miR-151a-5p promotes the proliferation and metastasis of colorectal carcinoma cells by targeting AGMAT

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Abstract. Colorectal carcinoma (CRC) is one of the most common types of digestive cancer. It has been reported that the ectopic expression of microRNAs (miRs) plays a critical role in the occurrence and progression of CRC. In addition, it has also been suggested that miR-151a-5p may serve as a useful biomarker for the early detection and treatment of different types of cancer and particularly CRC. However, the specific effects and underlying mechanisms of miR-151a-5p in CRC remain elusive. The results of the current study demonstrated that miR-151a-5p was upregulated in CRC cell lines and clinical tissues derived from patients with CRC. Functionally, the results showed that miR-151a-5p significantly promoted CRC cell proliferation, migration and invasion. Additionally, dual luciferase reporter assays verified that agmatinase (AGMAT) was a direct target of miR-151a-5p and it was positively associated with miR-151a-5p expression. Mechanistically, miR-151a-5p could enhance the epithelial-mesenchymal transition of CRC cells. Taken together, the results of the current study revealed a novel molecular mechanism indicating that the miR-151a-5p/AGMAT axis could serve a crucial role in the regulation of CRC and could therefore be considered as a potential therapeutic strategy for CRC.

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Abbreviations: CRC, colorectal carcinoma; AGMAT, agmatinase; TGF, transforming growth factor; GEDS, Gene Expression Display Server; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR; GFP, green fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; MMP, matrix metalloproteinase; EMT, epithelial-mesenchymal transition

Key words: miR-151a-5p, AGMAT, epithelial-mesenchymal transition, colorectal carcinoma

Introduction

Colorectal carcinoma (CRC) is one of the most common types of malignant tumors, ranking third in newly diagnosed cancer cases and cancer-related deaths, preceded by lung cancer and breast cancer in humans, thus seriously endangering the safety and lives of humans (1). In recent years, due to the rapid development of science and technology, and changes in people's lifestyles, there is a substantial increasing trend of CRC incidence in younger population. Although progress has been made in revealing the molecular mechanism and treatment of CRC, its prognosis remains very poor, possibly due to the rapid progression and development of CRC, chemoresistance and lack of effective screening and treatment approaches (2-4). Therefore, identifying specific diagnostic biomarkers and effective therapeutic targets for CRC is of great importance.

Agmatinase (AGMAT), located in mitochondria, is a key enzyme involved in polyamine metabolism pathway and is mainly distributed in the kidneys and liver (5). The polyamine metabolic disorder is commonly caused by the abnormal expression of enzymes involved in the metabolic pathway. Therefore, the abnormal expression of AGMAT could promote the occurrence and development of cancer via affecting polyamine metabolism. A previous study showed that AGMAT was highly expressed in lung adenocarcinoma tissues and it was closely associated with tumor stage and prognosis in patient with lung adenocarcinoma; furthermore, the study also demonstrated that AGMAT promoted inducible nitric oxide (NO) synthase expression via activating the MAPK and PI3K/Akt signaling pathways and inducing NO release to enhance lung cancer cell invasion and metastasis *in vitro* (6). Additionally, AGMAT could promote pancreatic cancer cell proliferation, invasion and metastasis via the TGF-β/Smad pathway (7). Although AGMAT plays an essential role in the metabolism of amino acids, its effects on CRC have not been previously investigated.

With the discovery of microRNAs (miRNAs or miRs), their role in the onset and progression of CRC has become

a significant area of interest (8). Increasing evidence has suggested that the dysregulation of miRNAs is involved in the development of several types of cancer, including CRC (9). miRNAs also play a significant role in different biological and cellular processes, such as in cell proliferation, differentiation, apoptosis, death and metastasis (10-13). miR-151a-5p is a well-characterized oncogenic miRNA, which is dysregulated in CRC and is therefore recognized as a promising biomarker for the early detection and treatment of CRC (14). As a member of the miR-151 family, miR-151a-5p is a small non-coding RNA molecule that acts as a proto-oncogene and is expressed in several types of cancer, such as lung cancer (15), prostate cancer (16) and lymphoblastic leukemia (17). It has been reported that miR-151a-5p is involved in numerous biological processes. For instance, a previous study suggested that miR-151a-5p could be involved in the regulation of cell respiration and ATP production in mitochondria via targeting cytochrome B (18). As a member of housekeeping miRNAs, miR-151a-5p was identified to be stably expressed in endothelial cells and macrophages in an inflammatory setting (19). Furthermore, Guo *et al* (20) showed that miR-151a-5p was upregulated in lung cancer, while miR-151a-5p silencing could inhibit the proliferation, invasion and migration, and induce the apoptosis of A549 lung cancer cells. These findings suggested that miR-151a-5p could be a potential therapeutic target for several types of cancer. Although miR-151a-5p could be used as a potential non-invasive serological diagnostic marker for the detection of CRC, the particular effects and mechanisms of miR-151a-5p in CRC remain unclear (14).

Therefore, the present study aimed to explore the effect and molecular mechanisms of miR-151a-5p in CRC *in vitro*.

Materials and methods

Datasets analysis. Co-expression analyses of the interaction between miR-151a-5p and AGMAT were downloaded from the starbase database (http://starbase.sysu.edu.cn/), including COAD samples (n=450). The potential binding sites between miR-151a-5p and AGMAT were downloaded from the miRmap database (http://www.mirmap.ezlab.org). The expression levels of miR-151a-5p in tumor tissues (n=254) were directly compared with those in adjacent normal tissues (n=8) using the expression data from ~32 common types of cancer downloaded from the Gene Expression Display Server (http://bioinfo.life.hust.edu.cn/web/GEDS/). Additionally, the expression levels of miR-151a-5p in tumor tissues (n=445) were also directly compared with those in adjacent normal tissues (n=8) downloaded from the UCSC Xena database (http://xena.ucsc.edu/).

Cell lines and culture. The human CRC cell lines HCT8, HCT116, HT29, SW480, SW620, LS174T and DLD1 were purchased from the American Type Culture Collection. In addition, the normal human colorectal cells NCM460 and 293T cells were conserved in the central laboratory of Shanghai Fengxian District Central Hospital. All cell lines were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution in a humidified incubator at 37˚C with 5% CO_2.

Plasmids and retroviral infection. The full-length sequence of the human AGMAT gene was subcloned into the pCD513B vector (Geneppl Technology Co., Ltd.) and transfected to generate cells stably overexpressing AGMAT. To stably knock down AGMAT in CRC cells, small hairpin RNAs (shRNAs) targeting AGMAT were synthesized and subcloned into the lentiviral pPLK-GFP + Puro vector (Geneppl Technology Co., Ltd.). The sequences of the shRNA clones used were listed in Table I. The concentrations of shRNA clones used were as follows: NC, 964 ng/μl; shAGMAT#1, 1,142 ng/μl; and shAGMAT#3, 795 ng/μl. The mixture of 1 ml DMEM, 10 μg DNA and 10 μl (μg) GM easy™ Lentivirus mix (Genomeditech) was co-transfected into 293T cells using 60 μl Hg transgene™ reagent (Genomeditech), according to the manufacturer's instructions (cat. no. GMeasy 40; Genomeditech). The lentiviral particles were collected at 48 h following cell transduction.

Generation of stable cell lines and miR-151a-5p transfection. When the cell density of ~80-90%, CRC cells seeded into 6-cm culture-plates were infected with filtered lentiviral particles (the 2nd generation system) plus 8 μg/ml Polybrene (Sigma-Aldrich; Merck KGaA) for 24 h. Following transduction, cells were treated with 0.5 μg/ml puromycin (Invitrogen; Thermo Fisher Scientific, Inc.) for >7 days to obtain stably transfected cells; afterwards, the stably transfected cells were maintained with 0.3 μg/ml puromycin. Subsequently, when reached ~60-70% confluency in the cell culture plate, HCT116 cells, SW480 cells and stably AGMAT expressing CRC cells were treated with a mixture containing 5 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), 100 pmol miR-151a-5p and control oligonucleotides, according to the manufacturer's instructions. Following transfection for 48 h, the relative expression levels of miR-151a-5p were determined by reverse transcription-quantitative PCR (RT-qPCR). The sequences of miR-151a-5p mimics or inhibitor used were listed in Table I (Genomeditech).

