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ORIGINAL RESEARCH

Long Non-Coding RNA C1QTNF1 Antisense RNA 1 Upregulates Hexokinase 2 by Sponging microRNA-484 to Promote the Malignancy of Colorectal Cancer

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Purpose: The long noncoding RNA *C1QTNF1 antisense RNA 1* (*C1QTNF1-AS1*) contributes to hepatocellular carcinoma development. However, its expression and roles in colorectal cancer (CRC) have not been fully explored. Therefore, this study determined the expression and roles of *C1QTNF1-AS1* in CRC and elucidated its detailed mechanism of action.

Methods: *C1QTNF1-AS1* expression in CRC tissues and cell lines was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). We used Cell Counting Kit-8, flow cytometry, cell migration and invasion assays, and a xenograft tumor model to test the effects of *C1QTNF1-AS1* on CRC malignancy. The associations among *C1QTNF1-AS1*, microRNA-484 (miR-484), and *hexokinase 2* (*HK2*) were explored using luciferase reporter assay, RNA immunoprecipitation, RT–qPCR, and Western blotting.

Results: *C1QTNF1-AS1* was overexpressed in CRC and related to poor prognosis. *C1QTNF1-AS1* interference inhibited CRC cell proliferation, migration, and invasion but induced apoptosis. Furthermore, *C1QTNF1-AS1* deficiency impaired tumor growth in vivo. Mechanistically, *C1QTNF1-AS1* adsorbed miR-484, thereby increasing the expression of its target *HK2*. Rescue experiments revealed that the effects of *C1QTNF1-AS1* deficiency in CRC cells were reversed by inhibiting miR-484 or upregulating *HK2*.

Conclusion: *C1QTNF1-AS1* drives CRC progression by sponging miR-484 and consequently upregulating *HK2*. The *C1QTNF1-AS1*/miR-484/*HK2* pathway may serve as a diagnostic and therapeutic target for CRC.

Keywords: competitive endogenous RNA, therapeutic target, hexokinase 2

Introduction

Colorectal cancer (CRC) is the third most frequent human cancer and the second leading cause of cancer-associated deaths globally.[1] Every year, ~1.2 million novel cases are diagnosed and 860,000 mortalities occur worldwide.[2] Surgical excision, chemoradiotherapy, and immunotherapy are the currently available effective therapeutic techniques for early stages of CRC.[3] Despite substantial improvements in diagnostic methods and therapies, the treatment outcomes of patients with advanced-stage CRC remain unsatisfactory.[4,5] Approximately 50–60% of CRC patients will experience recurrence and metastasis even after surgery, and it is now known that these are the major causes of death among patients with CRC.[6,7]

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Increased tumor suppressor gene inactivation and oncogene activation drive CRC pathogenesis by deregulating important signaling pathways; however, the detailed mechanisms have not been clearly elucidated.[8–10] Therefore, it is an essential and urgent need to acquire an in-depth comprehension of the molecular mechanisms associated with CRC oncogenesis and progression to develop promising new targets for CRC diagnosis, prognosis, and management.

Long noncoding RNAs (lncRNAs) comprise a family of transcripts longer than 200 nucleotides in length.[11] They lack protein-coding ability and are expressed in a highly context-specific pattern.[12] lncRNAs play key roles in normal physiological processes and pathological behaviors by controlling gene expression at the transcriptional and post-transcriptional levels.[13] In recent years, extensive studies have revealed that alterations in lncRNA expression are closely related to diverse human diseases, particularly cancers.[14] Increasing evidence has demonstrated that a number of lncRNAs are dysregulated in CRC, contributing to CRC genesis and progression.[15–17] LncRNAs can execute cancer-inhibiting or cancer-promoting activities during CRC oncogenesis and are directly involved in several malignant processes.[18,19]

MicroRNAs (MiRNAs) are a family of short noncoding RNA molecules with a length of approximately 17–24 nucleotides.[20] MiRNAs have been shown to actively regulate gene expression by base complementary pairing with the 3′-untranslated regions (3′UTRs) of their target genes, resulting in mRNA degradation or translational suppression.[21] Changes in miRNA expression in CRC have been widely reported, and their abnormal expression has been notably correlated with CRC tumorigenesis.[22–24] Recently, the competitive endogenous RNA (ceRNA) hypothesis was proposed and is now widely accepted.[25,26] lncRNAs as a type of ceRNA that work as molecular sponges for miRNAs, regulating target mRNAs at the posttranscriptional level.[27] Accordingly, a comprehensive understanding of the roles of lncRNAs and miRNAs in CRC may offer novel insights into CRC pathogenesis and thus improve the therapeutic options.

The lncRNA *C1QTNF1 antisense RNA 1* (*C1QTNF1-AS1*) has been reported to contribute to the tumorigenesis of hepatocellular carcinoma.[28,29] However, its expression and detailed functions in CRC are not well understood. This study aimed to determine *C1QTNF1-AS1*expression in CRC, investigate the important roles played by *C1QTNF1-AS1* in CRC cells, and elucidate the underlying molecular events.

Materials and Methods

Patients and Clinical Specimens

CRC tissues and corresponding adjacent normal tissues were collected from 71 patients admitted to the Jilin Cancer Hospital. No patients had been previously treated with preoperative anticancer therapies or presented with cancers other than CRC. The present study was approved by the Ethics Committee of Jilin Cancer Hospital and conducted in accordance with the Declaration of Helsinki. Moreover, written informed consent forms were collected from all participants. Following tissue excision, all tissues were immediately frozen and stored in liquid nitrogen until further use.

Cell Lines

Four human CRC cell lines, namely HCT116, HT29, SW480, and SW620, were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). HCT116 and HT29 cell lines were grown in McCOY's 5A medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific Inc.), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco; Thermo Fisher Scientific Inc.). L-15 medium (Gibco; Thermo Fisher Scientific Inc.) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin was used to culture the other two CRC cell lines.

A normal human colon epithelium cell line (FHC) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM:F12 medium (Gibco; Thermo Fisher Scientific Inc.) with 25 mM HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, 20 ng/mL human recombinant epidermal growth factor, and 10% FBS. The culture conditions for all cells were 95% humidity, 5% CO_2, and 37°C.

Small Interfering RNA (siRNA), Vector, miRNA Mimic, and miRNA Inhibitor Transfections

The siRNAs used to silence *C1QTNF1-AS1* expression (si-C1QTNF1-AS1) and negative control siRNA were designed and produced by RiboBio (Guangzhou, China). MiR-484

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mimic, negative control miRNA mimic (miR-NC), miR-484 inhibitor, and negative control (NC) inhibitor were obtained from GenePharma Co., Ltd. (Shanghai, China). The pcDNA3.1 vectors overexpressing *HK2* (pcDNA3.1-HK2) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). An empty pcDNA3.1 vector was used as the negative control for pcDNA3.1-HK2. Logarithmic growth phase CRC cells were inoculated in 6-well plates, and cell transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) after the cell density reached 80% confluence.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the supplier's protocol. For detecting *C1QTNF1-AS1* and *HK2* mRNA expression, total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Using the SYBR Premix Ex Taq™ Kit (Takara Biotechnology Co., Ltd.), qPCR was conducted on an ABI 7900 Real-Time PCR system (Applied Biosystems, Foster City, USA). *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) was used as an internal control, and *C1QTNF1-AS1* and *HK2* expression was normalized to GAPDH expression.

To measure miR-484 expression, reverse transcription was performed using a miScript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany); subsequently, the obtained cDNA was used as a template for PCR amplification using a miScript SYBR Green PCR kit (Qiagen GmbH). MiR-484 expression was normalized to *U6* small nuclear RNA expression. The $2^{-\Delta\Delta Ct}$ method was employed to analyze relative gene expression.

Subcellular Fractionation

The nuclear and cytosolic fractions of CRC cell suspensions were isolated using a Cytoplasmic & Nuclear RNA Purification Kit (Norgen, Belmont, CA, USA). Both fractions were subjected to RT–qPCR to determine the localization of *C1QTNF1-AS1* expression in CRC cells. *GAPDH* and *U6* were used as cytoplasmic and nuclear controls, respectively.[30–32]

Cell Counting Kit-8 (CCK-8) Assay

Twenty-four hours after transfection, the cells were harvested and seeded into 96-well plates at a density of 300 cells/well. After the addition of 10 μL CCK8 solution (Dojindo Laboratories Co., Ltd., Kumamoto, Japan), the cells were cultured in an incubator at 37°C with 5% CO_2 for an additional 2 h, followed by detecting the absorbance at 450 nm using a Multiskan Ex microtiter plate reader (Labsystems, Helsinki, Finland). The CCK-8 assay was conducted at 0, 24, 48, and 72 h after cell inoculation and proliferation curves were generated.

