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Original Article

LncRNA SNHG4 promotes malignant biological behaviors and immune escape of colorectal cancer cells by regulating the miR-144-3p/MET axis

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Abstract: Objective: This study aimed to explore the underlying mechanism of long noncoding RNA (lncRNA) SNHG4 regulating MET to participate in the malignant biologic behaviors and immune escape of colorectal cancer (CRC) by sponging miR-144-3p. Methods: CRC tissues were collected and the expression levels of lncRNA SNHG4, miR-144-3p, and MET were detected by quantitative real-time PCR (qRT-PCR). Then, the localization of lncRNA SNHG4 was studied by fluorescence in situ hybridization (FISH), and the regulatory relationship among lncRNA SNHG4, miR-144-3p, and MET was verified by dual-luciferase reporter assay. Next, cell counting kit-8 (CCK-8), Clone formation assay, and Transwell migration assay were carried out to evaluate cell proliferation, colony formation, and invasion, respectively. Flow cytometry was performed to evaluate cell apoptosis. Western blotting was applied to semi-quantify the expression levels of MET and PD-L1 in cells. Results: LncRNA SNHG4 expression was upregulated in CRC tissues. Knockdown of lncRNA SNHG4 suppressed the proliferation, colony formation and invasion of CRC cells (all $P<0.05$). LncRNA SNHG4 directly regulated miR-144-3p, by which either lncRNA SNHG4 knockdown or miR-144-3p overexpression can inhibit CD4+ T cell apoptosis (both $P<0.05$) to suppress immune escape. Either overexpression of lncRNA SNHG4 or knockdown of miR-144-3p activated PD-1/PD-L1 and induced CD4+ T cell apoptosis (both $P<0.05$). LncRNA SNHG4 targeted and regulated MET through the regulation of miR-144-3p, while overexpression of MET can partially reverse the effect of lncRNA SNHG4 knockdown on CD4+ T cells. Conclusion: LncRNA SNHG4 sponges miR-144-3p and upregulates MET to promote the proliferation, colony formation, invasion, and immune escape of CRC cells, leading to the progression of CRC.

Keywords: LncRNA SNHG4, miR-144-3p, MET, colorectal cancer, immune escape

Introduction

Colorectal cancer (CRC) is one of the top five cancers globally in terms of morbidity and mortality [1]. Currently, radical surgery is still an effective treatment of CRC, but due to the high metastatic rate and high recurrence rate, some patients still suffer relapse after treatment [2]. The pathogenesis of CRC is complex, which involves multiple molecular pathways. Thus, exploring effective molecular targets and biomarkers based on the molecular mechanism of CRC is of great value. Studies have found that immune escape plays a vital role in the development of cancer. Cancer cells and immune cells interplay in the tumor microenvironment (TME), which influences the anti-tumor activity of immune cells [3]. The crosstalk between programmed death receptor 1 (PD-1) and programmed death-ligand 1 (PD-L1) can trigger CD4+ T cell apoptosis, resulting in immune escape. Therefore, PD-1/PD-L1 serves as an immune checkpoint. Multiple studies have reported that the regulation of PD-1/PD-L1 is becoming an effective strategy for tumor immunotherapy [4].

Long non-coding RNAs (lncRNAs), generally at least 200 nucleotides in length, are long-chain RNAs without protein-coding potentials. Many studies have reported that lncRNAs can be used as biomarkers for the diagnosis as well as treatment of cancers [5]. A study reported that lncRNA SNHG4 can foster the proliferation,

migration, invasion, and epithelial-mesenchymal transition (EMT) and affect other malignant behaviors in gastric cancer cells [6]. However, whether lncRNA SNHG4 can affect the progression and immune escape of CRC has not yet been confirmed.

MicroRNAs (miRNAs), about 22 nucleotides in length, belong to a type of small noncoding RNAs that do not have the protein-coding capacity. It is generally known that miRNAs are competitively occupied by lncRNAs. miRNAs can also bind to mRNAs, leading to the degradation of target mRNAs, which consequently represses corresponding protein synthesis. In this way, the lncRNAs-miRNAs-mRNA regulatory network is established [7]. Many studies have reported that miRNAs participate in the progression and immune escape of cancer. For example, miR-200c-loaded nanoparticles can significantly downregulate the expression of PD-L1, inhibit immune escape, and reverse the epithelial-mesenchymal phenotype in gastric cancer cells [8]. Another study has found that miR-144-3p can target Bcl6, leading to the inhibition of CRC cells' proliferation by suppressing Wnt signaling [9]. Whether miR-144-3p can regulate PD-1/PD-L1 or play a role in the immune escape of CRC has not yet been elucidated.

MET, also known as c-Met or hepatocyte growth factor (HFG) receptor, is a tyrosine kinase. This study revealed that MET was a downstream target gene of miR-144-3p. A previous study showed that cyclic AMP-responsive element-binding protein 5 (CREB5) could induce the invasion and metastasis of CRC cells by activating MET [10]. Another study has reported that PD-L1 can upregulate MET expression in esophageal cancer tissues, and MET is involved in the immune escape of cancer cells [11].

Collectively, this study focused on exploring the regulation of lncRNA SNHG4 on miR-144-3p and MET, which participates in the immune escape and malignant progression of CRC via immune checkpoint PD-1/PD-L1.

Materials and methods

Tissue isolation

A total of 70 CRC tissues and corresponding adjacent normal tissues were obtained from Sichuan Mianyang 404 Hospital. Normal tissues were obtained from more than 3 cm away from the primary lesion. Patients who had received radiological or chemical treatment were not included in this study. All patients signed the informed consent. Our research was approved by the Ethics Committee of Sichuan Mianyang 404 Hospital. After isolation, the tissues were subjected to snap-frozen using liquid nitrogen and stored at -80°C for later use.

Bioinformatic analysis

The chip data were analyzed by Gene Expression Omnibus (GEO) database to obtain differentially expressed lncRNAs in CRC tissues. The expression of lncRNA SNHG4 in CRC was analyzed by Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/detail.php?gene). StarBase (http://starbase.sysu.edu.cn/) and LncBook (http://bigd.big.ac.cn/lncbook/lncrnas) databases were leveraged to acquire the target miRNAs of lncRNA SNHG14. miR-144-3p downstream target genes were studied by TargetScan (http://targetscan.org/vert_72/), StarBase (http://starbase.sysu.edu.cn/), and miRDB (http://mirdb.org) databases. KEGG analysis was performed by using DAVID database (http://david.ncifcrf.gov). The DisGeNET database (http://disgenet.org/search) was applied to search for risk genes relevant to CRC. The protein interaction was analyzed by STRING (http://string-db.org/cgi/input?sessionId=bQ1pVjvgOs39&input_page_show_search=on) database, while the immune analysis of genes was conducted by Timer (http://cistrome.shinyapps.io/timer/) database.

Cell culture

Normal colon cell line NCM4600 and human colon cancer cell lines (LOVO, CACO2, HT29, and SW620) were obtained from the Query Network for Microbial Species of China. All cells were cultured in an incubator with Dulbecco's modified Eagle medium (DMEM) (D5796, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) under the condition of 5% CO_2 and 37°C. Small interfering RNA (siRNA) plasmids for gene knockdown, pcDNA plasmids for gene overexpression, and miRNA mimics/inhibitors were provided by Shanghai Jima Pharmaceutical Co., Ltd. Lipofectamine 3000 transfection reagent (L3000001, Thermo Fisher, USA) was used to transfect CACO2 cells by following the manu-

Table 1. List of plasmid sequences

Name	Sequence (5'-3')
si-SNHG4	F: GTCAGCGAGCGAACCCAATTGGC
	R: CCGATCGGCAGCCGCGCGCGA
SNHG4	F: CCGCCGATAGCGAGCGACACCCCAAC
	R: AACCATCGAGCGGGGGCTCTCGCAAAC
MET	F: GATATCGCGCGATCACACACGGT
	R: AATCGCGCGCGAGCTCGCGCCTGG
miR-144-3p mimic	F: ACAGAGCATCAGCGCGCTTCTCC
	R: TACTGCCGCGCTCGGCGACCAAAC
miR-144-3p inhibitor	F: CCGCTATCAGCGATCTATCGCGCGCTC
	R: ACTTGGCTCTGCGTCATAGGACAATA

facturer's protocols. The sequences of siRNA, pcDNA, miRNA are provided in **Table 1**. Cells were collected 48 hours after transfection for subsequent experiments.

Fluorescence in situ hybridization (FISH)

The FISH probe of lncRNA SNHG4 was designed by Guangzhou Bersin Biotechnology Co., Ltd. (China). The FISH kit (Bes1001, Guangzhou Boxin, China) was used to perform hybridization experiments to determine the localization and expression of lncRNA SNHG4 according to the manufacturer's protocols, by which the staining results were observed under the microscope.

