Super-enhancer Associated Long Non-coding RNA AC005592.2 Promotes Tumor Progression by Regulating OLFM4 in Colorectal Cancer

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Abstract

Background: Super-enhancer-associated long non-coding RNAs (SE-lncRNAs) have been reported to play essential roles in tumorigenesis, but the fundamental mechanism of SE-lncRNAs in colorectal cancer (CRC) remains largely unknown.

Methods: A microarray was performed to identify the differentially expressed SE-lncRNAs between CRC tissues and peritumoral tissues. A novel SE-lncRNA AC005592.2 was selected from these differentially expressed SE-lncRNAs to explore its effects in the CRC development. Fluorescence quantitative real-time PCR (qRT-PCR) was used to assay the expression of AC005592.2 in CRC tissues and cell lines. Functional assays were applied to identify the biological effects of AC005592.2 in CRC cells. Furthermore, RNA-seq was employed to predict potential targets of AC005592.2.

Results: AC005592.2 was significantly increased in CRC tissues and cells. And the high expression of AC005592.2 was significantly associated with TNM stage and tumor differentiation of CRC patients. Knockdown of AC005592.2 suppressed CRC cell proliferation, invasion and migration, but promoted apoptosis, while AC005592.2 overexpression exerted precisely the opposite effects on CRC cells. Besides, AC005592.2 positively regulated the expression of olfactomedin 4 (OLFM4), which was also up-regulation in CRC tissues.

Conclusion: The findings suggested that AC005592.2 is a crucial promoter of CRC progression, and may serve as an attractive therapeutic target for CRC.

Highlights

- A total of 23 strikingly changed SE-lncRNAs were identified in CRC tissues compared with normal adjacent colorectal tissues.
- ACC005592.2 is significantly increased in CRC tissues and cells.
- ACC005592.2 serves as an oncogene in CRC progression by enhancing cell proliferation, migration, invasion and reducing cell apoptosis.
- ACC005592.2 positively regulated the expression of OLFM4 in CRC cells, and OLFM4 was also up-regulation in CRC tissues.

Background

Colorectal cancer (CRC) is one of the most common malignant tumors. According to global cancer statistics 2018, CRC ranks as the third cause of cancer-related morbidity (10.2%) and the second cancer of mortality (9.2%) in the world. The progression of CRC is closely related to the mutation of oncogenes and antioncogenes[1], and an enormous amount of research effort has been carried out on this field. However, there are still numerous oncogenes and antioncogenes that have not been studied yet, and their role in CRC is completely unknown. Therefore, further deciphering of the mechanism of some unknown

CRC-related genes may provide us with more effective therapeutic strategies to improve the overall survival rate of CRC patient.

Super-enhancers (SEs) are clusters of enhancers enriched with genomic regulatory elements [2, 3]. In multiple types of mammalian cells, SEs are closely related to essential lineage-specific genes that can be used to regulate gene expression and confirm cell-type specificity through increase gene transcription over vast genomic distances[2, 4]. Moreover, SEs can regulate the expression of oncogenes and other transcripts important for tumor pathogenesis[5, 6]. Super-enhancer associated lncRNAs (SE-lncRNAs) are a specific set of lncRNAs transcribed from SE genomic regions. Recent studies have revealed that SE-lncRNAs are usually emerging as master RNA regulators in diverse gene expression programs and activate the gene expression by mechanisms of transcription factor trapping, chromatin looping, chromatin modification, PolII loading, and release of transcriptional repressor[7–11]. SE-lncRNAs are intimately involved in regulating tumorigenesis[11, 12]. A good example is that CCAT1-L positively regulates MYC expression by mediating chromatin looping between the MYC promoter and its enhancers to promote CRC progression[11].

In this study, the differentially expressed SE-lncRNAs in four pairs CRC tissues and peritumoral tissues was analyzed by using human SE-lncRNA microarray, and a novel CRC-associated SE-lncRNA which named ACC005592.2 was identified. The up-regulation of ACC005592.2 was significantly correlated with TNM stage and tumor differentiation of CRC patients. Further studies found that ACC005592.2 plays an oncogenic role in CRC progression by promoting cell proliferation, migration, invasion and restricting apoptosis. Mechanism research showed that ACC005592.2 might exert its oncogenic actions via regulating olfactomedin 4 (OLMF4), what's more, SE-lncRNA AC005592.2 has not been reported to regulate OLFM4 expression during CRC progression in any other experimental models.

