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A novel long noncoding RNA, AC005062.1, acts as an oncogene in colorectal cancer cell by regulating MACC1

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ABSTRACT: A novel long noncoding RNA (lncRNA), AC005062.1, has shown an abnormal expression in bladder cancer and small cell lung cancer; however, it has never been studied in colorectal cancer (CRC). In this study, we explored the expression and role of AC005062.1 in CRC. The expression of AC005062.1 in CRC tissues and cell lines was determined using quantitative real-time PCR (qRT-PCR). *In vitro* experiments such as CCK-8 assay, flow cytometry, wound scratch test, and transwell assay were performed in HT-29 cells after knockdown of AC005062.1. Our results showed that AC005062.1 was significantly up-regulated in CRC tissues and positively correlated with advanced clinical stage, larger tumor size, lymphatic metastasis, distant metastasis, and vascular invasion. Knockdown of AC005062.1 in HT-29 cells significantly inhibited cell proliferation, migration, and invasion and promoted cell apoptosis and G1 arrest. mRNA and protein expression levels of Metastasis Associated in Colon Cancer 1 (MACC1), a neighboring gene of AC005062.1, were decreased after the knockdown of AC005062.1. Our study demonstrated that AC005062.1, which acts as an oncogene, may be involved in CRC tumorigenesis and progression by regulating MACC1.

KEYWORDS: colorectal cancer, long noncoding RNA, AC005062.1, metastasis associated in colon cancer 1, oncogene, tumorigenesis

INTRODUCTION

As a common gastrointestinal tract tumor, colorectal cancer (CRC) ranks third in incidence and second in mortality among all cancers according to the report of Global Cancer Statistics 2020 [1]. In recent years, although great advances have been achieved in our understanding of the CRC, the molecular mechanisms underlying CRC tumorigenesis and progression remain largely unclear. Thus, it is of great significance to study the molecular mechanisms behind CRC to improve the treatment of this fatal disease.

Recently, increasing evidence indicates that long noncoding RNAs (lncRNAs) play critical roles in many biological processes that are relevant to the development and progress of cancers such as cell proliferation, apoptosis, cell cycle, migration, invasion, and signal transduction [2]. A bunch of lncRNAs such as small nucleolar RNA host gene 16 (SNHG16) [3], flamingo non-coding RNA (FLANC) [4], long noncoding RNA regulating IL-6 transcription (LNRRIL6) [5], small nucleolar RNA host gene 6 (SNHG6) [6], Long intergenic noncoding RNA for IGF2BP2 stability (LINRIS) [7], LINC00483 [8], and LINC00460 [9] have been identified as tumor suppressors or tumor promoters which participated in the occurrence and development of CRC; however, there are still a large number of lncRNAs need to be elucidated. Previous studies have shown that AC005062.1 (ENSG00000243004), a novel lncRNA located on chromosome 7p21.1 between position 19,918,981 and 20,140,453 (GRCh38.p13), was differentially expressed in bladder cancer [10] and small cell lung cancer [11], but in CRC, it has never been studied.

In the present study, we demonstrated that AC005062.1 was up-regulated in CRC tissues and cells for the first time. We also found that the knockdown of AC005062.1 by small interfering RNA (siRNA) can influence the proliferation, migration, invasion, apoptosis, and cell cycle of HT-29 cells. In addition, our results suggested that AC005062.1 may cis-regulate the expression of Metastasis Associated in Colon Cancer 1 (MACC1).

MATERIALS AND METHODS

Patient samples

Sixty-two pairs of CRC tumor and para-cancer tissues were obtained from patients who had undergone surgery in our hospital from 2018 to 2020. All samples were stored at −80 °C until use. Clinicopathological features recorded in our clinical database, including age and gender of patients, tumor location, size, Union for International Cancer Control (UICC) stage, vascular invasion, lymphatic metastasis, distant metastasis, and histologic type, were retrieved and retrospectively analyzed by two independent investigators. Written informed consent was obtained from each enrolled patient. The protocol of this study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College.

