miR-20b-5p Functions as Tumor Suppressor microRNA by targeting CyclinD1 in Colon Cancer

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Research

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Abstract

Background

MicroRNA functions as an oncogenic regulator or tumor suppressor in various human tumors. Bioinformatics analysis revealed that miRNA-20b is related to the tumorigenesis, however, the function of miRNA-20b in colon cancer is still not clear.

Methods

Apoptosis, cell cycle, expression of CCND1/CDK/FOXM1 axis in HCT116 cells were examined by flow cytometry, western blot, and Immunohistochemistry. Wound-healing migration assay and transwell assay were performed to test the migration and invasion of tumor cells. Bioinformatics and microarray analysis was performed for further mechanical research. Subcutaneous xenograft mouse models was to verify the function of miR-20b-5p in vivo.

Results

We found that miRNA-20b-5p inhibited the cell cycle, migration and invasion of CC cells, but had no effect on cell apoptosis. CyclinD1(CCND1) was identified as a direct target of miR-20b-5p. Overexpression of miRNA-20b-5p downregulated CCND1 level in HCT-116 cells. Mechanistically, the inhibiton of cell cycle, migration and invasion of CC cells by miRNA-20b-5p is through regulating the CCND1/CDK4/FOXM1 axis. Additionally, we found that miRNA-20b-5p could inhibit the tumorigenesis in murine CC xenograft models in Balb/c nude mice.

Conclusion

Therefore, our findings demonstrated that miR-20b-5p may serve as a tumor suppressor in CC by negatively regulating CCND1 and that miR-20b-5p may be a potential therapeutic target for the management and treatment of colon cancer.

Background

Colon cancer(CC) accounts for about 10% of the global total diagnosed of cancers each year, and is the second most common cancer in women and the third most common cancer in men[7]. Accordingly, CC remains one of the leading causes of cancer-related death worldwide, and has high mortality rates above 40% in worldwide [9, 34].Thus, exploring the initiation, progression and metastasis of CC is of great importance for the development of effective treatments for patients with CC.

MicroRNAs, a class of endogenous non-coding single-stranded RNAs which contains approximately 19–25 nucleotides in length, can specifically bind to target genes and inhibit gene expression. Due to its extensive role in gene regulation, microRNAs can interact with many cellular networks and signaling pathways so as to play a crucial role in many physiological and pathophysiological processes, such as proliferation, differentiation, apoptosis, and even influence the occurrence and development of inflammation and tumors[29, 5, 24].

Based on the sequence homology and seed conservation, the miR-20b-5p belongs to miR-17 family, which contains six miRNAs: miR-17-5p, miR-20a-5p, miR-106b-5p, miR-20b-5p, miR-93-5p and miR-106a-5p[12]. So far, miR-20b-5p has been reported to be abnormally expressed in many human malignancies and it is also reported to act as a tumor suppressor miRNA in human breast cancer and papillary thyroid carcinoma via regulating the expression of VEGF and MAPK/ERK signaling pathways, respectively[13, 42]. Conversely, miR-20b-5p has been demonstrated to be negatively correlated with the prognosis of gastric cancer and hepatocellular carcinoma[16]. In summary, miR-20b-5p plays a complex role in various tumors through different target genes.

Based on previous database and bioinformatics analysis, we found that all miR-17 family members are highly expressed in gastrointestinal samples in comparison to normal intestinal tissue, except for miR-20b-5p, which is under-expressed in colorectal cancer[18, 33, 45]. In addition, there was a correlation between the expression level of miR-20b-5p and the clinical outcomes; the overall survival and progression-free survival rate of CC patients with high miR-20b-5p levels was significantly higher[39]. However, the underlying mechanisms of miR-20b-5p in regulation of the pathogenesis of CC remain poorly understood.

Therefore, the purpose of our study was to elucidate the biological functions and mechanisms of miR-20b-5p in CCs by using in vitro and in vivo experiments.

Materials And Methods

Cell Culture. The human colon cancer cell line (HCT-116, SW480 and HT29), normal intestinal epithelial cell line (HIEC), 293T cells and 3T3 cells were all obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, P.R. China). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin-streptomycin in cell incubator with 5% CO^2 at 37 °C. The culture medium was changed every other day. MicroRNA mimics or plasmids were transfected using Lipofectamine 2000 according to the manufacturer's instruction.

Lentivirus preparation and stable-transfected cell line. MiR-20b-5p was cloned into the HBLV-ZsGreen-PURO vector (Hanbio biotechnology Co., Ltd, Shanghai, China) and its auxiliary packaging original vector plasmid(pSPAX2 and pMD2G) were prepared. The three plasmid vectors were co-transfected into 293T cells, and replaced with complete medium 6 h after transfection, cultured for 48 and 72 h .Then, collect the cell supernatant rich in lentivirus particles, 4 ° C, 2000 × g, 10 min, remove the cell debris, and then

collect the virus supernatant, using ultra-isolation: 4 ° C, 82700 × g, centrifuge 120 min, and finally a high titer of lentiviral ultra-eluate was obtained. HCT116 cells were used when cell confluence rate reached 50%, virus was added into the culture medium with the polybrene. 48 h later, flow sorting technology was used to sort GFP positive cells and perform cell expansion.

RNA oligonucleotides transfection. MiR-20b-5p-mimics, miR-20b-5p inhibitor (Shanghai Tuoran Biological Technology Co., Ltd.), CCND1 siRNA or nonspecific siRNA (Santa Cruz Biotechnology) were transfected into the colon cancer cells respectively, using Lipofectamine 2000(Invitrogen, USA) according to the manufacturer's protocol. The sequences of the siRNA oligonucleotides were provided in Table 1. At 48 h after transfection, RNA and protein were isolated for the following experiments.

Apoptosis and cell cycle assay. Annexin V/7-AAD Apoptosis Detection Kit from BD was used to detect the apoptosis following the manufacturer's instruction. Anti-Ki67 and Hoechst were stained for cellcycle experiment. All flow cytometric analyses were performed on an LSR II Fortessa cytometer (BD Biosystems), and the data were analyzed using FlowJo software.

Wound-healing migration assay. Cells (1×10^5) were seeded into the culture insert(ibidi, German) in the middle of a 24-well plate Dish. After the cells are full of the insert area, the insert is removed with tweezers, a 500 μm wide scratch can be generated. The plate was incubated at 37° C in serum-free medium. The migration of cells into the wounded area was recorded every 4–6 h and photographed using an inverted microscope (magnification, x20).