Dual-luciferase reporter assay

Wild-type (WT) plasmids (SNHG4-WT, MET-WT) and mutant (MUT) plasmids (SNHG4-MUT, MET-MUT) were constructed, which were cloned into the pGL3 promoter vector (VT1726, Hunan Youbio, China). CACO2 cells were co-transfected with miR-144-3p negative control (NC)/mimic and corresponding WT/MUT pGL3 plasmid, respectively, followed by the incubation in a 24-well culture plate. Lipofectamine 3000 transfection reagent (L3000001, Thermo Fisher, USA) was applied for the transfection based on manufacturer's procedures. After 48 hours, a dual-luciferase reporter gene detection kit (GM-040501, Shanghai Jiman Biotechnology Co., Ltd., China) was applied to measure the relative luciferase activity of each group of cells.

Quantitative real-time PCR (qRT-PCR)

TRIzol RNA extraction reagent (15596026, Thermo Fisher, USA) was used to isolate RNAs from tissues and cells, followed by the application of a cDNA synthesis kit (11917010, Thermo Fisher, USA) for reverse transcription and 2× SYBR Green qPCR MasterMix (Beijing Sevenbio Innovation Biotechnology Co., Ltd., China) for PCR amplification. The ABI7000 detection system was used to stop the amplification (Thermo Fisher, USA). All steps were carried out based on the protocols in the kit. The relative expression level was quantified using $2^{-\Delta\Delta Ct}$ method. GAPDH and U6 expression levels were used as internal references. The primers were provided by Nanjing GenScript Biotechnology Co., Ltd. (China), which are listed in **Table 2**.

Cell counting kit-8 (CCK-8)

The CCK8 assay was performed to detect cell proliferation. Transfected cells were inoculated in a 96-well culture plate at a density 1×10^3 cells/well, which were cultured in an incubator. CCK8 solution (10 ml) was added to each well at 0 h, 24 h, 48 h, 72 h, and 96 h (CK04, Dojindo Laboratories, Japan). After incubation for 2 hours, the OD values at 450 nm wavelength were measured using a multimode microplate reader (Thermo Fisher, USA).

Clone formation assay

Transfected cells were cultured in a 6-well plate at a density of 5×10^2 cells/well, which were incubated in an incubator with 5% CO_2 at 37°C. After 2 weeks of culture, cells were fixed with 4% paraformaldehyde (PFA) followed by staining with 0.1% crystal violet (G1063, Beijing Solarbio Science & Technology Co., Ltd., China). Colony formation was observed and counted under a microscope.

Transwell migration assay

The pre-chilled Matrigel (354230, Shanghai Qcbio Science & Technology Co., Ltd., China) was placed in the upper chamber for the inoculation of CACO2 cells. The culture medium containing 10% FBS was transferred to the lower chamber. The whole Transwell chamber was incubated for 24 hours, after which the cells under the membrane were fixed by PFA then stained using 0.1% crystal violet. The cell invasion was observed under a microscope, and the number of invasive cells was counted.

Table 2. List of primer sequences

Gene name	Sequence (5'-3')
LncRNA SNHG4	F: AGTGTCGGCCGCATGCTACGG
	R: TCAAACGATCCGCGCTACGAGCG
miR-144-3p	F: GTCATACGAGCTACGAGCATCGA
	R: GTCAGCTACGCGAAACTAGCT
MET	F: GAAATCAGCTACGACTCG
	R: GTAAACTAGTCTCTTTATAT
PD-L1	F: AGTAGATCCTAGCTATCACGAGCT
	R: CTAGCTACGACGCGCGATCTACG
U6	F: AAATCGCGCTCAGCGACGATC
	R: CAACCATCGACTACGAGCT
GAPDH	F: CTCAGCATCGACGATCACGC
	R: CTAGCTGCATCGATCAGCGTC

Note: PD-L1: programmed death-ligand 1.

Flow cytometry

The transfected CACO2 cells were co-cultured with CD4+ T cells (PB009-2-C, AllCells Biotechnology Co., Ltd., Shanghai, China), followed by the addition of PD-1 antibody (ab52587, Abcam, UK) and PD-L1 antibody (ab205921, Abcam, UK). CD4+ T cell isolation kit (130-096-533, Beijing Nuowei Biotechnology Co., Ltd., China) was used to isolate CD4+ T cells from CACO2 cells [12]. Based on manufacturer's instructions, the percentage and apoptosis of CD4+ T cells were detected using Annexin V-FITC/PI Apoptosis Detection Kit (AD10, Tongren Chemical, Japan).

Western blotting

RIPA lysis buffer (R0278, Sigma-Aldrich, USA) was used to extract proteins from each group of cells, and the BCA method was applied for quantitative analysis. Isolated proteins were separated using a sodium dodecyl sulfate polyacrylamide gel (10%) electrophoresis (SDS-PAGE), which were transferred to a polyvinylidene fluoride (PVDF) membrane (IPVH00010, Sigma-Aldrich, USA). Then, the membrane was incubated by 5% non-fat milk for blocking. After one-hour blocking, the primary antibody MET (ab51067, Abcam, UK), PD-L1 (ab205921, Abcam, UK) or GAPDH (ab8245, Abcam, UK) was added to the membrane for overnight incubation at 4°C. The next day, the horseradish peroxidase (HRP)-conjugated secondary antibody IgG (ab133470, Abcam, UK) was added to incubate for 1 hour, after which chemiluminescence detection reagent (32209, Thermo Fisher, USA) was applied to visualize the protein bands. ImageJ software was used for the semi-quantification by analyzing the gray values of corresponding bands.

Statistical analysis

SPSS23.0 version software was applied for data analysis. Counted data in clinicopathological characteristics analysis were represented as n, and were tested by chi-square test or exact probability test. The quantitative data were expressed as mean ± standard deviation. If the data were normally distributed, the data analysis between the two groups was processed with a T-test, while the data analysis among multiple groups was done by the one-way analysis of variance (ANOVA). If the data did not follow normal distribution, the Mann-Whitney U test was used for data analysis between the two groups, while the Bonferroni method was used for data analysis between multiple groups. $P<0.05$ was considered significant.

Results

LncRNA SNHG4 expression is upregulated in CRC

Differentially expressed lncRNAs in 6 CRC tissues and corresponding normal tissues were firstly analyzed by microarray analysis, and the results showed that 434 lncRNAs were upregulated and 617 were downregulated in CRC (**Figure 1A**). The top 100 differentially expressed lncRNAs were selected to draw a heat map (**Figure 1B**), which showed that NR_003141 (lncRNA SNHG4) was significantly upregulated in CRC tissues. The GEPIA database also showed that lncRNA SNHG4 expression in colon adenocarcinoma (COAD) was significantly upregulated than that in the normal control group (**Figure 1C**). LncRNA SNHG4 expression in CRC was further verified through experiments and the results demonstrated that compared with adjacent normal tissues, lncRNA SNHG4 in CRC tissues was significantly upregulated (**Figure 1D**, $P<0.05$), which was consistent with results in cells. Moreover, the highest expression of lncRNA SNHG4 was found in CACO2 cells, which were selected for subsequent analysis (**Figure 1E**, all $P<0.05$). The receiver operator characteristic (ROC) figure showed that lncRNA SNHG4 was highly sensitive to the diagnosis of CRC (**Figure 1F**,

