LncRNA CARMN m6A Demethylation by ALKBH5 Inhibits Mutant p53-Driven Tumor Progression through miR-5683/FGF2

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Article

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Abstract

N-methyladenosine (m6A) is the abundant RNA modification in colorectal cancer. However, the biological significance of m6A methylation of LncRNA CARMN remains unknown in colorectal cancer, especially for mutant p53 Gain-of-function. Here, we found that CARMN reduced in the colorectal cancer patients with mutant p53, due to its rich m6A methylation, which promotes cancer proliferation, invasion, and metastasis \textit{in vitro} and \textit{in vivo}. Deeper investigation illustrates that ALKBH5 directly demethylated m6A level of CARMN at 477 sites, which maintains CARMN with a higher expression level. However, mutant p53 binds to the promoter of ALKBH5 to prevent its transcription, results in the high level m6A methylation of CARMN, subsequently read by YTHDF2/YTHDF3 and degraded. Meantime, overexpressing CARMN significantly suppressed colorectal cancer progression \textit{in vitro} and \textit{in vivo}. In addition, miR-5683 was identified as a direct downstream target of LncRNA CARMN, which plays anti-tumor effect through cooperating with CARMN to down-regulate FGF2 expression. Our study revealed the regulator and functional mechanism of CARMN in colorectal cancer with mutant p53, which may highlight a demethylation-based approach for the diagnosis and therapy of cancer.

INTRODUCTION

Current research indicates that approximately 90% of the human genome is translated into RNA, but less than 2% of it contains protein-coding genes [1, 2]. Despite being characterized as “junk DNA”, more than 80% of human and mouse genomes are translated into different kinds of RNA, such as LncRNAs (Long noncoding RNAs), miRNAs, siRNAs, and piRNAs [3]. LncRNAs are transcripts with more than 200 nucleotides but no obvious protein-coding capacity, which have been shown to be crucial at the transcriptional, translational, and post-translational levels in a variety of physiologic and pathologic conditions [4, 5]. It is clear that lncRNAs lack large open reading frames yet perform critical physiological functions as RNA molecules. The activity of lncRNAs is determined by the complicated interaction between RNA binding proteins and microRNAs mediated pathways in cancer. Furthermore, more and more lncRNAs are being found and described at a rapid pace in the human genome [6].

CARMN (cardiac mesoderm enhancer-associated noncoding RNA) has been identified as a highly abundant and conserved specific lncRNA in smooth muscle cells. Recently, CARMN was shown to be a host lncRNA for the MIR143/145 cluster, with functions in cardiac differentiation, smooth muscle cell differentiation, and phenotypic regulation [7, 8, 9, 10, 11, 12, 13, 14]. The early loss of CARMN has been identified as a crucial event that primers VSMCs (Vascular smooth muscle cells) towards a pro-atherogenic phenotype \textit{in vitro} and accelerates atherosclerosis progression \textit{in vivo} [15]. A novel therapeutic target for atherosclerosis beyond lipid reduction or anti-inflammatory interventions is provided by silencing CARMN, which inhibits VSMCs (Vascular smooth muscle cells) proliferation in atherosclerotic plaques [16]. However, there have been few studies of lncRNAs controlling mutant p53-induced colorectal cancer.
Recently, studies have implicated the significant roles of epigenetic alteration in the formation and progression of tumors. N6-ethyladenosine (m6A) is the most common epitranscriptome alteration in eukaryotic cells, which was initially discovered in the 1970s [17, 18, 19]. The alterations of m6A are the result of a dynamic and reversible process initiated by the m6A methyltransferase complex (MTC), which includes METTL3(methyltransferase-like 3), METTL14, WTAP (Wilms tumor 1-associated protein), and is demethylated by ALKBH5(AlkB homolog H5) and FTO (fat mass and obesity-associated protein) [20, 21, 22, 23, 24]. ALKBH5 promotes tumor growth by keeping the stemness of breast cancer cells, which as the major demethylase of m6A alteration [25].

The p53 mutation, cytogenetic abnormalities, and decreased overall survival and event-free survival in acute leukaemia are associated with the deletion of ALKBH5. The studies revealed that the role of ALKBH5 was conflicting in cancer [26, 27]. The stimulation of p53-induced ALKBH5 transcription regulated the m6A alterations in pancreatic cancer [27]. ALKBH5 was expressed less accompanied with knocked down or inhibited p53 transcriptional activity in CSCs (non-small-cell lung cancer-derived cancer stem-like cells) [28]. However, the features of ALKBH5-dependent m6A alteration and its pathogenic function remain unknown in mutant p53-induced colorectal cancer.

LINRIS (Long Intergenic Noncoding RNA for IGF2BP2 Stability) knockdown resulted in lower levels of IGF2BP2(insulin-like growth factor 2 mRNA-binding protein 2) in CRC cells, with MYC-mediated glycolysis [29]. The METTL3-mediated m6A medication of THAP7-AS1 increased its expression by the IGF2BP1-dependent pathway [30]. FTO demethylates LncRNA LINC00022 at the m6A position, which stimulates the formation of ESCC (esophageal squamous cell carcinoma) tumors in vivo [31]. The m6A level of XIST was significantly reduced and XIST expression was increased when METTL14 was knocked down [32]. The N6-methyladenosine modification mediated by METTL3 resulted in LINC00958 upregulation by stabilizing its RNA transcript [33]. The function of CARMN could suppress bladder cancer proliferation by the miR-1275/AXIN2/Wnt/β-catenin pathway [34]. CARMN could enhance cancer cell death by sponging miR-125a to upregulate p53 in endometrial carcinoma [35]. It is necessary to explore how CARMN regulates colorectal cancer with mutant p53 development.

The TP53 mutation takes on a critical role as a target of genomic instability, which is linked to accelerated tumor growth and a low patient survival rate. LncRNAs have critical functions in colorectal cancer maintenance. However, it is uncertain whether mutant p53-regulated LncRNAs are involved in colorectal cancer. This study identified LncRNA CARMN controlled by p53R273H that are specific in colorectal cancer. Over-expressing CARMN inhibits the proliferation of tumor with mutant p53-induced colorectal cancer. Besides, m6A is an enzyme involved in LncRNAs stability. It is uncertain what role it plays in TP53-mutant colorectal cancer. The m6A demethylase ALKBH5 is low expressed in mutant p53 induced colorectal cancer. It was demonstrated that mutant p53 binds to the promoter of ALKBH5 to inhibit its transcription, which lowers the level of its protein production. CARMN was found and described as a major regulator of colorectal cancer by directly demethylated with the ALKBH5, which was a suppressive modulator of colorectal cancer proliferation and differentiation. Additionally, YTHDF2 and YTHDF3 were shown to be downregulated in response to CARMN overexpressed. CARMN and ALKBH5
promoted colorectal cancer patients’ tumorigenesis via the p53/ALKBH5/CARMN/miR-5683 pathway. These findings could shed light on the functions of m6A methylation in colorectal cancer patients with p53R273H mutation.