Methods

Clinical samples

A total of 33 pairs of CRC tissues and peritumoral tissues were obtained from patients who underwent surgical resection in the Affiliated Cancer Hospital of Nanjing Medical University (Nanjing, China). None of these patients underwent radiotherapy, preoperative chemotherapy or other tumor-specific therapies. All fresh tissues were stored in −80°C until use.

Arraystar human SE-LncRNA microarray

Arraystar human SE-LncRNA microarray is global profiling of SE-lncRNAs and protein-coding mRNA, which about 7753 SE-lncRNAs and 7040 coding mRNA. The microarray analysis was performed by Kangcheng Biology Engineering (Shanghai, China) following the arraystar standard protocol. Briefly, four CRC tissues and peritumoral tissues were selected to profiled the expression of SE-lncRNAs. The dysregulation of SE-lncRNAs were identified and analyzed according to the criteria of fold change >2 and

P-value < 0.05. The raw data have been uploaded to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The GEO accession numbers are GSE15102.

Cell culture

The CRC cell lines HCT-116 (RRID: CVCL_0291), SW480 (RRID: CVCL_0546), SW620 (RRID: CVCL_0547), HCT-8 (CVCL_2515), HT-29 (RRID: CVCL_0320), LoVo (RRID: CVCL_0399), HCT-15 (RRID: CVCL_0292) and normal human colon epithelial cell line (FHC, RRID: CVCL_3688) were purchased from the American Type Culture Collection (ATCC). These cells were cultured in 90% Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Invitrogen, USA) at 37 °C incubator containing 5% CO_2.

RNA extraction and reverse transcription

Total RNA was extracted from tissues and cells with the TRIzol reagent (Qiagen, USA). The RNA quantity and quality were assessed by NanoDrop ND-2000 spectrophotometer (Thermo, USA). The integrity of RNA was confirmed by 1% agarose gel electrophoresis. And then RNA was reversely transcribed as cDNA using the Prime-Script RT reagent with gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions.

Fluorescence quantitative real-time PCR analysis

The fluorescence quantitative real-time PCR (qRT-PCR) analysis was performed using the SYBR Green Master Mix kit (TaKaRa, Japan) on a life Quantstudio 6 Flex system (Applied Biosystems, USA with the following conditions: 95°C for 10 min and 40 cycles of 95°C 15 s, 60°C 60s. The relative mRNA expression was calculated by $2^{-\Delta\Delta CT}$ method, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control of CT value. The primer sequences were showed in Supplementary Table S1.

Protein-coding potential

The protein-coding potential of AC005592.2 isoform (ENST00000510311.1,ENSG000002231185.2) was assessed by using Coding Potential Assessment Tool (CPAT, http://lilab.research.bcm.edu/cpat/), Coding Potential Calculator (CPC, http://cpc.cbi.pku.edu.cn/)[13] and PhyloCSF[14, 15]. Here, the UCSC genome browser may serve as an alternative to viewing PhyloCSF scores for AC005592.2 by copying the URL.

Subcellular localization analysis

The separation of nucleus and cytoplasm fractions was performed with the PARIS^{TM} kit (Invitrogen, USA) according to the manufacturer's instructions. Then mRNA expression of AC005592.2 in the nucleus and cytoplasm was testified by qRT-PCR. CT values of AC005592.2 were compared to GAPDH in the nucleus, and were compared to U6 in cytoplasm.

siRNA transfection

The three siRNAs targeting AC005592.2 (siRNA-78 sense strand: GGAAGCUAGUAGAAGAUUUTT and anti-sense strand: AAAUCUUCUACUAGCUUCCTT; siRNA-273 sense strand: GAAUGGCACUUUGGACAAUTT and anti-sense strand: AUUGUCCAAAGUGCCAUUCTT; siRNA-402 sense strand: GGAGUAGGCUGACCAGUUATT and anti-sense strand: UAACUGGUCAGCCUACUCCTT) and scrambled negative control siRNA (siRNA-NC, siRNA-NC sense strand: UUCUCCGAACGUGUCACGUTT and anti-sense strand: ACGUGAGCACUUCGGAGAATT) were synthesized and purchased from GenePharma (Shanghai, China). The Lipofectamine RNAi MAX kit (Invitrogen, USA) was used to transfected siRNA into CRC cells according to the manufacturer's instructions. Two of the three siRNA sequences were selected for further studies based on the knockdown efficiency, as confirmed by qRT-PCR.

Construction and infection of vectors for AC005592.2 over-expressing lentivirus

The vectors for AC005592.2 over-expressing lentivirus and negative control were designated and constructed as LV5-AC005592.2 and LV5-NC by GenePharma (Shanghai, China). CRC cells were infected with LV5-AC005592.2 and LV5-NC in the presence of 5 μg/mL polybrene. After 24 h, the supernatant was replaced with fresh culture medium, then cultured for 48-72 h. the expression of AC005592.2 infected cells was validated by qRT-PCR.