Cell culture and transfection

Human normal colorectal FHC cell line and CRC cell lines (HT-29, Caco-2, LoVo, HCT-15, and SW480) (Cell bank of the Chinese Academy of Sciences, Shanghai,

China) were maintained in DMEM medium supplied with 10% fetal bovine serum (FBS) and cultured in an incubator with 5% CO_2 at 37 °C. The 100 nM siRNA targeting AC005062.1 (si-AC005062.1, sense: 5′-GAGAAGAGCUAUUGUAAGAUU-3′, antisense: 5′-UCUUACAAUAGCUCUUCUCUU-3′) or control siRNA (si-Ctrl, Sense: 5′-UAAGGCUAUGAAGAGAUACUU-3′, antisense: 5′- GUAUCUCUUCAUAGCCUUAUU-3′) obtained from Shanghai Shenggong Biological Company (Shanghai, China) was transfected into HT-29 cells using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) under the instructions of the manufacturer.

Quantitative real-time PCR (qRT-PCR)

After quality control, total RNA extracted from tissues and cells using Trizol was reversed to cDNA using the Evo M-MLV RT Kit with gDNA Clean for qPCR (Accurate Biotechnology Co., Ltd., Hunan, China). qRT-PCR was then performed on an ABI7500 PCR system (Applied Biosystems, USA). The relative expression of AC005062.1 and MACC1 was calculated using the $2^{-\Delta\Delta Ct}$ method. Primers used in this study are listed in Table 1.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 assay was employed to explore the impact of AC005062.1 knockdown on cell proliferation. Briefly, siRNA transfected HT-29 cells were seeded in 96-well plates and cultured for 0 h, 12 h, 24 h, 36 h, 48 h, and 60 h, respectively. Then, 10 μl of CCK8 reagent was added to each well and incubated for 1 h. The absorbance value at 450 nm was measured using a microplate reader.

Flow cytometry

Flow cytometry was performed to evaluate the impact of AC005062.1 knockdown on cell apoptosis and cell cycle. Briefly, siRNA transfected HT-29 cells were harvested after 48 h culture, washed with PBS, and then stained with an Annexin V-fluorescein isothiocyanate propidium iodide (FITC/PI) apoptosis detection kit (BD Biosciences, USA) or a PI staining kit (MultiSciences (Lianke) Biotech Co., Ltd., Hangzhou, China). After that, samples were detected using the FACSCalibur Flow Cytometer (Becton Dickinson, USA) and analyzed with the appropriate software.

Wound scratch test

Wound scratch test was used to determine the impact of AC005062.1 knockdown on cell migration. Briefly, siRNA transfected HT-29 cells were seeded in 6-well plates and cultured to 90–95% confluence; then, cells were streaked vertically with 100 μl tip. Next, cells were washed with PBS and incubated in a serum-free medium for 24 h. Images were captured with an inverted microscope (Leica, Germany) and analyzed using Image-J software. The wound closure rate was defined as: (initial scratch area − 24 h scratch area)/initial scratch area. Relative wound closure (%) = wound closure rate of other sample/wound closure rate of No. 1 sample in the si-Ctrl group × 100 %.

Transwell assay

A Transwell assay was done to assess the impact of AC005062.1 knockdown on cell invasion. Briefly, siRNA transfected HT-29 cells were inoculated in the upper chamber coated with matrigel (BD Biosciences) and cultured with serum-free medium. Medium supplemented with 10% FBS was placed in the lower chamber. Cells were cultured in an incubator for 24 h, fixed with polyformaldehyde, stained with 0.5% crystal violet, and pictured with an inverted microscope (Leica). Migrating cells were counted in five random fields per sample.

Relationship between AC005062.1 and MACC1 in CRC

As cis-regulation of neighboring genes is a well-known mode of action of lncRNAs, we investigated whether AC005062.1 can directly regulate MACC1 expression. First, RNA-RNA CoExpression between AC005062.1 and MACC1 was performed using ENCORI Pan-Cancer Analysis Platform (https://starbase.sysu.edu.cn/panGeneCoExp.php) [12]. Second, qRT-PCR and western blot analysis were performed to determine the impact of AC005062.1 knockdown on the expression of MACC1 in HT-29 cells.

Western blot analysis

Proteins were extracted from HT-29 cells using RIPA lysis buffer and quantified by BCA assay. After being separated by SDS-PAGE electrophoresis, proteins were transferred to PVDF membrane and incubated with primary antibodies against cleaved caspase-3 (CST, 1:1000), Bax (CST, 1:1000), Bcl-2 (CST, 1:1000), MACC1 (CST, 1:1000), and GAPDH (CST, 1:1000) overnight at 4 °C. Secondary antibody was then added and incubated at room temperature for 1 h. Chemiluminescent signals were detected using the Novex™ ECL Chemiluminescent Substrate Reagent Kit (Invitrogen).