Flow Cytometry

The transfected cells were collected, rinsed with pre-cooled phosphate buffer solution (Gibco, Thermo Fisher Scientific Inc.), and subjected to apoptotic analysis using an Annexin V–Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (BioLegend, San Diego, CA, USA). After centrifugation, the supernatant was discarded, and the resultant cells were resuspended in 100 μL of flow cytometry binding buffer. Cells were double-stained with 5 μL of Annexin V-FITC and 5 μL of propidium iodide, and the proportion of apoptotic cells was measured using a flow cytometer (FACScan™, BD Biosciences, Franklin Lakes, NJ, USA).

Cell Migration and Invasion Assays

To determine cell migration, transfected cells after 48 h of incubation were mixed with FBS-free culture medium to obtain a single-cell suspension. The upper chambers of 24-well Transwell plates (BD Biosciences) were loaded with 100 μL of cell suspension containing 5×10^4 cells. A volume of 600 μL culture medium supplemented with 20% FBS was added into the lower chambers. After 24 h of incubation at 37°C, the migrated cells were fixed in 4% paraformaldehyde and stained using 0.5% crystal violet. The stained cells were then photographed and counted under a light microscope (Olympus Corporation, Tokyo, Japan) at a magnification of ×200. To examine cell invasion, the Transwell plates were coated with Matrigel (BD Biosciences) and incubated at 37°C for 3 h in an incubator. The same experimental procedures were followed as described above for the migration assay.

Xenograft Tumor Model

The short hairpin RNA (shRNA) targeting *C1QTNF1-AS1* (sh-C1QTNF1-AS1) and negative control shRNA (sh-NC) were produced by RiboBio and inserted into the lentivirus vector pLKO.1 vector. The generated vectors, namely pLKO.1-sh-C1QTNF1-AS1 and pLKO.1-sh-NC, alongside psPAX2 and pMD2.G were transduced into 293T

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cells (Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences). After 48 h, the lentiviruses expressing either sh-C1QTNF1-AS1 or sh-NC were introduced into SW480 cells. Cells stably expressing sh-C1QTNF1-AS1 or sh-NC were selected with puromycin (5 μg/mL; Sigma-Aldrich; Merck KGaA).

BALB/c nude mice (male, aged 4–6 weeks) were purchased from Shanghai SLAC Laboratory Animal, Co., Ltd. (Shanghai, China) and subcutaneously injected with 1×10^6 SW480 cells stably expressing sh-C1QTNF1-AS1 or sh-NC. The tumor size was recorded every 7 days, and tumor volume was calculated using the following formula: Volume = 0.5 × width^2 × length. At 28 days post-injection, all mice were euthanized through cervical vertebrae luxation, and the resulting tumor xenografts were resected, weighed, and stored for further use. The animal experimental protocol was approved by the Ethics Committee of Jilin Cancer Hospital and complied with the Animal Protection Law of the People's Republic of China-2009 for experimental animals.

Bioinformatic Analyses

miRDB (http://mirdb.org/) was used to identify target miRNAs of *C1QTNF1-AS1*. The putative targets of miR-484 were predicted using three bioinformatics tools: miRDB, TargetScan (http://www.targetscan.org/), and starBase 3.0 (http://starbase.sysu.edu.cn/). The expression of miR-484 in CRC was analyzed using YM500v3 (http://120.110.158.132:8787/ym500v3/).

RNA Immunoprecipitation (RIP) Assay

RIP assays were conducted using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). CRC cells were harvested via centrifugation and treated with RIP buffer. The resultant cell lysate was incubated overnight at 4°C with magnetic beads conjugated to human anti-Argonaute 2 (Ago2) or control IgG antibody (Millipore). Proteinase K was used to digest protein from the immunoprecipitates. Finally, RT–qPCR was performed to quantify *C1QTNF1-AS1* and miR-484 enrichment in the immunoprecipitated RNA.

Luciferase Reporter Assay

The fragments of *C1QTNF1-AS1* (420 bp) harboring the wild-type (wt) target sequence of miR-484 and mutant (mut) *C1QTNF1-AS1* fragments were amplified using RT–qPCR and inserted into the pmirGLO dual-luciferase reporter vector (Promega Corporation, Madison, WI, USA). The resultant luciferase reporter vectors were termed C1QTNF1-AS1-wt and C1QTNF1-AS1-mut, respectively. The same experimental procedures were used to construct HK2-wt and HK2-mut luciferase reporter vectors (420 bp). The primers were designed as follows: C1QTNF1-AS1-wt, 5′-CCGCTCGAGAGAGAACTAGAGGCTGCAGCG−3′ (forward) and 5′-GCTCTAGACCAGTGGCCAGGGAGGCTGGAT-3′ (reverse); C1QTNF1-AS1-mut, 5′-CCGCTCGAG AGAGAACTAGAGGCTGCAGCG−3′ (forward) and 5′-GCTCTAGACCAGTGGCCAGGGAGGCTGGAT-3′ (reverse); HK2-wt, 5′-CCGCTCGAGGTAATCCCCCTTGCCAAATTC−3′ (forward) and 5′-GCTCTAGAACATGTGCGCCCCGCTGAGAT−3′ (reverse); and HK2-mut, 5′-CCGCTCGAGGTAATCCCCCTTGCCAAATTC−3′ (forward) and 5′-GCTCTAGAACATGTGCGCCCCGCTGAGAT−3′ (reverse).

CRC cells were seeded into 24-well plates with a density of 1.5×10^5 cells per well. The wt (1.6 μg) or mut (1.6 μg) reporter vectors alongside miR-484 mimic (40 pmol) or miR-NC (40 pmol) were cotransfected into CRC cells using Lipofectamine® 2000 (2 μL). We quantified the luciferase activity using a dual-luciferase reporter assay system (Promega Corporation) and normalized to Renilla activity. Luciferase and renilla activities were quantified employing GloMax Luminometer (Promega Corporation).

Western Blotting

Cultured cells were lysed using the RIPA Lysis Buffer (Beyotime Institute of Biotechnology; Shanghai, China), and total protein concentration was detected using bicinchoninic acid assay (Beyotime Institute of Biotechnology). SDS–PAGE (10%) was used to resolve equal amounts of protein. The membranes were blocked with 5% milk in TBS–Tween-20 buffer (TBST) for 2 h at room temperature prior to overnight incubation at 4°C with primary antibodies against HK2 (cat. No. ab209847; Abcam, Cambridge, UK) or GAPDH (cat. No. ab128915; Abcam). Subsequently, the membranes were incubated with the goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (cat. No. ab150077; Abcam) at room temperature for 2 h. After 3 rinses with TBST, the protein bands were visualized using the PierceTM ECL Western blotting substrate (ThermoFisher Scientific). GAPDH was considered the loading control for normalization.

Microsatellite Instability (MSI) Analysis

The MSI status of tumors was tested using a multiplex PCR method. A total of four mononucleotide repeat markers, including *BAT26, NR21*, *NR27* and *CAT25*, were analyzed.

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Tumors presenting MSI in ≥1 mononucleotide repeat marker were classified as MSI phenotype, whereas those without MSI were classified as non-MSI phenotype.

Statistical Analysis

All data were presented as mean ± standard deviation from at least three independent replicates. The correlations of *C1QTNF1-AS1* expression with clinical variables of patients with CRC were analyzed using chi-squared test. Survival curves were obtained using the Kaplan–Meier method, and the Log rank test was applied to determine the differences between curves. Pearson's correlation coefficient was used to evaluate the expression relationship between the two genes in CRC tissues. Differences between two groups were examined using Student's *t*-test. One-way ANOVA followed by Tukey's post hoc test was used for comparisons among multiple groups. $P < 0.05$ was considered as statistically significant.