Dual luciferase reporter assay. The luciferase reporter constructs were generated by inserting the wild-type (WT) or mutant (MUT) 3' untranslated region (3' UTR) of AGMAT into the pGL3 luciferase vector (Geneppl Technology Co., Ltd.). Subsequently, the stable AGMAT overexpressing CRC cells were co-transfected with 2 μg WT or MUT AGMAT-3' UTR, *Renilla* Luc, miR-151a-5p mimics or miR-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions (Shanghai Yeasen Biotechnology Co., Ltd.). Following transfection for 48 h, the firefly and *Renilla* luciferase activities were determined by spectrophotometry using the Dual Luciferase Reporter Assay System (Glomax 96; Promega Corporation). The luciferase activity was then calculated. The results are expressed as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

Cell counting kit-8 (CCK-8) assay. The cell proliferation ability was assessed using CCK-8 assay (Dojindo Molecular Technologies, Inc.), according to the manufacturer's protocol. Briefly, treated CRC cell lines were seeded into a 96-well plate at a density of 10^3 cells/well in 100 μl DMEM supplemented

Table I. The sequences of specific primers used for shRNA and miR-151a-5p.

Primer	Sequence (5'→3')
shAGMAT#1	CGATGTGAATGTCAATCTTTA
shAGMAT#3	CGGGAAGAATCAGTGATGCTT
NC	GTTCTCCGAACGTGTCACGTT
miR-151a-5p mimic NC	UUGUACUACACAAAAGUACUG
miR-151a-5p mimics	UCGAGGAGCUCACAGUCUAGU
miR-151a-5p inhibitor NC	CAGUACUUUUGUGUAGUACAA
miR-151a-5p inhibitor	ACUAGACUGUGAGCUCCUCGA

sh-, short hairpin; NC, negative control; miR, microRNA.

with 10% FBS and were then cultured for 10, 24, 48 and 72 h at 37˚C with 5% CO_2. Subsequently, cells were supplemented with 10 μl CCK-8 solution, followed by incubation for an additional 1 h in the dark. Finally, the optical density (OD) of each well was measured at a wavelength of 450 nm using a microplate reader (BioTek Instruments, Inc.).

Colony formation assay. For colony formation assays, treated CRC cells were seeded into a 12-well plate at a density of $3x10^3$ cells/well and cultured in DMEM supplemented with 10% FBS for 7 or 10 days. The formed colonies were fixed with 4% paraformaldehyde at room temperature for 30 min and were then stained with 0.1% crystal violet at room temperature for 1 h followed by washing with ddH_2O. Images of the formed colonies were captured under an inverted light microscope (Olympus Corporation). Finally, the number of colony cells (>50 cells per cell colony) were counted using ImageJ software (version 1.52a; National Institutes of Health).

Cell migration and invasion assay. The migration and invasion abilities of HCT116 and SW480 cells transfected with the aforementioned plasmids/miR mimics/inhibitors for 48 h were evaluated using Transwell chambers (0.8 μm; cat. no. 353097; Corning, Inc.). Briefly, treated HCT116 and SW480 cells at a density of 10^5 cells/well were added to the upper chamber of the Transwell chamber coated or not with Matrigel (0.8 μm; cat. no. 354480; Corning, Inc.). The upper chambers of the Transwell chamber with Matrigel were precoated with 500 μl medium without serum for 1 h at 37˚C with 5% CO_2. For migration assays, the lower chamber was supplemented with 600 μl medium with 10% FBS, cells were cultured for 48 h, while for the invasion assays, the lower chamber was supplemented with 600 μl medium with 20% FBS, cells were cultured for 24 h, the upper chamber was supplemented without serum. The migratory or invasive cells were fixed with 4% paraformaldehyde at room temperature for 30 min and were then stained with 0.1% crystal violet at room temperature for 1 h followed by ddH_2O washing. Finally, migratory or invasive cells were observed and images were captured under a fluorescence microscope (magnification, x100).

RT-qPCR analysis. Total RNA was extracted from treated cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The nuclear and cytoplasmic extracts were isolated using the cytoplasmic and nuclear RNA purification kit (cat. no. 21000; Norgen Biotek Corp.). Subsequently, 1 μg RNA was reverse transcribed into cDNA using the Evo M-MLV RT Mix kit with gDNA Clean for qPCR (cat. no. AG11728; Accurate Bio-Medical Technology Co., Ltd.) and the miRNA 1st strand cDNA synthesis kit (by stem-loop) (cat. no. MR101-02; Vazyme Biotech Co., Ltd.). qPCR was performed on the quantum Studio 6 real-time PCR system (Thermo Fisher Scientific, Inc.) using the SYBR Green premix Pro Taq HS qPCR kit (Rox plus) (cat. no. AG1718; Accurate Bio-Medical Technology Co., Ltd.). The thermocycling conditions used for reverse transcription PCR were as follows: For gDNA clean, 42˚C 2 min, 4˚C; for synthesis of first strand cDNA of miR-151a-5p, 25˚C for 5 min, 50˚C for 15 min, 85˚C for 5 min; for synthesis of common primer cDNA, 37˚C for 15 min, 85˚C for 5 min, 4˚C. The thermocycling conditions used for qPCR were as follows: For miR-151a-5p, 95˚C for 5 min, 95˚C 10 sec, 60˚C for 5 min; for common primers, 95˚C for 30 sec, 95˚C for 5 sec, 60˚C for 30 sec. The relative mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta Cq}$ method (21). GAPDH served as an internal reference gene for AGMAT. The primers for U1 were purchased from Guangzhou RiboBio Co., Ltd. The primer sequences used are listed in Table II (Sangon Biotech Co., Ltd.).

Immunofluorescence assay. The transfected cells were cultured in 48-well plates at a density of $6x10^4$ cells/well for 24 h. Following fixation with 4% paraformaldehyde at room temperature for 30 min, the cells were permeabilized with 1% Triton X-100 at room temperature for 15 min followed by blocking with 3% BSA (cat. no. 4240GR100; Biofroxx; neoFroxx) at 37˚C for 1 h. After cell incubation with primary antibodies at 4˚C overnight, the cells were treated with the corresponding secondary Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (Thermo Fisher Scientific, Inc.) at room temperature for 30 min in the dark. Nuclei were counterstained with DAPI (5 mg/ml; cat. no. C1006; Beyotime Institute of Biotechnology) at room temperature for 20 min in the dark. The stained cells were observed under a fluorescence microscope (magnification, x200). The antibodies used for immunofluorescence are listed in Table III.

Western blot analysis. Total proteins were extracted from treated CRC cells using RIPA lysis buffer (New cell & Molecular Biotech Co., Ltd.) supplemented with phosphatase inhibitors (Beyotime Institute of Biotechnology) and protein concentration was measured with the BCA Protein Assay Kit (cat. no. P0012; Beyotime Institute of Biotechnology). Subsequently, the proteins (10-20 μl/lane, 25 mg/ml) were separated by 10% SDS-PAGE (New cell & Molecular Biotech Co., Ltd.) and were then transferred onto a nitrocellulose membrane (Pall Corporation). Following blocking with 5% skimmed milk (Beyotime Institute of Biotechnology) at room temperature for 1 h, the membrane was incubated with primary antibodies at 4˚C overnight. After incubation with the corresponding secondary antibodies at room temperature

Table II. The sequences of specific primers used for reverse transcription-quantitative PCR.