CCK8 assay

Cell proliferation was examined with the CCK-8 detection kit (Dojindo, Japan) according to the manufacturer protocol. Briefly, the CRC cells with different treatments were replanted in 96-well plates at a density of 5×10^3 cells/well, and then incubated with 10 μl CCK8 solution for 37°C 2 h. The proliferation index was measured every 24 h to 96 h at 450 nm absorbance.

Transwell assay

Cell migration and invasion assays were performed using Falcon Cell Culture Insert (BD Biosciences, USA), the 8.0 μm pore polycarbonate membranes of invasion assays coated with Matrigel (BD Biosciences, USA). Briefly, approximately 4×10^4 cells with different treatments were seeded into the upper

chamber with 0.2 mL serum-free DMEM, and the lower chamber was added 0.6 mL DMEM containing 10% FBS as a chemoattractant. After further incubation for 24–48 h, CRC cells that penetrate to the other side of membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of cells was counted under a light microscope as cell migration or invasion ability

Cell apoptosis analysis

Cell apoptosis was performed using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Multi Sciences, China) according to the manufacturer's instructions. Briefly, CRC Cells were harvested and washed in cold phosphate-buffered saline (PBS). The washed cells were centrifuged and resuspended using 1X annexin-binding buffer to obtain a cell density of 1×10^6 cells/ml. Then add Alexa Fluor 488 annexin V and 100 μg/mL PI working solution to the cell suspension. After incubation at room temperature for 15 minutes, add 1X annexin-binding buffer. Finally, the stained cells were analyzed by flow cytometry.

Western blot analysis

The CRC cells were collected in the radio-immunoprecipitation (RIPA) lysis buffer (Biovision, USA) to extract cellular protein, and the protein concentration was detected with BCA protein assay kit (Thermo, USA). Total lysis was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto PVDF membranes (Millipore, USA). The membranes were blocked in 5% nonfat dry milk for 1 h at room temperature, and incubated with 1:1000 Human Olfactomedin-4 (OLFM4) antibody (Affinity Biosciences, USA, RRID: AB_2846459) or 1:5000 GAPDH antibody (Abcam, USA, RRID: AB_2049706) at 4°C overnight. After being incubated with Goat Anti-Rabbit IgG H&L (HRP) secondary antibody (Abcam, USA, RRID: AB_955417) for 1 h at room temperature, the protein bands were detected by ECL method (Millipore, USA)

RNA sequencing array and bioinformatics analysis

Three siRNA ACC00552.2 transfected HT-29 cells and three negative control were selected for RNA-Sequencings (RNA-seq) to find downstream target genes of AC005592.2. Whole RNA-seq was performed by Guangzhou RiboBio (Guangzhou, China) using Illumi-naHiSeq 3000 platform. All the differentially expressed genes (fold change > 2, *P*-value < 0.05) were used for hierarchical clustering, volcano plots, gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses. *P*-value < 0.05 was considered as the threshold to define significant enrichment of the genes GO and KEGG enrichment analysis.

Statistical analysis

Statistical analyses were performed by GraphPad Prism v6.0 (GraphPad Software, Inc. La Jolla, CA, USA) and SPSS Statistics Version 20.0 (SPSS, Chicago, IL, USA). The unpaired 2-tailed student's *t*-test was used to evaluate significant significance between two groups. Statistical differences for more than two groups were determined by two-way ANOVA and Multiple *t*-test. All data were represented as the mean ± SD for triplicate independent measurements. The value of $P < 0.05$ was considered to be a statistical difference.

Results

Expressional profiles of SE-lncRNA and mRNA in CRC

To analyze the roles of SE-lncRNAs in CRC, an SE-lncRNA microarray was performed to profile the differentially expressed SE-lncRNAs and mRNA in four CRC tissues and peritumoral tissues. From boxplot line diagrams of SE-lncRNA and mRNA (Fig. 1a, b), the results showed that the distribution of SE-lncRNAs and mRNAs signal values were in proper symmetrical analysis, and SE-lncRNAs at lower levels than mRNA in CRC, which consistent with previous reports in other tissues[16]. Additionally, a total of 23 differentially expressed SE-lncRNAs were identified between the two groups, including 15 up- and 8 down-regulated SE-lncRNAs (fold change>2, *P*-value < 0.05) in CRC tissues relative to peritumoral tissues (Fig. 1c). These data confirmed that the expression of SE-lncRNA undergoes a change that cannot be ignored during CRC tumorigenesis. A total of 165 (91 up- and 74 down-regulated) differentially expressed mRNA were also identified (fold change > 1.5, *P*-value < 0.05) between the two groups (Fig. 1d, e), which will help us to search potential target genes and further explore the biological function of SE-lncRNA in CRC.