Results

C1QTNF1-AS1 is Upregulated in CRC, and Depleted C1QTNF1-AS1 Inhibits the Malignant Processes

To determine the functions of *C1QTNF1-AS1* in CR its expression in 71 pairs of CRC tissues and corresponding adjacent normal tissues was determined using qRT–qPCR. The data presented a drastic increase in the levels of *C1QTNF1-AS1* in CRC tissues when compared to those of corresponding normal tissues (Figure 1A). Furthermore, *C1QTNF1-AS1* expression was evidently higher in all four tested CRC cell lines (HCT116, HT29, SW480, and SW620) than in the normal human colon epithelium cell line FHC (Figure 1B). Using the median *C1QTNF1-AS1* value in CRC tissues as the cutoff value, all enrolled CRC patients were classified into either C1QTNF1-AS1-low (n = 35) or C1QTNF1-AS1-high (n = 36) expression groups. Increased *C1QTNF1-AS1* expression was associated with tumor size (P = 0.026), lymphatic metastasis (P = 0.020) and tumor-node-metastasis (TNM) stage (P = 0.042) (Table 1), but presented none association with gender, age, tumor location, tumor side, *N/KRAS* gene status, or MSI status (all $P > 0.05$). In addition, patients with CRC that exhibited high levels of *C1QTNF1-AS1* tended to have shorter overall survival than patients with low levels of *C1QTNF1-AS1* (Figure 1C, P = 0.033).

Since *C1QTNF1-AS1* was highly expressed in CRC and closely related to poor prognosis, we speculated that *C1QTNF1-AS1* may exert important actions during CRC genesis and progression. HCT116 and SW480 cells were transfected with si-C1QTNF1-AS1 or si-NC. The RT–qPCR data showed that the inhibitory effect of si-C1QTNF1-AS1#3 on *C1QTNF1-AS1* expression in both cell lines was more substantial than those of si-C1QTNF1-AS1#1 and si-C1QTNF1-AS1#2 (Figure 1D); hence, si-C1QTNF1-AS1#3 was used in the subsequent experiments. *C1QTNF1-AS1* depletion decreased the proliferation of HCT116 and SW480 cells (Figure 1E), as evidenced by the CCK-8 assays. Furthermore, flow cytometric analysis revealed that loss of *C1QTNF1-AS1* increased the ratio of apoptotic HCT116 and SW480 cells (Figure 1F). Moreover, interference of *C1QTNF1-AS1* resulted in an obvious decrease in the migration (Figure 1G) and invasion (Figure 1H) of HCT116 and SW480 cells. Altogether, these data provide evidence that *C1QTNF1-AS1* plays a cancer-promoting role in the oncogenicity of CRC.

C1QTNF1-AS1 Functions as a miR-484 Sponge in CRC Cells

LncLocator (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/), an lncRNA subcellular localization predictor, identified that *C1QTNF1-AS1* was located in the cytoplasm (Figure 2A). Furthermore, subcellular fractionation followed by RT–qPCR confirmed that *C1QTNF1-AS1* was primarily distributed in the cytoplasm of HCT116 and SW480 cells (Figure 2B), suggesting that *C1QTNF1-AS1* may control gene expression at the posttranscriptional level. It is widely accepted that cytoplasmic lncRNAs can function as miRNA sponges by competitively binding to miRNAs, releasing their downstream target mRNAs.[27]

Using miRDB, 26 miRNAs had a reverse complementary sequence to *C1QTNF1-AS1* (Figure 2C). Among these, eight miRNAs (miR-219b-5p, miR-212-5p, miR-615-3p, miR-488-5p, miR-525-5p, miR-520a-5p, miR-484, and miR-346) were selected as experimental subjects based on their known biological roles in cancer.[33–39] To confirm these predictions, RT–qPCR was performed to measure the expression of these miRNAs in HCT116 and SW480 cells after *C1QTNF1-AS1* knockdown. MiR-484 expression was increased in HCT116 and SW480 cells upon *C1QTNF1-AS1* depletion, whereas the expressions of the other miRNAs was unaffected (Figure 2D). Subsequently, YM500v3 was used to predict the expression status of miR-484 in CRC. MiR-484 was found to be weakly expressed in CRC tissues (Figure 2E). To confirm this

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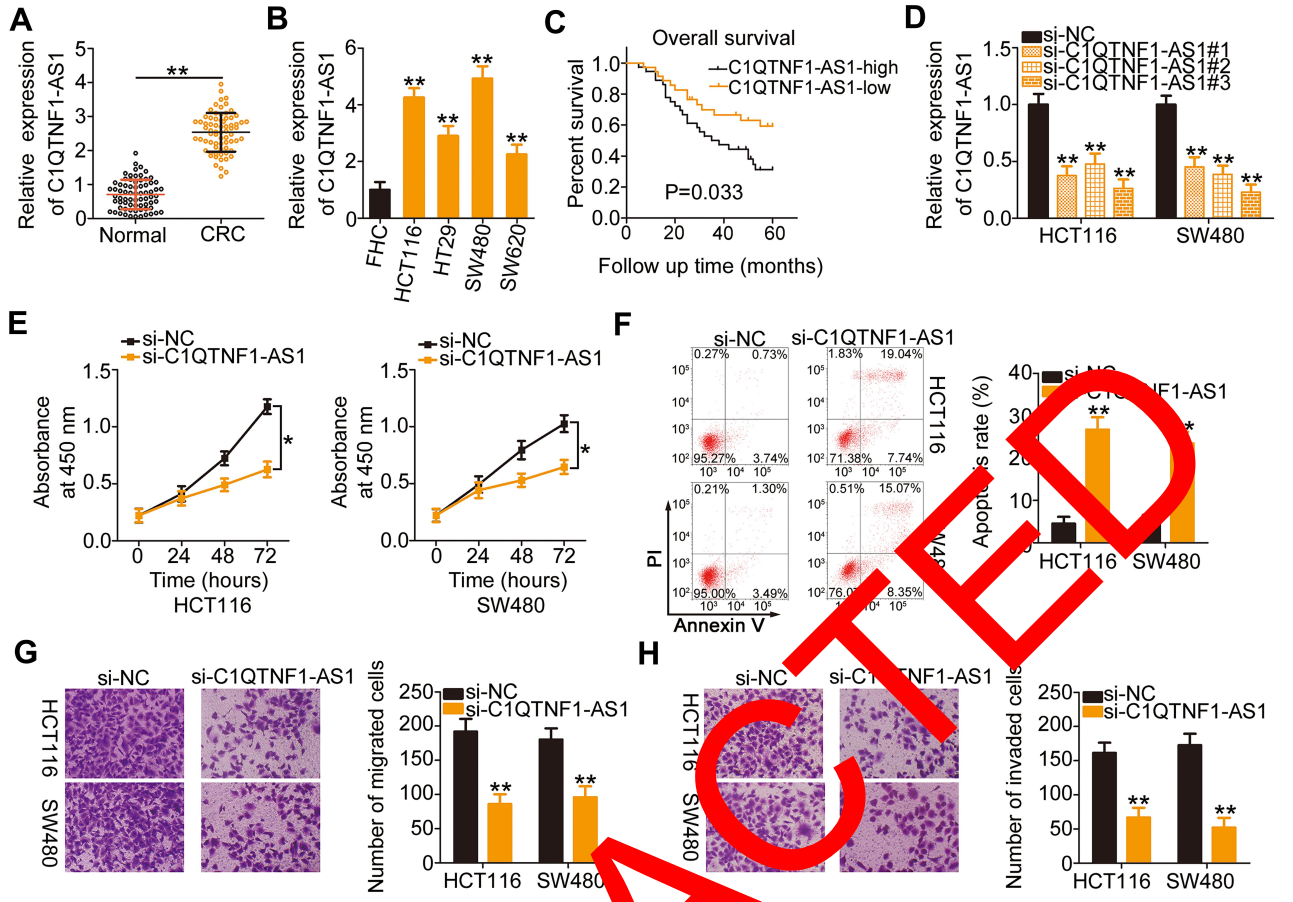

Figure 1 *C1QTNF1-AS1* is highly expressed in CRC, and *C1QTNF1-AS1* depletion inhibits the malignant behaviors of CRC cells. (**A**) *C1QTNF1-AS1* expression in 71 pairs of CRC tissues and corresponding adjacent normal tissues was validated using RT–qPCR (Student's *t*-test). Each sample contained three replicates and the assay was repeated three times. (**B**) RT–qPCR was performed to measure *C1QTNF1-AS1* expression in different human CRC cell lines and the normal human colon epithelium cell line FHC (One-way ANOVA). Each sample contained three replicates and RT-qPCR was repeated three times. (**C**) The Kaplan–Meier method was used to examine the overall survival of patients with CRC in *C1QTNF1-AS1*-low (n = 35) or *C1QTNF1-AS1*-high (n = 36) expression groups (Log rank test). (**D**) The interfering efficacies of three siRNAs designed to silence endogenous *C1QTNF1-AS1* expression were determined using RT–qPCR in HCT116 and SW480 cells (One-way ANOVA). Each sample contained three replicates and RT-qPCR was repeated three times. (**E**) The effects of *C1QTNF1-AS1* silencing on HCT116 and SW480 cell proliferation was detected using the CCK-8 assay (Student's *t*-test). Each group contained five replicates. CCK-8 assay was repeated three times. (**F**) Flow cytometric analysis was used to quantify the ratio of apoptotic HCT116 and SW480 cells after *C1QTNF1-AS1* downregulation (Student's *t*-test). Each group contained three replicates, and the assay was repeated three times. (**G** and **H**) Cell migration and invasion assays showed the migratory and invasive capacities of C1QTNF1-AS1-deficient HCT116 and SW480 cells (Student's *t*-test). Each group contained three replicates, and the assay was repeated three times. *P < 0.05 and **P < 0.01.