**MATERIALS AND METHODS**

**Cell lines and tissue treatment**

Human colorectal cancer cell lines (HCT116, sw480, sw620, HT29) were purchased from ATCC. Cells were incubated with DMEM modified medium (Gibco), which contains 10% FBS in the 37°C incubator with 5% CO2 atmosphere (Thermo Fisher Scientific).

**Data extraction and analysis**

Data of RNA sequencing (RNA-Seq), miRNA sequencing (miRNA-Seq), masked somatic mutation, and clinical information of colon patients and rectum patients were retrieved from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). A total of 449 individuals with colon and 94 with rectum were included in the current study, which respectively had 212 mutant TP53 patients, 237 wild-type patients in the colon and 68 mutant TP53 patients, 26 wild-type patients in the rectum. Level 4 reverse-phase protein array data of patients were obtained from The Cancer Proteome Atlas (TCPA) (https://www.tcpaportal.org/). DeSeq2 and EdgeR R packages were used to analyze and process the expression data of LncRNAs, miRNAs, and mRNAs expression. Volcano plots were visualized by the ggpur and ggthemes packages in R software, and survival curve plots were made by the survival and survminer packages in R software.

DemiRNAs targeted with DeLncRNAs were searched from the LncBase v3 online tool (https://diana.e-ce.uth.gr/Lncbasev3/), and the potential mRNAs targeted by DEMiRNAs were predicted by the miRWalk (http://mirwalk.umm.uni-heidelberg.de/). Protein-protein interaction (PPI) networks between the DemRNAs were constructed by the online tool STRING (http://string-db.org/). The Cytoscape (version 3.8.2) was used to calculate the core value of DemRNAs in PPI networks and visualize the LncRNA–miRNA–mRNA network.

**Structure determination**

To study the structure of CARMN, the website (http://rna.tbi.univie.ac.at/) of IncRNAs of secondary structure prediction was predicted.

**Western blotting**

RIPA lysis buffer (Beyotime Institute of Biotechnology) combined with proteinase and phosphatase inhibitors was used to lyse the collected cells on ice for 30 minutes (Selleck). A bicinchoninic acid (BCA) protein test kit was used to determine the protein content (Thermo Fischer Scientific). The proteins from each sample were then transferred to PVDF membranes and blocked for 1 hour at room temperature with 5% no-fat milk. The PVDF membranes were incubated in the antibody solution overnight at 4°C. The
membranes were then rinsed three times for 10 minutes with PBST. This was followed by a one-hour incubation in secondary antibodies and rinsed three times for 10 minutes with PBST. It was detected by an Ultra High Sensitivity ECL KiSt (GLPBIO, #GK10008) using Odyssey infrared imaging system (LICOR, Lincoln, NE).

**Real-time quantitative PCR**

Total RNA was extracted from cancer cells using the RNA isolater Total RNA Extraction Reagent Kit (Vazyme). The NanoDrop ND-1000 was used to measure the quality of RNA. Additionally, the RNA integrity was evaluated by the conventional denaturing agarose electrophoresis. Hiscript III All-in-one RT SuperMix Perfect for qPCR Kit (Vazyme) was used to create cDNA from 1ug of total RNA by reverse transcription. The ChamQ Universal SYBR qPCR Master Mix Kit (Vazyme) was used to conduct the RT-qPCR test. Table 1 contains the primer sequences for the gene.

**Chromatin immunoprecipitation**

The Chromatin immunoprecipitation experiment was carried out with specified regents and protocol by ChIP Assay Kit (Beyotime Institute of Biotechnology). Formaldehyde was used to cross-link the colorectal cells for 10 minutes, followed by 5 minutes with glycine solution at room temperature. Then, the cells were placed on ice before being lysed in SDS lysis buffer. To obtain 200-1000bp DNA fragments, the genomic DNA was sonicated in an ultrasonic breaker machine. To eliminate cross-links between protein and genomic DNA, the cell lysates were treated with NaCl at 65°C for four hours. Immunoprecipitations were probed overnight at 4°C with p53 antibody (Proteintech) as described [36]. Next, the DNA-protein complex was washed three times at 4°C. The products for PCR detection were obtained after DNA has been purified by the universal DNA purification kit (Tiangen). Table 2 showed the primer sequence for ALKBH5 from the predicted three locations utilized in this investigation.

**RNA-Binding Protein Immunoprecipitation (RIP) Assay**

Colorectal cancer cells were seeded in culture plates for 24 hours before being co-transfected by Lipofectamine 2000 with GFP-CARMN, and vector. Cells were detected for RNA immunoprecipitation after 48 hours using FTO, METTL3, and ALKBH5 antibody from the EZ-Magna RIP™ Kit (Millipore). RT-PCR was conducted on pure RNA complexes, and co-isolated RNA-binding proteins were identified by Real-time quantitative PCR.

**M6A-RNA immunoprecipitation (MeRIP) assay**

Total RNA was collected from sw480 cells transfecting plasmids (overexpressing or down-regulating ALKBH5) or empty vector control, and genomic DNA was removed using DNase (Vazyme). Following mRNA purification and fragmentation, the fragments were immunoprecipitated with m6A antibody using EpiQuik™ CUT&RUN m6A RNA Enrichment Kit (EpiGentek Group, #P-9008).

**Luciferase reporter assay**
Colorectal cancer cells were seeded in 24-well plates for 24 hours before being co-transfected with the luciferase reporter vector, Renilla vector. A luciferase reporter, the Renilla luciferase construct, and either miR-5683 control, miR-5683 mimics, or miR-5683 inhibitors were transfected into sw480 cells by Lipofectamine 2000 (Invitrogen). Using a Dual-Lumi™ Luciferase Reporter Gene Assay Kit (Beyotime Institute of Biotechnology), the ratios of firefly and Renilla luciferase activities were measured after 48 hours transfection. Then, enriched m6A modified mRNA was identified using qRT-PCR.