Gene name	Sequence (5'→3')
GAPDH	F: TTGGTATCGTGGAAGGACTCA
	R: TGTCATCATATTTGGCAGGTT
microRNA-151a-5p	F: CGCGTCGAGGAGCTCACAG
	R: AGTGCAGGGTCCGAGGTATT
AGMAT	F: CTTGTCGAAGTTTCACCACCGTA
	R: CTTTGGGGAGAGCACATAGCATC
AZIN2	F: CAACTCAGCCTTGGACCTGTACTTC
	R: CTGCTCCGTGGATGGTTTCTTCTG
AMD1	F: CCCGACGCAAACCAAGGATCTG
	R: TTCAACAGGGGAACCAGTGCTTTC
ODC1	F: CACTGTTGCTGCTGCCTCTACG
	R: GGTTCTGGAATTGCTGCATGAGTTG
OAZ1	F: TCTCCCTCCACTGCTGTAGTAACC
	R: TGACTATTCCCTCGCCCACCTG
SRM	F: GATGATCGCCAACCTGCCTCTC
	R: ATCTCACACTGGACCACGGACTC
SMS	F: TTGGCAGAGAGTGATTTGGCATATACC
	R: CCACCTCCCAGAATGAGTACATCTTTG
SAT1	F: TGGTTGCAGAAGTGCCGAAAGAG
	R: ATAACTTGCCAATCCACGGGTCATAG
SMOX	F: CACACCCTCACCTACCCACCTG
	R: CACTGCCTCGTCATCACACTTCTC
PMF1	F: GCGATGACACAGCAAATCTATGACAAG
	R: AGGCATTCAAGACAGCTTCTAGGTTC
TP53	F: GCCCATCCTCACCATCATCACAC
	R: GCACAAACACGCACCTCAAAGC
PAOX	F: TTCCAGTGTCGGTAGAGTGTGAGG
	R: ATCTTCCTGATTGCTTCTGCCTTCTC

AGMAT, agmatinase; F, forward; R, reverse.

for 1 h, the immunoreactive bands were visualized using an ECL Kit (Vazyme Biotech Co., Ltd.) and images were captured under the Tanon 4600 system (Tanon Science and Technology Co., Ltd.). The density of each band was measured using a computer-assisted imaging analysis system (Adobe Systems Inc.). The expression levels of the target proteins were normalized to those of β-tubulin. The following primary and secondary antibodies were used: Anti-AGMAT, anti-MMP2, anti-MMP9, anti-N-cadherin, anti-E-cadherin, anti-Vimentin, anti-β-catenin, HRP-conjugated Affinipure Goat anti-Mouse IgG (H+L) and HRP-conjugated Affinipure Goat anti-Ribbit IgG (H+L) (Table IV). The protein expression levels were assessed using ImageJ software (National Institutes of Health).

Statistical analysis. All experiments were repeated at least in triplicate. Data are expressed as the mean ± SD. All statistical analyses were carried out using GraphPad Prism 8 (GraphPad Software, Inc.). The differences between two groups were compared using unpaired Student's t-test. Comparisons among multiple groups were conducted using with one- or two-way ANOVA followed by Dunnett's multiple comparisons test used as post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-151a-5p is upregulated in CRC cell lines and tissues. To explore the pathophysiological relevance of miR-151a-5p in human CRC, the expression levels of miR-151a-5p were determined in CRC and adjacent normal tissues obtained from UCSC Xena database and the Gene Expression Display Server. Therefore, compared with normal colorectal tissues, miR-151a-5p was significantly upregulated in CRC tissues (Fig. 1A and B). These data suggested that miR-151a-5p was upregulated in CRC tissues and could therefore regulate the occurrence and development of CRC via acting as a tumor-promoting gene. In addition, the expression levels of miR-151a-5p were further detected in several CRC cell lines by RT-qPCR. The analysis showed that the expression levels of miR-151a-5p were higher in CRC cell lines compared with normal colorectal cells. Among all CRC cell lines examined, SW480 cells exhibited the highest miR-151a-5p expression levels, while HCT116 cells the lowest one (Fig. 1C). Therefore, HCT116 and SW480 cells were selected for the subsequent experiments. Furthermore, the transfection efficiency of miR-151a-5p in CRC cells was also verified (Fig. 1D and E).

miR-151a-5p promotes CRC cell proliferation, migration and invasion. To further uncover the potential role of miR-151a-5p in CRC, HCT116 or SW480 cells were transfected with miR-151a-5p mimics or inhibitor, respectively. Following cell transfection for 48 h, the cell proliferation ability was assessed by CCK-8 and colony formation assays. The results showed that compared with the NC group, the change in the overexpression levels of miR-151a-5p significantly promoted the proliferation of HCT116 cells in time-dependent manner (Fig. 2A), while as predicted, the proliferation capacity of SW480 cells transfected with miR-151a-5p inhibitor was significantly inhibited (Fig. 2B). In addition, the effect of miR-151a-5p on the proliferation of HCT116 and SW480 cells was evaluated by colony formation assay. The results demonstrated that the overexpression levels of miR-151a-5p promoted the proliferation of HCT116 cells (Fig. 2C). By contrast, the proliferation of SW480 cells was significantly inhibited after transfection with miR-151a-5p inhibitor (Fig. 2D). The enhanced migration and invasion abilities of CRC cells could also serve a key role in the growth of CRC. Therefore, to further evaluate the effect of miR-151a-5p on CRC cell invasion and migration, Transwell assays coated with Matrigel or not, respectively, were used. The data revealed that compared with the corresponding NC group, the migration and invasion abilities of HCT116 cells were significantly increased following cell transfection with miR-151a-5p mimics (Fig. 2E-G). However, the opposite effect was observed in miR-151a-5p-depleted SW480 cells (Fig. 2H-J). The aforementioned findings indicated that miR-151a-5p could significantly enhance the proliferation, migration and invasion abilities of CRC cells.

miR-151a-5p enhances the epithelial-mesenchymal transition (EMT) of CRC cells. Epithelial cells are characterized by

Table III. Antibodies used for immunofluorescent staining.

Target	Supplier	Cat. no.	Dilution	Isotype	Host species
E-cadherin	Proteintech Group, Inc.	20874-1-AP	1:200	IgG	Rabbit
Vimentin	Proteintech Group, Inc.	10366-1-AP	1:200	IgG	Rabbit

Table IV. Antibodies used for western blot analyses.

Primary antibodies

Target	Supplier	Cat. no.	Dilution	Isotype	Host species	MW (KDa)
AGMAT	Abcam	Ab231894	1:2,000	IgG	Rabbit	38
MMP2	Proteintech Group, Inc.	10373-2-AP	1:1,000	IgG	Rabbit	60
MMP9	Proteintech Group, Inc.	10375-2-AP	1:1,000	IgG	Rabbit	92
N-cadherin	Proteintech Group, Inc.	22018-1-AP	1:2,000	IgG	Rabbit	130
E-cadherin	Proteintech Group, Inc.	20874-1-AP	1:5,000	IgG	Rabbit	135
Vimentin	Proteintech Group, Inc.	10366-1-AP	1:2,000	IgG	Rabbit	54
β-Catenin	Proteintech Group, Inc.	51067-2-AP	1:5,000	IgG	Rabbit	92
β-Tubulin	Proteintech Group, Inc.	66240-1-Ig	1:10,000	IgG2a	Mouse	55

Secondary antibodies

Target	Supplier	cat. no.	Dilution
HRP-conjugated Affinipure Goat anti-mouse IgG (H+L)	Proteintech Group, Inc.	SA00001-1	1:5,000
HRP-conjugated Affinipure Goat anti-rabbit IgG (H+L)	Proteintech Group, Inc.	SA00001-2	1:5,000

mesenchymal phenotype and changes in the expression of three significant biomarkers, including E-cadherin, vimentin and N-cadherin, eventually leading to reduced cell adhesion, loss of polarity and tight junction (22). Matrix metalloproteinases (MMPs) are proteins involved in the degradation of base membranes, thus affecting cell metastasis during the development of malignant tumors (23). Therefore, western blot analysis was performed and the result revealed that miR-151a-5p overexpression in HCT116 cells downregulated E-cadherin and upregulated N-cadherin, vimentin and β-catenin. Correspondingly, western blot analysis was also performed to evaluate the effect of miR-151a-5p on the expression levels of metastasis-related MMPs, such as MMP2 and MMP9. The results demonstrated that compared with the NC group, miR-151a-5p enhanced the protein expression levels of both MMP2 and MMP9 in HCT116 cells. By contrast, miR-151a-5p silencing in SW480 cells exhibited the opposite effect (Fig. 3A-C). To further determine the expression of EMT-related proteins in CRC cell lines, the expression of E-cadherin and vimentin was assessed by immunofluorescence analysis. The immunofluorescence analysis results demonstrated that miR-151a-5p overexpression in HCT116 cells downregulated E-cadherin and upregulated vimentin. However, miR-151a-5p silencing upregulated E-cadherin and downregulated vimentin (Fig. 3D). The aforementioned results verified that miR-151a-5p could promote EMT in CRC cells.