Abbreviations: C1QTNF1-AS1, C1QTNF1 antisense RNA 1; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8; si-C1QTNF1-AS1, small interfering RNA targeting C1QTNF1-AS1; si-NC, negative control small interfering RNA; PI, propidium iodide.

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prediction, RT–qPCR was performed to determine miR-484 expression in 71 CRC tissue pairs and corresponding adjacent normal tissues. CRC tissues presented lower miR-484 expression than that in adjacent normal tissues (Figure 2F). Notably, Pearson's correlation coefficient analysis showed that there was a negative correlation between miR-484 and *C1QTNF1-AS1* expression in the 71 CRC tissues (Figure 2G; r = −0.7619, P < 0.0001).

Next, the luciferase reporter assay was performed to examine the physical binding relationship between *C1QTNF1-AS1* and miR-484 in CRC cells. The wild-type and mutant binding sites between *C1QTNF1-AS1* and miR-484 were shown in Figure 2H. The upregulation of miR-484 clearly reduced the luciferase activity of C1QTNF1-AS1-wt harboring the wild-type miR-484 binding sequences; however, the suppression of luciferase activity by miR-484 mimic was abrogated by the mutated binding sequences (Figure 2I). Furthermore, the results of RIP assays showed that *C1QTNF1-AS1* and miR-484 were remarkably enriched in the Ago2-containing microribonucleoprotein complexes (Figure 2J). It is generally known that Ago2 is a core component of the RNA-induced silencing complex, and is required for miRNA-mediated gene silencing. MiRNA executes its roles by directly binding to Ago2, and putative

Table 1 Correlation Between *C1QTNF1-AS1* Expression and Clinical Variables in Patients with CRC

Clinical Variables	C1QTNF1-AS1-High Group (n = 36)	C1QTNF1-AS1-Low Group (n = 35)	P
Gender			0.238
Male	14	19	
Female	22	16	
Age (years)			0.813
<60	17	15	
≥60	19	20	
Tumor location			0.462
Rectum	11	14	
Colon	25	21	
Tumor side			0.238
Right-side tumor	14	19	
Left-side tumor	22	16	
NRAS gene status			0.493
Mutation	2	0	
No mutation	34	35	
KRAS gene status			0.260
Mutation	6	2	
No mutation	30	33	
MSI status			[illegible]
MSI	1	0	
Non-MSI	35	35	
Tumor size (cm)			0.026*
<5	18	27	
≥5	18	8	
Lymphatic metastasis			0.020*
Absence	20	29	
Presence	16	6	
TNM stage			0.042*
I–II	20	28	
III–IV	16	7	

Note: *P < 0.05 by the chi square test.
Abbreviations: TNM, tumor-node-metastasis; MSI, microsatellite instability.

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miRNA targets can be isolated from this complex after Ago2 co-immunoprecipitation. The results of RIP assay implied the direct interaction between *C1QTNF1-AS1* and miR-484 in CRC cells. Altogether, *C1QTNF1-AS1* functioned as a miR-484 sponge in CRC cells.

MiR-484 is a Tumor-Inhibiting miRNA and Directly Targets HK2 in CRC Cells

To elucidate the detailed functions of miR-484 in CRC cells, miR-484 mimic or miR-NC was transfected into HCT116 and SW480 cells, and the transfection efficiency was determined using RT–qPCR (Figure 3A). The proliferative abilities of HCT116 and SW480 cells were obviously impaired after transfection with miR-484 mimic (Figure 3B). In addition, the upregulation of miR-484 drastically increased the apoptosis of HCT116 and SW480 cells, as demonstrated by flow cytometry (Figure 3C). Furthermore, cell migration (Figure 3D) and invasion (Figure 3E) strikingly decreased in HCT116 and SW480 cells after miR-484 overexpression.

A potential miR-484 binding site located in the 3′-UTR of *HK2* (Figure 3F) was identified using three bioinformatics tools, including miRDB, TargetScan, and StarBase 3.0. A luciferase reporter assay was conducted to further confirm this prediction. MiR-484 mimic or miR-NC together with HK2-wt or HK2-mut reporter plasmids were transfected into HCT116 and SW480 cells. MiR-484 overexpression caused a significant decrease in luciferase activity of HK2-wt in HCT116 and SW480 cells, whereas the HK2-mut-induced luciferase activity did not change in response to miR-484 mimic cotransfection (Figure 3G). Subsequently, the mRNA and protein levels of *HK2* in miR-484-overexpressing HCT116 and SW480 cells were determined using RT–qPCR and Western blotting, respectively. As illustrated in Figure 3H and I, *HK2* mRNA (Figure 3H) and protein (Figure 3I) levels were lower in HCT116 and SW480 cells transfected with miR-484 mimic than in the control cells. Next, we examined the association between miR-484 and *HK2* in CRC tissues. *HK2* mRNA expression was elevated in CRC tissues (Figure 3J) and inversely correlated with miR-484 expression (Figure 3K; $r = -0.7004$, $P < 0.0001$). These results demonstrate that miR-484 performs tumor-suppressive activities during CRC progression, and *HK2* is a direct target of miR-484 in CRC cells.

C1QTNF1-AS1 Plays a Carcinogenic Role by Regulating a miR-484/HK2 Axis

After identifying *HK2* as a direct target of miR-484, we wondered whether *C1QTNF1-AS1*was implicated in the regulation of *HK2*. Transfection with si-C1QTNF1-AS1 caused a statistically significant decrease ($P < 0.01$) in *HK2* mRNA (Figure 4A) and protein (Figure 4B) levels in HCT116 and SW480 cells. Thereafter, we performed rescue experiments to investigate whether *C1QTNF1-AS1* controls *HK2* expression in CRC cells by sponging miR-484. First, RT–qPCR analysis was used to verify the efficiency of miR-484 inhibitor transfection (Figure 4C).