**Nuclear/cytoplasmic isolation**

Thermo Fisher’s NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (#78835) were used to isolate nuclear/cytoplasmic material according to the manufacturer’s procedure. Cytoplasmic and nuclear fractions were separated for RNA extraction. Respectively, human β-actin and U6 were utilized as control of cytoplasmic and nuclear RNA.

**RNA pulldown**

The sw480 cells lysates were treated with Biotin labeled probes of CARMN manufactured by Tsingke (Beijing, China), and then streptavidin beads were used to draw them down (Thermo Scientific Pierce). Then, western blot assay was performed on the precipitated ALKBH5 protein.

**Colony formation assay**

Colorectal cancer cells were planted at a density of 1*10^3 cells per well in six pore plates. After two weeks of incubation, colonies were fixed and stained with 0.1% Crystal Violet solution (Beijing Solarbio Science & Technology). The number of colonies was counted.

**Cell flow cytometry**

The number of labeled cells was determined using a BD FACS Aria III flow cytometer (Catalog#: BD FACS Aria™ III, BD Biosciences, Hercules, CA, USA).

**Xenograft studies**

Six-week-old female the nude mice were inoculated with 1×10^6 cells (shVector and Overexpressed CARMN sw480 cells) for each flank. The tumors were measured every other day. shVector (Empty vector) and OE-CARMN xenografts were established and measured.

**Statistical analysis**

The data was graphically plotted using the R package and the GraphPad Prism version 6.02 software. The Kaplan-Meier log-rank test was used to compare patient survival curves. The T-test and two-way ANOVA were used to analyze group differences. The value of p < 0.05 was regarded as statistically significant for two-sided statistical tests.

**RESULTS**
CARMN was down-regulated by Mutant p53 through ALKBH5.

According to our previous finding, mutant p53 enhances colon cancer malignant growth and immune evasion by the PHLPP2/AKT/PD-L1 pathway [36]. To investigate the expression of LncRNAs in colorectal cancer with mutant p53, p53 missense mutation profile data was obtained from the Cancer Genome Atlas using the R maftools program (TCGA-COAD dataset). The data from the volcano plot of IncRNAs expression signals were utilized to examine the p53 mutational landscape of 473 samples based on clinical characteristics (Fig. 1A). For these samples, CARMN expression was down-regulated with a significant difference. Next, we wonder to investigate whether CARMN expression affects colorectal cancer patients’ clinical progression. Kaplan-Meier analysis showed that colorectal cancer patients of later periods with lower CARMN levels had shorter overall survival (Fig. 1B). The second structure of CARMN was displayed (Supplementary Figure S1A). To confirm the coding transcripts from the non-coding transcript, the Coding-potential calculator coding potential assessment tool showed CARMN has a very weak protein-coding potential (Supplementary Figure S1B). Then, to identify the relation of mutant p53 and CARMN, mutant p53R273H was knocked down in sw480 and HT29 cells using shRNA, which showed higher expression levels of CARMN. By the contrast, mutant p53R273H was overexpressed in sw480 and HT29 cells, which showed lower expression of CARMN, compared with that of the control group (Fig. 1C-1D).

The expression of p53 is controlled both transcriptionally and post-transcriptionally by N-6-methyladenosine (m6A) RNA methylation [37]. The function of enzyme N6methylase (m6A) in TP53-mutant colorectal cancer is unknown, which is crucial for mRNA stability, translation, and splicing. To study the role of m6A modification in colorectal cancer cells with mutant p53, the results of an m6A dot blot assay indicated that global m6A RNA levels of p53 mutation were clearly increased (Fig. 1E). Furthermore, it was showed that the effect of p53 expression and m6A methylation in sw480 cells (Supplementary Figure S1C). In addition, the clinical findings of ALKBH5, FTO, and METTL3 mRNA expression were summarized in colorectal patients of wild-type and mutant p53 (Supplementary Figure S1D-S1E). The mRNA expression of ALKBH5 was observed negatively correlated with mutant p53 in colorectal cancer patients (Fig. 1F-1G). Similarly, the protein levels of ALKBH5 were lower, with higher level mutant p53 expression in colorectal cancer cells (Fig. 1H). The trends of FTO (Supplementary Figure S1D) and METTL3 (Supplementary Figure S1E) mRNA expression with p53 were barely no statistically significant in the above samples. Next, the influence of ALKBH5 on m6A methylation was studied by using the dot blot assay. As a result, the m6A methylation levels of RNA significantly declined after over expressing ALKBH5, while increased evidently in the ALKBH5 knocking down group (Fig. 1I). Concurrently, IHC assays were performed in mutant p53R175H of colorectal cancer samples. The analysis of Ki67 and m6A showed that strong signals specifically surrounded the mutant p53 tissue. Besides, ALKBH5, FTO, METTL3, METTL14, METTL16, and WTAP were detected in a section of colorectal cancer tissue (Supplementary Figure S1F). These findings indicated that ALKBH5 demethylation upregulated CARMN expression, which inhibited colorectal cancer progression with mutant p53.

**Mutant p53 transcriptionally decreased the expression of ALKBH5 by binding to its promoter.**
Studies have shown that ALKBH5 was revealed to be positively correlated with wild type p53 in lung cancer by the Gene Expression Profiling Interactive Analysis (GEPIA) web tool. Further, p53 affected ALKBH5 transcription to influence the global m6A methylation level [28]. The stimulation of ALKBH5 transcription by p53 operated as a feedback loop to control the m6A modification in pancreatic cancer [27]. However, the relation between mutant p53 and ALKBH5 has not been reported. The fluorescence imaging of GFP-p53R273H and mCherry-ALKBH5 was performed to study their subcellular localization in sw480 cells. As shown in Fig. 2A, the colocalization of GFP-p53R273H and mCherry-ALKBH5 in the same cells was observed. To investigate if mutant p53 could bind to the promoter of ALKBH5 and pinpoint the exact binding sites, three possible binding sites were generated by bioinformatics analysis (Fig. 2B). Then, we detected the activity of different regions of the ALKBH5 promoter (Fig. 2C-2E). As a result, p53 activated the proximal −1481bp region of ALKBH5. We identified a p53 binding site in this region (Fig. 2D). ALKBH5 promoter activation was completely inhibited when the mutant p53 was knocked down, suggesting a direct binding between mutant p53 and this region. Additionally, as a transcription factor, p53’s binding motif on the promoter of ALKBH5 was shown (Supplementary Figure S2A-S2B). Therefore, the ChIP experiment revealed that mutant p53 coupled to the binding sites of the ALKBH5 promoter.