Cell transwell assays. Cells (5 × 104) were re-suspended in serum-free medium and seeded at the upper chamber. 500 μl medium with 10% FBS was added to the lower portion of the chamber. After 24 h of incubation, cells were stained with crystal violet staining solution (Beyotime, Nantong, China) then counted and photographed (five views per well). All experiments were performed in triplicate.

Quantitative real-time PCR. RNA samples were isolated using TRIzol Reagent (Invitrogen), according to the manufacturer's procedures. SYBR Green PCR reagents were purchased from Takara, and RT-PCR was performed following the manufacturer's protocol in an ABI 7500 PCR machine (Applied Biosystems). The detailed primer sequences were listed in Table 1.

Western Blotting. Cell lysates were collected in SDS lysis buffer (ddH_2O 37 ml, 10% SDS 10 ml, Ph7.5 Tris-HCL 3 ml). Protein concentration was tested by BCA protein assay according to manufacturers' instruction (Thermo Fisher Scientific, USA). Proteins were loaded on 8–12% SDS-PAGE gel and transferred to PVDF membrane. Primary antibodies used for western blot analysis were CCND1(2978S, Cell Signaling Technology), CCND3(2936, Cell Signaling Technology), CDK4(12790S, Cell Signaling Technology),CDK6(3136S, Cell Signaling Technology), FOXM1(20459, Cell Signaling Technology), p-FOXM1(14655, Cell Signaling Technology) and beta-actin (AC-40)(Sigma-Aldrich, USA). Secondary antibodies used were HRP-linked goat anti-rabbit/mouse IgG (Cell Signaling technology, MA). Immune complexes were visualized with ECL Western Blotting Detection Reagents (Merck Millipore, USA) and captured with an image Reader (Amersham Imager 600, GE healthcare Life Sciences).

Dual-luciferase reporter system analysis. The 3'UTR sequence of CCND1 weas subcloned into the pSicheck2-control vector (Promega) to construct pSicheck2-CCND1-WT. QuickChange sitedirected mutagenesis kit (TOYOBO) was used to construct the specifically mutated CCND1 gene 3'UTR fragment, which named vectors, pSicheck2-CCND1-Mut1, pSicheck2-CCND1 -Mut2, pSicheck2-CCND1-Mut1 + 2, respectively. Co-transfect the vectors, miR-20b-5p mimics and control to 293T cells by using Lipofectamine 2000 (Invitrogen), harvest the cells in 48 h and analyzed for luciferase activity using Dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Transfection was repeated three times in triplicate.

Subcutaneous xenograft mouse models. HCT116 which stably transfected with miR-20b-5p expression vector or control vector (1 × 107 cells/0.2 mL PBS) were injected subcutaneously into the left and right flanks of 6-week-old female Balb/c nude mice (n = 6/group), respectively. Tumor size was measured every 2 days for 4 weeks using a digital caliper. Tumor volume (mm3) was estimated by measuring the longest(L) and shortest(W) diameters of the tumor and use the following formula to calculate (tumor volume = $L*W^2/2$). At 4 weeks after implantation, the mice were sacrificed and the weights of their xenograft tumors were determined. The excised tissues were fixed in 10% neutral-buffered formalin for further investigation.

Immunohistochemistry. The excised tumor tissues were fixed in 10% neutral-buffered formalin, and then 4% paraformaldehyde-fixed paraffin-embedded tumor were stained with CCND1(2978S, Cell Signaling Technology) antibody at a concentration of 1:100.

Microarray analysis. HCT-116 cells were transfected with miR-20b-5p-NC and miR-20b-5p-mimics according to the manufacture of lipofectamine 2000. 48 h later, total RNA was extracted using TRIzol Reagent (15596-018; Life Technologies), following the manufacturer's instructions. Array hybridization (901229) and scan (00-00212; both from Affymetrix) were performed according to the manufacturer's instructions. Raw data were normalized using MAS 5.0 algorithm, Gene Spring Software 11.0 (Agilent Technologies, Santa Clara, CA).Gene function classification and KEGG analysis were performed.

Bioinformatics prediction. The method we used to predict the related potentially target CCND1 was according to the prediction of the bioinformatical software online including Target Scan (www.targetscan.org) and miRDB(www.mirdb.org).

Statistical Analysis. The results are expressed as the mean ± SEM. Statistics were performed using GraphPad Prism 8.0 software. Unpaired Student's t tests (only two groups), One-Way ANOVA (more than two groups) and Two-Way ANOVA (for tumor growth analysis) were used to calculate P values where appropriate. $P < 0.05$ was considered statistically significant. Error bars indicate the standard deviation in all the Figures.

Table 1
Primer sequences used in the present study.

miR-20b-5p-5p forward	**5’- ACACTCCAGCTGGGCAAAGTGCTCATAGT – 3’**
miR-20b-5p-5p reverse	5’- TGGTGTCGTGGAGTCG – 3’
U6 forward	5’-CTCGCTTCGGCAGCACA-3'
U6 reverse	5’-AACGCTTCACGAATTTGCGT-3’
CCND1 forward	5’-AGTTCATTTC- CAATCCGCCC-3’
CCND1 reverse	5’-TTTCCGTGGCACTAG- GTGTC-3’
E2F1 forward	5’-CAAGAAGTCCAAGAACCACATCC-3’
E2F1 reverse	5’-AGATATTCATCAGGTGGTCCAGC-3’
CDK4 forward	5’- GCCCTCAAGAGTGTGAGAGTC-3’
CDK4 reverse	5’- CACGAACTGTGCTGATGGGA-3’
CDK6 forward	5’-GGACTTTCTTCATTCACACCG – 3’
CDK6 reverse	5’- GACCACTGAGGTTAGGCCA-3’
GAPDH forward	5’- ACCTGACCTGCCGTCTAGAA – 3’
GAPDH reverse	5’- TCCACCACCCTGTTGCTGTA-3’

Results

miR-20b-5p was down-regulated in colon cancer. MiR20b is encoded by the miR-106a-363 cluster which located on human chromosome X and belongs to the miR-17 family, together with the miR-17-92 cluster and the miR-106b-25 cluster[19]. Through The Cancer Genome Atlas (TCGA) data and bioinformatics analysis, we identified that only one miR-17 family member, miR-20b-5p, was significantly decreased in gastrointestinal tumor samples in comparison to normal intestinal tissue, especially in colon cancer. The level of any other member (miR-17-5p, miR-20a-5p, miR-106b-5p, miR-93-5p and miR-106a-5p) was higher (Fig. 1A)[45]. The results of Real time-PCR showed that compared with normal intestinal epithelial cells, the level of miR-20b-5p was lower in colon cancer cell lines, especially in HCT116 cells (Fig. 1B). To study the role of miR-20b-5p in colon cancer, we used colon cancer cell line HCT116 to construct a stable transgenic cell line that overexpressed miR-20b-5p. The cell transfection efficiency was as high as 99% (Fig. 1C).