Statistical analysis

SPSS 19.0 software (SPSS Inc., USA) was used for statistical analyses. The Chi-square test or student's *t*-test was used for comparison between two groups, as appropriate. *p*-value less than 0.05 was considered statistically significant.

RESULTS

Upregulation of AC005062.1 in CRC tissues and cells

To determine the expression of AC005062.1 in CRC tissues and cells, qRT-PCR was performed.

Table 1 Primer sequences used in this study.

Gene name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
AC005062.1	TTCATCAGTGGGGTTCACCAG	AAGTTGTGCAGGTCAAGAAGC
MACC1	AGCAGTTGGAAGCAGGTGAA	ACTGTGCAACTGGTTCACCA
GAPDH	TCCACCCATGGCAAATTCCA	GCCTTCTCCATGGTGGTGAA

MACC1: Metastasis associated in colon cancer 1.

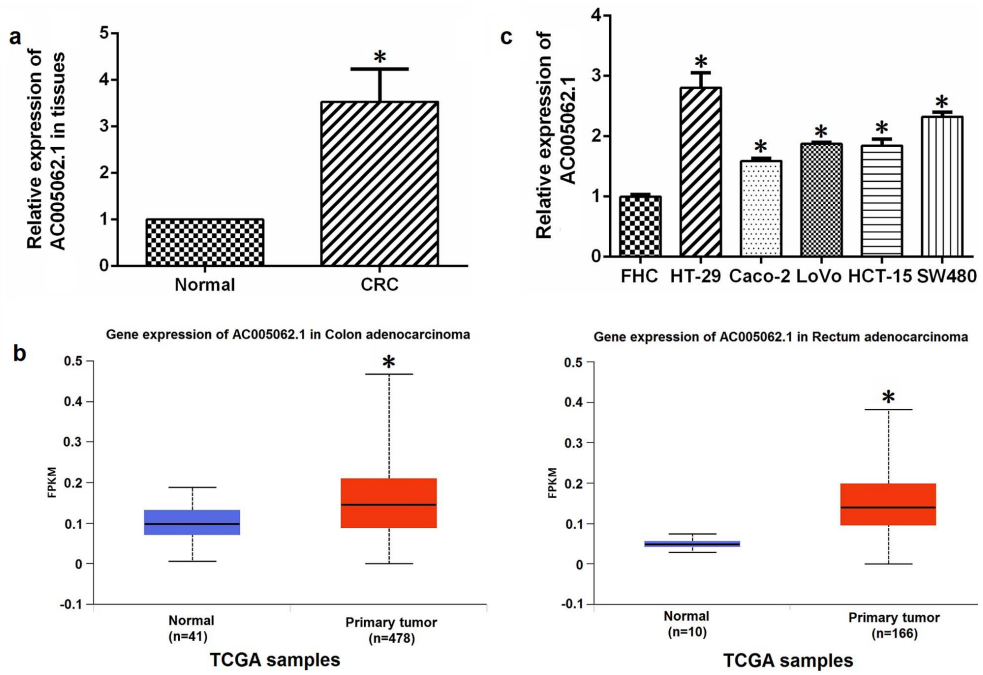

Fig. 1 Significant up-regulation of AC005062.1 expression in CRC tissues and cells. Up-regulation of AC005062.1 (a) in CRC tissues compared with para-cancer tissues determined by qRT-PCR, (b) in colon adenocarcinoma and rectum adenocarcinoma compared with normal tissues from TCGA data using the UALCAN platform, (c) in CRC cell lines compared with normal colorectal FHC cell line. CRC: colorectal cancer; TCGA: The Cancer Genome Atlas (* $p < 0.05$).