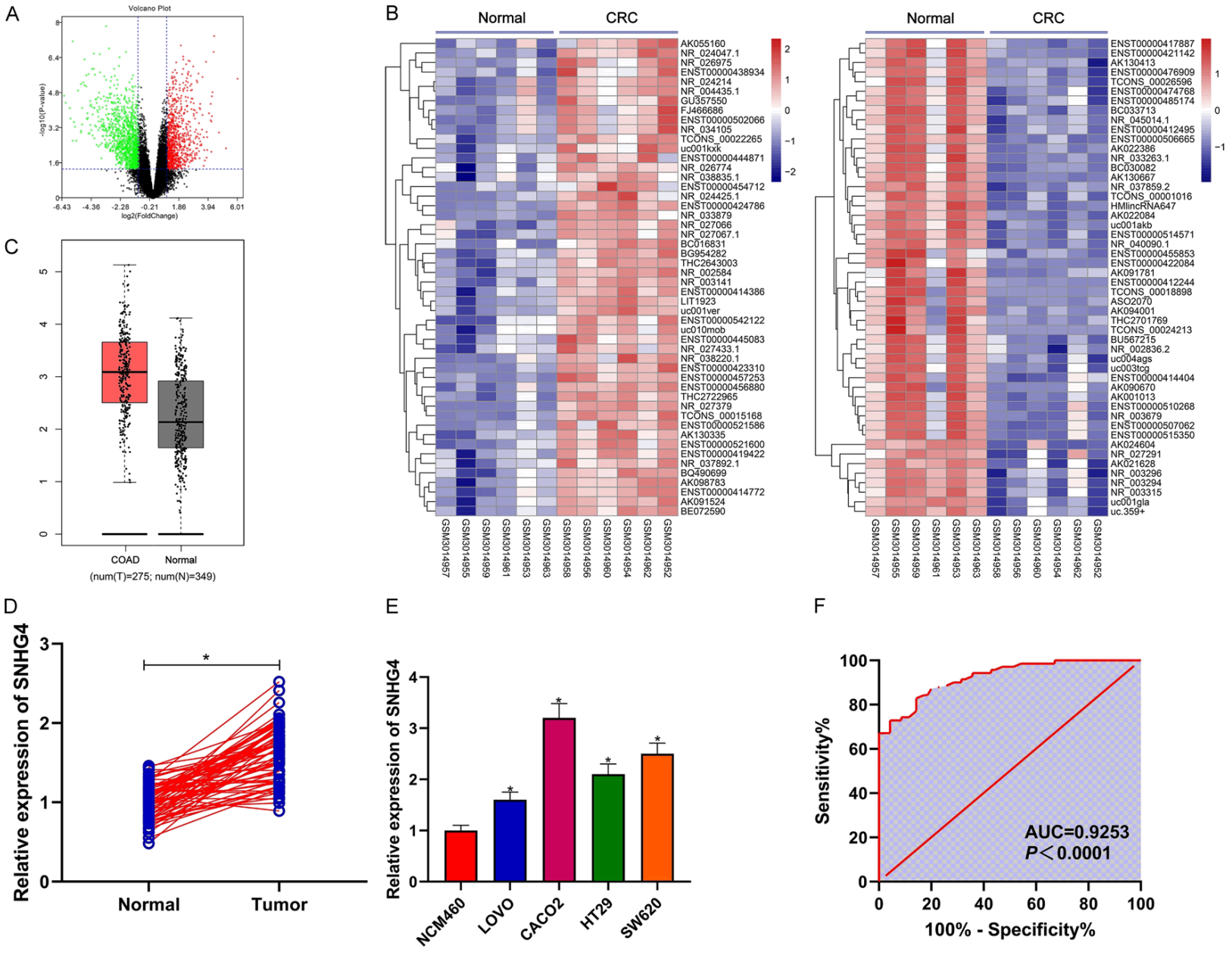

A
Volcano Plot
-log10(P-value)
log2(FoldChange)
B
Normal
CRC
C
COAD
Normal
(num(T)=275; num(N)=349)
D
Relative expression of SNHG4
Normal
Tumor
E
Relative expression of SNHG4
NCM460
LOVO
CACO2
HT29
SW620
F
Sensitivity%
100% - Specificity%
AUC=0.9253
P＜0.0001

Figure 1. LncRNA SNHG4 may be correlated to the progression of CRC. A: Volcano map to show 434 upregulated lncRNAs and 617 downregulated lncRNAs in CRC; B: The top 100 differentially expressed lncRNAs to draw a heat map; C: GEPIA database to show the significantly upregulated expression of lncRNA SNHG4 in COAD; D: qRT-PCR to detect the expression of lncRNA SNHG4 in CRC tissues; E: qRT-PCR to detect the expression of lncRNA SNHG4 in CRC cells; F: ROC curve to show the sensitivity of lncRNA SNHG4 in the diagnosis of CRC. Compared with normal tissues and cells, *P<0.05. CRC: colorectal cancer; lncRNAs: long non-coding RNAs; GEPIA: gene expression profiling interactive analysis; COAD: colon adenocarcinoma; qRT-PCR: quantitative real-time PCR; ROC: receiver operator characteristic.

Table 3. Correlation between lncRNA SNHG4 expression and clinicopathologic characteristics

Clinicopathologic data	Case	SNHG4 expression		P
		High (n)	Low (n)	
Total	70	36	34	
Age (years)				0.3387
<65	34	15	19	
≥65	36	21	15	
Gender				0.2927
Male (n)	20	8	12	
Female (n)	50	28	22	
TNM staging				0.0112
I-II (n)	23	17	6	
III-IV (n)	47	19	28	
Carcinoembryonic antigen level				0.4556
<5 ng/mL (n)	25	11	14	
≥5 ng/mL (n)	45	25	20	
Tumor size (cm)				0.0166
<5 (n)	29	20	9	
≥5 (n)	41	16	25	
Lymphovascular invasion				0.4556
No (n)	45	25	20	
Yes (n)	25	11	14	

P<0.05). Based on the median expression level of lncRNA SNHG4 in the tissues of these 70 patients, the tissues were grouped into a high lncRNA SNHG4 expression group and low lncRNA SNHG4 expression group. The analysis of the correlation between lncRNA SNHG4 expression and clinicopathologic characteristics revealed that lncRNA SNHG4 expression was correlated to TNM staging and tumor sizes (**Table 3**). These results indicated that lncRNA SNHG4 may be involved in CRC development.

Knockdown of lncRNA SNHG4 can inhibit cell growth and invasion

LncRNA SNHG4 silencing plasmid was designed to study the effect of lncRNA SNHG4 on the proliferation and invasion of CACO2 cells. First, the transfection efficiency of the knockdown was tested. In comparison to the si-NC group, si-SNHG4 had significantly downregulated lncRNA SNHG4 expression level (**Figure 2A**, P< 0.05). CCK8, Clone formation assay, and Transwell migration assay indicated that cell proliferation, colony formation, and invasion of the si-SNHG4 group were all significantly inhibited in comparison to the si-NC group (**Figure 2B-D**, P<0.05). The data revealed that lncRNA SNHG4 knockdown suppressed cell growth and invasion.

LncRNA SNHG4 functions by targeting miR-144-3p

Many studies reported that lncRNA was localized to the cytoplasm, and it functioned as a sponge of miRNA to play a role in cancer. First, the FISH experiment verified that lncRNA SNHG4 was highly expressed in the cytoplasm (**Figure 3A**), where it can play a role as the sponge of miRNAs. The target miRNAs of lncRNA SNHG4 were derived from the online predictions of LncBook and StarBase, which were intersected and visualized by a Venn diagram (**Figure 3B**) including hsa-miR-500b-5p, hsa-miR-1277-5p, hsa-miR-144-3p, hsa-miR-186-5p, hsa-miR-511-3p, hsa-miR-499b-5p, and hsa-miR-362-5p. In these target miRNAs, studies reported that hsa-miR-144-3p was downregulated in CRC, so hsa-miR-144-3p was selected for this study [13]. Based on the specific binding site of lncRNA SNHG4 and miR-144-3p (**Figure 3C**), a dual-luciferase reporter assay was designed. The results showed that compared with the SNHG4-WT+miR-144-3p NC group, the luciferase activity of the SNHG4-WT+miR-144-3p mimic group was notably decreased (**Figure 3D**, P<0.05). The detection of miR-144-3p expression in both cell lines and tissues revealed that miR-144-3p expression in CRC tissues was sig-