MiR-20b-5p inhibits proliferation, migration, and invasion of CC cells in vitro. In order to identify whether miR-20b-5p affected the survival of HCT116 cells, apoptosis and cell cycle experiments were performed. Our data showed that, miR-20b-5p had no impact on apoptosis(Fig. 2A–B). However, we found that the percentage of cells in the G1 phase was increased in the miR-20b-5p overexpressed group compared with

that in the control group, and the S phase percentage was significantly decreased (Fig. 2C-D). Overall, these findings suggest that miR-20b-5p inhibits the proliferation of HCT116 cell line by blocking cell cycle progression at the G1/S transition, but not influences cell apoptosis.

Next, we sought to further determine whether miR-20b-5p has any other impact on migration and invasion of colon cancer cell, we conducted the wound-healing migration assay and transwell assay. As shown in Fig. 2E-2F, we found that the width of the wound was significantly broader in miR-20b-5p overexpressed CC cells compared with that in control group. Furthermore, the results of transwell assay indicate that over-expression of miR-20b-5p inhibits the invasiveness of HCT-116 cell. Taken together, these data indicate that over-expression of miR-20b-5p can inhibit cell cycle, migration, and invasion, but not apoptosis of CC cells.

MiR-20b-5p suppresses tumor growth in vivo. To further investigate the effect of miR-20b-5p on tumor growth in vivo, we injected miR-20b-5p-overexpressed or control HCT-116 cells into the left flank of mice (n = 6–8) and tracked their growth. Our data showed that, the tumors from miR-20b-5p-overexpressed cells grow slower than xenografts derived from control cells (Fig. 3A); The average weight and volume of the miR-20b-5p-overexpressed tumors were significantly lower than those of control tumors (Fig. 3B and 3C). These results indicate that miR-20b-5p plays a role in inhibiting the growth of colon cancer in vivo.

MiR-20b-5p significantly affects the cell cycle pathway of colon cancer cells. In order to further explore the mechanism by which miR-20b-5p suppresses the growth of colon cancer cells, We transfected miR-20b-5p mimics into HCT-116 cells, extracted RNA after 48 hours, verified the expression of miR-20b-5p by RT-PCR, and performed microarray analysis. Array analysis showed that, according to foldchange > 1.5 times, a total of 971 genes were up-regulated and 116 genes were down-regulated after overexpression of miR-20b-5p(Figure 4A). GO analysis found that, after overexpression of miR-2b-5p, numerous of cell biological functions, cell components, and molecular functions are affected, details were shown in Fig. 4B. We further performed KEGG analysis and found that overexpression of miR-20b-5p significantly affects the cell cycle, MAPK signaling pathway, p53 signaling pathway, and VEGF signaling pathway ,which play an important role in the pathogenesis and metastasis of colon cancer, especially the cell cycle pathway(Fig. 4C). This is consistent with our previous in vitro research results.

CCND1 is a direct target gene of miR-20b-5p. To further identify the target genes of miR-20b-5p, we first check the expression level of genes that are closely related to the cell cycle G1 / S transition by RT-PCR. Our data showed that the mRNA level of CCND1 was decreased significantly in miR-20b-5p-overexpressed group(Fig. 5A). Western blot and immunohistochemistry further confirmed that CCND1 protein levels are also significantly down-regulated in miR-20b-5p-overexpressed cells (Fig. 5B-C). Then we used TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org /microrna /home.do) to confirm whether CCND1 was one of putative targets of miR-20b-5p. We found that in the 3'UTR of CCND1, there were two miR-20b-5p binding sites. Next, luciferase report assays were performed to verify the direct inhibitory effect of miR-20b-5p on CCND1. MiR-20b-5p over-expression significantly suppressed the luciferase activity of constructs containing the wild-type 3'-UTR-CCND1 (3'-UTR WT), while

this effect was largely compromised when the miR-20b-5p binding sites in the 3'UTR of CCND1 were mutated (3'-UTR MUT1 and 3'-UTR MUT2, Fig. 5D-E). Based on the above results, we confirmed that CCND1 is a direct target gene of miR-20b-5p.

MiR-20b-5p regulates the CCND1/CDK4/FOXM1 axis to influence the cell cycle, migration and invasion of CC cells. CCND1 has been reported to be overexpressed in so many solid tumors, and is recognized as a driver oncogene of solid tumors[28]. In our study, we confirmed that CCND1 has a higher expression in colon cancer through the TCGA dataset analysis(Fig. 6A). In addition, higher CCND1-expressed patients have shorter survival compared to low CCND1-expressed group, although there is no statistical difference (P = 0.0661)(Fig. 6B). CCND1 always associates with the cyclin-dependent kinases (CDKs)CDK4 or CDK6 to control the tumor progression through the G1 phase, the G1-to-S phase transition, so as to control cell cycle progression. Our data showed that CCND1 retained the ability to interact with downstream protein partners and to activate CDK-kinase activity. In HCT116 cells which are overexpressed with miR-20b-5p, CDK4 expression is consistent with CCND1 expression level, but not CDK6(Fig. 6C). To further explore the molecular mechanism, we transfected the pCMV-CCND1 or empty vector to HCT116 cells, which is stably overexpressed with miR-20b-5p or vector. We found that miR-20b-5p reduces level of CCND1, and ectopic expression of CCND1 can partically restore the CCND1 expression, followed with the same tendency of FOXM1 phosphorylation,but has no effect on total FOXM1 protein level(Fig. 6D). Inversely, HCT116 cells were treated with miR-20b-5p inhibitor (antisense RNA) in conjunction with knockdown of CCND1. CDK4 and FOXM1 phosphorylation levels are positively correllated with the changes of CCND1.Therefore, we have reasons to speculate that miR-20b-5p regulates the CCND1 / CDK4 / FOXM1 axis, leading to cell cycle arrest, inhibition of cell migration and invasion, thereby preventing tumor from progression.

Discussion

In the past decades, robust evidences were provided to demonstrate that miRNAs can be upregulated or downregulated in different tumour tissues. Furthermore, miRNAs were testified to be an oncogenic miRNAs by inhibiting the expression of tumoursuppressor genes, or as tumour-suppressive miRNAs by inhibiting oncogene expression. In our study, we uncover that miR-20b-5p plays an important role in regulation the cell cycle, migration, invation of CC cells in vitro, and inhibit the tumor growth of CC in vivo. We establish a defined role of miR-20b-5p as a tumor suppressor miRNA in colon cancer.