AGMAT is a target gene of miR-151a-5p and is positively associated with miR-151a-5p expression. Polyamines, such as putrescine, spermidine and spermine, produced during the metabolic process, are low molecular weight, aliphatic and polycationic nitrogen compounds containing two or more amino groups. Currently, the application of new technologies has increased our understanding of the genetics and potential molecular biology of polyamine function in normal and tumor cells. Recognition of the effect of the increased polyamine levels on tumor transformation and progression has deepen our understanding of the significant role of polyamines in cancer, thus providing reasonable targets for intervention (24). It has been reported that miRNAs can regulate tumor-associated proteins in CRC (25,26). However, the association between genes and miRNAs is not a one-to-one correspondence. The results of the current study showed that following miR-151a-5p overexpression or silencing in CRC cell lines, the levels of the polyamine metabolism-related metabolites and key enzymes significantly altered, while the most differences were observed in the expression levels of AGMAT at the molecular level (Fig. 4A and B). These findings indicated that miR-151a-5p could promote polyamine metabolism via regulating AGMAT. Therefore, subsequently, the present study aimed to explore whether miR-151a-5p could regulate CRC progression via targeting AGMAT. Bioinformatic analysis using the starbase database predicted that AGMAT encompassed a binding site for miR-151a-5p and revealed that AGMAT was positively

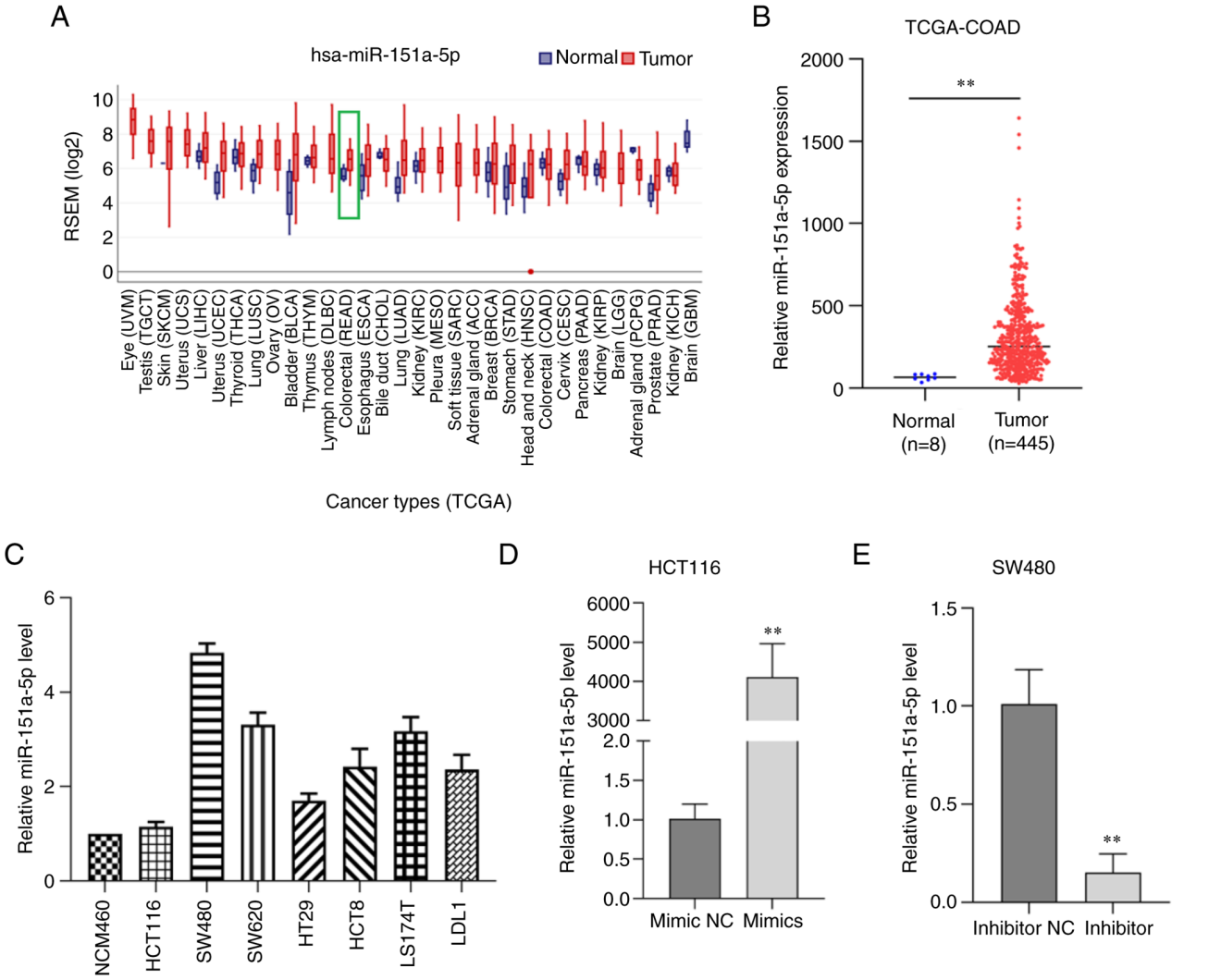

Figure 1. miR-151a-5p is sparsely expressed in CRC cell lines and tissues. (A) The expression levels of miR-151a-5p in CRC tissues (n=254) vs. adjacent normal tissues (n=8) in the Gene Expression Display Server database are shown. (B) The expression levels of miR-151a-5p in CRC tissues (n=445) vs. adjacent normal tissues (n=8) in The UCSC database are presented. (C) The expression levels of miR-151a-5p in CRC cell lines were assessed by RT-qPCR. **P<0.01 vs. NCM460 cells. (D) The transfection efficiency of miR-151a-5p in HCT116 cells transfected with miR-151a-5p mimics were determined using RT-qPCR. **P<0.01 vs. mimic NC. (E) The transfection efficiency of miR-151a-5p in SW480 cells transfected with miR-151a-5p inhibitor were assessed using RT-qPCR. **P<0.01 vs. inhibitor NC. All data are expressed as the mean ± SD (n=3). miR-151a-5p, microRNA-151a-5p; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

associated with miR-151a-5p expression in CRC (Fig. 4C). In addition, it was further predicted using the miRmap database that the potential miR-151a-5p binding site was encompassed in AGMAT-3' UTR (Fig. 4D). Previous studies suggested that the majority of miRNAs in the cytoplasm could act as tumor suppressors, thus resulting in gene silencing via inhibiting gene translation (27). By contrast, other studies showed that several miRNAs, located at the nucleus, could positively regulate gene transcription via binding to and activating their target enhancers (28,29). In the present study, RNA isolation from nuclear and cytoplasmic extracts revealed that miR-151a-5p was mainly localized in the nucleus, as evidenced by RT-qPCR analysis (Fig. 4E and F). To further verify whether miR-151a-5p could regulate the expression of AGMAT via binding to AGMAT-3' UTR, a dual luciferase reporter assay was performed. As revealed in Fig. 4G, the luciferase activity of AGMAT-3' UTR was significantly increased in cells transfected with miR-151a-5p mimics. However, miR-151a-5p overexpression showed a modest effect on luciferase activity in cells transfected with mutated putative binding site. For the convenience of follow-up tests, the stable AGMAT expression cell lines were designed, namely, the overexpression of AGMAT in HCT116 cells and the knockdown of AGMAT in SW480 cells. The transfection efficiency of AGMAT in stable transmutation cells was determined by RT-qPCR and western blot analysis. ShAGMAT#1 was selected from the AGMAT-stably knocked down SW480 cells due to the best knockdown effect (Fig. S1). Furthermore, RT-qPCR and western blot analysis were carried out to reveal the relationship between miR-151a-5p and AGMAT expression. The results identified that the mRNA expression levels of AGMAT were significantly reduced in AGMAT-stably overexpressing HCT116 cells transfected with miR-151a-5p inhibitor (Fig. 5A). However, the opposite effect was observed in the AGMAT-stably knocked down SW480 cells transfected with miR-151a-5p mimics (Fig. 5B). Western blot analysis