Our results showed that the AC005062.1 expression was prominently increased in the CRC tissues as compared with para-cancer tissues (Fig. 1a). Next, TCGA lncRNA analysis was performed using the UALCAN platform (http://ualcan.path.uab.edu/analysis-lncRNA.html) to verify the expression of AC005062.1 in CRC tissues [13]. Results showed that AC005062.1 was up-regulated in both colon adenocarcinoma (COAD) tissues and rectum adenocarcinoma (READ) tissues as compared with normal tissues (Fig. 1b). Furthermore, AC005062.1 was significantly up-regulated in HT-29, Caco-2, LoVo, HCT-15, and SW480 cells as compared with FHC cell (Fig. 1c). Because HT-29 cell line has the highest expression of AC005062.1, we chose this cell line to perform following *in vitro* experiments.

Correlation of AC005062.1 expression and CRC clinicopathological features

To explore the correlation between AC005062.1 expression and the clinicopathological features, patients were divided into two groups based on the median AC005062.1 expression. Our results showed that there were no significant differences between these two groups with regard to age, gender, location, and pathologic differentiation, whereas patients in the high AC005062.1 expression group had advanced clinical stages and larger tumor size than patients in the low AC005062.1 expression group. Besides, lymphatic metastasis, distant metastasis, and vascular invasion were more frequently observed in the high AC005062.1 expression group (Table 2). Our results suggested that over-expression of AC005062.1 may play an important role in the development and metastasis of CRC.

Inhibition of CRC cell proliferation, migration, and invasion by AC005062.1 knockdown

To investigate the functional role of AC005062.1 in CRC, siRNA was transfected into HT-29 cells. qRT-PCR revealed that the expression of AC005062.1 was significantly decreased after si-AC005062.1 transfec-