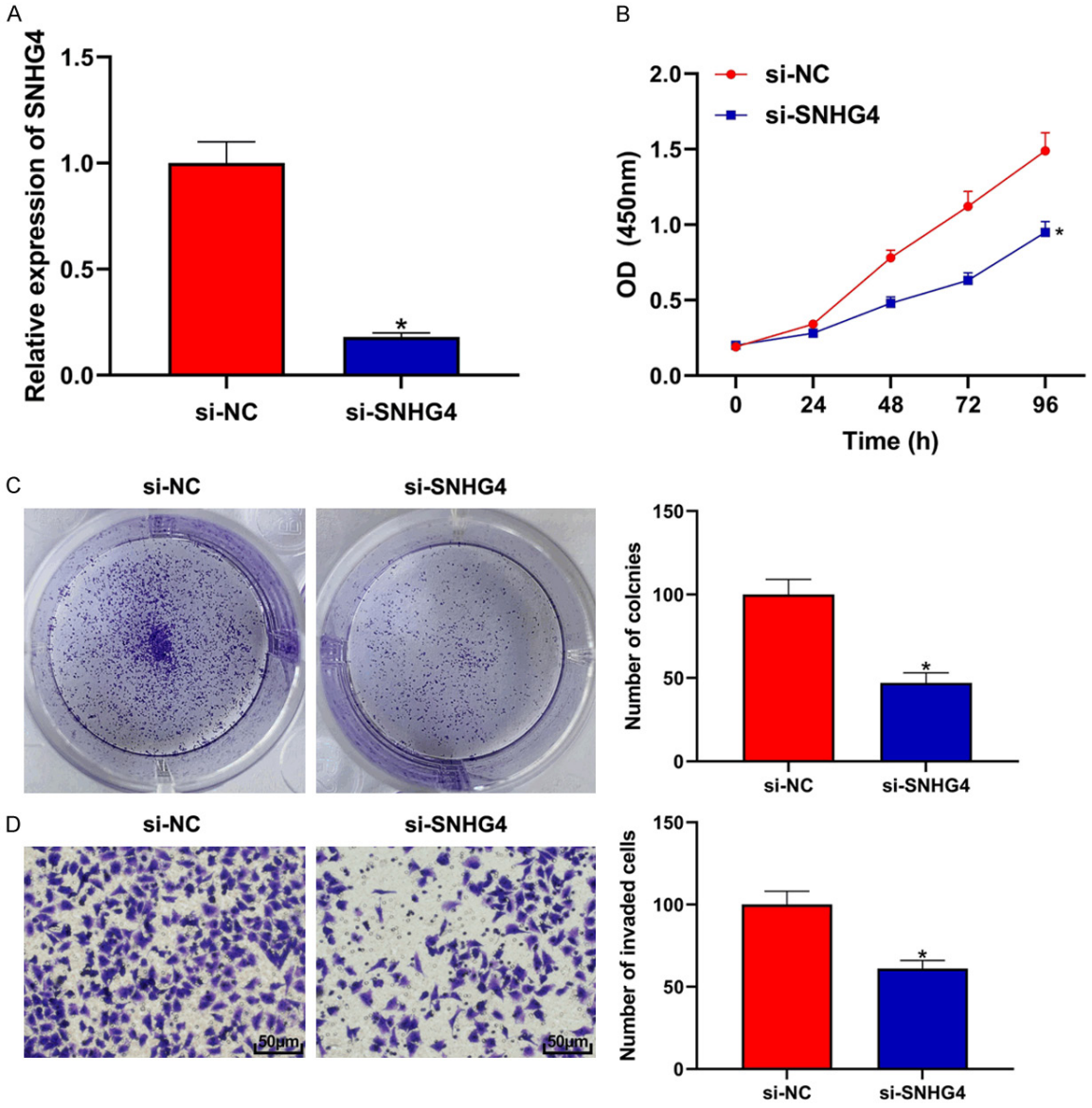


Figure 2. Knockdown of lncRNA SNHG4 can inhibit cell growth and invasion. A: Transfection efficiency of silencing lncRNA SNHG4; B: CCK8 to detect the cell proliferation; C: Clonogenic assay to detect cell colony formation; D: Transwell assay to detect cell invasion (×200). Compared with the si-NC group, *P<0.05.

nificantly downregulated compared to the corresponding normal tissues (**Figure 3E**, P<0.05). Compared with NCM460 cells, miR-144-3p expression was all significantly downregulated in CRC cell lines, of which CACO2 cells showed the most significant downregulation of miR-144-3p expression (**Figure 3F**, all P<0.05). Correlation analysis revealed that miR-144-3p was negatively correlated with lncRNA SNHG4 expression (**Figure 3G**, P<0.05). Knockdown of lncRNA SNHG4 upregulated miR-144-3p expression (**Figure 3H**, P<0.05). These data demonstrated that lncRNA SNHG4 targets miR-144-3p and plays a regulatory role in CRC.

LncRNA SNHG4/miR-144-3p induces interaction between CACO2 cells and CD4+ T cells and triggers CD4+ T cell apoptosis through the PD-1/PD-L1 immune checkpoint

Studies showed that SNHG1 of the SNHG family could promote the immune escape of breast cancer cells [14]. Another member of the SNHG family in this study, lncRNA SNHG4, may also

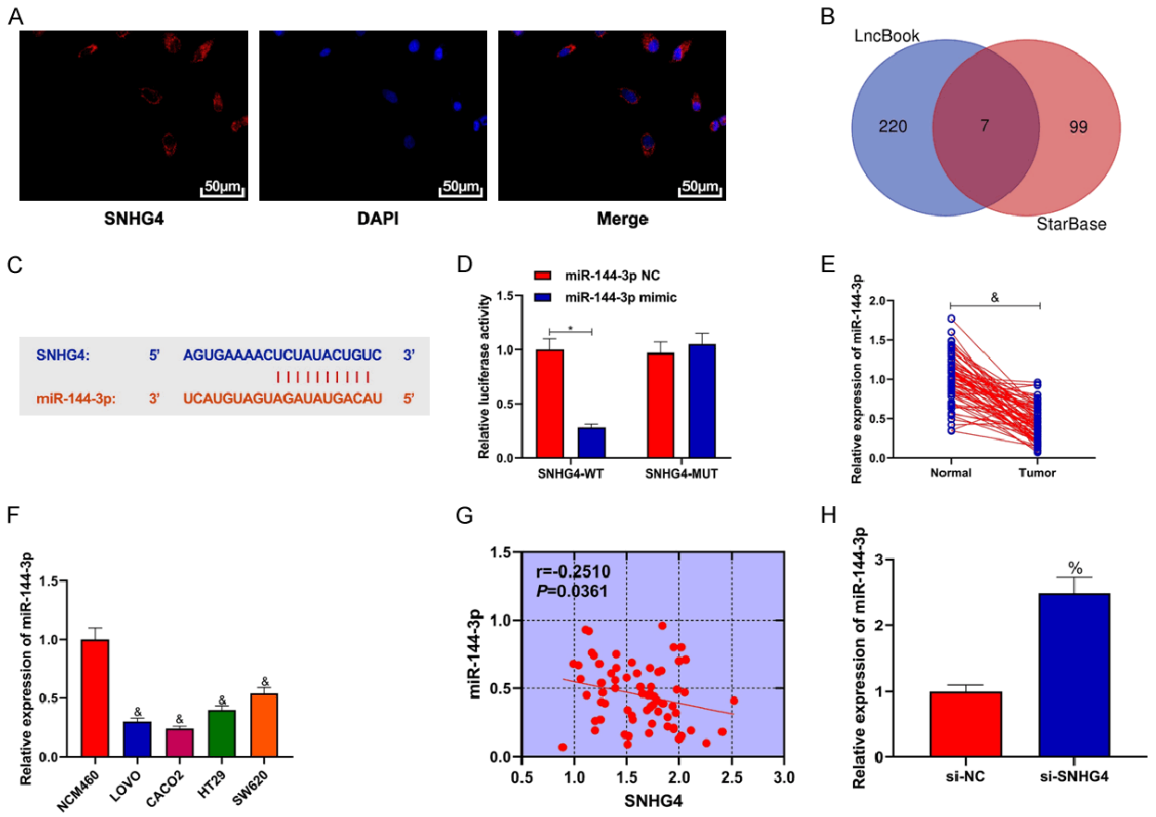
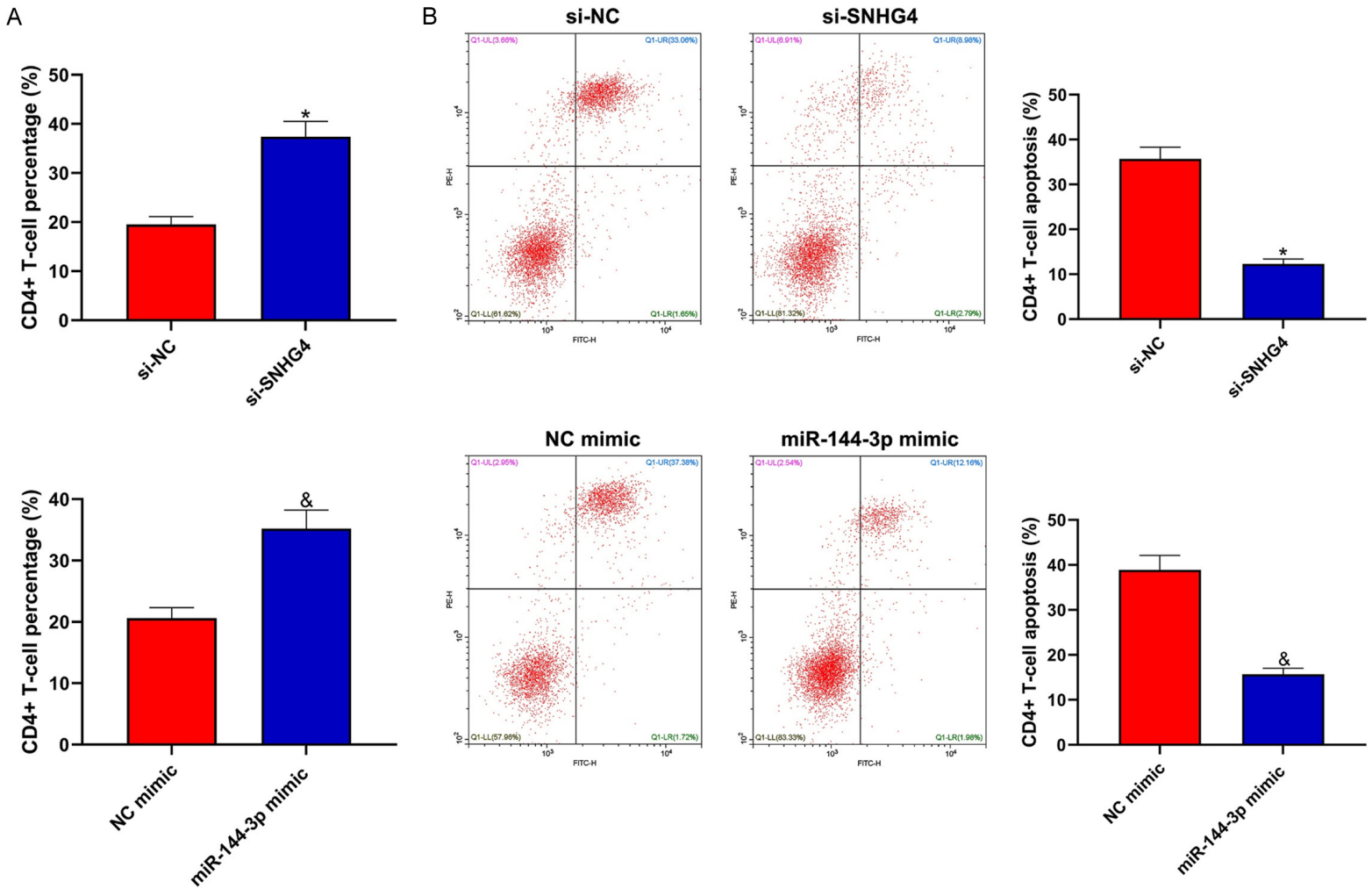