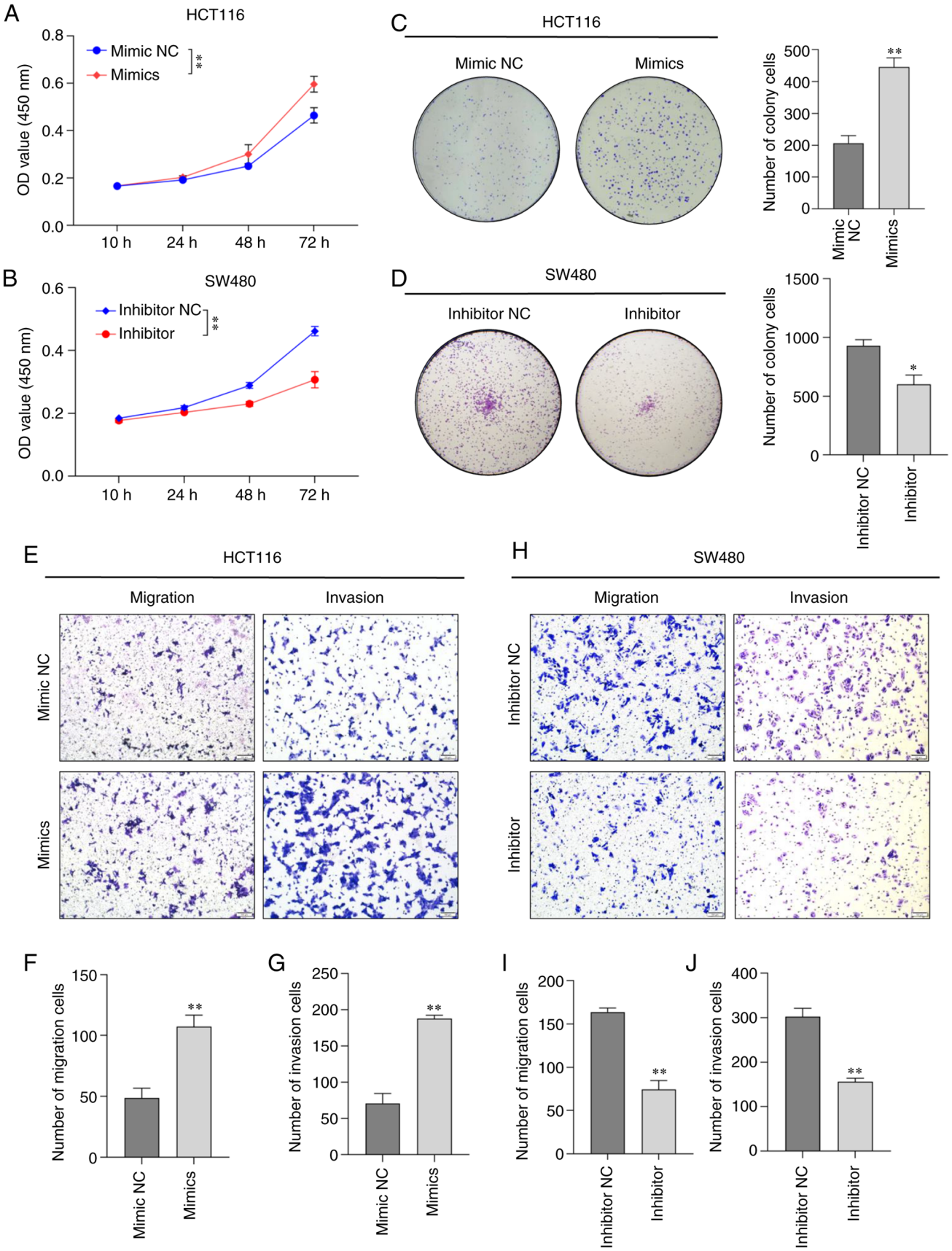

Figure 2. miR-151a-5p promotes CRC cell proliferation, migration and invasion. (A and B) The proliferation ability of (A) HCT116 and (B) SW480 cells transfected with miR-151a-5p mimics and inhibitor, respectively, was assessed by Cell Counting Kit-8 assay at the indicated time points. (C and D) Colony formation assays were also performed to evaluate the proliferation ability of (C) HCT116 and (D) SW480 cells transfected with miR-151a-5p mimics and inhibitor, respectively. (E-J) The migration and invasion abilities of (E) HCT116 and (H) SW480 cells transfected with miR-151a-5p mimics and inhibitor, respectively, were examined. The migration ability of (F) HCT116 and (I) SW480 cells was evaluated by Transwell chambers without Matrigel. The invasion ability of (G) HCT116 and (J) SW480 cells was determined using Transwell chambers coated with Matrigel. *P<0.05 and **P<0.01 vs. mimic NC or inhibitor NC. All data are expressed as the mean ± SD (n=3). miR-151a-5p, microRNA-151a-5p; CRC, colorectal cancer; NC, negative control.