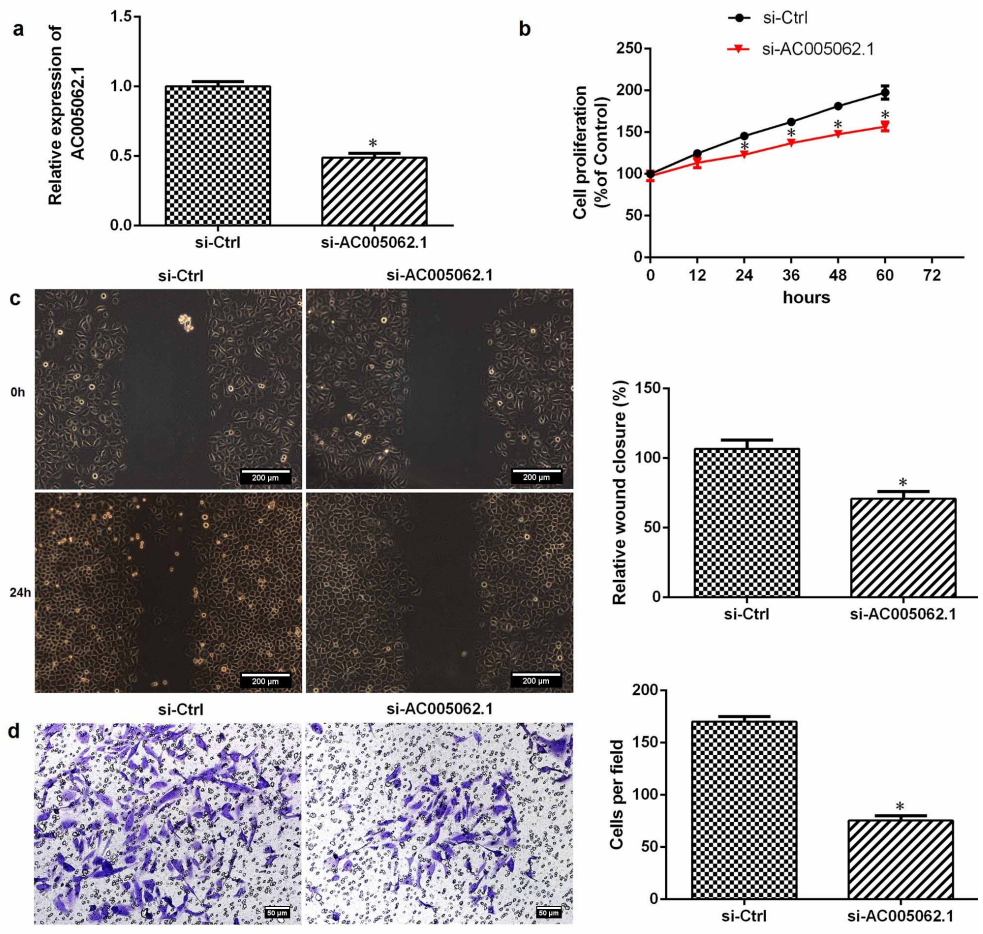

Fig. 2 Inhibition of CRC cell proliferation, migration, and invasion by AC005062.1 knockdown. (a) Inhibition of AC005062.1 expression after si-AC005062.1 transfection ($n = 3$); Effects of AC005062.1 knockdown (b) on proliferation of HT-29 cells via CCK-8 assay ($n = 5$), (c) on cell migration of HT-29 cells via wound scratch test ($\times 40$, scale bar = 200 μm) ($n = 3$), (d) on cell invasion of HT-29 cells via transwell assay ($\times 200$, scale bar = 50 μm) ($n = 3$). si-Ctrl: control siRNA; si-AC005062.1: siRNA targeted AC005062.1. At least three independent experiments were performed (* $p < 0.05$).

tion (Fig. 2a). CCK-8 assay showed that, as compared with the si-Ctrl group, the proliferation of the si-AC005062.1 group was significantly inhibited (Fig. 2b). Wound scratch test showed a significantly delayed wound closure in the si-AC005062.1 group as compared with the si-Ctrl group (Fig. 2c). Transwell assay revealed that the invasion ability of HT-29 cells was inhibited after si-AC005062.1 transfection (Fig. 2d). Taken together, our results demonstrated that knockdown of AC005062.1 can inhibit CRC cell proliferation, migration, and invasion.

Promotion of CRC cell apoptosis and G1 arrest by AC005062.1 knockdown

Flow cytometric results showed that the cell apoptosis rate was significantly increased in the si-AC005062.1 group as compared with the si-Ctrl group ($15.25 \pm 0.35\%$ and $5.95 \pm 0.99\%$, respectively, $p <$ 0.05) (Fig. 3a). Western blot results showed that the protein expressions of cleaved caspase-3 and Bax were increased while Bcl-2 was decreased after knockdown of AC005062.1 (Fig. 3b). As for cell cycle, flow cytometry results revealed that knockdown of AC005062.1 in HT-29 cells resulted into G1 phase cell cycle arrest (Fig. 3c). Taken together, our results demonstrated that knockdown of AC005062.1 can promote CRC cell apoptosis and G1 arrest.

Positive cis-regulation of MACC1 expression by AC005062.1

As shown in the Ensembl database, MACC1 is a neighboring gene (within a 100 kb window) of AC005062.1 (Fig. 4a). RNA-RNA CoExpression analysis showed a positive correlation between AC005062.1 and MACC1 in both COAD ($r = 0.646$, $p = 5.39\text{e-}57$) and READ ($r = 0.634$, $p = 3.82\text{e-}20$) (Fig. 4b). Both qRT-PCR