To detect the function of ALKBH5 in our system, cell viability was analyzed by cell counting kit-8 at 1 day, 2 days, 3 days, and 4 days, respectively (Fig. 2G-2H). Consistent with recent report [27], knocking down ALKBH5 significantly increased the proliferation and migration rate, while contrary results were observed when ALKBH5 was overexpressed in sw480 cells (Fig. 2I, and 2K). Next, flow cytometry analysis revealed that depletion of ALKBH5 induced cell cycle arrest at the S phase (Fig. 2J). The cell scratch and migration tests showed that after 24 and 48 hours of culture, the scratch distance and migration rate of sw480 cells decreased with knocking down ALKBH5 (Fig. 2L and 2M). Taken together, these findings suggest that mutant p53 directly bound to the promoter of the ALKBH5 region to inhibit its transcription, down-regulated ALKBH5 protein level and accelerated colorectal cancer progression.

ALKBH5 demethylated m6A CARMN and maintained its expression level by YTHDF2.

To study the relationship of CARMN with ALKBH5 in colorectal cancer with mutant p53, an RNA immunoprecipitation (RIP) assay was performed and the results revealed that CARMN interacted with ALKBH5 in sw480 cells (Fig. 3A-3C). Further, the EMSA (electrophoretic mobility shift assay) analysis showed that FAM labeled CARMN oligos interacted with ALKBH5 proteins (Fig. 3D and Supplementary Figure S3A). Additionally, the consensus motif search yielded the GGACT sequence, which has been identified as a consensus methylation motif by others [38, 39]. A consensus motif was discovered, which was relatively obvious in the CDS of ALKBH5 (https://meme-suite.org/meme/tools/meme) (Fig. 3E). According to the RNA pull-down test, the binding between ALKBH5 and biotin-labeled CARMN decreased in the antisense of CARMN (Fig. 3F). To visualize CARMN-ALKBH5 interaction, GFP-CARMN and mCherry-ALKBH5 were transfected in sw480 cells and their colocalization was observed (Fig. 3G). Importantly, the potential specific m6A site of CARMN that was implicated by ALKBH5 was predicted using the SRAMP website (http://www.cuilab.cn/sramp/), and three candidates of m6A modification sites with high
confidence was obtained (Fig. 3H). The secondary structure of CARMN was shown and three potential m6A modification sites were predicted, which was verified by m6A RIP assay that 477 m6A site was the right one (Fig. 3I-3J, and Supplementary Figure S3B).

Further, overexpressed or knocked down ALKBH5 increased or decreased the amounts of CARMN respectively in sw480 cells (Fig. 3J, 3K). Studies have shown that YTHDF proteins may influence essential biological processes connected to m6A RNA methylation [40, 41]. To investigate whether YTHDFs were involved in the m6A modification of CARMN, an RNA immunoprecipitation (RIP) assay was performed and the results revealed that an abundance of CARMN was presented in the complex pulled down by YTHDF2 and YTHDF3 (Fig. 3L and 3M). It indicated that CARMN interacted with YTHDF2 and YTHDF3. Then, the effect of YTHDF2 and YTHDF3 on the stability of CARMN was tested. As shown in Fig. 3N-3Q, knocking down YTHDF2 or YTHDF3 significantly increased CARMN expression. Additionally, ALKBH5 protein was exclusively detected in the nucleus fractions, and YTHDF2 and YTHDF3 were localized in the cytoplasmic fractions (Supplementary Figure S3C). Furthermore, the immunofluorescence detection showed that YTHDF2 and YTHDF3 gave us an identical result (Fig. 3R). In summary, the m6A modification of CARMN was demethylated by ALKBH5 and then bound to YTHDF2 and YTHDF3 in cytoplasmic fractions.

**CARMN inhibits the proliferation, invasion, and metastasis of colorectal cancer with mutant p53.**

The lack of lnc273-31 or lnc273-34 significantly delayed colorectal cancer of mutant p53R273H initiation and tumorigenic in vivo [42]. To clarify the function of CARMN in colon cancer cells with mutant p53, it was overexpressed by pcDNA3.1 plasmid or knocked down by synthesized specific shRNAs (Fig. 4A-4C). As a result, cell viability and colony formation decreased significantly after overexpressing CARMN, which increased markedly after knocking down CARMN (Fig. 4D, 4E, 4G and 4H). Similar results were obtained from scratch assay (Fig. 4I and Supplementary Figure S4H) and the transwell invasion (Fig. 4J). Moreover, CARMN inhibited the growth of colorectal cancer cells via inducing S phase cell cycle arrest and apoptosis (Fig. 4F). These results indicated that CARMN inhibited colon cancer cell proliferation and metastasis. Interestingly, CARMN had a negative feedback on mutant p53 expression (Supplementary Figure S4A), which might be through the transcriptional regulation. These results above indicated that CARMN prevents colorectal cancer with mutant p53 from proliferation and migration.

To gain insight into where CARMN was distributed throughout the subcellular environment, we observed the presence of CARMN by fluorescence imaging in a single cell (Fig. 4K). Furthermore, bioinformatics prediction and the nuclear/cytoplasmic RNA separation test findings revealed that CARMN was predominantly found in the nucleus (Fig. 4L, and Supplementary Figure S4D-S4G).

The levels of caspase 3 and c-caspase 3, typical indicators of mitochondria-mediated apoptosis, were significantly down-regulated by knocking down CARMN. While the activations of bcl-xl and bcl-2 increased, which were detectable in sw480 and sw620 cells (Fig. 4M-4N). The contrary trends of caspase 3, c-caspase 3, bcl-xl and bcl-2 were observed with overexpressing CARMN (Fig. 4Q-4R). In parallel, whether CARMN affected autophagy was also studied. As shown in Fig. 4O and 4P, ULK1 and LC3
decreased obviously when CARMN was knocked down. However, p62 decreased as the direct downstream targets of autophagy. Contrary results were obtained when CARMN was overexpressed (Fig. 4O-4P). Together, the above results indicated that CARMN induced apoptosis and autophagy at the same time.

MiR-5683 suppressed the progression of colorectal cancer at downstream of CARMN.