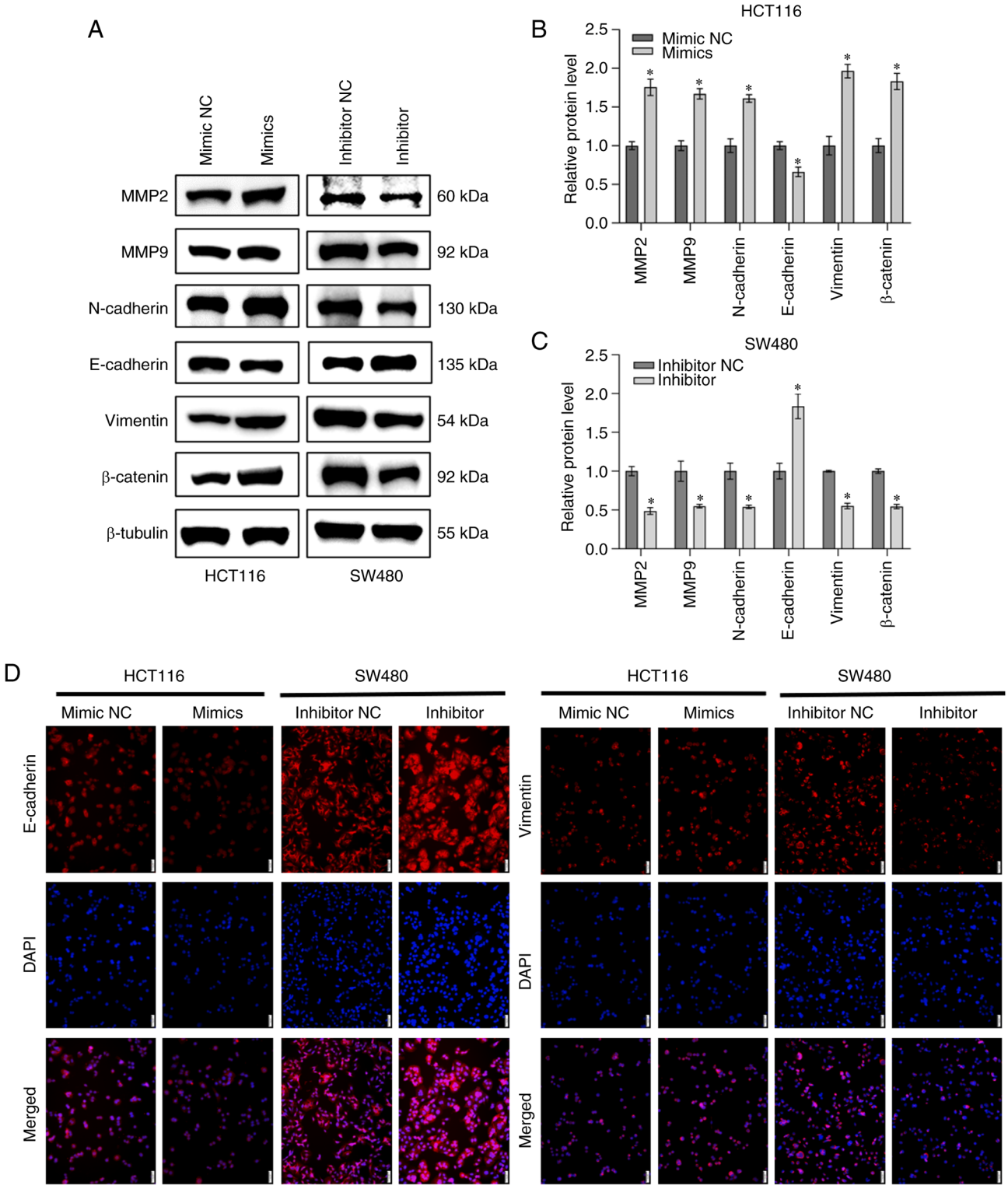

Figure 3. miR-151a-5p enhances the epithelial-mesenchymal transition of CRC cells. (A) The protein expression levels of E-cadherin, vimentin, β-catenin, N-cadherin, MMP2 and MMP9 were detected in (B) HCT116 and (C) SW480 cells using western blot analysis. (D) The expression levels of E-cadherin and vimentin in CRC cells transfected with miR-151a-5p mimics or inhibitor were determined by immunofluorescence assay. *P<0.05 vs. mimic NC or inhibitor NC. All data are expressed as the mean ± SD (n=3). miR-151a-5p, microRNA-151a-5p; CRC, colorectal cancer; NC, negative control.

further verified the positive regulatory association between miR-151a-5p and AGMAT (Fig. 5C and D). This indicated that miR-151a-5p could promote the expression of AGMAT in CRC cells. The aforementioned data suggested that AGMAT could be a potential target of miR-151a-5p and could be positively associated with miR-151a-5p expression in CRC.

miR-151a-5p promotes the effects of AGMAT on the proliferation, migration and invasion of CRC cells. To further verify whether miR-151a-5p could regulate the potential functions of AGMAT in CRC, AGMAT-stably expressing HCT116 or SW480 cells were transfected with miR-151a-5p inhibitor or mimics. Following transfection for 48 h, the

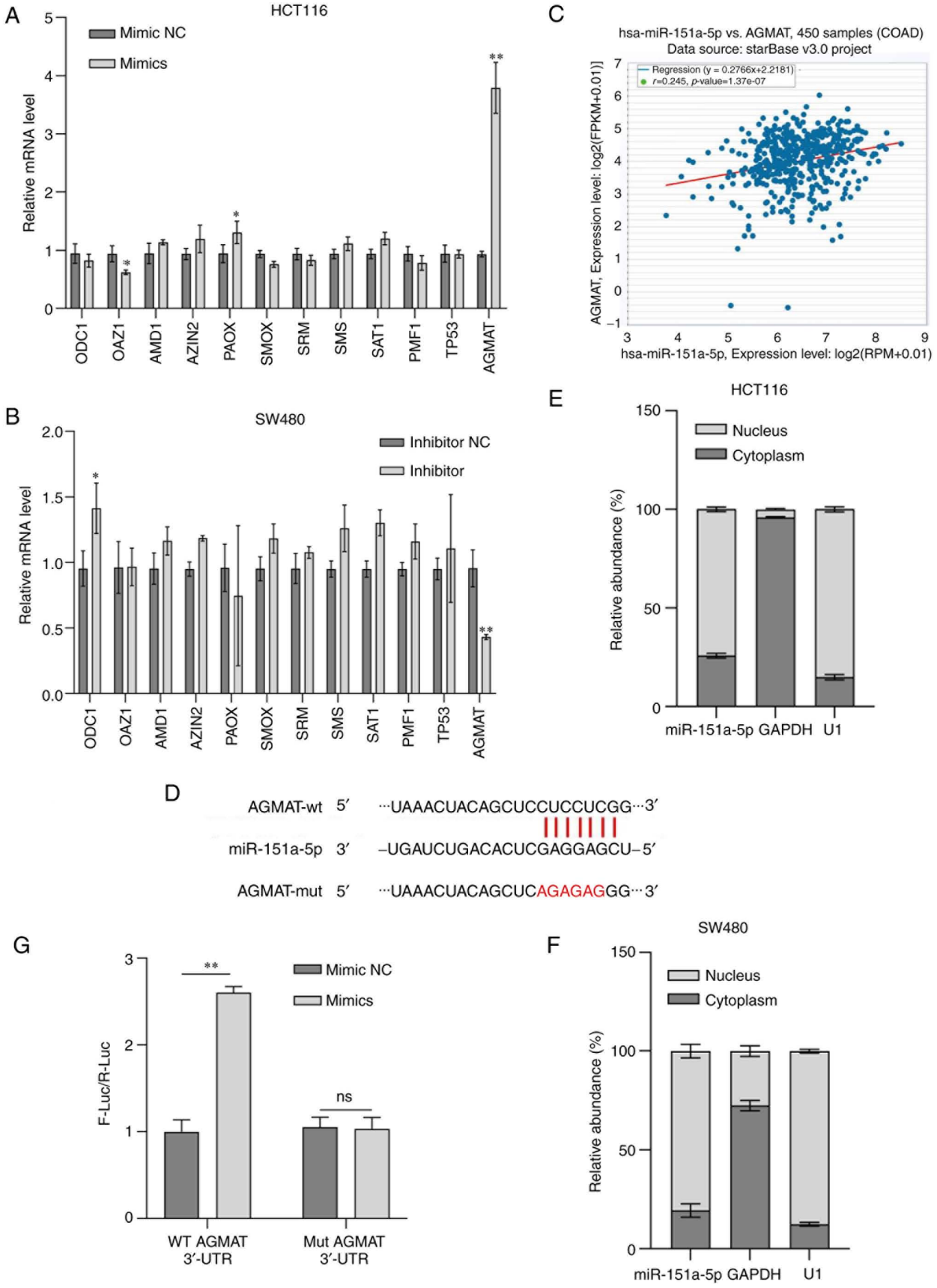

Figure 4. AGMAT is a target gene of miR-151a-5p and is positively associated with miR-151a-5p expression. (A and B) The mRNA expression levels the of polyamine metabolism-related enzymes in (A) HCT116 and (B) SW480 cells transfected with miR-151a-5p mimics and inhibitor, respectively, were determined by RT-qPCR. (C) The association between miR-151a-5p and AGMAT expression is shown. (D) The binding sites between miR-151a-5p and the 3' untranslated region of AGMAT are illustrated. (E and F) The expression levels of miR-151a-5p in nuclear and cytoplasmic extracts isolated from (E) HCT116 and (F) SW480 cells were detected by RT-qPCR. (G) The binding activity of miR-151a-5p on AGMAT was evaluated using the dual luciferase reporter assay. *P<0.05 and **P<0.01 vs. mimic NC or inhibitor NC. All data are expressed as the mean ± SD (n=3). AGMAT, agmatinase; miR-151a-5p, microRNA-151a-5p; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; ns, not significant.