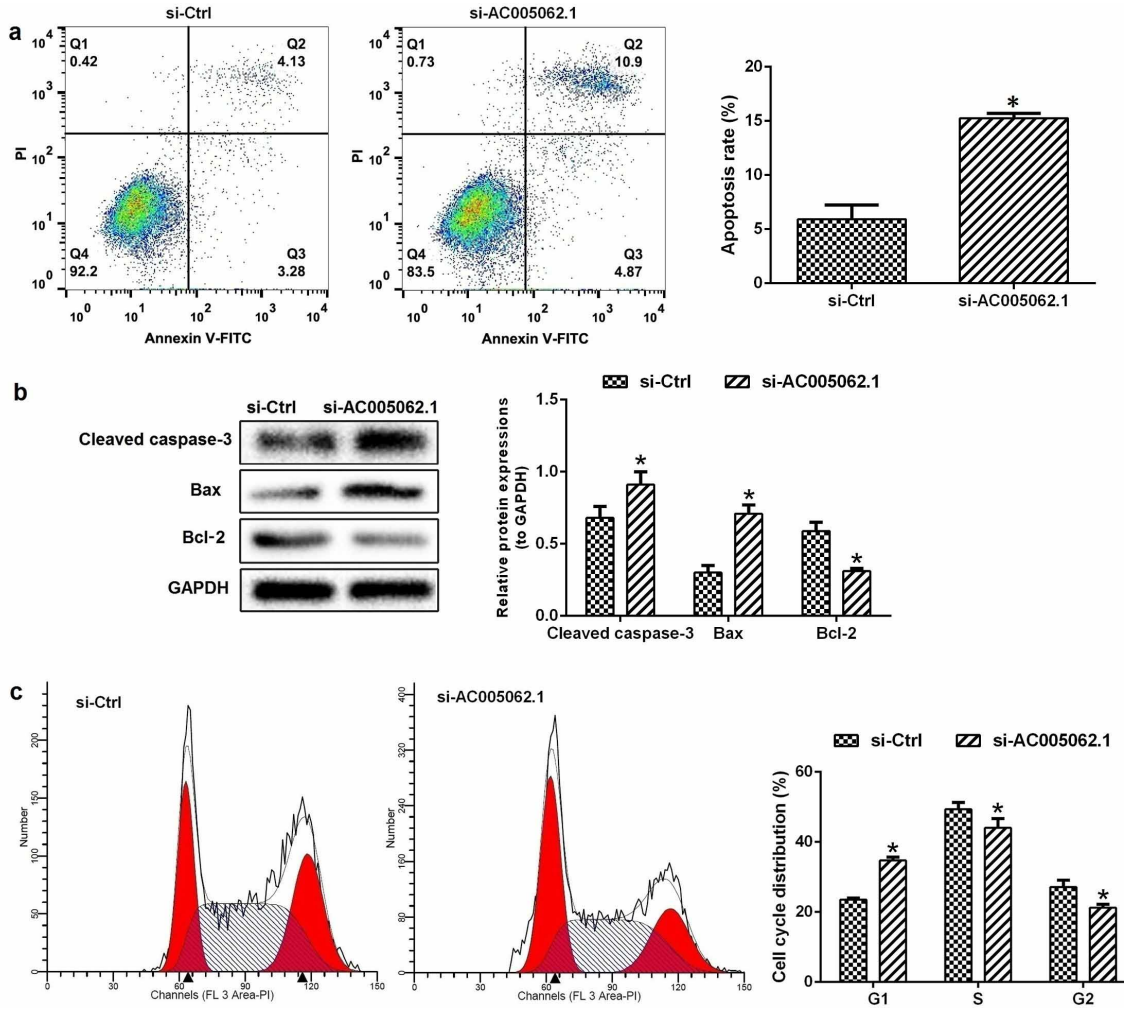

Fig. 3 Promotion of CRC cell apoptosis and G1 arrest by AC005062.1 knockdown. Effects of AC005062.1 knockdown (a) on cell apoptosis of HT-29 cells via flow cytometry ($n = 3$), (b) on protein expression of cleaved caspase-3, Bax, and Bcl-2 of HT-29 cells via western blot analysis ($n = 3$), (c) on cell cycle via flow cytometry ($n = 3$). si-Ctrl: control siRNA; si-AC005062.1: siRNA targeted AC005062.1. At least three independent experiments were performed (* $p < 0.05$).

and western blot experiments demonstrated that the expression of MACC1 was decreased after knockdown of AC005062.1 (Fig. 4c). Taken together, our results suggested that AC005062.1 may positively regulate the expression of MACC1 by a cis-regulatory model.

DISCUSSION

To the best of our knowledge, this is the first study to explore the expression and role of AC005062.1 in CRC. In our research, we demonstrated that AC005062.1 was up-regulated in CRC tissues and positively correlated with advanced clinical stage, larger tumor size, lymphatic metastasis, distant metastasis, and vascular invasion. We also found AC005062.1 was up-regulated in CRC cells; knockdown of this lncRNA in HT-29 cells by siRNA significantly inhibited cell proliferation, migration, and invasion and promoted cell apoptosis and G1 arrest. Besides, our results revealed that AC005062.1 may positively cis-regulate the expression of MACC1.