MiRNA-20b belongs to the miR-106a-363 cluster, which is loctaed on the X chromosome, together with miR-17-92 and miR-106b-25 clusters, form a large family called the miR-17 family[19, 37]. It is well known that the miR-17 family, as an oncomiRs, is involved in the pathogenesis of a variety of solid tumors,such as prostate cancer, breast cancer and so on[8, 6, 25]. However, the role of miR-20b-5p in the pathogenesis of cancers is controversial. MiR-20b-5p has a tumor suppressive role in gastric cancer(GC) through regulation of the PI3K / AKT / mTOR signaling pathway[36]. Also, miR-20b-5p suppressed TGF-β1-induced migration and invasion of prostate cancer by up-regulating E-cadherin and down-regulating vimentin, leading to TGF-β1-induced inhibition of epithelial-to-mesenchymal transition (EMT) [31]. In spite of that,

miR-20b-5p plays important roles in tumorigenesis of esophageal cancer via regulation of PTEN expression[40], and also functions as an oncogene in breast cancer via targeting EGR1[17]. There is limited data on expression of this miRNA in colon cancer. The study performed by Xie JianGuo et al. found that miR-20b-5p reduces 5-FU resistance to induce apoptosis in vitro by suppressing ADAM9 / EGFR in CC cells[10]. And very few data showed that miR-20b-5p is under-expressed in colon cancer[33], and the expression level of miR-20b-5p is negatively correlated with long progression-free survival (PFS) and overall survival (OS) [39]. But its underlying mechanism is not clear. Our study demonstrated that overexpression of miR-20b-5p prevents cell proliferation, cell migration and tumor growth in HCT-116 tumor xenograft experiments. We confirmed the anti-tumor effects of miR-20b-5p in HCT116 cells in vitro and in vivo, supporting the tumor suppressor role of this miRNA.

Microarray results show that overexpression of miR-20b-5p can affect a variety of signaling pathways, including some that are very important for the development of colon cancer, such as MAPK signaling pathway, p53 signaling pathway, and VEGF signaling pathway, but the most obvious one is the cell cycle signaling pathway. Our investigation has also identified CCND1, which is critical for the cell cycle progression, as a target of miR-20b-5p in colon cancer, specifically. This is in agreement with our microarray analysis. Although previous studies have shown that miR-20b-5p can regulate the cell cycle in bladder cancer, there is no direct experimental evidence[30]. For the first time, we have demonstrated that CCND1 is a target gene of miR-20b-5p in colon cancer.

CCND1 is an important regulator of the G0/G1 transition in the cell cycle, and is often abnormally expressed in cancer and is a biomarker for cancer phenotype and disease progression[38]. CCND1 is an established human oncogene that is commonly overexpressed in tumor tissues, especially in lung cancer, melanoma and oral squamous cell carcinoma[32]. In addition, CCND1 regulates many of the key processes involved in cancer development and maintenance, including the DNA damage response [14], migration[27, 20, 11]. From TCGA dataset, we found that CCND1 was also elevated in colon cancer. However, CCND1 expression has no correlation with tumor stages. The data showed that the higher CCND1 level, the shorter survival time, although it is not statistically significant. However, we cannot underestimated the importance of CCND1 in the development of colon cancer. The most-well-known function of CCND1 is to activate the cyclin-dependent kinases (CDKs) CDK4 and CDK6, so as to promote cell proliferation[26]. The CCND1/CDK4/6 complex can phosphorylate some transcription factors that activate or inhibit the expression of genes needed for cell cycle progression. Besides this, the migration of epithelial cells, and macrophages was reduced under the circumstances of absence of CCND1[27, 21]. In our study, we confirmed that miR-20b-5p decreased the expression of CCND1, and also affected the activation of CDK4, but not CDK6.

Forkhead Box M1 (FOXM1) is critically required for the cell cycle transition from G1 to S phase, once stabilized by phosphorylation, FOXM1 maintains G1/S phase expression, suppresses the levels of reactive oxygen species (ROS), and protects cancer cells from senescence, so as to promote tumor progression [41], and plays a critical role in the process of tumorigenesis, as observed in several cancer

types[15, 23]. In addition, FOXM1 also plays a critical role in the metastasis of prostate cancer[22], breast cancer[43] and colon cancer through reversal of epithelial-to-mesenchymal transformation[44] or maintenance of mitochondrial function [35]. Previous investigation confirmed that in melanoma cells, FOXM1 acts as a common critical phosphorylation substrate of CCND1-CDK4/6 complexes [2]. Our study demonstrates that in colon cancer, CCND1/CDK4 maybe affect the tumorigenesis of colon cancer by phosphorylating FOXM1. However, further research is needed to explore the specific mechanism.

In conclusion, this report is the first to elucidate that miR-20b-5p acts as a tumor suppressor miRNA in the pathogenesis of CC via negatively regulating the CCND1/CDK4/FOXM1 axis prominently. Although our work focuses on miR-20b-5p, other miRNAs are also predicted to regulate CCND1, such as miR-15a and miR-16, whose expression are inversely correlated with CCND1 expression in prostate and lung cancers[3, 4]. Further work may elucidate how much other miRNAs contribute to CCND1 regulation. However, more work still needs to be done to establish whether miR-20b-5p/CCND1 axis can be effectively targeted, and if so, this will be more useful therapeutically.

Conclusion

Our data uncover that miR-20b-5p inhibited the cell cycle, migration, invation of colon cancer (CC) cells in vitro, and inhibit the tumor growth of CC in vivo for the first time. Mechanistically, CCND1/CDK4/FOXM1 axis was regulated by miRNA-20b-5p.Taken together, our findings highlight that miRNA-20b-5p plays a critical role in the progression and metastasis of colon cancer, nd that miR-20b-5p may be a potential therapeutic target for the management and treatment of colon cancer.

Abbreviations

CC: colon cancer; miR：microRNA; CCND1: CyclinD1; TCGA:The Cancer Genome Atlas; FOXM1:Forkhead Box M1; ROS :reactive oxygen species; CDKs :cyclin-dependent kinases; PFS: progression-free survival; OS:overall survival; EMT :epithelial-to-mesenchymal transition; GC: gastric cancer.

Declarations

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Competing interests: The authors declare that they have no competing interests.

Authors' contributions: Hui Yang and Jian Lin designed and performed the experiments, and Hui Yang was a major contributor in writing the manuscript, data collection and data analysis . Jinling Jiang, Jun Ji and Chao Wang provided assistance in experiment material. Jun Zhang provided help in designing the study. All authors read and approved the final manuscript.