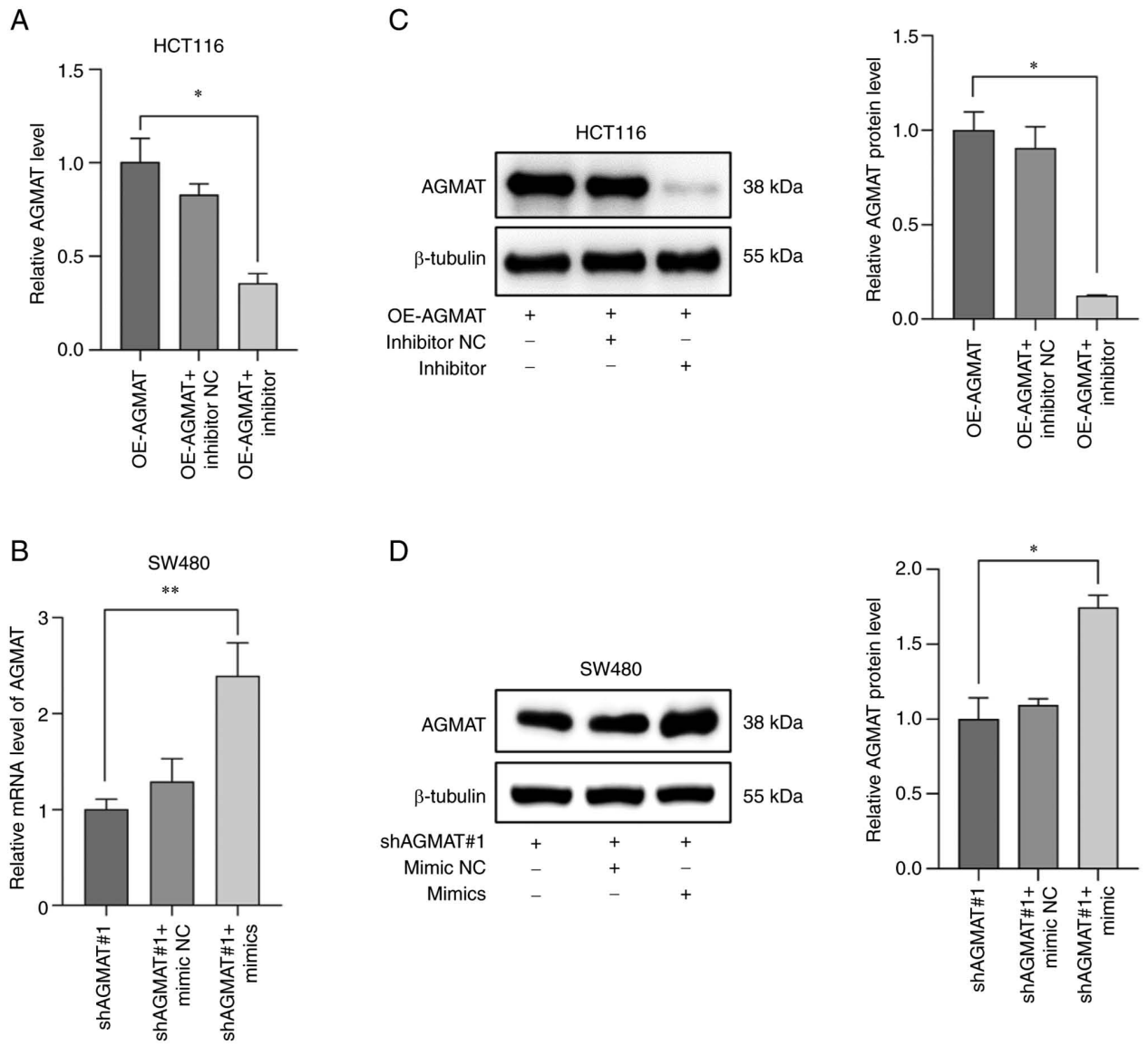

Figure 5. miR-151a-5p promotes the expression of AGMAT in colorectal cancer cells. (A) The mRNA expression levels of AGMAT in AGMAT-stably overexpressing HCT116 cells transfected with miR-151a-5p inhibitor was determined using RT-qPCR. (B) The mRNA expression levels of AGMAT in AGMAT-stably knocked down SW480 cells transfected with miR-151a-5p mimics was determined using RT-qPCR. (C) The protein expression levels of AGMAT in AGMAT-stably overexpressing HCT116 cells after transfection with miR-151a-5p inhibitor were evaluated using western blot analysis. (D) The protein expression levels of AGMAT in AGMAT-stably knocked down SW480 cells after transfection with miR-151a-5p mimics were evaluated using western blot analysis. *P<0.05 and **P<0.01 vs. mimic NC or inhibitor NC. All data are expressed as the mean ± SD (n=3). AGMAT, agmatinase; miR-151a-5p, microRNA-151a-5p; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; OE, overexpression; sh-, short hairpin; NC, negative control.

proliferation ability of AGMAT-stably expressing CRC cells was evaluated by CCK-8 and colony formation assays. Therefore, compared with the NC group, the proliferation of the AGMAT-stably overexpressing HCT116 cells was significantly decreased after transfection with miR-151a-5p inhibitor in a time-dependent manner (Fig. 6A). However, the significant opposite result of the proliferation was observed in a time-dependent manner in the AGMAT-stably knocked down SW480 cells transfected with miR-151a-5p mimics (Fig. 6B). Additionally, the effect of miR-151a-5p targeting AGMAT on the proliferation of the AGMAT-stably overexpressing HCT116 or SW480 cells was also evaluated by colony formation assays. As expected, the proliferation ability of the AGMAT-stably overexpressing HCT116 cells transfected with miR-151a-5p inhibitor was significantly decreased (Fig. 6C), but the significant opposite result of the proliferation was observed in a time-dependent manner in the AGMAT-stably knocked down SW480 cells transfected with miR-151a-5p mimics (Fig. 6D). To further explore whether miR-151a-5p could regulate CRC cell invasion and migration via targeting AGMAT, Transwell invasion and migration assays were performed. The results showed that compared with the NC group, the migratory and invasive abilities of the AGMAT-stably overexpressing HCT116 cells transfected with miR-151a-5p inhibitor were significantly decreased (Fig. 6E-G). The completely opposite effect was obtained in the AGMAT-stably knocked down SW480 cells transfected with miR-151a-5p mimics (Fig. 6H-J). The aforementioned results indicated that miR-151a-5p could enhance the biofunction of CRC cells via targeting AGMAT.

