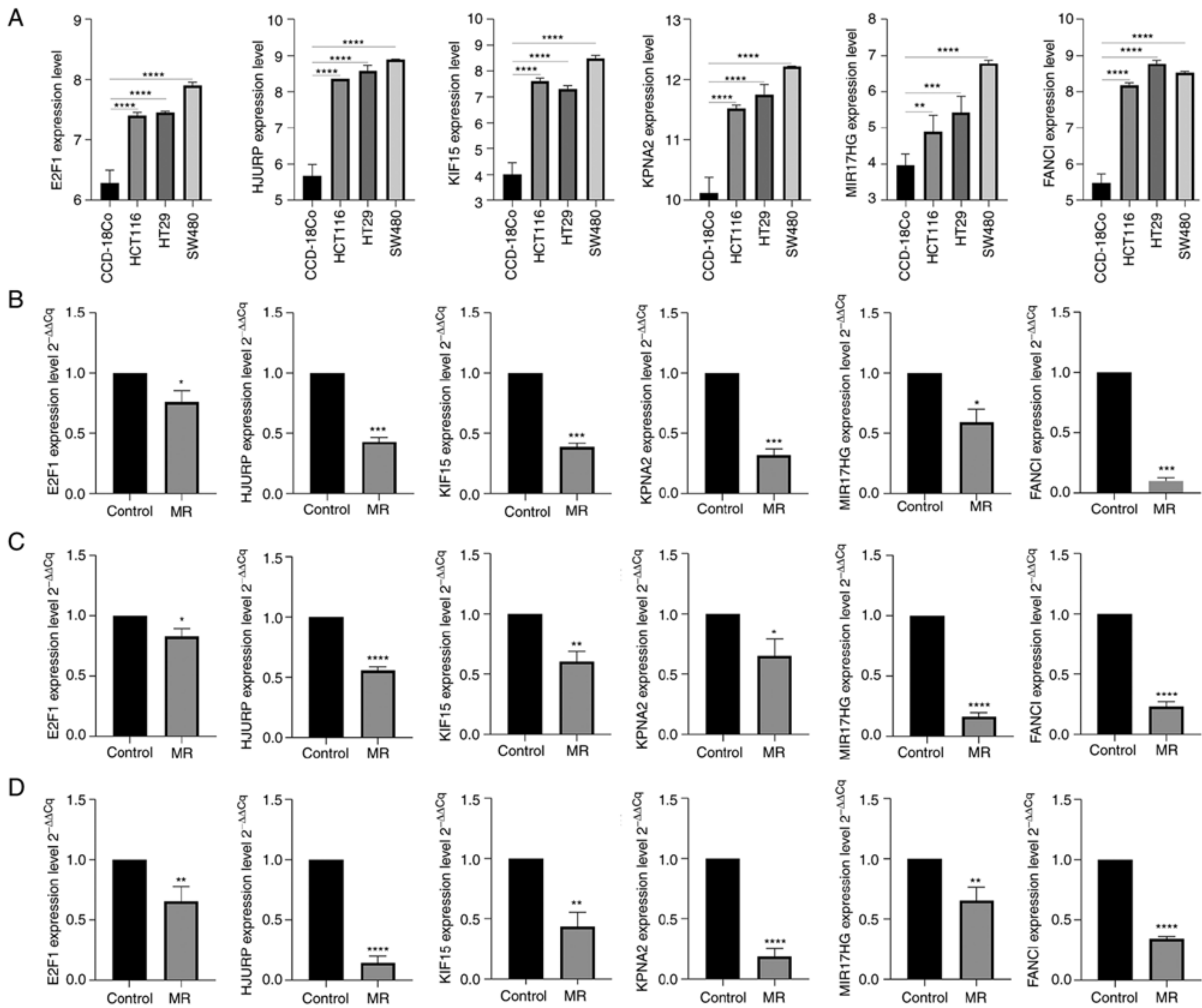

Figure 8. Methionine restriction downregulated six key genes. (A) The expression of six key hub genes in HCT116, HT29 and SW480 colon cancer cell lines were significantly higher than those in CCD-18co colon fibroblast cell lines. (B-D) Reverse transcription quantitative PCR showed that methionine restriction decreased the expression of six hub genes in HCT116, HT29 and SW480 colon cancer cell lines. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.001$.

stimulate malignant changes in the intestinal tissues of mice. Hanley *et al* (15) and Komninou *et al* (16) successively found that restricting methionine intake inhibited the progression of colon cancer. At present, the drug that targets methionine is methioninase, which can catalyze the α- and γ-cleavage reactions of methionine to produce α-butyric acid, methyl mercaptan and ammonia. Methioninase can cooperate with traditional chemotherapy drugs to treat colon cancer and exert a improved therapeutic effect (8,17,18). The study of Machover *et al* (19) showed that the possible mechanism of methioninase-induced tumor suppression is that it can decrease the methylation level of proto-oncogene cyclin-dependent kinase inhibitor 2A and increase its expression. The present study explored the effect of MR on colon cancer cells. The results showed that MR could inhibit the proliferation, metastasis and invasion of colon cancer cells and promote their apoptosis, which also reflects the methionine-dependent phenotype of colon cancer cells.