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Figures

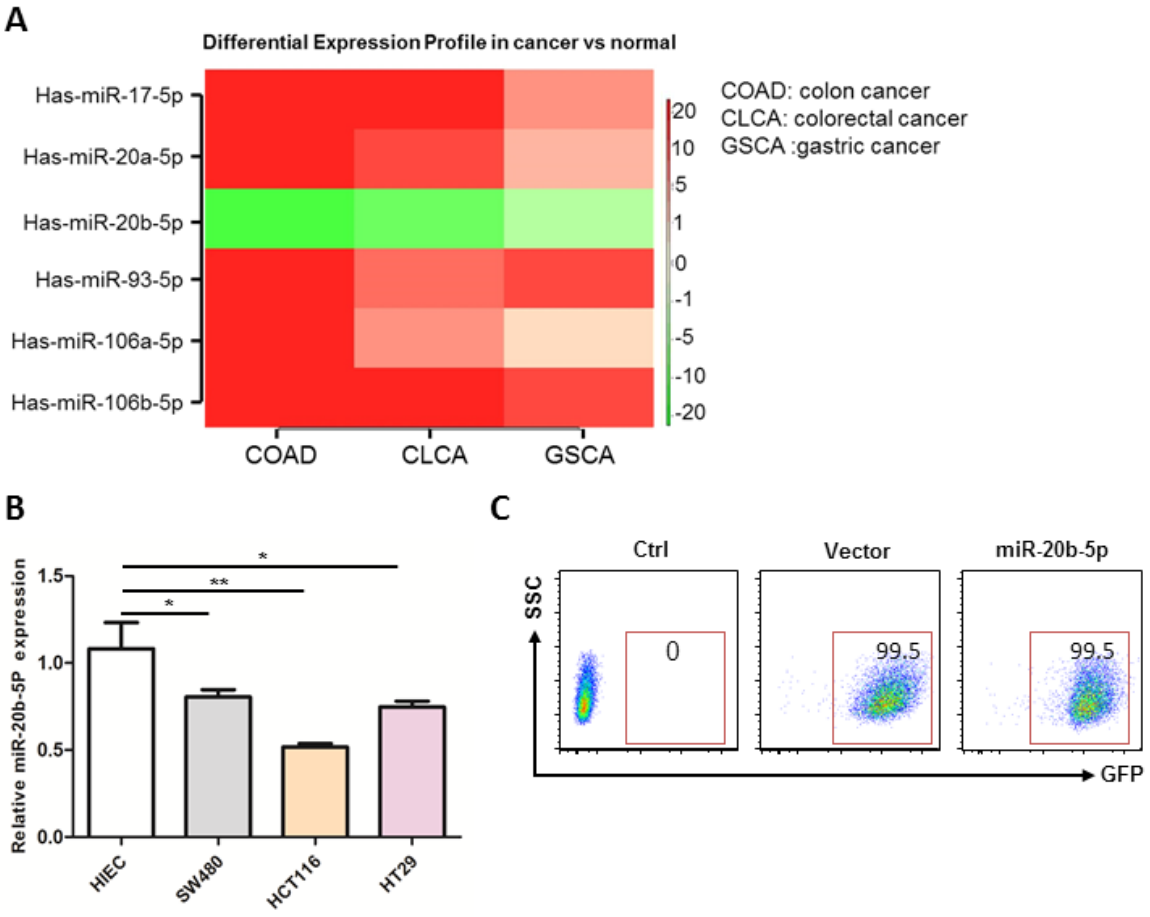

Figure 1

The expression of miR-20b-5p in colon cancer was decreased. (A)The heatmap of expression of miR-17 family in gastrointestinal carcinoma.(B)The relative expression of miR-20b-5p in normal intestinal epithelial cells and colon cancer cell lines. (C) The representative flow cytometric analysis of transfected efficiency of lenti-virus in HCT-116 cell line. Data are expressed as mean ± s.e.m. *P ≤ 0.05, **P ≤ 0.01.