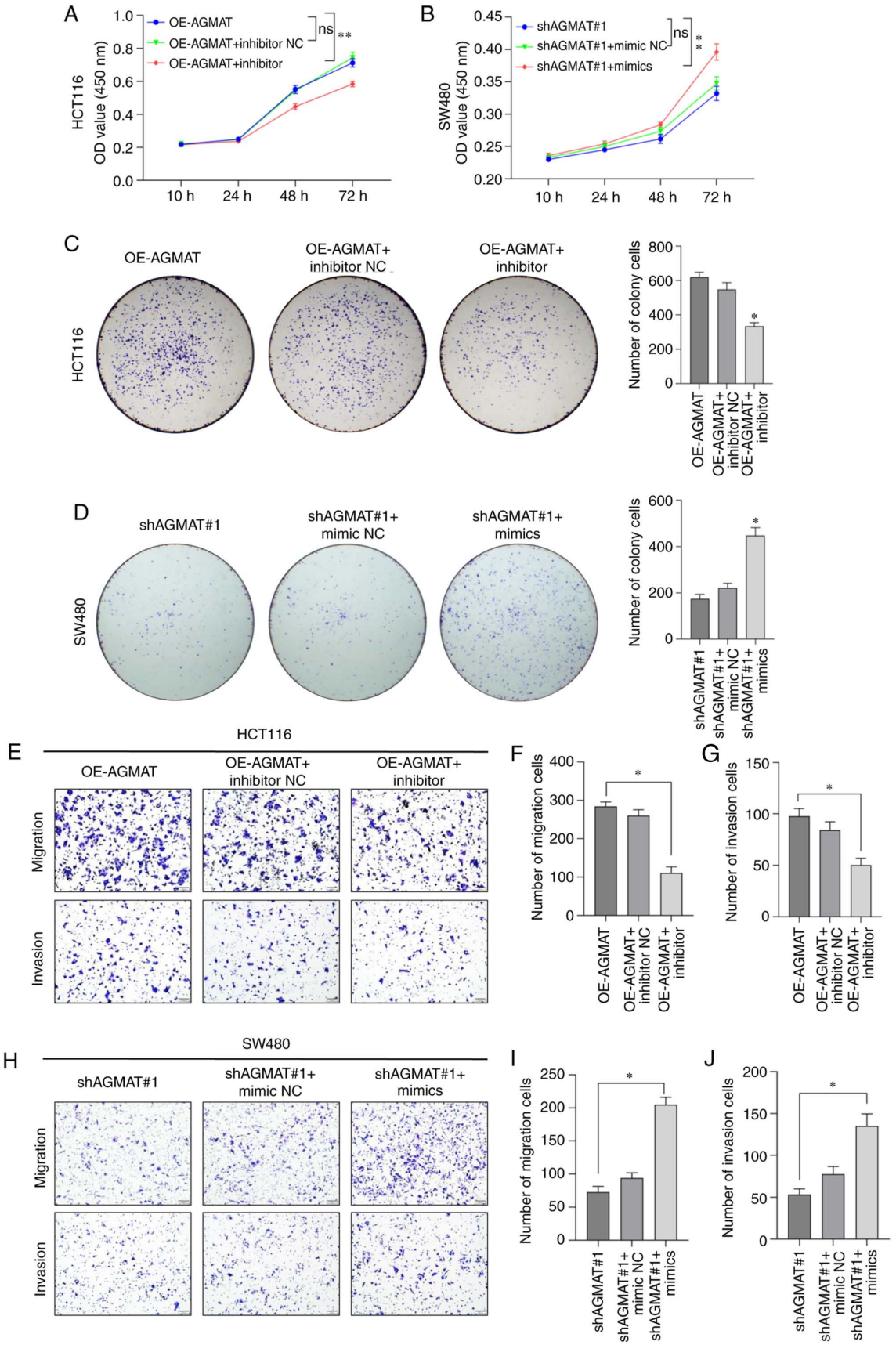

Figure 6. miR-151a-5p promotes the effects of AGMAT on colorectal cancer cell proliferation, migration and invasion. (A and C) The proliferation ability of AGMAT-stably overexpressing HCT116 cells transfected with miR-151a-5p inhibitor was assessed by (A) CCK-8 assay and (C) colony-formation assays. (B and D) The proliferation ability of AGMAT-stably knocked down SW480 cells transfected with miR-151a-5p mimics was assessed by (B) CCK-8 and (D) colony formation assays. (E-J) The migratory and invasive abilities of (E-G) AGMAT-stably overexpressing HCT116 cells transfected with the miR-151a-5p inhibitor and (H-J) AGMAT-stably knocked down SW480 cells transfected with the miR-151a-5p mimics were assessed by Transwell migration and invasion assays. *P<0.05 and **P<0.01 vs. the OE-AGMAT or shAGMAT#1 group. All data are expressed as the mean ± SD (n=3). AGMAT, agmatinase; miR-151a-5p, microRNA-151a-5p; CCK-8, Cell Counting Kit-8; OE, overexpression; sh-, short hairpin; NC, negative control.

Discussion

Emerging evidence has suggested that miRNAs can mediate the development of several diseases, such as CRC, through multiple genes and biological pathways (30-32). It has been reported that miRNAs are abnormally expressed in CRC tissues, thus serving as powerful and promising biomarkers for the early screening and treatment of CRC (33,34). For example, Lopez-Camarillo *et al* (35) investigated the expression of several miRNAs, known as metaMIRs, which are involved in the initiation of cell invasion and metastasis via targeting numerous proteins associated with several cellular processes. Therefore, the aforementioned study highlighted the potential use of metaMIRs as novel specific markers for evaluating cancer progression. MicroRNAs play an important role in regulating gene expression under normal and pathological conditions, such as cancer (36). The majority of miRNAs act as tumor suppressors, thus promoting gene silencing through translational suppression, which mainly occurs in the cytoplasm (27). However, the aberrant overexpression of other miRNAs, known as super-enhancers, commonly localized in the nucleus, can promote tumor occurrence, growth and/or metastasis (28,29). It has been reported that several miRNAs can act as biomarkers in CRC via negatively regulating the expression of different genes. However, the application of miRNAs as super-enhancers in clinical practice remains limited. Previous studies demonstrated that miR-151a-5p could be used as a potential non-invasive serological diagnostic marker for the diagnosis of CRC (14) and endometrial cancer (37). However, the effect and underlying mechanism of miR-151a-5p on CRC remains unclear. Consistent with previous findings, the results of the current study revealed that miR-151a-5p was significantly upregulated in CRC. Additionally, miR-151a-5p overexpression enhanced the proliferation ability of CRC cells. Furthermore, the results demonstrated that the tumor-promoting effects of miR-151a-5p were mediated via improving the migration and invasion abilities of CRC cells. MMPs are significant calcium- and zinc-dependent proteolytic enzymes, which can degrade the components of the extracellular matrix and basement membrane. These proteins are involved in cell invasion and metastasis during cancer progression (38). The present study verified that miR-151a-5p overexpression notably reinforced the migration and invasion abilities of CRC cells and increased the protein expression levels of MMP2 and MMP9. In addition, miR-151a-5p promoted the EMT of CRC cells.

miRNAs are a class of endogenous small single-stranded non-encoding RNAs with a length of ~18-24 nucleotides, completely or incompletely complementary with the 3' UTR of their target mRNAs. miRNAs can negatively regulate the expression of their target genes at the post-transcriptional level (39,40). In the present study, bioinformatic analysis revealed that the AGMAT 3' UTR encompassed a complementary binding site for miR-151a-5p and AGMAT positively regulated miR-151a-5p expression. To further explore the combined effect of miR-151a-5p and AGMAT on the occurrence and development of CRC, a dual luciferase reporter assay was performed to verify that miR-151a-5p could directly target AGMAT. Similar to arginase, AGMAT, as a metal hydrolase, belongs to the urea hydrolase superfamily. The catalytic activity of AGMAT depends on Mn^{2+} ions (41). Agmatine, an endogenous polyamine located in mitochondria, is catalyzed by L-arginine decarboxylase and hydrolyzed by agmatine enzyme to form urea and putrescine. Previous studies suggested that AGMAT could not only regulate the biological effects of agmatine in mammalian cells (42), but it could also be considered as an alternative mechanism for polyamine biosynthesis (5). Several studies have reported the regulatory mechanisms of AGMAT and its analogs in several diseases (43-45). However, the role of AGMAT in cancer remains elusive. Recent studies showed that AGMAT could play a significant role in lung adenocarcinoma (6) and pancreatic adenocarcinoma (7). In the present study, AGMAT was identified to be positively associated with miR-151a-5p expression. In addition, transfection of AGMAT-stably knocked down CRC cells with miR-151a-5p mimics promoted the proliferation, migration and invasion of CRC cells. The current study was the first to demonstrate that miR-151a-5p could play a significant role in regulating the proliferation and metastasis of CRC cells via targeting AGMAT.

In conclusion, the current study demonstrated that miR-151a-5p was significantly upregulated in CRC tissues and cell lines, while its ectopic expression promoted the proliferation, migration and invasion of CRC cells via targeting AGMAT. Furthermore, miR-151a-5p could enhance the EMT processes of CRC cells. Therefore, the miR-151a-5p/AGMAT axis could serve as a potential therapeutic target for the diagnosis and treatment of CRC.

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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

YX, YZ, LC and XL contributed to the experiments. CW and MH were responsible for the bioinformatic analysis and the experiment result analysis. YX and XZ confirm the authenticity of all the raw data. XL and YX contributed to writing the manuscript. JC and XZ designed project ideas, provided technical guidance and revised the manuscript. All authors have read and approved the final version of the 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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