AC005592.2 is highly expressed in CRC tissues and cells

In these differentially expressed SE-lncRNAs, it can be found that AC005592.2 was remarkably up-regulated in CRC (fold change = 3.984, *P*-value = 0.022). To confirm the microarray analysis findings, 33 pairs of CRC tissues were collected, as well as the paired peritumoral tissues. The results of qRT-PCR showed that AC005592.2 expression in CRC tissues was significantly higher than in peritumoral tissues (fold change = 3.128, *P*-value = 0.0054, Fig. 2a). Similarly, the AC005592.2 expression was higher in CRC cell lines than in cell line FHC (Fig. 2b). The cell lines HCT-116 and HT-29 harbored higher AC005592.2 expression were selected for future research. To further assessed the clinical-pathological association of AC005592.2 in CRC patients, the patients were divided into two groups via the median values: high AC005592.2 expression group (above the median) and low AC005592.2 expression group (below the median). It was found that patients in high AC005592.2 expression group more likely to be in advanced TNM stage (*P*-value = 0.037) and poor tumor differentiation (*P*-value = 0.026), but no significant association with other clinical parameters (Table 1).

Table 1
Correlations between AC005592.2 and clinicopathological characteristics in 33 CRC cases

Characteristics	AC005592.2 expression		
	Low (n=16)	High (n=17)	P-value
Age			1.000
<60	7	7	
≥60	9	10	
Gender			1.000
Female	8	8	
Male	8	9	
Tumor size			0.1721
< 5 cm	11	10	
≥ 5 cm	5	7	
T stage			0.166
T1-T2	9	5	
T3-T4	7	12	
Lymph node metastasis			0.728
N0	10	9	
N1–2	6	8	
Distant metastasis			0.688
M0	13	12	
M1	3	5	
TNM stage			0.037*
I-II	12	6	
III-IV	4	11	
Tumor differentiation			0.026*
Well/moderately	14	8	
Poorly	2	9	

* P-value < 0.05

The AC005592.2 is super enhancer-associated long non-coding RNA mainly localized in the nucleus

AC005592.2, also named SPRY4 anti-sense RNA 1 (SPRY4-AS1), whose genomic sequence (ENSG00000231185) has six transcripts, namely ENST00000510311.1, ENST00000443800.1, ENST00000515288.1, ENST00000414314.1, ENST00000425963.1 and ENST00000514303.1, which represent distinct annotated isoforms of a single lncRNA gene. Among them, the transcript ENST00000510311.1 that mapped to chr5:141,704,858-141,843,619 with a length of 591bp, is a CRC-related SE-lncRNA obtained from microarray results in this study (Fig. 2c). Furthermore, AC005592.2 was classified as a noncoding RNA with coding probability 0.0174 and 0.158 predicted by CPAT and C PC, respectively, as well as the resulting of PhyloCSF, which indicated no evidence for AC005592.2 translation of any possible ORF (Fig. 2d). In addition, Subcellular localization analysis showed that AC005592.2 is mostly located in the nucleus of CRC cells (Fig. 2e), which suggests the role of AC005592.2 mainly at the level of transcription.

Knockdown of AC005592.2 inhibits CRC cell proliferation, invasion, and migration, induces apoptosis

To study the potential effects of AC005592.2 on CRC progression, the loss-of-function assays were performed to evaluate the effects of AC005592.2 knockdown in CRC cell lines. Three AC005592.2 siRNAs including siRNA-402, siRNA-273 and siRNA-78, were designed to transfect HCT-116 and HT-29 cells, the results showed that the expression level of AC005592.2 was significantly reduced following three siRNA transfection when compared to siRNA-NC transfection (Fig. 3a). Moreover, siRNA-402 and siRNA-273 with higher silence efficiency were selected for future functional assays. The CCK-8 assay showed that AC005592.2 knockdown effectively attenuated cell proliferation compared with the control group (Fig. 3b). Transwell assays showed that the number of invaded and migrated cells were significantly suppressed after AC005592.2 knockdown (Fig. 3c). The flow cytometry assay results showed that the silence of AC005592.2 could promote cell apoptosis effectively (Fig. 3d).