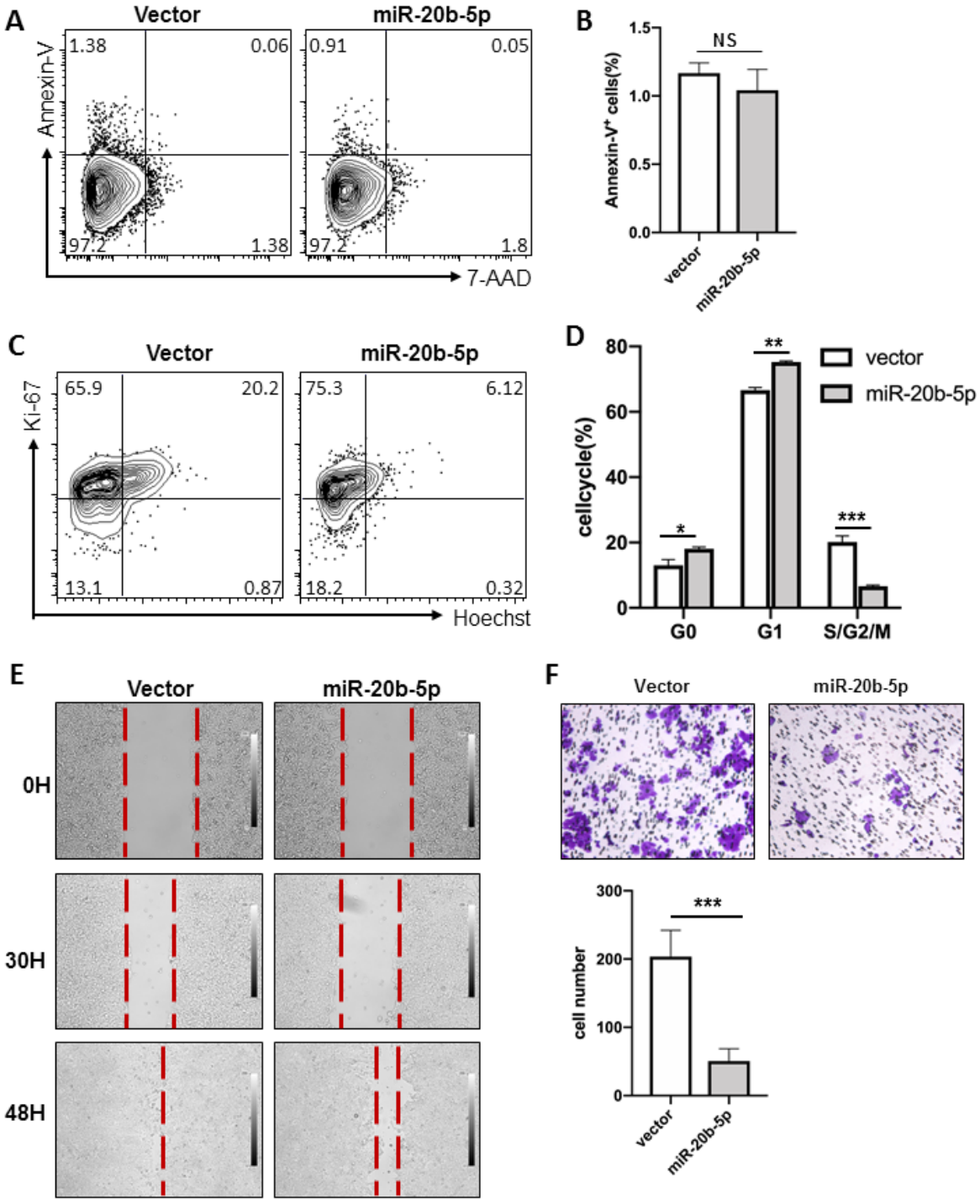

Figure 2

miR-20b-5p inhibits the cell cycle, migration and invasion, but does not affect the apoptosis of colon cancer cell. HCT-116 cells which stably transfected with miR-20b-5p expression vector or control vector were used for following experimants. (A-B)Cell viability was tested by using Annexin V/7-AAD staining. Representative flow cytometric analysis of apoptosis(A) and statistical histogram was shown right(B). (C-D)Ki-67/Hoechst were double stained and flow cytometric assay was used to analysis the cell

proliferation. Results of flow cytometric analysis(C) and percentages of G0/G1/G2-S-M were shown(D). (E-F)Wound-healing migration assay(E) and cell transwell assay(F) were performed to testify the effect of miR-20b-5p in migration and invasion in vitro. HCT116 cells expressing miR-20b-5p or negative control were plated. Representative microscopy images (x20) were shown. Data are presented as the mean±SE from three independent experiments performed in triplicate. Data are expressed as mean ± s.e.m. *P ≤ 0.05, **P ≤ 0.01.

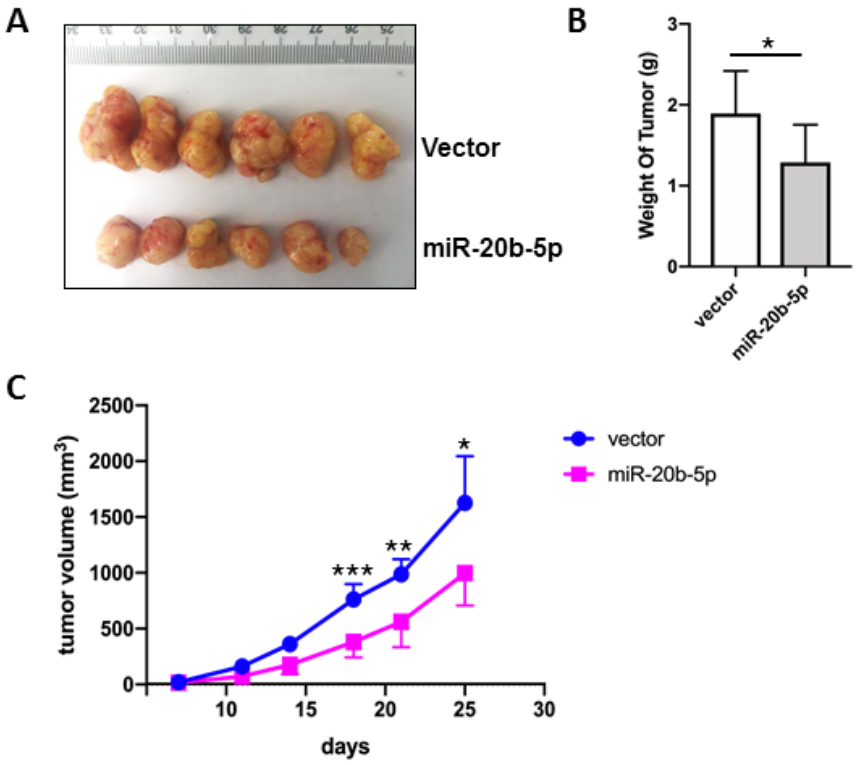

Figure 3

MiR-20b-5p decreases tumor volume in mice xenografts. 1 × 107 HCT-116 cells stably transfected with miR-20b-5p expression vector or control vector were subcutaneously injected into two flanks of 6 wk female Balb/c nude mice(n=6), and tumor volume was measured during the course of the experiment. (A) Gross images of tumor size.(B) Weight of tumors excised from 6 mice in each group. (C) The tumor volumes in the HCT116- HBLV-ZsGreen-miR-20b-5p group were lower than those in the HCT116-HBLV-ZsGreen-empty group. Data are expressed as mean ± s.e.m. *P ≤ 0.05, **P ≤ 0.01.