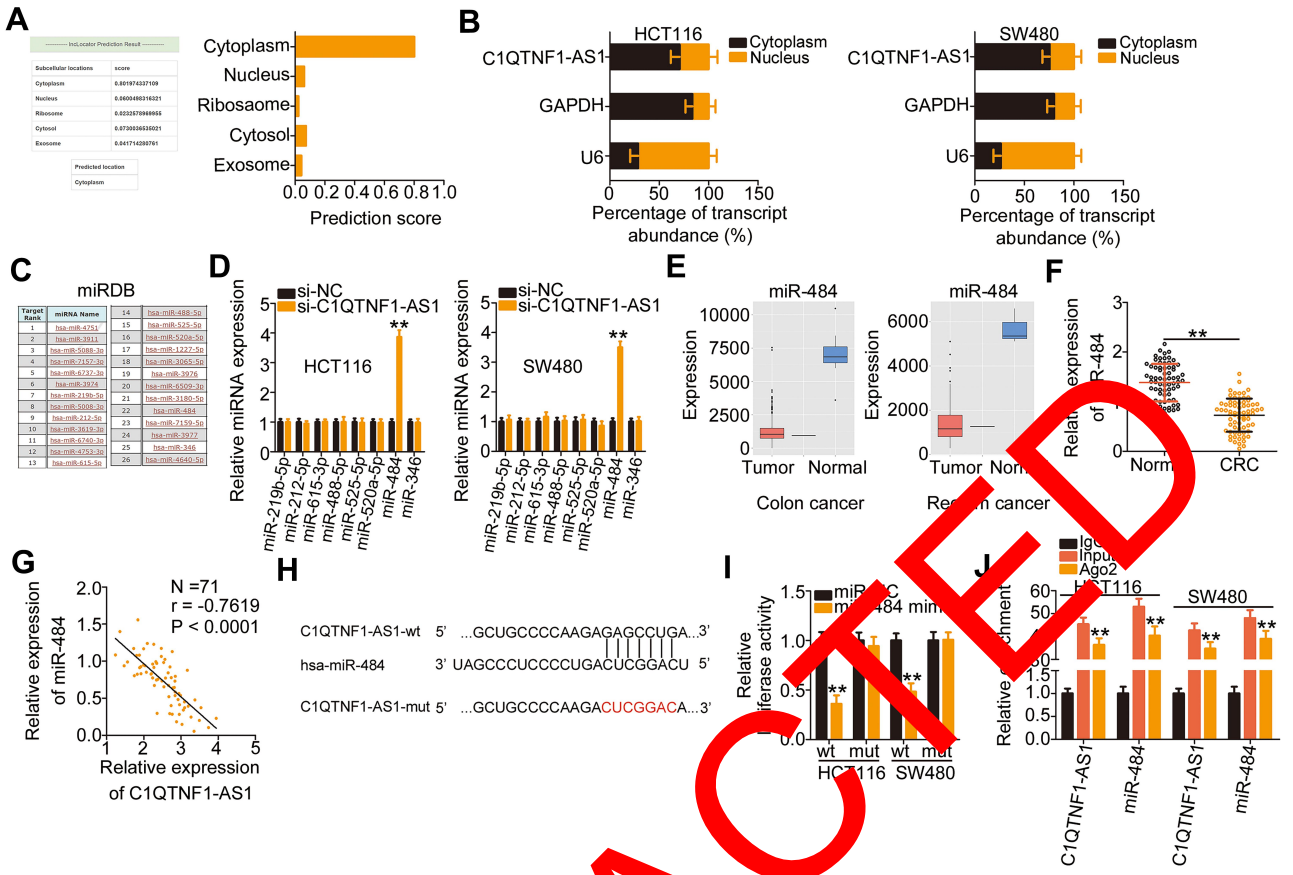

Figure 2 *C1QTNF1-AS1* functions as a miR-484 sponge in CRC cells. (**A**) LncLocator predicted that *C1QTNF1-AS1* was primarily located in the cytoplasm. (**B**) Subcellular fractionation assays followed by RT–qPCR analysis evaluated the location of *C1QTNF1-AS1* in HCT116 and SW480 cells. (**C**) The miRNAs that may interact with *C1QTNF1-AS1* were predicted using miRDB. (**D**) The expression of miR-219b-5p, miR-212-5p, miR-615-3p, miR-488-5p, miR-525-5p, miR-520a-5p, miR-484, and miR-346 was detected using RT–qPCR in HCT116 and SW480 cells after si-C1QTNF1-AS1 or si-NC transfection (Student's *t*-test). Each sample contained three replicates and RT-qPCR was repeated three times. (**E**) The expression level of miR-484 in colon adenocarcinoma (left) and rectal adenocarcinoma (right) was predicted using YM500v3. (**F**) MiR-484 expression was detected using RT–qPCR in 71 pairs of CRC tissues and adjacent normal tissues (Student's *t*-test). Each sample contained three replicates and RT-qPCR was repeated three times. (**G**) Pearson's correlation coefficient was used to analyze the correlation between miR-484 and *C1QTNF1-AS1* expression in the 71 CRC tissues (Pearson's correlation coefficient analysis). (**H**) The wild-type binding site between *C1QTNF1-AS1* and miR-484 was presented using bioinformatics analyses. The mutant binding sequences were also shown. (**I**) Luciferase reporter assays were conducted to confirm the direct binding between miR-484 and *C1QTNF1-AS1* in CRC cells. Luciferase activity was detected in HCT116 and SW480 cells after cotransfection with miR-484 mimic or miR-NC and C1QTNF1-AS1-wt or C1QTNF1-AS1-mut (Student's *t*-test). Luciferase reporter assay was repeated three times with three replicates. (**J**) RIP assays were conducted in HCT116 and SW480 cells, and the relative expression of miR-484 and *C1QTNF1-AS1* in the immunoprecipitates was measured using RT–qPCR (Student's *t*-test). RIP assay contained three replicates, and was repeated three times. **P < 0.01.

Abbreviations: C1QTNF1-AS1, C1QTNF1 antisense RNA 1; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miRNA, microRNA; wt, wild-type; mut, mutant; RIP, RNA immunoprecipitation; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; U6, U6 small nuclear RNA; Ago2, Argonaute 2; IgG, immunoglobulin G; miR-NC, negative control miRNA mimic; si-C1QTNF1-AS1, small interfering RNA targeting C1QTNF1-AS1; si-NC, negative control small interfering RNA.

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si-C1QTNF1-AS1, together with miR-484 inhibitor or NC inhibitor, was cotransfected into HCT116 and SW480 cells. The expression levels of *HK2* mRNA (Figure 4D) and protein (Figure 4E) were significantly reduced by *C1QTNF1-AS1* downregulation, which was recovered by miR-484 inhibition, suggesting that *C1QTNF1-AS1*positively regulated *HK2* expression in CRC cells by sponging miR-484.

To further understand the roles of *C1QTNF1-AS1*, two rescue experiments were conducted to determine whether *C1QTNF1-AS1* exerts an oncogenic role in CRC cells by regulating the miR-484/*HK2* axis. First, HCT116 and SW480 cells were transfected with si-C1QTNF1-AS1 in the presence of miR-484 inhibitor or NC inhibitor, and these cells were used in functional rescue experiments. The reduced cell proliferation (Figure 4F) and enhanced cell apoptosis (Figure 4G) by si-C1QTNF1-AS1 were reversed by miR-484 inhibitor cotransfection. Similarly, cell migratory (Figure 4H) and invasive (Figure 4I) abilities were impaired after C1QTNF1-AS1 knockdown but restored after miR-484 inhibition. The *HK2* overexpression vector pcDNA3.1-HK2 was also used in rescue experiments, and the overexpression