Figure 3. Verification of a targeting relationship between lncRNA SNHG4 and miR-144-3p. A: FISH experiment to detect the localization and expression of lncRNA SNHG4 (×200); B: Overlapped target miRNAs of lncRNA SNHG4; C: Specific binding site of lncRNA SNHG4 and miR-144-3p; D: Dual-luciferase reporter assay to verify the targeting relationship; E: qRT-PCR to detect the expression of miR-144-3p in CRC tissue; F: qRT-PCR to detect the expression of miR-144-3p in CRC cells; G: LncRNA SNHG4 and miR-144-3p correlation analysis; H: Effect of knockdown of lncRNA SNHG4 on the expression of miR-144-3p. Compared with the miR-144-3p NC group, *P<0.05; compared with normal tissues and cells, &P<0.05; compared with the si-NC group, %P<0.05. FISH: fluorescence in situ hybridization; CRC: colorectal cancer; qRT-PCR: quantitative real-time PCR.

be correlated to the immune escape of cancer cells. CACO2 cells and CD4+ T cells were co-cultured to mimic the tumor microenvironment, by which the apoptosis and survival rate of the cells were evaluated by flow cytometry. The results showed that either knockdown of lncRNA SNHG4 or miR-144-3p overexpression considerably increased the percentage of CD4+ T cells and decreased the apoptosis rate of CD4+ T cells (**Figure 4A**, **4B**, all P<0.05). PD-1/PD-L1 was used as an immune checkpoint, which usually induces CD4+ T cell apoptosis and triggers immune escape. In this study, the PD-1/PD-L1 antibody was added to the co-culture of CACO2 cells and CD4+ T cells to block the effect of PD-1/PD-L1. The data revealed that either lncRNA SNHG4 overexpression or inhibition of miR-144-3p reduced the percentage of CD4+ T cells while increasing the apoptosis rate of CD4+ T cells. These phenomena were partially reversed after the addition of PD-1/PD-L1 antibody (**Figure 4C**, **4D**, all P<0.05). Our data indicated that lncRNA SNHG4/miR-144-3p induced an interaction between CACO2 cells and CD4+ T cells, and triggered CD4+ T cell apoptosis through the PD-1/PD-L1 immune checkpoint.

LncRNA SNHG4/miR-144-3p targets and regulates MET

To explore how lncRNA SNHG4/miR-144-3p regulates PD-1/PD-L1, StarBase, TargetScan, and miRDB online prediction databases were first leveraged to find the downstream target genes of miR-144-3p. A Venn diagram was established to visualize the overlapped genes (**Figure 5A**). A total of 573 potential targets were analyzed by KEGG pathway (**Figure 5B**), by which the genes in cancer-related pathways


A
B
si-NC
si-SNHG4
CD4+ T-cell percentage (%)
CD4+ T-cell apoptosis (%)
NC mimic
miR-144-3p mimic
*
&
PE-H
FITC-H

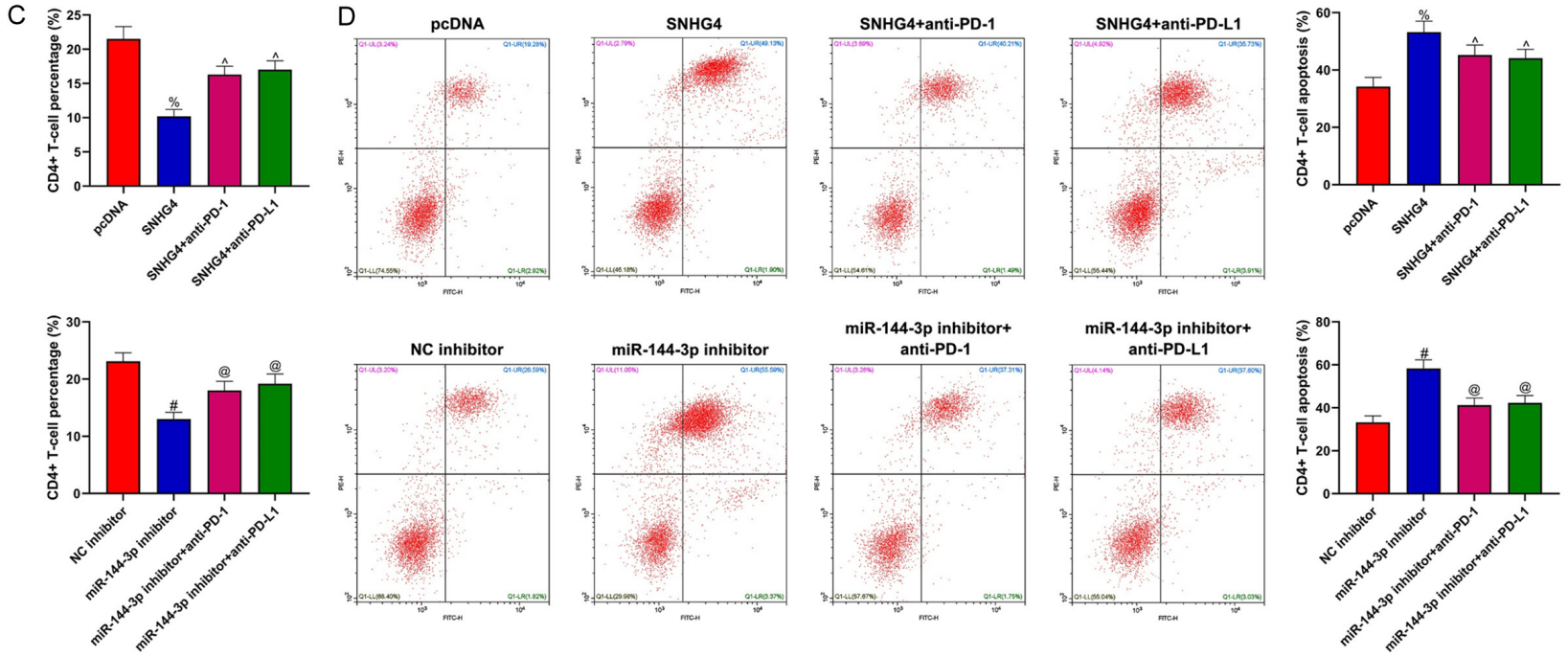


Figure 4. LncRNA SNHG4/miR-144-3p induces an interaction between CACO2 cells and CD4+ T cells and CD4+ T cell apoptosis through the PD-1/PD-L1 immune checkpoint. A: Flow cytometry to detect the percentage of CD4+ T cells; B: Flow cytometry to detect the apoptotic rate of CD4+ T cells; C: Flow cytometry to detect the effect of PD-1/PD-L1 antibody on the percentage CD4+ T cells; D: Flow cytometry to detect the effect of PD-1/PD-L1 antibody on the apoptotic rate of CD4+ T cells. Compared with the si-NC group, $^{*}P<0.05$; compared with the NC mimic group, $^{\&}P<0.05$; compared with the pcDNA group, $^{\%}P<0.05$; compared with the SNHG4 group, $^{\wedge}P<0.05$; compared with the NC inhibitor group, $^{\#}P<0.05$; compared with the miR-144-3p inhibitor group, $^{@}P<0.05$.

were selected for further investigation. The top 5 risk genes for CRC derived from DisGeNET and KEGG pathway-enriched genes were subjected to the gene-disease association analysis, which showed that PTEN, MET, FOS, and FN1 were all strongly associated with CRC risk genes (**Figure 5C**). Previous studies found that MET played a role in the promotion of CRC, thus, MET was selected as the target of this study [15]. The targeted regulatory relationship between miR-144-3p and MET was verified by Dual-luciferase reporter assay. The data demonstrated that the luciferase activity of cells in the MET-WT+miR-144-3p mimic group was remarkably decreased in comparison to the MET-WT+miR-144-3p NC group (**Figure 5D**, P<0.05). The analysis of qRT-PCR showed that MET expression in CRC tissue was significantly upregulated from that of the corresponding normal tissues (**Figure 5E**, P<0.05). In comparison to NCM460 cells, MET expression in CRC cell lines was all upregulated (**Figure 5F**, all P<0.05). Pearson correlation coefficient analysis demonstrated that MET was positively correlated with lncRNA SNHG4 expression in CRC tissues, but it was negatively correlated with miR-144-3p expression (**Figure 5G**, all P<0.05).