Overexpression of AC005592.2 promotes CRC cell proliferation, invasion, and migration, inhibits apoptosis

Furthermore, the gain-of-function assays were also used to confirm the acting roles of AC005592.2 overexpression in CRC cells. As compared to LV5-NC infection, the AC005592.2 expression was

significantly increased in HCT-116 and HT-29 cells following LV5-AC005592.2 infection (Fig. 4a). The functional assays showed that AC005592.2 overexpression significantly increased the proliferation of HCT-116 and HT-29 cells (Fig. 4b), while the invasive and migratory potentials of HCT-116 and HT-29 cells were effectively induced (Fig. 4c). Similarly, the overexpression of AC005592.2 in CRC cells also effectively inhibited cell apoptosis (Fig. 4d).

The potential downstream signaling of AC005592.2

To explore the molecular mechanisms of AC005592.2 in promoting CRC progression, the RNA-seq assays were performed to analyze gene expression changes induced by AC005592.2 silencing. Hierarchical clustering showed systematic variations in the genes between the groups of siRNA-402 and siRNA-NC (Fig. 5a). A total of 579 dysregulated genes, including 437 up- and 142 down-regulation genes, were revealed following AC005592.2 knockdown by volcano plot (fold change > 2, *P*-value < 0.05) (Fig. 5b). Furthermore, GO analysis was performed to analyze the related biological process (BP), cellular component (CC) and molecular function (MF) of these identified genes (Fig. 5c). The AC005592.2-regulated genes were mainly involved in following pathways: for BP: single−organism process, cellular process, single-organism cellular process; for CC cell: cell, cell part, intrinsic component of membrane; for MF: binding, protein binding, transmembrane transporter activity. Similarly, KEGG pathway enrichment analyses also revealed the AC005592.2 enrichment was associated with genes in Neuroactive ligand-receptor interaction, cAMP signaling pathway, Nicotine addiction, Glutamatergic synapse (Fig. 5d).

AC005592.2 directly regulates OLFM4 expression in CRC cells

From the intersection of the 579 dysregulated genes with 165 differentially expressed mRNA identified from SE-lncRNA microarray, four candidate genes (MLEC, DSCAML1, OLFM4, HAS1) were selected (Fig. 6a). The results of qRT-PCR showed that OLFM4 expression was exhibited the biggest fold change in HCT-116and HT-29 cells, whether the AC005592.2 gene was knockdown or overexpressed (Fig. 6b, c). WB analysis showed that AC005592.2 down-regulation significantly increased the protein expression levels of OLFM4 in HCT-116 and HT-29 cells (Fig. 6b). In addition, OLFM4 was also significantly increased in CRC tissues (fold change = 6.918, *P*-value = 0.0017, Fig. 6e), which consistent with the data obtained from SE-lncRNA microarray (fold change = 34.033, *P*-value = 0.0036) and RNA-seq (fold change = 2.287, *P*-value = 0.0034). These results suggested that OLFM4 is a downstream-regulated gene by AC005592.2 in CRC cells.

Discussion

The carcinogenesis of CRC is a complex process, that generally considered to involve the activation of oncogenes or the inactivation of antioncogenes[17]. Increasing evidences indicated that SE-lncRNAs are

closely related to the development of multiple cancers[12], including CRC[11], and is expected to provide new therapeutic targets for CRC. Arraystar human SE-LncRNA microarray is designed to profile lncRNAs transcribed from SE regions. With the high-performance workflow and the in-depth SE-lncRNA annotation, the microarrays produce rich lncRNA profiling data superior to RNA-seq, and reveal the relationships of complex SE-lncRNA biology and regulatory with some transcription factor or cancer-related genes. In this study, the SE-LncRNA microarray was used and identified a set of differentially expressed SE-lncRNAs related to CRC. Among these SE-lncRNAs, high expression of AC005592.2 in CRC tissues was positively related with TNM stage and tumor differentiation.

AC005592.2, also known as SPRY4-AS1, is located on chromosome5q31.3and is an anti-sense RNA of SPRY4. In further studies, it was found that knockdown of AC005592.2 could inhibit CRC cell proliferation, invasion and migration, but promote apoptosis, while overexpression of AC005592.2 exerted exactly the opposite effects in CRC cells. Therefore, AC005592.2 has made a crucial contribution to the carcinogenesis of CRC. Forasmuch as regulating the expression of adjacent genes by cis is one of the most important mechanisms of SE-lncRNA[11, 18, 19]. We first tried to explore the molecular mechanisms of AC005592.2 in CRC by analyzing the genes that overlapped with AC005592.2 or within 50 KB of its transcription start site, but without success. The basic principle of trans acting prediction target gene is that the function of lncRNA is related to its co-expressed protein-coding genes[20]. This study tried to predict the target gene of AC005592.2 by WGCNA co-expression analysis, but still failed. Finally, RNA-seq assays were carried out to explore AC005592.2-regulated genes and pathways, it was found that there are four genes MLEC, DSCAML1, OLFM4, HAS1 in both dysregulated genes obtained from RNA-seq assays and differentially expressed mRNA identified from SE-lncRNA microarray. Further qRT-PCR and WB validated OLFM4 may be a potential target of AC005592.2. Therefore, it is reasonable to believe that OLFM4 is a target gene of AC005592.2.