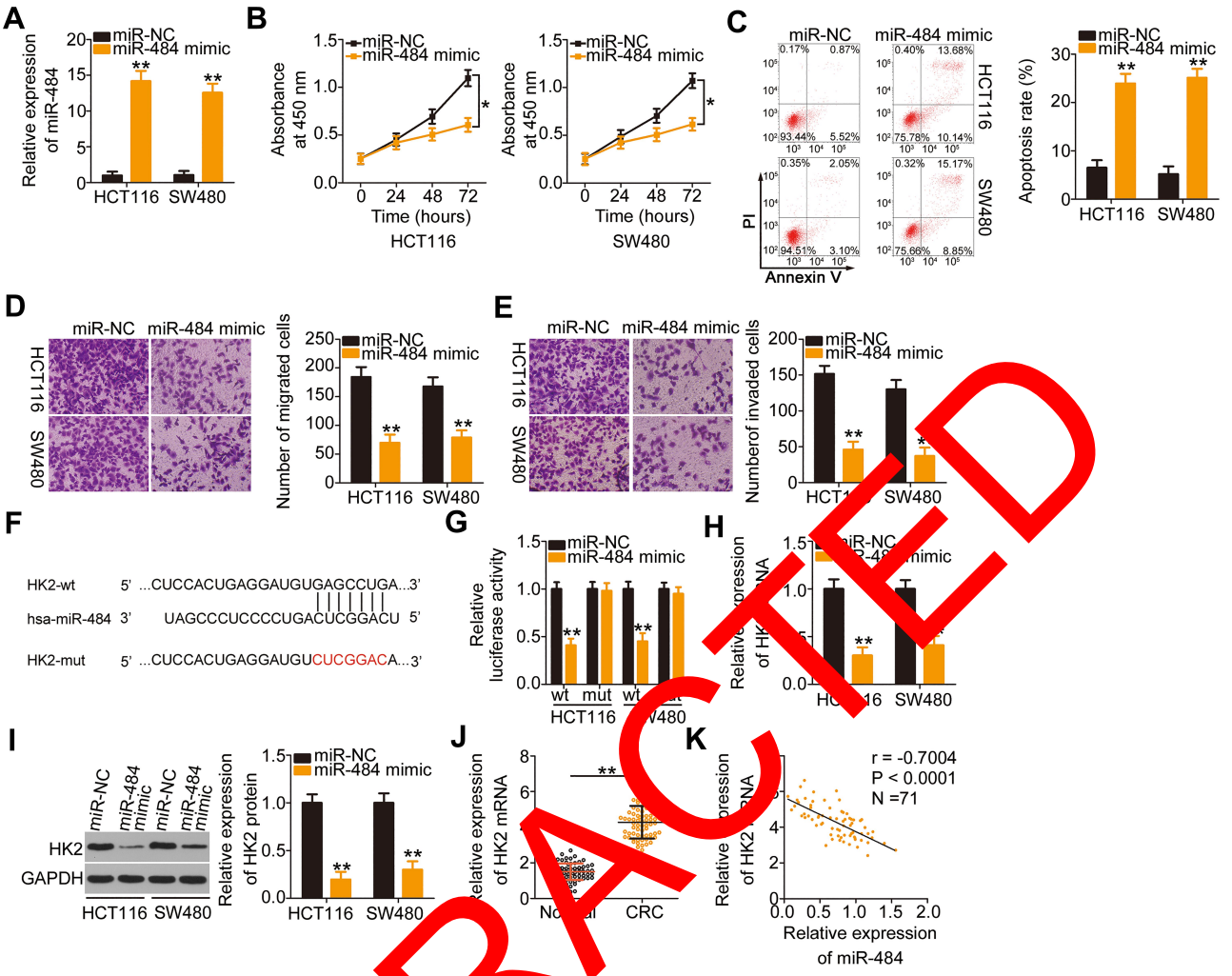

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Figure 3 *HK2* is a direct target of miR-484 in C cells. (-qPCR analysis was used to test the transfection efficiency of miR-484 mimic in HCT116 and SW480 cells (Student's *t*-test). Each sample contained th replicates ar T-qPCR was repeated three times. (**B** and **C**) CCK-8 assays and flow cytometric analysis were used to measure the proliferation and apoptosis T116 and SW cells after miR-484 upregulation (Student's *t*-test). CCK-8 assay contained five replicates and flow cytometric analysis had three replicate both as were repeated hree times. (**D** and **E**) The migratory and invasive abilities of miR-484 mimic- or miR-NC-transfected HCT116 and SW480 cells were analyzed by cell mi tion and invasion assays (Student's *t*-test). Each group contained three replicates, and the assay was repeated three times. (**F**) The predicted wild-ty and mutated miR-4 inding sites in the 3′-UTR of *HK2* were presented. (**G**) The interaction between miR-484 and the 3′-UTR of *HK2* in CRC cells was validated b iciferase reporter assay. H 116 and SW480 cells were transfected with miR-484 mimic or miR-NC alongside HK2-wt or HK-2 mut, and then the luciferase activit as detect using a dual-luciferase reporter assay system (Student's *t*-test). Luciferase reporter assay was repeated three times, with three replicates. (**H** and **I**) RT–q and W stern blotting analyses showed the expression of *HK2* mRNA and protein in HCT116 and SW480 cells after miR-484 mimic or miR-NC transfection (Student's *t*-t both assays re repeated three times. (**J**) Relative *HK2* mRNA expression in the 71 pairs of CRC tissues and corresponding adjacent normal tissues w ed using T-qPCR tudent's *t*-test). Each sample contained three replicates and RT-qPCR was repeated three times. (**K**) The relationship between miR-484 and 2 mRN in the 71 C ssues was determined using Pearson's correlation coefficient analysis. *P < 0.05 and **P < 0.01.

Abbrevia ons: CRC, orectal cance RT-qPCR, reverse transcription-quantitative polymerase chain reaction; wt, wild-type; mut, mutant; HK-2, hexokinase 2; GAPDH, glyceraldeh 3-pho enase; miR-484, microRNA-484; miR-NC, negative control microRNA mimic; PI, propidium iodide.

efficiency of pcD A3.1-HK2 was determined using Western blotting (Figure 5A). Cell proliferation was suppressed by reduced C1QTNF1-AS1 in HCT116 and SW480 cells, whereas this suppressive effect was impaired by the restoration of *HK2* (Figure 5B). Strikingly, the rate of HCT116 and SW48 cell apoptosis increased by si-C1QTNF1-AS1 was recovered by pcDNA3.1-HK2 introduction (Figure 5C). Similarly, the loss of *C1QTNF1-AS1* hindered HCT116 and SW480 cell migration (Figure 5D) and invasion (Figure 5E), which was rescued by *HK2* overexpression. Thus, these results collectively uncovered that *C1QTNF1-AS1* played cancer-promoting roles in CRC cells by sponging miR-484 and consequently increasing *HK2* expression.

C1QTNF1-AS1 Depletion Inhibits Tumor Growth of CRC Cells in vivo

Lastly, a xenograft tumor model was used to validate the role of *C1QTNF1-AS1* in promoting CRC cell growth