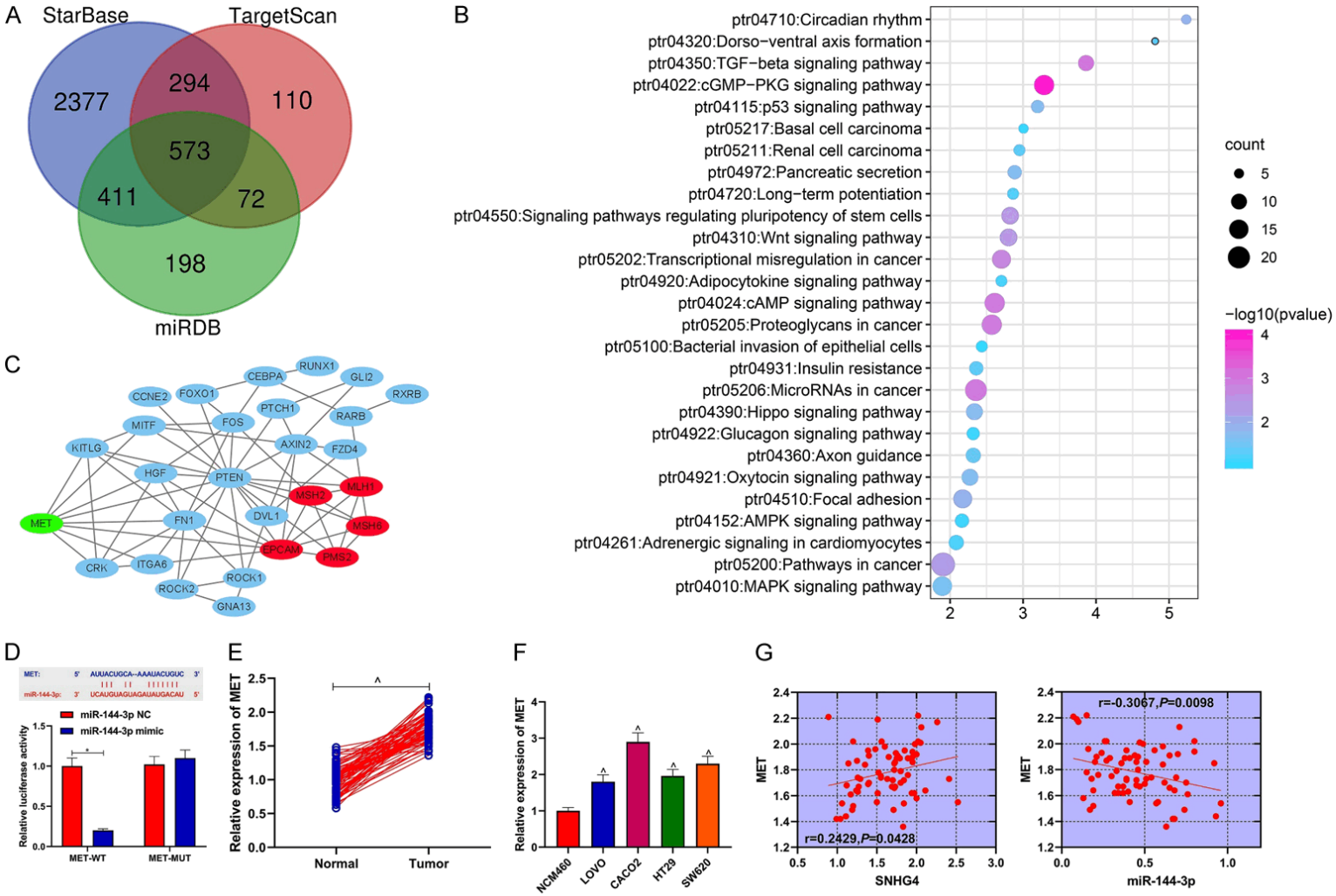
In addition, this study found that MET was related to immune infiltration and showed the strongest correlation with CD4+ T cells (**Figure 5H**), suggesting that it facilitates the attraction of more immune cells to exert immune responses in tumors. Studies showed that the expression of MET and PD-L1 had a significant correlation in metastatic melanoma, and PD-L1 overexpression can considerably enhance the expression level of MET [16, 17]. This study found that knockdown of lncRNA SNHG4 downregulated the mRNA and protein expressions of MET and PD-L1, while the addition of miR-144-3p inhibitor partially compromised this effect (**Figure 5I**, all P<0.05). These results demonstrated that lncRNA SNHG4 targeted and regulated MET/PD-L1 through miR-144-3p in CRC.

LncRNA SNHG4 inhibits CD4+ T cell activity through MET and promotes the progression of CACO2 cells

A rescue experiment was performed to study whether lncRNA SNHG4 can affect the progression of CRC by regulating MET using CACO2 cells. First, it was confirmed that knockdown of lncRNA SNHG4 downregulated MET and PD-L1 expression, while overexpression of MET partially upregulated their expression (**Figure 6A**, all P<0.05). After CACO2 cells were co-cultured with CD4+ T cells, flow cytometry revealed that lncRNA SNHG4 knockdown upregulated the percentage of CD4+ T cells and reduced the rate of CD4+ T cell apoptosis. Overexpression of MET partially reversed this effect (**Figure 6B**, all P<0.05). CCK8 assay demonstrated that lncRNA SNHG4 knockdown inhibited the cell proliferation, while overexpression of MET partially reversed this effect (**Figure 6C**, all P<0.05). The clone formation assay showed that lncRNA SNHG4 knockdown inhibited cell colony formation, whereas MET overexpression partially reversed this effect (**Figure 6D**, all P<0.05). Transwell migration assay revealed that lncRNA SNHG4 knockdown inhibited cell invasion; however, overexpression of MET partially reversed this effect (**Figure 6E**, all P<0.05). These data indicated that lncRNA SNHG4 inhibited CD4+ T cell activity by regulating MET and promoted the progression of CACO2 cells.