OLFM4, also known as hGC-1 or GW112, was first cloned from human myeloblasts. As a secreted glycoprotein, OLFM4 is a family of olfactomedin and strongly expressed in the stomach, small intestine, colon, prostate and bone marrow [21]. Previous studies have revealed that OLFM4 is closely related to several gastrointestinal malignancies including CRC[22, 23], its roles in the progression of CRC involved anti-inflammation, proliferation, differentiation, apoptosis and cell adhesion[24]. For example, Seko N et al. examined the expression and distribution of OLFM4 in CRC by immunohistochemistry and found that of 34% of CRC cases were positive for OLFM4 cytoplasmic staining [25]; Liu W et al. reported OLFM4 overexpression could alter the morphology and cortical actin distribution of HT-29 cells, and decrease cell adhesion and migration[26]. In addition, the up-regulation of OLFM4 was often detected in highly differentiated and early-stage CRC, while in some poorly differentiated late tumor-node-metastasis stage and metastatic CRC, down-regulation or no expression was more frequently detected[26]. Interestingly, OLFM4 down-regulation patients with CRC have better overall survival than that OLFM4 up-regulation [25]. Moreover, CRC progression can be attenuated by blocking the Wnt/β-catenin signaling pathway via OLFM4 negative regulation [27]. In this study, AC005592.2 positively regulated the expression of OLFM4 in CRC cells, and OLFM4 was up-regulation in CRC tissues. Combining with the present works of OLFM4,

we have a hypothesis thatAC005592.2 may contribute to CRC progression by regulating OLFM4, in which multiple mechanisms might be involved.

Molecular targeted drugs with the high tumor-targeting ability and low side effects have become a research hotspot of anti-tumor therapies. At present, the study of targeted drugs mainly follows the principle of treating a molecule acts on one target to treat one tumor. However, the tumor is a disease characterized by multiple molecular pathological changes and various signal pathways imbalances[28, 29], tumor cells can adapt to new signal pathways by self-modifying mutation, so many of single-target drugs failed to reach the original expectancy[30–32]. The combination works to a certain degree solved this problem, but there are some limitations to this approach, such as complicated measurement design and drug interactions. The development of a single molecule, which simultaneously regulating multiple mechanisms, not only achieves more potent therapeutic effects, but also avoids the problems caused by combined medication[33, 34]. In further investigations, a large number of experiments will be carried out to verify AC005592.2 contribute to CRC related to multiple mechanisms, which is of considerable significance to explore new therapies for CRC.

Conclusions

In summary, this is the first study to evaluate the role of AC005592.2 in CRC systematically. AC005592.2 is upregulated in CRC tissues and its overexpression may be associated with CRC progression. Therefore, these findings enable us to reasonably conclude that AC005592.2 is an oncogene in CRC, and may serve as a target for new therapies in CRC, which will bring a new opportunity for CRC patients.

Abbreviations

CRC: colorectal cancer; SE-lncRNAs: super-enhancer associated long non-coding RNAs; qRT-PCR: fluorescence quantitative real-time PCR; OLFM4: olfactomedin 4; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NC: negative control; NCBI: National Center for Biotechnology Information; GEO: Gene Expression Omnibus; ATCC: American Type Culture Collection; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; SD: standard deviation; PBS: phosphate-buffered saline; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GO: gene ontology; KEGG: Kyoto encyclopedia of genes and genomes.

Declarations

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Authors' contributions

FY and LPY concepted and designed the study. HHC and PJ collected, stored and managed clinical specimens. LPY and HHC performed experiments, collected and analyzed the data. LT and FY gave intelligent advice and provided technical and material support. LPY wrote the original draft. All authors reviewed the previous versions of the manuscript. All authors read and agreed to the published version of the manuscript.

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Competing Interests

The authors declare that they have no conflict of interest.

Ethics approval and consent to participate

This study was approved by the Clinical Research Ethics Committee of Nanjing Medical University and the ethical permit number is (2019)843. Written informed consent of all the atients were obtained for research purposes.