Methionine restriction is a promising means of cancer suppression, but there are few reports on the analysis of the potential mechanism of methionine inhibiting colon cancer growth. Using public data, the genes that may be regulated by MR were analyzed and enrichment analysis was performed on these genes. Among the upregulated genes, the focus was placed on MAPK phosphatase activity and protein tyrosine/threonine phosphatase activity. The MAPK cascade is a signal transduction component that serves a key role in converting extracellular stimuli into cellular responses through the phosphorylation of different substrates. The MAPK signaling pathway controls various cellular processes, such as growth, differentiation, proliferation, survival and death. The activation of this signal depends on the simultaneous phosphorylation of both threonine (T) and tyrosine (Y) sites (20). To study methionine on MAPK signaling, Xin *et al* (21) used proteomics methods and found that the expression of p38 increased following MR in gastric cancer. P38 is inseparable from the oncogene-induced senescence of tumor cells and can also serve a tumor suppressor role (20). Some of the downregulated genes of MR were enriched in glycolysis process, ATPase activity and drug metabolism. There are differences in energy metabolism between tumor and normal cells, and tumor cells can preferentially produce a large amount of lactic acid through aerobic

glycolysis to obtain the required energy. By inhibiting the energy production of tumor cells, this approach can play an effective anti-tumor effect. In gastric cancer, MR is found to reduce the activity of aerobic glycolysis and induce tumor cell apoptosis (22). The reduction in tumor productivity leads to the inactivation of a number of ATPases and drug metabolism depends on a variety of ATP-dependent transport enzymes (23). Among them, P-gp glycoprotein serves an important role in tumor resistance and it uses ATP to supply energy to metabolize cell-energy drugs (23). Xin *et al (*24) found that MR induces the downregulation of the expression of resistance-related protein P-gp, which enhances the drug sensitivity of gastric cancer.

Based on bioinformatics analysis, MR was found to affect the important transcription factor E2F1 and the key lncRNA MIR17HG. In addition, four independent prognostic genes of colon cancer regulated by MR were identified based on Lasso and multivariate Cox regression, among which FANCI and HJURP can be considered as risk genes. E2F1 is a member of the E2F transcription factor family, which can bind to DNA with dimer partner protein through the E2 recognition site 5'-TTTC[CG]CGC-3' found in the promoter region of various genes. E2F1 is directly involved in several types of cancer with poor prognosis and has been shown to be a key cancer biomarker (25). E2F1 not only promotes the proliferation and metastasis of rectal cancer, but also contributes to aerobic glycolysis, repressurization and oxidative metabolism and promotes anabolic metabolism (26). MIR17HG is an miR-17-92 cluster host gene lncRNA, which participates in cell proliferation and growth by regulating the cell growth phenotype. Studies on cervical cancer, non-small cell carcinoma and osteosarcoma have shown that MIR17HG acts as an oncogene in tumors, inhibiting its expression, resulting in tumor cell proliferation, metastasis and drug resistance (27-29). The study by Xu *et al* (30) on colon cancer showed that MIR17HG is closely associated with tumor immunity. First, it competitively sponges miR-375, thereby increasing the expression of nuclear factor κB/V-Rel Avian reticuloendotheliosis viral oncogene homolog A and then upregulating programmed death receptor 1, RELA can also directly bind to the MIR17HG promoter region to form a positive feedback loop to activate MIR17HG transcription.

The FANCI gene serves a key role in maintaining chromosome stability and repairing DNA double-strand breaks by homologous recombination. It repairs inter-strand DNA crosslinks through homologous recombination and induces FANCL to promote Fanconi anemia, complementation group D2 monoubiquitination and participates in the recruitment of DNA repair sites (31). HJURP is a histone chaperone involved in the recruitment of *de novo* histone H3 variants CenH3 (CENP-A) and CENP-C in nucleosomes, which can regulate the amplification of centromeric chromatin and chromosomal stability (32). HJURP has been confirmed as a key prognostic gene in breast cancer, hepatocellular carcinoma, advanced serous ovarian cancer, colon cancer and prostate cancer (33-37). Studies have shown that HJURP promotes tumor cell proliferation (38). For example, Wang *et al* (38) showed that HJURP activates Mouse Double Minute 2 homolog transcription by regulating the recruitment of H3K4me2 in its promoter region to inhibit the expression of p53 and promote pancreatic cancer cell proliferation. Chen *et al* (39) found that HJURP destabilizes p21 through MAPK/ERK1/2 and protein kinase B/glycogen synthase kinase-3β pathways, thereby regulating nuclear cytoplasmic translocation and ubiquitin-mediated p21 degradation and promoting tumor cell proliferation. In addition, studies have shown that HJURP can promote epithelial-mesenchymal transition of tumor cells by regulating the Wnt/β-catenin pathway and Sphingosine kinase 1 (40,41). In addition, studies by Cao *et al* (42) and Yuan *et al* (43) successively confirmed that HJURP affects the oxidative stress and cell cycle arrest of tumor cells by regulating the peroxisome proliferator-activated receptor γ-sirtuin 1 feedback loop. In the present study, it was confirmed that E2F1, MIR17HG, FANCI and HJURP are highly expressed in colon cancer tissues. In addition, six hub genes expression decreased significantly following MR, suggesting that MR may exert a tumor suppressing effect through the above pathways.

In conclusion, it was verified *in vitro* that MR inhibits the proliferation, metastasis and invasion of colon cancer. Using bioinformatics, the potential molecular biological mechanisms affecting the progression of colon cancer were analyzed and the key target genes regulated by MR were identified. However, there are still some defects in this study, including: The mechanism by which MR regulates the expression of crucial genes is not yet clear and the mechanisms by which these genes influence colon cancer progression remain to be elucidated. Addressing these deficiencies will be the focus of future research work.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ performed the bioinformatics analysis and wrote the manuscript; ZC performed the experiments and data analysis; CL designed the entire project, supervised and supported the completion of the work, and made revisions to the manuscript and approved for publication. LZ and CL confirm the authenticity of all the raw data. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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