Discussion

Many studies have reported that lncRNAs play a regulatory function in CRC. For example, lncRNA CASC15 can promote CRC by regulating miR-582-5p [18]. LncRNA FLVCR-AS1 promotes the development of CRC by regulating the miR-381/RAP2A axis [19]. Studies have found that other members of lncRNA SNHG4 homologous family are involved in the process of cancer immune escape. For instance, lncRNA SNHG14 regulates miR-152-3p to promote the progression and immune escape of diffuse large B-cell lymphoma [20]. Our study demonstrated that lncRNA SNHG4 expression was upregulated in CRC specimens through microarray and biological prediction analyses. Many previous studies have shown the carcinogenic effect of lncRNA SNHG4, such as lncRNA SNHG4 sponging miR-204-5p to upregulate the expression of RUNX2, resulting in the invasion and progression of renal cancer [21]. LncRNA SNHG4 induces the growth and invasion of neuroblastoma by functioning as the sponge of miR-377-3p [22]. Our paper is the first to investigate the role of lncRNA SNHG4 in CRC, which shows that knockdown of lncRNA SNHG4 can repress CRC cell proliferation and invasion, as well as inhibit CD4+ T cell apoptosis and immune escape. PD-1 antibody or PD-L1 antibody both can suppress the inhibitory effect of silencing lncRNA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miRDB
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B
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ptr04320:Dorso−ventral axis formation
ptr04350:TGF−beta signaling pathway
ptr04022:cGMP−PKG signaling pathway
ptr04115:p53 signaling pathway
ptr05217:Basal cell carcinoma
ptr05211:Renal cell carcinoma
ptr04972:Pancreatic secretion
ptr04720:Long−term potentiation
ptr04550:Signaling pathways regulating pluripotency of stem cells
ptr04310:Wnt signaling pathway
ptr05202:Transcriptional misregulation in cancer
ptr04920:Adipocytokine signaling pathway
ptr04024:cAMP signaling pathway
ptr05205:Proteoglycans in cancer
ptr05100:Bacterial invasion of epithelial cells
ptr04931:Insulin resistance
ptr05206:MicroRNAs in cancer
ptr04390:Hippo signaling pathway
ptr04922:Glucagon signaling pathway
ptr04360:Axon guidance
ptr04921:Oxytocin signaling pathway
ptr04510:Focal adhesion
ptr04152:AMPK signaling pathway
ptr04261:Adrenergic signaling in cardiomyocytes
ptr05200:Pathways in cancer
ptr04010:MAPK signaling pathway
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C
RUNX1
CEBPA
GLI2
RXRB
CCNE2
FOXO1
PTCH1
RARB
MITF
FOS
KITLG
AXIN2
FZD4
HGF
PTEN
MLH1
MSH2
MET
FN1
DVL1
MSH6
EPCAM
PMS2
CRK
ITGA6
ROCK1
ROCK2
GNA13
D
MET: 5' AUUACUGCA--AAAUACUGUC 3'
miR-144-3p: 3' UCAUGUAGUAGAUAUGACAU 5'
miR-144-3p NC
miR-144-3p mimic
Relative luciferase activity
MET-WT
MET-MUT
E
Relative expression of MET
Normal
Tumor
F
Relative expression of MET
NCM460
LOVO
CACO2
HT29
SW620
G
MET
SNHG4
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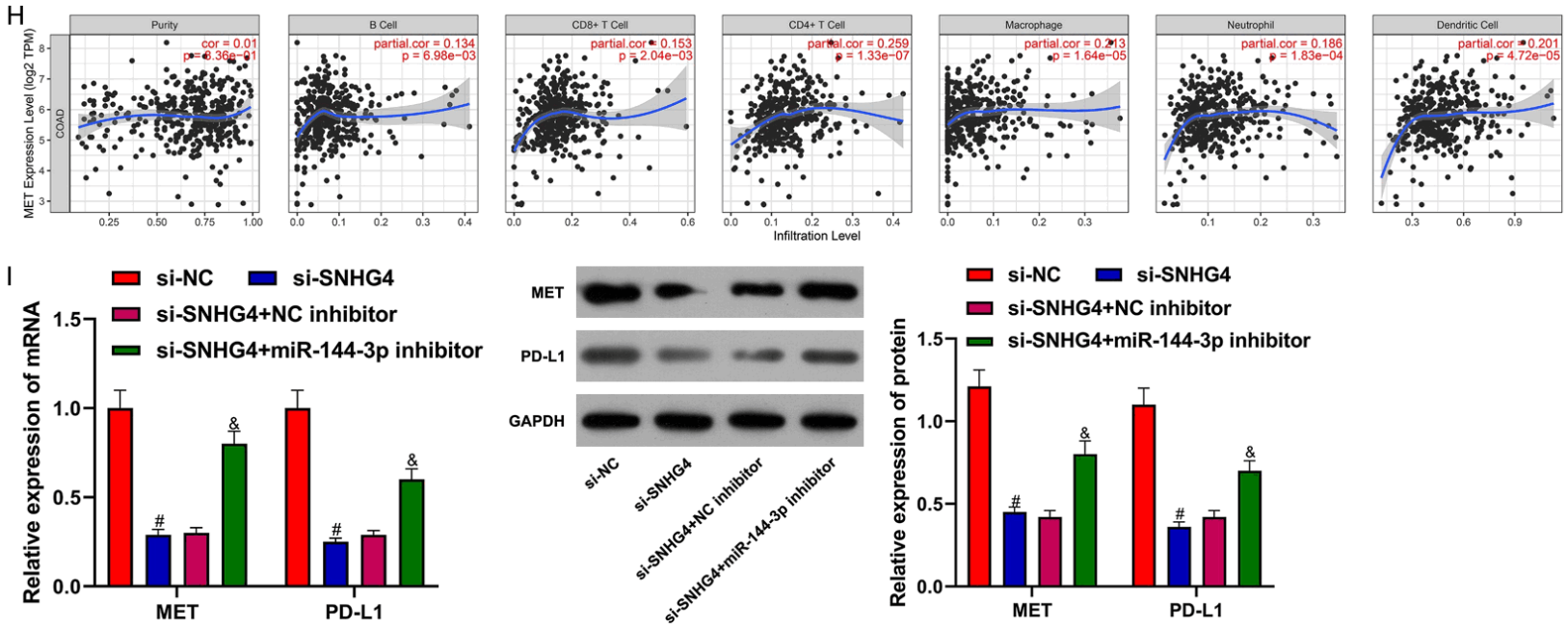
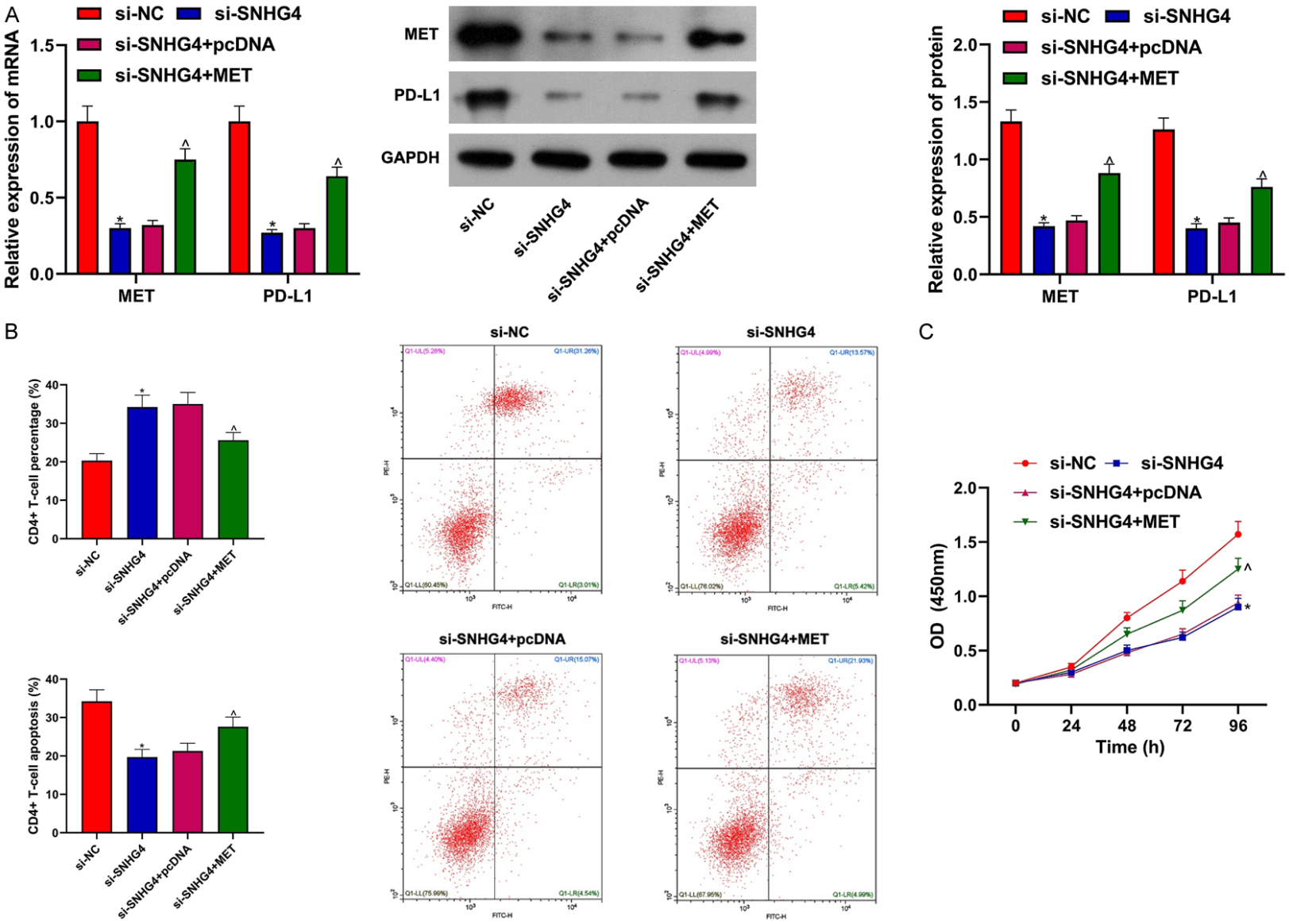


Figure 5. LncRNA SNHG4/miR-144-3p targets and regulates MET. A: Venn diagram to show the number of overlapped target genes of miR-144-3p; B: KEGG analysis of overlapped target genes; C: Analysis of the interaction between overlapped target genes and CRC risk genes (red representing CRC risk genes); D: The dual-luciferase report assay to verify the targeting relationship between miR-144-3p and MET; E: qRT-PCR to detect the expression of MET in CRC tissue; F: qRT-PCR to detect the expression of MET in CRC cells; G: Correlation analysis of MET and lncRNA SNHG4 and miR-144-3p; H: Immune analysis of MET using the Timer online website; I: The effect of lncRNA SNHG4 and miR-144-3p on the expression of MET/PD-L1. Compared with the miR-144-3p NC group, $^{*}P<0.05$; compared with normal tissues and cells, $^{\wedge}P<0.05$; compared with the si-NC group, $^{\#}P<0.05$; compared with the si-SNHG4+NC inhibitor group, $^{\&}P<0.05$. CRC: colorectal cancer; qRT-PCR: quantitative real-time PCR.