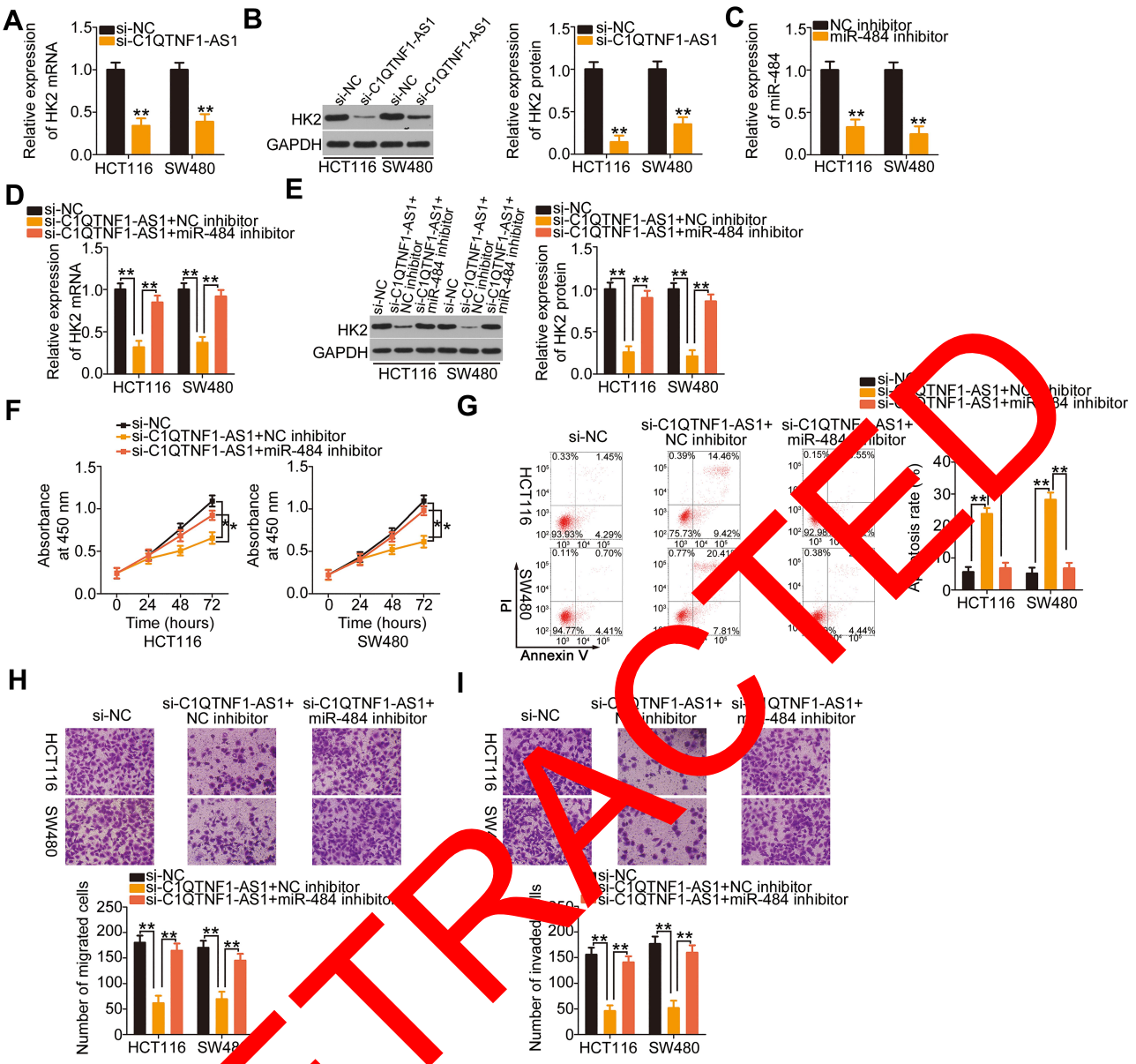

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Figure 4 The cancer-inhibiting effects of *C1QTNF1-AS1* knockdown on HCT116 and SW480 cell proliferation, apoptosis, migration, and invasion were impaired by miR-484 inhibition. (**A** and **B**) *HK2* mRNA and protein was quantified in HCT116 and SW480 cells with *C1QTNF1-AS1* knockdown (Student's *t*-test). Both assays were repeated three times. (**C**) The inhibition efficiencies of miR-484 inhibitor were determined using RT–qPCR in HCT116 and SW480 cells (Student's *t*-test). Each sample contained three replicates and RT-qPCR was repeated three times. (**D** and **E**) C1QTNF1-AS1-depleted HCT116 and SW480 cells were further cotransfected with miR-484 inhibitor or NC inhibitor, and cotransfected cells were subjected to RT–qPCR and Western blotting for the measurement of *HK2* mRNA and protein expression (One-way ANOVA). Each sample contained three replicates. Both assays were repeated three times. (**F** and **G**) The proliferation and apoptosis of HCT116 and SW480 cells treated as described above were evaluated using CCK-8 assays and flow cytometric analysis, respectively (One-way ANOVA). CCK-8 assay contained five replicates and flow cytometric analysis had three replicates. Both assays were repeated three times. (**H** and **I**) Cell migration and invasion assays detected the migration and invasion of HCT116 and SW480 cells after the introduction of C1QTNF1-AS1 and miR-484 inhibitor or NC inhibitor (One-way ANOVA). Both assays contained five replicates and were repeated three times. *P < 0.05 and **P < 0.01.

Abbreviations: RT-qPCR, reverse transcription-quantitative polymerase chain reaction; HK-2, hexokinase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miR-484, microRNA-484; NC inhibitor, negative control inhibitor; C1QTNF1-AS1, C1QTNF1 antisense RNA 1; CCK-8, Cell Counting Kit-8; si-C1QTNF1-AS1, small interfering RNA targeting C1QTNF1-AS1; si-NC, negative control small interfering RNA; PI, propidium iodide.

in vivo. Nude mice were subcutaneously inoculated with SW480 cells stably expressing sh-C1QTNF1-AS1 or sh-NC. A significant decrease in tumor volume (Figure 6A and B) and weight (Figure 6C) was identified in the tumor xenografts that originated from C1QTNF1-AS1-depleted SW480 cells. At 4 weeks post-injection, the nude mice were euthanized and tumor xenografts were collected for molecular detection. Tumors derived from cells with *C1QTNF1-AS1* stably knocked down revealed decreased *C1QTNF1-AS1*expression (Figure 6D) and increased miR-

484 (Figure 6E) expression on RT–qPCR analysis. Furthermore, Western blotting indicated that the *HK2* protein (Figure 6F) level was downregulated in the tumor xenografts that originated from C1QTNF1-AS1-depleted SW480 cells. Altogether, these results demonstrated the promotive effects of *C1QTNF1-AS1* on tumor growth of CRC cells in vivo, which was executed via the regulation of the miR-484/HK2 axis.

Discussion

Over the last few years, lncRNAs have received considerable attention because of their important roles in human cancers.[40–42] An increasing number of studies have reported that several lncRNAs are differentially expressed in CRC and contribute to colorectal carcinogenesis and cancer.[43–45] LncRNAs promote or suppress diverse malignant processes in CRC, playing oncogenic or tumor-suppressing roles.[46–48] A number of lncRNAs have been identified to be dysregulated in CRC, but the regulatory network of lncRNAs continues to remain unclear, requiring further exploration. In this study, we first detected the expression of *C1QTNF1-AS1* in CRC and determined the relationship between the clinicopathological parameters and *C1QTNF1-AS1* expression among CRC patients. Subsequently, we performed functional analyses to investigate the regulatory roles of *C1QTNF1-AS1* during CRC progression. Furthermore, the mechanisms underlying the cancer-promoting actions of *C1QTNF1-AS1* in CRC cells were elucidated in detail.

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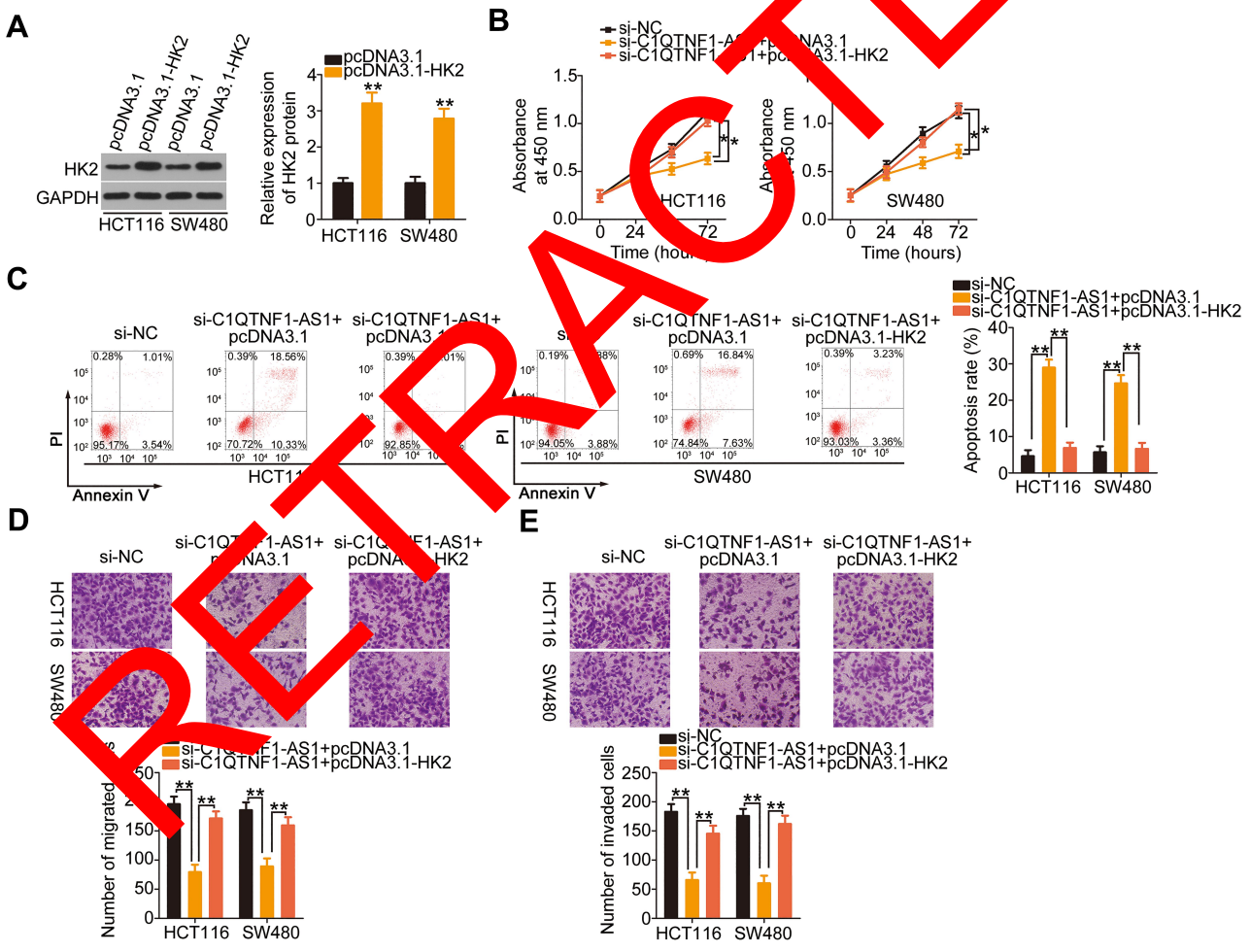

Figure 5 *HK2*upregulation rescues the function of si-C1QTNF1-AS1 in HCT116 and SW480 cells. (**A**) Western blotting was used to measure *HK2* protein expression in HCT116 and SW480 cells after pcDNA3.1 or pcDNA3.1-HK2 transfection (Student's *t*-test). Western blotting was repeated three times. (**B–E**) si-C1QTNF1-AS1, in parallel with pcDNA3.1 or pcDNA3.1-HK2, was cotransfected into HCT116 and SW480 cells. Cell proliferation, apoptosis, migration, and invasion were determined using the CCK-8 assay, flow cytometric analysis, and cell migration and invasion assays, respectively (One-way ANOVA). All assays contained at least three replicates and were repeated three times. *P < 0.05 and **P < 0.01.