MiRNAs expression was linked to gain-of-function of mutant p53 in patients with head and neck squamous cell carcinoma (HNSCC) patients [43]. Herein, the miRNAs expression was screened with a significant difference from the volcano plot in the p53 mutational landscape of colorectal cancer samples, which was analyzed using the R packages (Fig. 5A). Additionally, we predicted miRNAs that could bind with CARMN using the publicly available websites (https://starbase.sysu.edu.cn/starbase2/mirLncRNA.php) and selected the top miRNAs of miR-5683 for further analysis (Fig. 5B). Consistently, mutant p53R273H inhibited the expression of miR-5683, whereas miR-5683 was obviously up-regulated by silencing mutant p53R273H in colon cancer cells (Fig. 5D-5E). Moreover, we discovered that overexpression of miR-5683 reduced luciferase activity of wild-type CARMN but not the Mut-CARMN utilizing dual-luciferase reporter assays (Fig. 5C and Supplementary Figure S5A). It indicated that CARMN directly “sponges” miR-5683. To investigate the potential role of miR-5683 in colorectal cancer cells with mutant p53, miR-5683 mimics or miR-5683 inhibitor was transfected into cells to overexpress or knock down miR-5683 (Fig. 5F-5G, Supplementary Figure S5D-S5F). As a result, cell viability declined with miR-5683 overexpression, while risen after it was knocked down (Fig. 5H and 5I), suggesting that miR-5683 suppressed colorectal cancer cell growth. This conclusion was also confirmed by the EDU immunofluorescence staining (Supplementary Figure S5G). Moreover, the flow cytometry experiment confirmed S arrest in the miR-5683 inhibition group, while the group of miR-5683 mimics resulted in a lower proportion of mutant p53 cells in the S phase (Fig. 5K). In addition, transwell migration assays revealed that miR-5683 inhibited colorectal cancer cell metastasis (Fig. 5J).

To further determine the function of miR-5683 with mutant p53, various markers of apoptosis and autophagy were detected. Overexpressed miR-5683 extremely decreased the level of Bcl-xl and Bcl-2. Whereas inhibition of miR-5683 increased the level of Bcl-xl and Bcl-2 and blocked the c-caspase 3 induction (Fig. 5L, and Supplementary Figure S5H). Interestingly, miR-5683 mimics did change with LC3 induction (Fig. 5M, and Supplementary Figure S5I). Furthermore, after transfecting the RFP-GFP-LC3 plasmid into sw480 cells and treating them with miR-5683 mimics, GFP degradation and RFP/GFP elevation were observed, which implied the activation of autophagy (Fig. 5N). Interestingly, miR-5683 overexpression suppressed mutant p53R273H production while inhibition of it clearly boosted mutant p53R273H (Supplementary Figure S5B-S5C). These results demonstrated that miR-5683 had a negative feedback on mutant p53 expression, as CARMN did. These suggested that miR-5683 inhibits cell progression and contributes to apoptosis and autophagy in colorectal cancer with mutant p53.

MiR-5683 inhibited colon cancer growth and mutant p53 activity through degrading FGF2 mRNA.
Over 2000 miRNAs have been found in humans, and they may regulate one-third of the mRNAs [44, 45, 46]. To find out genes sharing the regulatory role of miR-5683 with CARMN, we predicted the target genes of miR-5683 using the miRWalk database (Fig. 6A). Then, the STRING database depicted these significantly different predicted mRNAs with mutant p53 by protein-protein interaction (PPI) network. The discovery of miRNA-mRNA target interactions is critical for understanding the regulatory network mediated by miR-5683 (Fig. 6B-6C). Among them, the core genes with a fold change were screen out and investigated by the Cytoscape database (Fig. 6D-6E, and Supplementary Figure S6A-S6B). Hence, FGF2 caught our attention because the regulatory network mediated by miR-5683 was relatively significant among the predicted target genes. Additionally, Kaplan-Meier survival analysis revealed that patients with high FGF2 expression had worse survival (Fig. 6F).

The function of mutant p53 increases the activation of surrounding fibroblasts to inhibit autophagy, accompanied with higher FGF2 [47]. We compared the FGF2 levels in TCGA between the p53 mutant and wild-type groups, and found that the p53 mutant group of colorectal cancer had higher FGF2 level, p = 0.0046 (Fig. 6G). Furthermore, the relationship between mutant p53 and FGF2 was studied. The result showed that overexpressed mutant p53 R273H evidently up-regulated the expression of FGF2 (Fig. 6H).

To explore how FGF2 exerts its function, the subcellular localization of FGF2 was firstly detected, and showed mainly in the nuclear (Fig. 6I). Next, the colony formation assay and CCK-8 experiments indicated that FGF2 promoted colorectal cancer cell proliferation (Fig. 6J, 6K, and 6N). FGF2 could protect cells against other kinds of death such as apoptosis or necrosis through autophagy suppression [48]. To further explore the mechanism behind, apoptosis and autophagy were analyzed. The results showed that overexpressing FGF2 distinctly increased Bcl-xl/Bcl-2/p62 expression, while obviously decreased the expression of caspase 3/ULK1/LC3II (Fig. 6L-6M, 6O-6P). Contrary trends were observed when FGF2 was knocked down (Fig. 6L-6M, 6O-6P), which demonstrated that FGF2 inhibited both apoptosis and autophagy. Surprisingly, FGF2 had a positive feedback on mutant p53 expression (Fig. 6L-6M). These findings indicated that miR-5683 down-regulates FGF2 and activates apoptosis and autophagy.

**CARMN collaborates with miR-5683 to down-regulates FGF2 and induce autophagy.**

Our results have demonstrated that miR-5683 down-regulating FGF2 expression on both protein and mRNA level (Supplementary Figure S5H, S5I, S7A and S7B), which verified that FGF2 was the target gene of miR-5683. Interestingly, as the upstream regulator of miR-5683, CARMN overexpressing also reduced FGF2 activity (Fig. 7A and Supplementary Figure S7C). According to Zhao Y et al., the role of multiple autophagic pathways in targeting and degrading mutant p53 proteins [49]. MiRNAs generated from mutant p53 play a critical role in autophagy inhibition [50]. To further reveal their relation and function, CARMN and miR-5683 were co-overexpressed, which led to a further reduction of FGF2 and mutant p53 expression on both mRNA and protein level, compared with that of the CARMN overexpressing only group (Fig. 7A and 7H). Meantime, p62 also had a further reduction while LC3II had a further increase, and more YFP-LC3 puncta was observed under the same condition (Fig. 7H and 7C). These results indicated that
CARMN and miR-5683 have a synergistic effect on FGF2 inhibition, autophagy induction and negative feedback of mutant p53.