A
si-NC
si-SNHG4
si-SNHG4+pcDNA
si-SNHG4+MET
Relative expression of mRNA
MET
PD-L1
GAPDH
Relative expression of protein
B
CD4+ T-cell percentage (%)
CD4+ T-cell apoptosis (%)
C
OD (450nm)
Time (h)

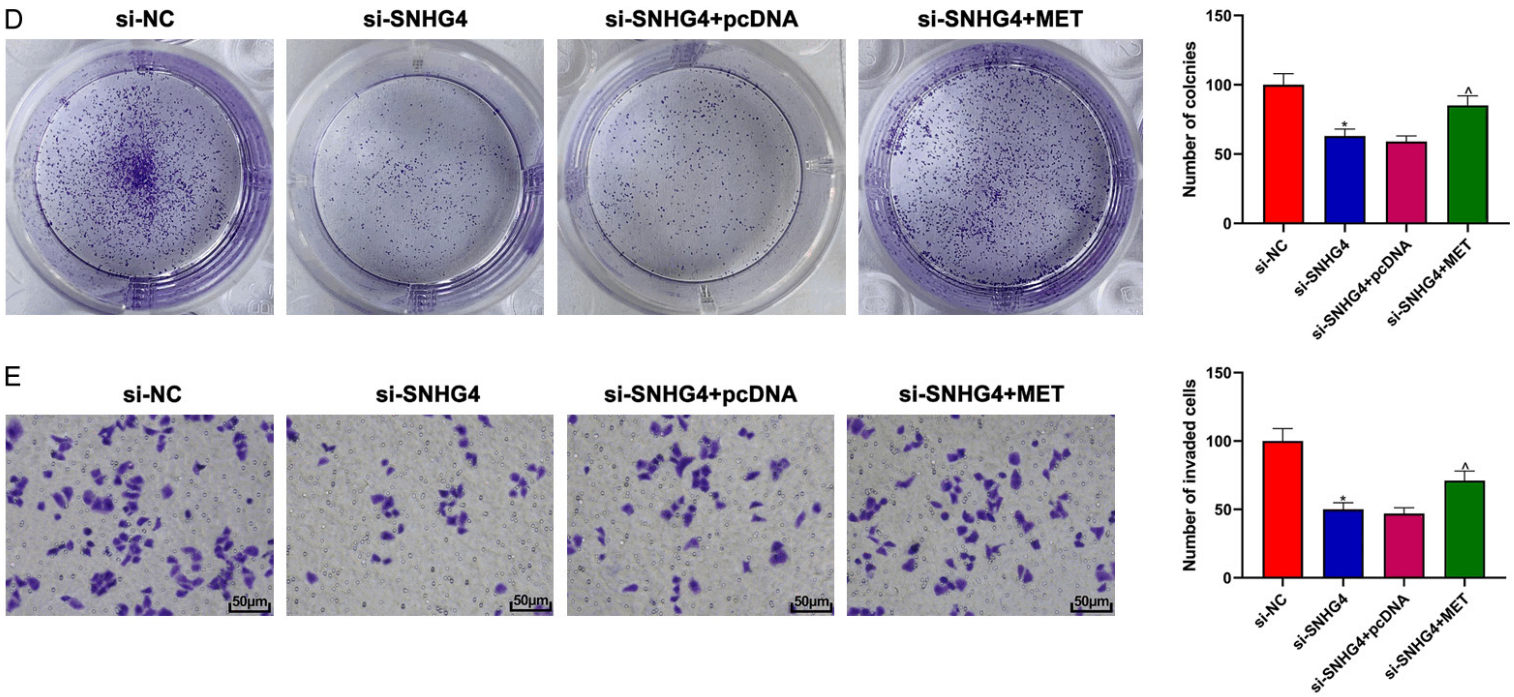


Figure 6. LncRNA SNHG4 inhibits CD4+ T cell activity through MET and promotes the progression of CACO2 cells. A: The effect of lncRNA SNHG4 on the expression of MET and PD-L1; B: Flow cytometry to analyze of CD4+ T cell activity and apoptosis; C: CCK8 to detect cell proliferation; D: Clonogenic assay to detect cell colony formation; E: Transwell migration assay to detect the cell invasion (×200). Compared with the si-NC group, *P<0.05; compared with the si-SNHG4+pcDNA group, ^P<0.05. PD-L1: programmed death-ligand 1.

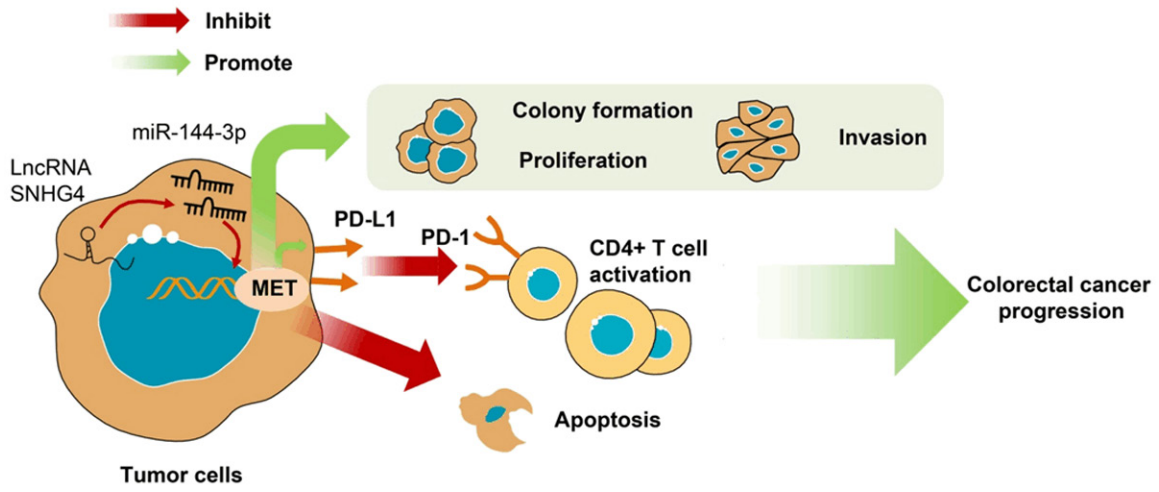


Figure 7. LncRNA SNHG4 regulates the miR-144-3p/MET axis to promote the immune escape and progression of colorectal cancer.

SNHG4 on immune escape, suggesting that knockdown of lncRNA SNHG4 inhibits immune escape by downregulating PD-1/PD-L1.

This study has also elucidated the mechanism of lncRNA SNHG4 in CRC. As shown in previous studies, lncRNA SNHG4 acted as a sponge to bind miR-206 and regulated the function of YWHAZ in cervical cancer [23]. Our study has confirmed that lncRNA SNHG4 targets and binds to miR-144-3p through both bioinformatics analysis and experiments as well as elucidated that miR-144-3p expression was downregulated in CRC samples and negatively correlated with the expression of lncRNA SNHG4. A previous study [9] has shown that miR-144-3p inhibits the proliferation of CRC cells by suppressing the Wnt/β-catenin signaling, which is consistent with our data. Our results have also revealed that miR-144-3p overexpression can inhibit the apoptosis of CD4+ T cells, and the addition of PD-1 antibody or PD-L1 antibody can compromise the inhibitory effect on immune escape by miR-144-3p overexpression.

In addition, this study has also found MET as a key regulatory factor by analyzing downstream targets of miR-144-3p. Studies have shown that MET is related to immune escape. The combination treatment with rapamycin and honokiol can inhibit the growth of renal cancer cells induced by c-MET and inhibit PD-L1 to repress immune escape [24]. SOX13 promotes CRC cell metastasis by activating SNAI2 and c-MET [25]. KPNB1 inhibits the growth of CRC tumors by downregulating the expression of MET [26]. This study has shown that MET and CD4+ T cells are positively correlated in CRC tissues through the Timer database analysis. Additionally, MET can accumulate more CD4+ T cells *in vivo*. Our study has also revealed that MET is highly expressed in CRC tissues, which is positively correlated with lncRNA SNHG4 expression, but is negatively correlated with miR-144-3p expression. The inhibitory effect on the malignant behavior and immune escape of CRC cells by silencing lncRNA SNHG4 can be partially compromised by the overexpression of MET, indicating that lncRNA SNHG4 promotes CRC progression and immune escape by upregulating MET.

However, this research still has some limitations. First, only cell line experiments were carried out, whereas the *in vivo* experiments would be required to verify the effect of lncRNA SNHG4. Secondly, this study only collected 70 pairs of tissues; therefore, the small sample size and certain differences between patients may bias the results. In addition, other mechanisms by which lncRNA SNHG4 functions warrant future studies.

In summary, lncRNA SNHG4 is upregulated in CRC, knockdown of lncRNA SNHG4 can inhibit the progression and immune escape of CRC cells, and lncRNA SNHG4 regulates PD-1/PD-L1 immune checkpoint through miR-144-3p/MET axis, leading to the progression of CRC (**Figure 7**). Our study may provide novel biomarkers for the diagnosis and targeted immunotherapy of CRC.

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Disclosure of conflict of interest

None.

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