In recent years, a growing body of evidence has demonstrated that lncRNAs play an essential role in various cancers by regulating gene expression at transcriptional, post-transcriptional, and epigenetic levels. For instance, Li et al found that lncRNA ENST00000626052 (named ENSTa) regulated the trans-acting KIF19 gene and may be served as a novel biomarker for oral squamous cell carcinoma (OSCC) [14]. With the development in experimental techniques, taking next-generation sequencing for example, more and more dysregulated lncRNAs have been identified in CRC; however, the potential roles and mechanisms for most lncRNAs still remain unclear. AC005062.1 is a novel lncRNA with unknown

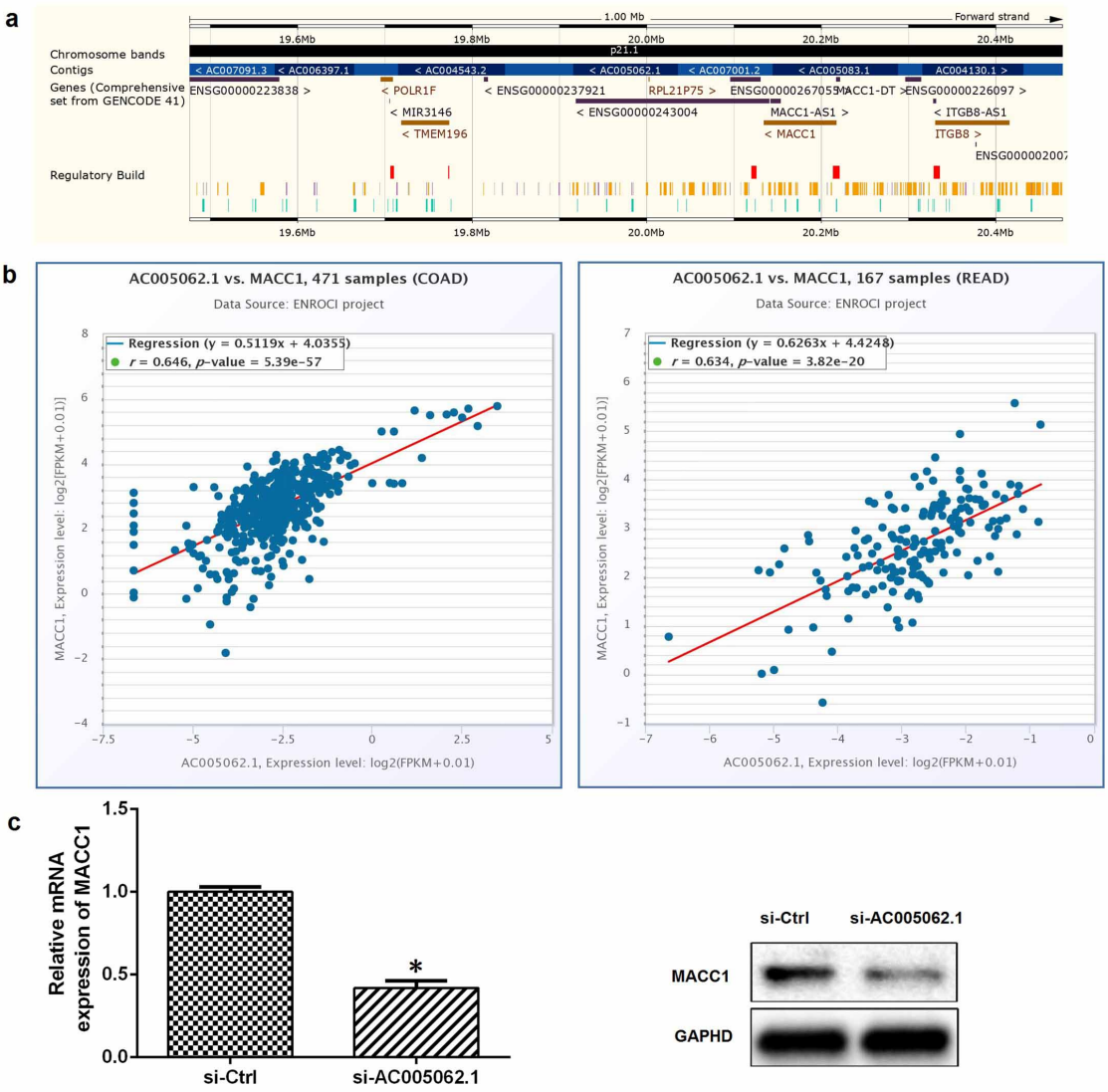

Fig. 4 Positive cis-regulation of MACC1 expression by AC005062.1. (a) Chromosomal location of AC005062.1 and MACC1 as shown in Ensembl database; (b) RNA-RNA CoExpression analysis showing a positive correlation between AC005062.1 and MACC1 in both COAD and READ samples; (c) Decrease in the expression of MACC1 both at mRNA and protein levels by AC005062.1 knockdown ($n = 3$). MACC1: metastasis associated in colon cancer 1; COAD:colon adenocarcinoma; READ: rectum adenocarcinoma; si-AC005062.1: siRNA targeted AC005062.1. At least three independent experiments were performed (* $p < 0.05$).