FGF2 has been reported to promote tumorigenesis via stimulating the PI3K/Akt signaling pathway [42, 49, 50]. And mTOR is the critical downstream effector of the Akt pathway and upstream of p70 S6 kinase [51]. Thus, it was selected for further analysis. To clarify the function of CARMN and miR-5683, the overexpression and interference efficiency of CARMN, p53, and FGF2 were assessed by qPCR analysis. The assay showed that overexpression of CARMN and miR-5683 in sw480 cells dramatically decreased p53 expression compared with the overexpressed CARMN group. Interestingly, overexpression of CARMN and miR-5683 could inhibit FGF2 expression than that in the overexpressed CARMN group (Fig. 7A-7B). After overexpression of CARMN, miR-5683, and treatment with YFP-LC3, we detected more distribution of LC3 in living cells, suggesting the autophagy synergistic effect of CARMN and miR-5683 (Fig. 7C).

As a result, we investigated the ability of modulation of the CARMN-miR-5683-FGF2 axis to influence Akt pathway activation. To investigate the prospective autophagic strategies of targeting mutant p53 in cancer, we transfected sw480 cells with CARMN, miR-5683, and FGF2. The results showed that CARMN and miR-5683 were related to Akt/mTOR signaling pathway, which plays a pivotal role in oncogenesis of colorectal cancer cells mutant p53. Our result revealed that phosphorylated Akt (P-Akt1) decreased in CARMN-transfected cells compared with control cells. To investigate the effect of CARMN and miR-5683 on the mTOR pathway, phosphorylated mTOR (P-mTOR) was detected. P-mTOR was reduced in CARMN-transfected cells compared with vectors. Conversely, P-mTOR was increased in inhibition of CARMN cells than vectors. The promotion effect of CARMN and miR-5683 on the Akt/mTOR pathway was abolished in miR-5683 inhibited or FGF2 overexpressed cells (Fig. 7D-7H). These findings strongly suggested that CARMN and miR-5683 could promote Akt/mTOR pathway.

**CARMN suppressed colon cancer growth in vivo and could be used as a potential tumor inhibitor.**

To verify the effect of CARMN on colorectal cancer growth in vivo, vector and GFP-CARMN were overexpressed in sw480 cells, which were then subcutaneously inoculated in the right armpit of the nude mouse to construct xenografts tumor models, respectively (Fig. 8A-8B). Significantly lower tumor volume was detected in sw480/GFP-CARMN group compared to that of sw480/vector group (Fig. 8D), while the weight of these mice in two groups had almost no difference (Fig. 8C). The result indicated that CARMN suppressed colorectal cancer growth in vivo, in the presence of mutant p53. Collectively, our results demonstrated that mutant p53 transcriptionally down-regulated ALKBH5 expression, which led to higher level m6A methylation of CARMN, subsequently degraded by YTHDF2/3 (Fig. 8E). On the other hand, CARMN directly interacted with miR-5683 and they had a synergistic effect on colorectal cancer growth suppression, through degrading FGF2 mRNA to inhibit Akt/mTOR pathway and induce apoptosis/autophagy. Additionally, both CARMN and miR-5683 had a negative feedback while FGF2 had a positive feedback on mutant p53 expression (Fig. 8E).

**DISCUSSION**
TP53 mutation gains-of-function is accompanied by tumor development and low patient survival rate. Our findings demonstrated that mutant p53 could bind the promoter of PHLPPL to inhibit its expression. In addition, the results revealed that colorectal cancer patients with mutant p53 had high levels of AKT phosphorylation and PD-L1 expression [36]. The apoptosis of p53-dependent traditional drugs was eliminated in mutant p53 colon cancer cells, accompanied with increased cell viability. It showed that mutant p53 could inactivate PUMA transcription to promote tumor chemo-resistance [52]. In this study, we identified ALKBH5 as a post-transcription target of mutant p53 and a deacetylated regulator of CARMN, and demonstrated CARMN/miR-5683/FGF2 axis played a crucial role in apoptosis and autophagy in vitro and in vivo for the first time. Mechanically, mutant p53 could bind to the ALKBH5 promoter to restrict its transcription, which led a m6A modification level increase of CARMN to trigger its degradation. CARMN correlated with miR-5683 to down-regulate the expression of FGF2, and finally initiated autophagy. Our results indicated the crucial role for ALKBH5 activation and CARMN induction as a novel target for the therapy of colorectal cancer with mutant p53.

Mutant p53 loses its ability to suppress tumor development and gain-of-function activities by accelerating tumor growth. A poor clinical prognosis is connected with TP53 mutation, which is present in around half of all colorectal cancer cells. Mutant p53 is a significant factor in 5FU resistance of colorectal cancer [52]. Currently, lncRNAs have been identified as important regulators of tumor development and progression, including apoptosis, DNA damage response, and cell proliferation and invasion [53]. The findings suggest that MALAT1 plays an important role in the regulation of VEGFA isoform production in breast cancer cells harboring gain-of-function mutant p53 and ID4 proteins [54]. However, the potential role of lncRNAs in colorectal cancer with mutant p53 is poorly understood. Here, 222 samples with mutant p53 and 251 samples with wild-type p53 from colorectal cancer patients were analyzed. Most of the samples with mutant p53 expressed a low level of CARMN (Fig. 1A), accompanied with an extremely low level of ALKBH5 (Fig. 1G). Meantime, patients with lower CARMN levels had shorter overall survival in later period colorectal cancer with mutant p53 (Fig. 1B). Therefore, CARMN may serve as a tumor suppressor with mutant p53 of colorectal cancer.