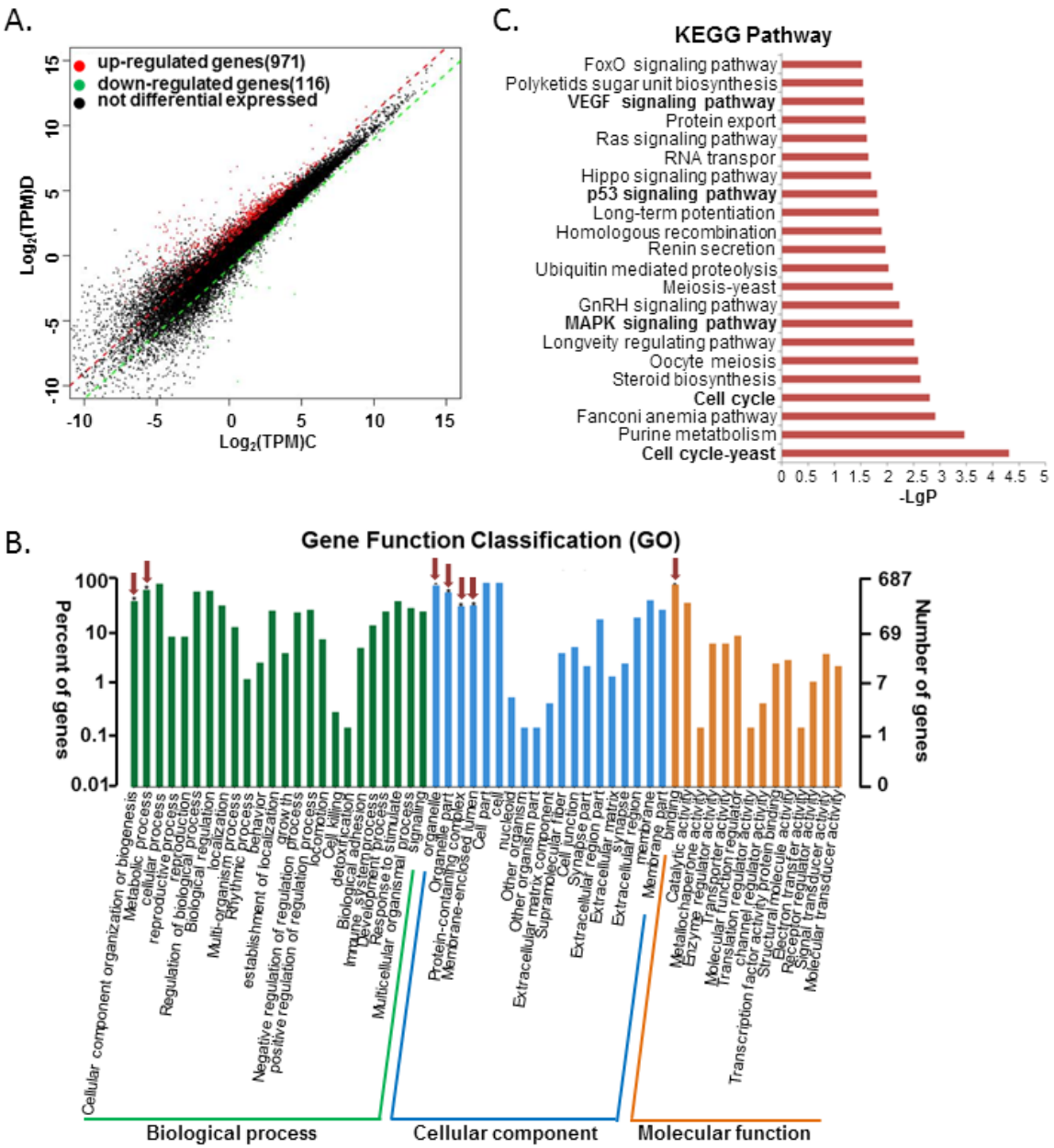

Figure 4

MiR-20b-5p affects multiple signaling pathways related to colon cancer, particularly the cell cycle pathway. HCT-116 cell line transfected with miR-20b-5p-mimics or negative control, RNA was extracted for microarray analysis. (A) Scatter plot comparing global gene-expression profiles of miR-20b-5p overexpressed or control group. Transcripts with a log2 (fold change) > 1.5 and adjusted P value < 0.01 are shown in red (increased expression, 971 genes) or black (decreased expression, 116 genes). (B) GO analyses of the RNAseq data as indicated. (C) Histogram shows the KEGG analysis for the signaling pathways that were significantly different between miR-20b-5p overexpressed and control group.