functions. Although it was found to be differentially expressed in bladder cancer [10], small cell lung cancer chemotherapy insensitivity [11], and human dopamine neuron differentiation [15], no functional studies have investigated the biological effects of AC005062.1. In the current study, up-regulated AC005062.1 was found in CRC tissues, which was further confirmed by TCGA data analysis using the UALCAN platform. Furthermore, we found AC005062.1 expression was closely related to advanced clinical stage, larger tumor size, lymphatic metastasis, distant metastasis, and vascular invasion, but not to age, gender, location, and pathologic differentiation. To further explore the role of AC005062.1 in CRC, *in vitro* experiments such as CCK-8 assay, wound scratch test, transwell assay, and flow cytometry were performed, and our results demonstrated that knockdown of AC005062.1 expression by siRNA exhibited an obvious effect on proliferation, migration, invasion, apoptosis, and cell cycle of HT-29 cells. Taken together, our study confirmed that the up-regulation of AC005062.1 participates in CRC development and progression.

Table 2 Correlation of AC005062.1 expression and CRC clinicopathological features.

Clinicopathological feature	AC005062.1 expression				*p*-value
	High ($n = 31$)		Low ($n = 31$)		
	n	%	*n*	%	
Age, years					
⩽ 50	13	41.9	11	35.5	0.602
> 50	18	58.1	20	64.5	
Gender					
Male	20	64.5	19	61.3	0.793
Female	11	35.5	12	38.7	
Location					
Colon	19	61.3	16	51.6	0.442
Rectal	12	38.7	15	48.4	
UICC stage					
I/II	15	48.4	23	74.2	0.037
III/IV	16	51.6	8	25.8	
Tumor size, cm					
⩽ 3	11	35.5	19	61.3	0.042
> 3	20	64.5	12	38.7	
Lymphatic metastasis					
Yes	14	45.2	6	19.4	0.030
No	17	54.8	25	80.6	
Distant metastasis					
Yes	12	38.7	5	16.1	0.046
No	19	61.3	26	83.9	
Vascular invasion					
Yes	17	54.8	9	29.0	0.039
No	14	45.2	22	71.0	
Pathologic differentiation					
Poor	10	32.3	7	22.6	0.694
Moderate	13	41.9	15	48.4	
Well	8	25.8	9	29.0	

CRC: Colorectal cancer.

MACC1 gene, located on chromosome 7 (7p21.1) between position 20,134,655 and 20,217,404 (GRCh38.p13), was first reported in 2009 in human colon cancer tissues [16]. After the discovery, MACC1 has been established as a key regulator for tumor progression and metastasis in a broad variety of solid cancers such as CRC [17], gastric cancer [18], hepatocellular carcinoma [19], breast cancer [20], cervical cancer [21], and ovarian cancer [22]. In CRC, MACC1 was over-expressed [16, 23, 24] and has been reported to be involved in various fundamental biological processes such as cell proliferation, invasion, and metastasis through various mechanisms [25, 26]. In the current study, we confirmed AC005062.1 can cis-regulate the expression of MACC1 based on the distance and expression correlation of these two genes. The functions of AC005062.1 in CRC may be relevant with its role in regulating MACC1.

However, there are some limitations in this study due to fund shortages. First, only the HT-29 cell line was used to knockdown the expression of AC005062.1 in *in vitro* experiments. Second, the knockdown of MACC1 in HT-29 cells should be done to better explore the role of AC005062.1-MACC1 axis in CRC.

CONCLUSION

In summary, our work demonstrated that AC005062.1 was up-regulated in CRC tissues and cells. AC005062.1 knockdown significantly inhibited cell proliferation, migration, and invasion and promoted cell apoptosis and G1 arrest in HT-29 cells. AC005062.1, acting as an oncogene, may be involved in CRC tumorigenesis and progression by regulating MACC1. In future, more studies are warranted to further elucidate the roles and mechanisms of AC005062.1 in cancers.

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