There also exists an opinion that blocking p53 mRNA m6A modification by S-adenosyl homocysteine or siRNA-mediated METTL3 inhibition increases hepatocellular carcinoma susceptibility to chemotherapy [55]. According to Uddin et al., m6A modification at the p53 pre-mRNA leads to p53R273H mutant protein expression. Suppressing RNA methylation and ceramide glycosylation may be an effective cancer therapeutic approach for targeting TP53 missense mutations [56]. Besides, ALKBH5 levels were found to be high in the wild-type p53 group, while the p53 mutation group had low levels of ALKBH5 in pancreatic cancer, according to the analysis of the TCGA datasets. Furthermore, it was identified that wild type p53 could bind the ALKBH5 promoter to activate the ALKBH5 transcription [27]. In recent years, there is a little study that m6A modification with mutant p53 in cancer therapy. Nevertheless, it is collusive that m6A modification of lncRNAs with mutant p53 in colorectal cancer. Our results showed that mutant p53 blocked ALKBH5 promoter activity, three p53 binding sites were predicated on the promoter of ALKBH5, and ChIP assay results showed that one of these binding sites directly interacted with mutant p53 (Fig. 2B-2E). This mutant p53 binding site is different from the wild type p53 binding site that Guo et al.
reported [27]. The result further confirmed that mutant p53 interacts with the promoter of ALKBH5 (Fig. 2A). In this study, we focus on the demethylase of ALKBH5 regulates the methylation of CARMN in colorectal cancer with mutant p53. In contrast to the antisense CARMN group, biotin-labeled CARMN obviously pulled down ALKBH5 (Fig. 3F). In addition, a significant upregulation of CARMN was observed following ALKBH5 overexpression (Fig. 3K), which suggests that ALKBH5 plays a critical role in removing the methylation of CARMN.

Though cell migration and dedifferentiation may be trigged by CARMN-mediated regulation of miRNAs, CARMN could influence human coronary arterial smooth muscle cells (hCASMCs) proliferation independently of miR-143 and miR-145 [15]. MiR143HG (CARMN) was shown to suppress miR-1275 levels, which directly targeted AXIN2 to modulate the Wnt/catenin pathway [57]. The mechanism of CARMN regulating colorectal cancer with mutant p53 was investigated, and we found that CARMN co-operated with miR-5683 to exert its function (Fig. 8B-8C). The role of miR-5683 has been confirmed to suppress gastric cancer by targeting the gene pyruvate dehydrogenase kinase 4 (PDK4) [58]. In this study, it was confirmed that miR-5683 downregulated the expression of wild type CARMN group, while this effect was abrogated by mutant sequence of CARMN in dual-luciferase reporter assays (Fig. 5C).

Moreover, to assess their effect, we checked the expression of FGF2, as a target of miR-5683 (Fig. 6). There are several regulators that might be involved in FGF2 expression in different cancers. Circ001422 and miR-195-5p increased FGF2 expression to accelerate osteosarcoma tumorigenesis and metastasis [59]. While in this study, FGF2 was significantly downregulated by the synergistic effect of CARMN and miR-5683 (Fig. 7A, 7D), which might be through Akt/mTOR pathway (Fig. 7C, 7E-7H).

**CONCLUSIONS**

CARMN loss is associated with poor clinicopathological characteristics and prognosis of colorectal cancer with mutant p53. Overexpression of CARMN reduces the cell proliferation, migration, and colorectal cancer with mutant p53, whereas CARMN knockdown facilitates mutant p53 with colorectal cancer progression. Demethylation of CARMN and increase in its level underlie the effect of ALKBH5 in an m6A-YTHDF2/YTHDF3-dependent manner.

**Abbreviations**

LncRNA, long noncoding RNA; CARMN, cardiac mesoderm enhancer-associated noncoding RNA;

**Declarations**

**AVAILABILITY OF DATA AND MATERIALS**

All data needs to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
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Not applicable.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

N.L., X.J. designed experimental approaches, performed experiments, analyzed data, and co-wrote the manuscript; J.L., G.Z., M.J. and G.J. performed experiments; Y.Z. analyzed data, provided oversight and critical expertise, and co-wrote the manuscript.

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CONSENT FOR PUBLICATION

Not applicable.

References


**Figures**
Figure 1

Downregulation of LncRNA CARMN is associated with high methylation levels in colorectal cancer with mutant p53. (A) A volcano plot displayed the DE-LncRNAs between TP53 mutant and wild-type patients in CRC. (B) The plot of the survival curve showed the low expression of CARMN led to a poor prognosis in advanced colorectal patients with mutant p53. (C, D) The qPCR assays were used to analyze CARMN RNA levels in sw480 and HT29 cells transfected with sh-p53R273H or OE-p53R273H. (E, I) m6A dot blot
assays of sw480 cells with knockdown or overexpression of p53-R273H (E) or ALKBH5 (I) were designed to measure the m6A level from total RNA diluted with 100ng, 200ng, and 400ng (E) or 200ng, 400ng and 800ng (I), methylene blue (MB) staining worked as a loading control. (F) The mRNA expression of demethylase ALKBH5 between mutant and wild-type p53 was visualized by R studio from the database TCGA. (G) The mRNA expression of ALKBH5 in colon cancer patients with mutant p53. (H) The relative protein level of ALKBH5 in sw480 cells transfected with sh-p53R273H or OE-p53R273H.
Figure 2

Mutant p53 inhibits the transcription of ALKBH5 to promote the malignant proliferation of colorectal cancer with mutant p53. (A) The live sw480 cells transfected with GFP-p53R273H and mCherry-ALKBH5 were imaged by confocal microscopy. (B) Three potential mutant-p53 binding sites on the ALKBH5 promoter were shown in the diagram. (C-E) A specific binding site was demonstrated by ChIP. DNAs were pull-downed by ALKBH5 or IgG antibody. The input served as an internal positive control. (F) The relative protein level of ALKBH5 in sw480 cells transfected with OE-ALKBH5 or si-ALKBH5. (G-I) The CCK8 assays (G, H) and colony formation assays (I) were obtained to measure the effect on sw480 cells transfected with OE-ALKBH5 or si-ALKBH5. (J) Cell cycle distribution was obtained by flow cytometry in sw480 cells overexpressed ALKBH5. (K-M) The EdU assays (K), wound-healing assays (L), and transwell migration assays (M) were applied to compare the cell proliferation or migration ability in sw480 cells transfected with OE-ALKBH5 or si-ALKBH5.
Figure 3

ALKBH5 combines with LncRNA CARMN to remove its methylation modification, thereby regulating its expression. (A-C) RIP assays confirmed the association between CARMN and FTO, METTL3, and ALKBH5 in sw480 cells. (D) The concentration of ALKBH5 protein was mixed with FAM-labeled CARMN. (E) Motif analysis was made by the online tool DREME to identify “ATGCC” as the m6A consensus motif of ALKBH5. (F) Proteins obtained from the RNA pull-down experiment were used to measure the
quantification of ALKBK5 in sw480 cells transfected with Biotin labeled ALKBH5. (G) The co-localization of CARMN and ALKBH5 was observed by confocal microscopy in sw480 transfected with GFP-CARMN and mCherry-ALKBH5. (H) The online tool SRAMP was used to analyze methylation modification sites of CARMN. (I, J) MeRIP assays interpreted the m6A sites which ALKBH5 combined with CARMN in sw480 cells transfected with OE-ALKBH5 or si-ALKBH5. (K) The qPCR assays were used to analyze CARMN and ALKBH5 RNA levels in sw480 cells transfected with OE-ALKBH5 or si-ALKBH5. (L, M) The MeRIP assays displayed the reader which could combined with the CARMN in SW480 cells by YTHDF2 and YTHDF3 antibodies. (N-Q) The expression of CARMN in sw480 or HT29 cells transfected with siYTHDF2 or siYTHDF3. (R) The co-localization of YTHDF2 and YTHDF3 with nucleus were observed by Immunofluorescence in sw480 cells.
Figure 4