Abbreviations: HK-2, hexokinase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; C1QTNF1-AS1, C1QTNF1 antisense RNA 1; si-C1QTNF1-AS1, small interfering RNA targeting C1QTNF1-AS1; si-NC, negative control small interfering RNA; CCK-8, Cell Counting Kit-8; PI, propidium iodide.

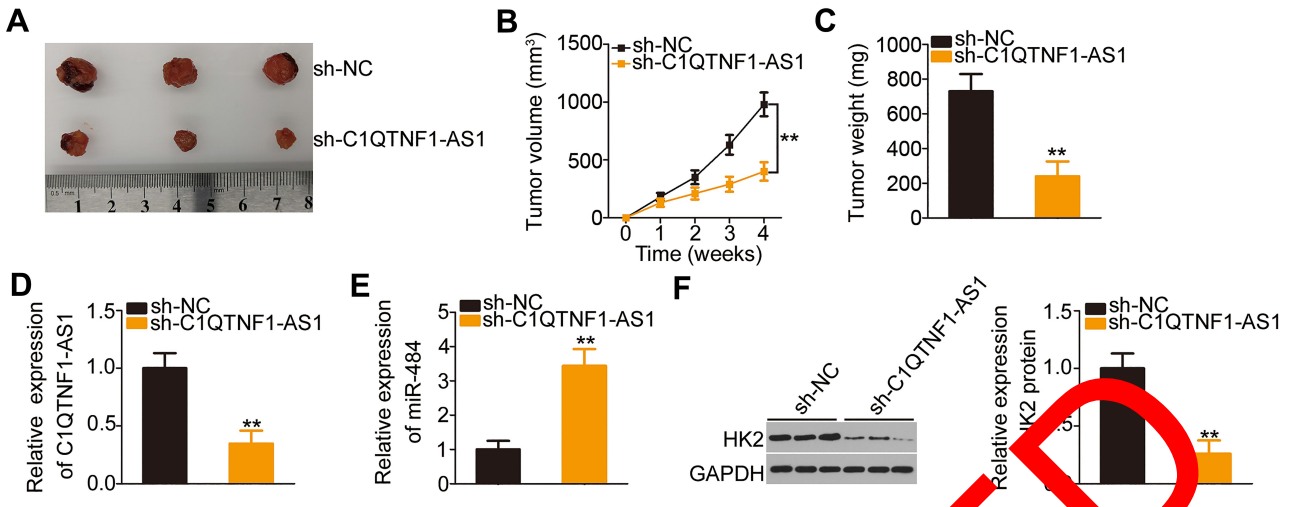

Figure 6 Downregulation of *C1QTNF1-AS1* suppresses tumor growth in vivo. (**A**) The representative images of tumor xenografts formed in SW480 cells stably expressing sh-C1QTNF1-AS1 or sh-NC. (**B**) The growth curves were plotted according to the tumor volumes recorded (Student's *t*-test). Each group contained three nude mice. (**C**) The weights of tumor xenografts were detected (Student's *t*-test). Each group contained three nude mice. (**D** and **E**) RT–qPCR revealed the expression of *C1QTNF1-AS1*and miR-484 in tumor xenografts derived from sh-C1QTNF1-AS1 or sh-NC stably-transfected SW480 cells (Student's *t*-test). Each group contained three nude mice. Each sample contained three replicates and RT-qPCR was repeated three times. (**F**) The protein level of *HK2* in tumor xenografts was determined using Western blotting (Student's *t*-test). Each group contained three nude mice. Western blotting was repeated three times. **$P < 0.01$.

Abbreviations: HK-2, hexokinase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; C1QTNF1-AS1, C1QTNF1 antisense RNA 1; sh-C1QTNF1-AS1, short hairpin RNA targeting C1QTNF1-AS1; sh-NC, negative control short hairpin RNA; miR-484, microRNA-484.

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C1QTNF1-AS1 is downregulated in hepatocellular carcinoma.[28,29] Functionally, *C1QTNF1-AS1* upregulation attenuates cell proliferation, migration, and invasion and induces cell apoptosis in hepatocellular carcinoma.[28,29] In addition, ectopic *C1QTNF1-AS1* expression hampers the tumor growth of hepatocellular carcinoma in vivo.[28,29] However, the expression and roles of *C1QTNF1-AS1* have not been fully explored in CRC. Here, RT–qPCR analysis confirmed that *C1QTNF1-AS1* expression in CRC tissues increased compared with that in adjacent normal tissues. Interestingly, the analysis of clinical data revealed that an increased level of *C1QTNF1-AS1* was related to larger tumor size, higher incidence of lymphatic metastasis, more advanced TNM stage, and shorter overall survival, suggesting that *C1QTNF1-AS1* may be an oncogenic lncRNA that facilitates CRC progression. A series of experiments uncovered that *C1QTNF1-AS1* depletion suppressed CRC cell proliferation, invasion, and migration in vitro. Furthermore, interference of *C1QTNF1-AS1* increased cell apoptosis in vitro and reduced cell growth in vivo.

With regard to the mechanism, lncLocator and subcellular fractionation analysis demonstrated that *C1QTNF1-AS1* was enriched in the cytoplasm of CRC cells, suggesting that *C1QTNF1-AS1* may control gene expression at the posttranscriptional level. Accumulating studies have revealed the extensive interactions among lncRNA-miRNA-mRNA regulatory pathways involving ceRNAs, wherein lncRNAs can recover the expression and roles of miRNAs by sequestering miRNAs.[49–51] In our study, we used bioinformatics analysis to screen putative miRNAs that may interact with *C1QTNF1-AS1*. MiR-484 was identified to potentially interact with *C1QTNF1-AS1*, and this prediction was confirmed using the luciferase reporter and RIP assays. In addition, RT–qPCR analysis showed that the knockdown of *C1QTNF1-AS1* increased miR-484 expression in CRC cells. Furthermore, miR-484 was weakly expressed in CRC and exhibited a reverse correlation with *C1QTNF1-AS1* expression in CRC tissues.

Next, the putative targets of miR-484 were explored, and we confirmed that *HK2* was a direct target of miR-484 using luciferase reporter assays, RT-PCR analysis, and Western blotting. The regulatory relationship between *C1QTNF1-AS1*, miR-484, and *HK2* was then evaluated in detail. Our results showed that *C1QTNF1-AS1* downregulation reduced *HK2* expression in CRC cells at both mRNA and protein levels. In subsequent rescue assays, we found that miR-484 inhibition could partially abrogate the regulatory actions of *C1QTNF1-AS1* knockdown on *HK2* expression in CRC cells. Overall, these findings revealed that *C1QTNF1-AS1* is a type of ceRNA that functions as an miR-484 sponge and thereby increases *HK2* expression.

MiR-484 has been reported to be aberrantly expressed in a variety of human cancers, including CRC.[52] In CRC, miR-

484 was downregulated and closely correlated with the tumor stage.[53] *HK2*, a key metabolic enzyme,[54] was identified as the direct downstream target of miR-484 in CRC cells. In this study, miR-484 inhibition or *HK2* restoration diminished the impacts of *C1QTNF1-AS1* deficiency on the aggressiveness of CRC cells, suggesting that *C1QTNF1-AS1*partially executed its cancer-promoting actions in CRC cells by modulating a miR-484/*HK2* axis. Therefore, these results identified a novel ceRNA pathway in CRC involving *C1QTNF1-AS1*, miR-484, and *HK2*. The *C1QTNF1-AS1*/miR-484/*HK2* pathway may contribute to CRC pathogenesis.

In the study, a total of 6 mice was used in xenograft tumor model, and each group contained three nude mice. The number of mice per group was low, and it was a limitation of our study. We will resolve it in the near future.

Conclusion

In conclusion, this study revealed the abnormally high expression of *C1QTNF1-AS1* in CRC and its clinical relevance. *C1QTNF1-AS1* drove the progression of CRC by adsorbing miR-484 and consequently upregulating *HK2*. The *C1QTNF1-AS1*/miR-484/*HK2* pathway may be a potential therapeutic target for the diagnosis and treatment of CRC.

Ethics Approval and Informed Consent

The present study was approved by the Ethics Committee of Jilin Cancer Hospital and conducted in accordance with the Declaration of Helsinki. Moreover, written informed consent forms were collected from all participants. The animal experimental protocol was approved by the Ethics Committee of Jilin Cancer Hospital and complied with the Animal Protection Law of the People's Republic of China-2009 for experimental animals.

Disclosure

The authors declare that they have no competing interests.

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