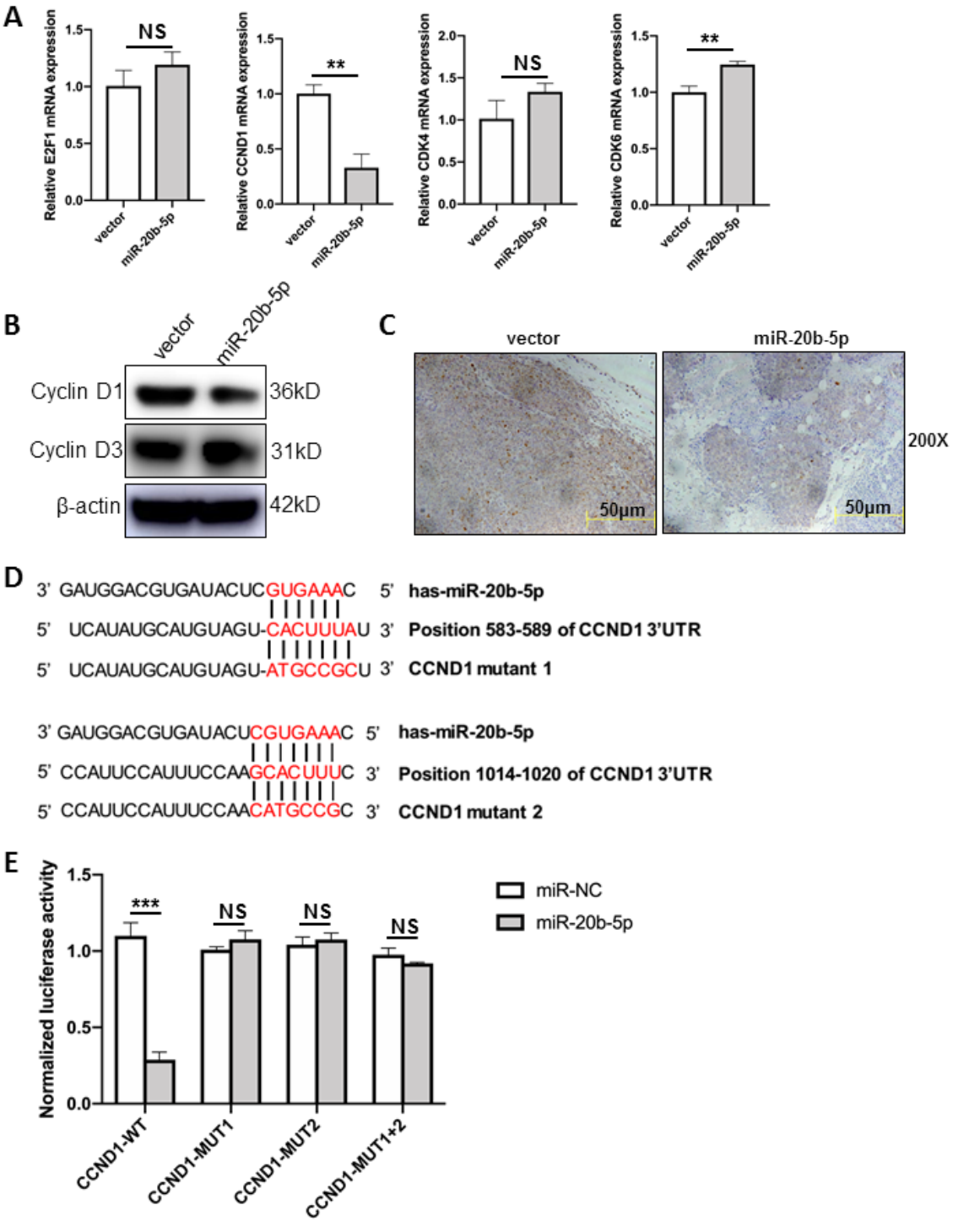

Figure 5

CCND1 is a direct target of miR-20b-5p. (A) The HCT116 cells were transfected with miR-20b-5p-mimic or mimic-con (final concentration, 50 nM). After 48 h, the mRNA level of E2F1,CCND1,CDK4 and CDK6 were determined by RT-PCR and normalized GAPDH. (B) CCND1 protein level was determined by western blot and normalized β-actin. (C) Immunohistochemical results showed that CCND1 expression was significantly reduced in miR-20b-5p overexpressing tumor tissues. (D) Schematic representation of miR-20b-5p binding to CCND1 3'UTR and respective mutagenesis performed to disrupt miR-20b-5p binding sites. (E) 293T cells were cotransfected with CCND1-3'UTR-WT, CCND1-3'UTR-mut1, CCND1-3'UTR-mut2, and CCND1-3'UTR-mut 1+2, together with miR-20b-5p-mimic or mimic-con. After 48 h, luciferase activity was determined and normalized to Renilla luciferase activity. Data are presented as the mean±SE from three independent experiments performed in triplicate. Data are expressed as mean ± s.e.m. *$P \leq 0.05$, **$P \leq 0.01$.

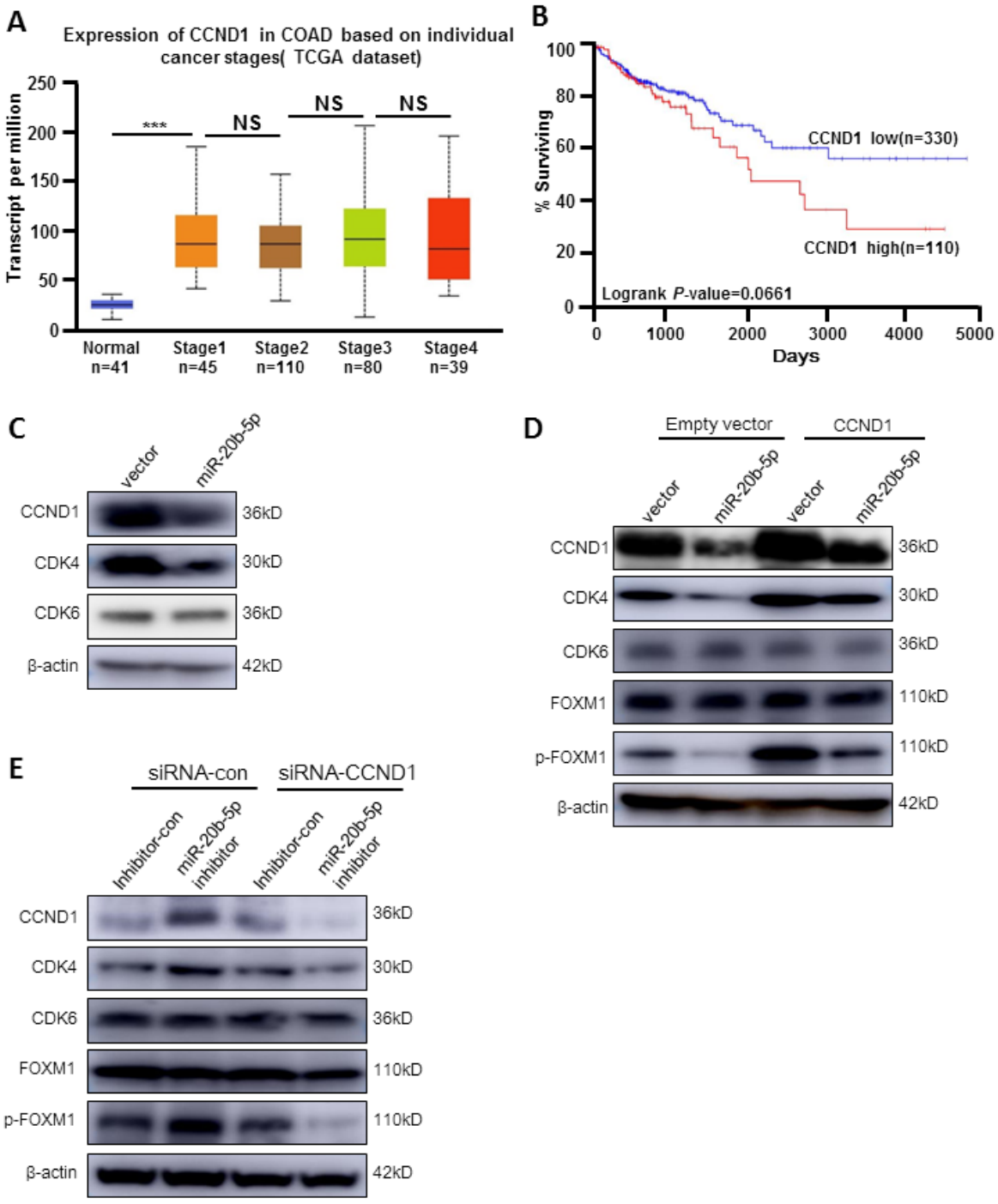

Figure 6

miR-20b-5p is a negative regulator of CCND1/CDK4/FOXM1 signaling pathway. (A) TCGA data shown the expression of CCND1 in individual colon cancer stages(http://ualcan.path.uab.edu/index.html). (B) Kaplan–Meier estimates of survival based on CCND1 expression level in colon cancer in The Cancer Genome Atlas. High levels of CCND1 in colon cancer has worse survival than CCND1-low-expression group (Cutoff High 25% vs Cutoff low 75%, P=0.0661). Data was analyzed using OncoLnc

(www.oncolnc.org)[1]. (C) Protein Expression of CCND1, CDK4, CDK6 and β-actin were detected by western blot in miR-20b-5p ectopic expressed cells or control group. (D) Empty vector or pCMV-CCND1 were transfected into stably expressed miR-20b-5p/NC-HCT116 cells respectively for 48h. The expression of CCND1, CDK4, CDK6, FOXM1, p-FOXM1 and β-actin were detected by western blot. (E) HCT116 cells were co-transfected with siRNA-CCND1/siRNA-con and miR-20b inhibitor/inhibitor control respectively, protein was collected 48 hours later. Representative western blot showed the levels of CCND1, CDK4, CDK6, FOXM1, p-FOXM1 and β-actin.