Downregulation of CARMN inhibits autophagy and apoptosis, thus promoting proliferation and migration of CRC cells in vitro. (A-C) RNA expression of CARMN in sw480 or HT29 cells transfected with shRNA-CARMN or OE-CARMN. (D-E) CCK8 assays were obtained to measure the effect on sw480 cells transfected with shRNA-CARMN or OE-CARMN. (F) Cell cycle distribution was obtained by flow cytometry in sw480 transfected with shRNA-CARMN or OE-CARMN. (G) EdU assay was obtained to observe the cell
proliferation ability in sw480 transfected with OE-CARMN or shRNA-CARMN. (H) Colony formation assays were obtained to measure the effect on sw480 cells transfected with shRNA-CARMN or OE-CARMN. (I-J) Wound-healing (I) and transwell migration assays (J) were used to observe the cell migration ability in sw480 cells transfected with OE-CARMN or shRNA-CARMN in sw480 cells. (K) The location of CARMN was imaged by confocal microscopy in sw480 transfected with GFP-CARMN. (L) The expression of CARMN in the subcellular fractions of colorectal cancer with mutant p53 was detected by RT-PCR. U6 and actin were utilized as nuclear and cytoplasmic markers, respectively. (M, N) The apoptosis-related proteins were detected after the CARMN was knockdown in sw480 and sw620 cells. (O, P) Knockdown of CARMN made the effect on cell autophagy in sw480 and sw620 cells. (Q, R) Expression of Bcl-xl, Bcl-2, Caspase3, and C-Caspase3 following CARMN overexpressed in sw480 and sw620 were evaluated by western blotting. (S, T) Expression of ULK1, P62, and LC3 following CARMN overexpressed in sw480 and sw620 were evaluated by western blotting.
Figure 5

MiR-5683 combines with LncRNA CARMN, and downregulation of it enhances the proliferation capability and tumor growth of colorectal cancer with mutant p53. (A) A volcano plot displayed the DE-miRNAs between TP53 mutant and wildtype patients in colorectal cancer. (B) The Venn diagram was drawn to take an intersection for miRNAs by the bioinformatics tool Venn. (C) The association between miR-5683 and CARMN was determined by the luciferase activities in sw480 cells co-transfected with WT-CARMN or...
MUT-CARMN and miR-5683 mimics. (D-E) The expression of miR-5683 was measured by RT-PCR in HCT116−/− cells transfected with OE-p53-273 (D) and sw480 cells transfected with shRNA-p53 (E). (F-G) The expression of miR-5683 was measured by RT-PCR in sw480 and sw620 cells transfected with miR-5683 mimics (F) or miR-5683 inhibitor (G). (H-J) The proliferation and migration abilities of sw480 cells transfected with miR-5683 mimics or miR-5683 inhibitor were tested by CCK8 and transwell migration assays. (K) Cell cycle distribution was obtained by flow cytometry in sw480 cells transfected with miR-5683 mimics or miR-5683 inhibitor. (L, M) The apoptosis-related and autophagy-related proteins were detected in sw620 cells by western blotting. (N) The sw480 cells co-transfected with GFP-mRFP-LC3 and miR-5683 mimics or GFP-mRFP-LC3 and miR-5683 inhibitor were observed by confocal microscopy.
Figure 6

FGF2 is correlated with miR-5683 and makes a promotional effect on tumor progression. (A) Upregulation (red) and downregulation (blue) genes were displayed on the volcano plot with mutant p53 of colorectal cancer. (B) Protein-protein interaction (PPI) networks between the De-mRNAs were constructed by the online tool STRING. (C) The PPI network was be simplified by Cytoscape, which was used to calculate the degree value of De-mRNAs in PPI networks. (D) The core genes in this PPI network. (E) The Venn plot was
used to obtain core genes which combined with miR-5683. (F) The survival plot made by the online tool GEPIA revealed higher expression of FGF2 resulted in lower survival. (G) The box diagram displayed the expression of FGF2 in TP53 mutant and wildtype patients. (H) The expression of FGF2 was measured by RT-PCR in HCT116/− cells transfected with OE-p53-273. (I) The location of FGF2 was observed by confocal microscopy in sw480 cells transfected with GFP-FGF2. (J, K, N) Transwell migration assays (J) and CCK8 assays (K, N) were used to detect the effect on sw480 cells transfected with shRNA-FGF2 or OE-FGF2. (L, M) Expression of ULK1, P62, LC3 were obtained by western blotting in sw480 and sw620 with FGF2 overexpressed or knockdown. (O, P) Overexpression of FGF2 suppressed autophagy in CRC cells.
Figure 7

CARMN and miR-5683 combine to down regulate the expression of FGF2. (A) RT-PCR assays were used to calculate the expression of FGF2, p53 and CARMN in sw480 cells transfected with OE-CARMN. (B) FGF2 and p53 expression level were reduced effectively in sw480 cells co-transfected with OE-CARMN and miR-5683 mimics. (C) The cells were observed by confocal microscopy in sw480 co-transfected with YFP-LC3 and GFP-CARMN or YFP-LC3, GFP-CARMN and miR-5683 mimics. (D-H) CARMN and miR-5683
overexpression in SW480 cells reduced the phosphorylation of mTOR and AKT1, inducing the downregulation of FGF2 and P53, enhancing cell autophagy.

**Figure 8**

CARMN mediates in vivo antitumor effects. (A-D) Nude mice were used to established xenograft model with shVector or OE-CARMN tumor. (C-D) tumors pictures in this test, (C) mice weight or (D) tumor volume.
was measured. *P<0.05. (E) Schematic representation of ALKBH5 mediated CARMN induction to regulate miR-5683/FGF2 signaling pathway.

**Supplementary Files**

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