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Figures

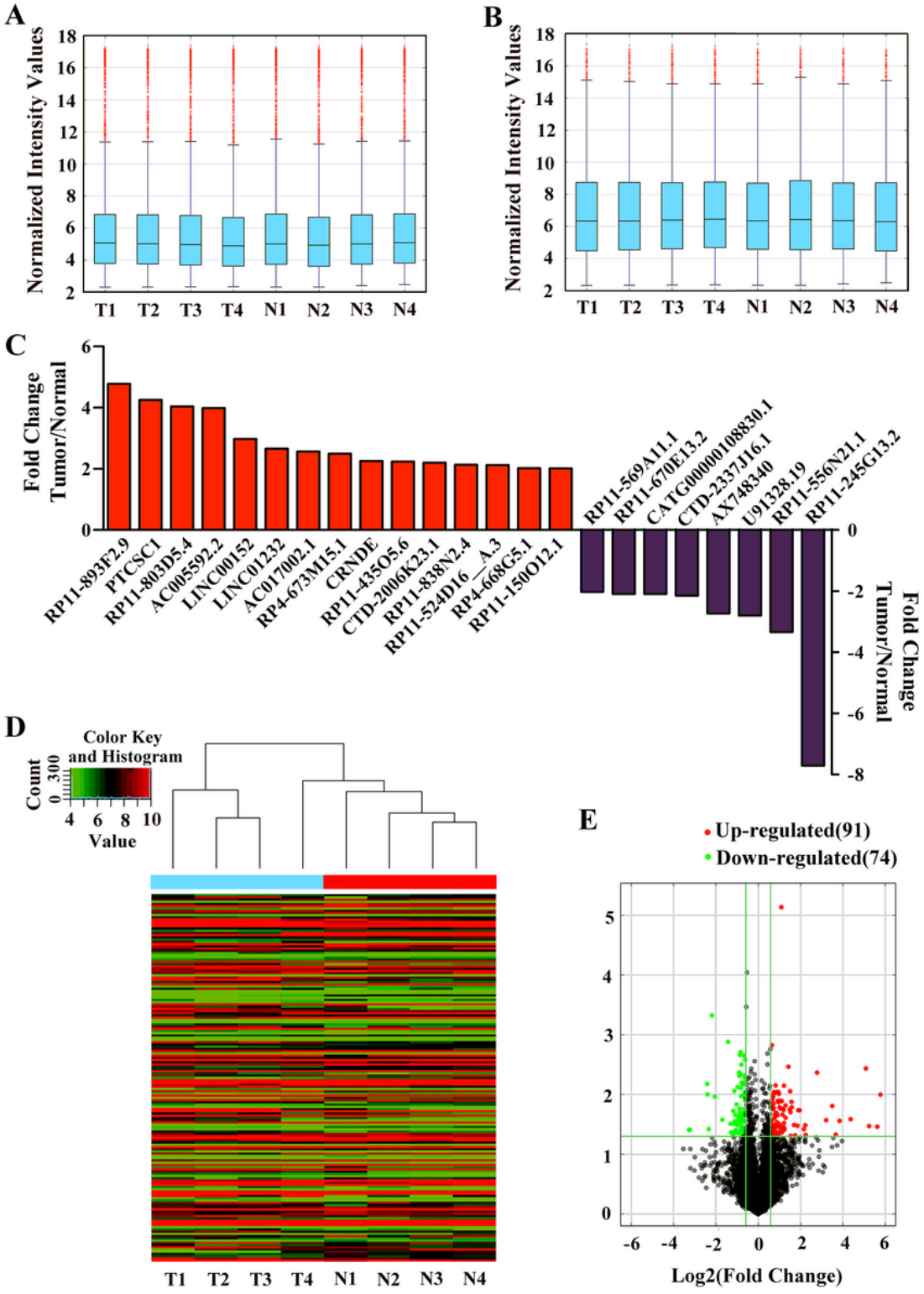
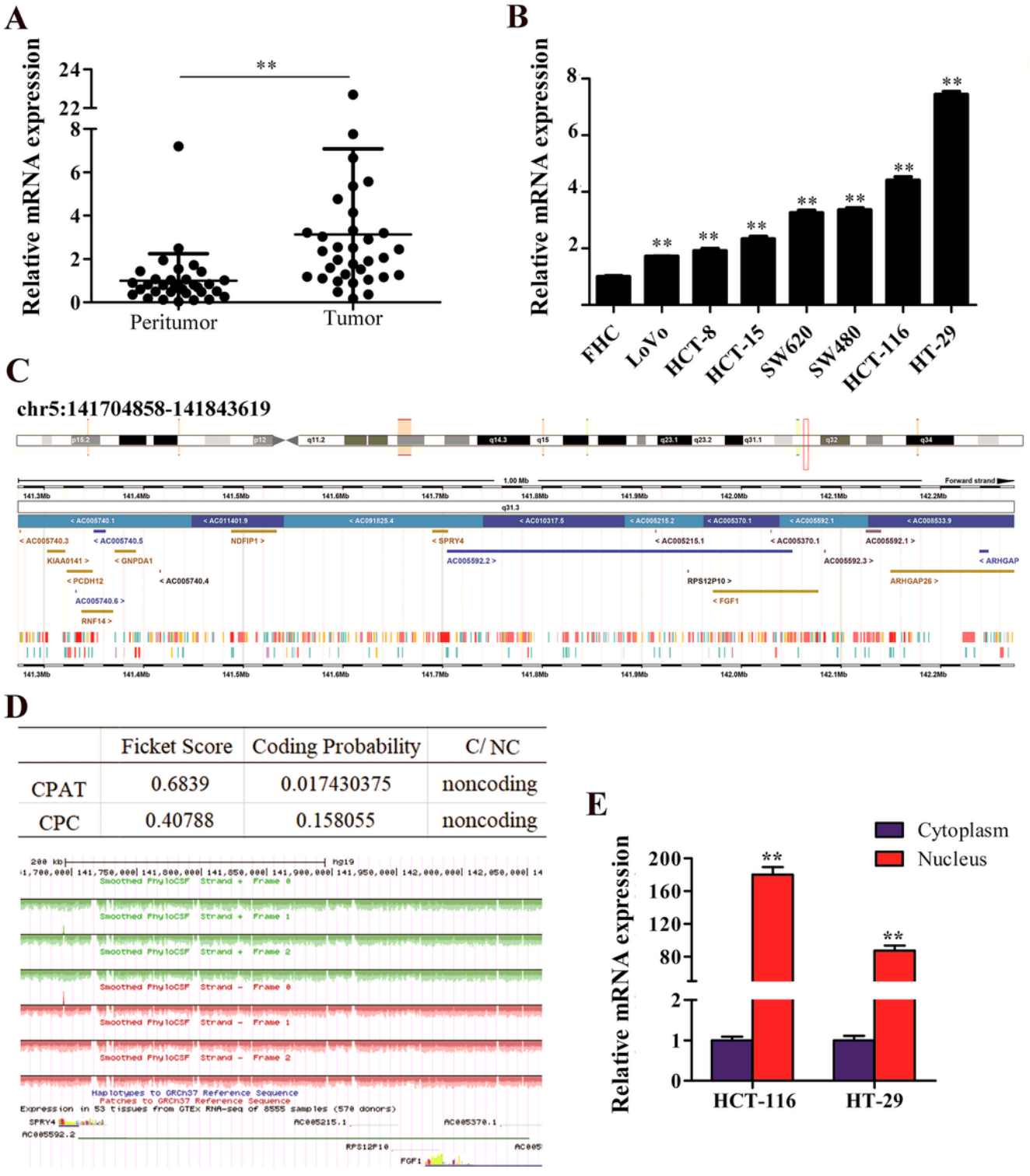


Figure 1

Expressional profiles of SE-lncRNA and mRNA in CRC. a The distribution of SE-lncRNAs signal values from microarray analysis was assessed by boxplot line diagrams. b The boxplot line diagrams of mRNAs. The boxplot analysis indicated that distribution of SE-lncRNAs and mRNAs signal values were in good symmetrical analysis and SE-lncRNAs at lower levels than mRNAs in CRC. c A total of 23 differentially expressed SE-lncRNAs, including 15 up- and 8 down-regulated SE-lncRNAs in CRC tissues

relative to peritumoral tissues (fold change > 2, P-value < 0.05). d Hierarchical cluster analysis of mRNAs that were differentially expressed (fold change > 1.5, P-value < 0.05) in CRC and peritumoral tissues. e Volcano plots visualizing the differentially expressed mRNAs. T: Tumor; N: Normal



	Ficket Score	Coding Probability	C/ NC
CPAT	0.6839	0.017430375	noncoding
CPC	0.40788	0.158055	noncoding

Figure 2

A SE-lncRNA AC005592.2, which was highly expressed in CRC tissues and cells, mostly located in nucleus. a The AC005592.2 expression in CRC tissues was significantly higher than peritumoral tissues. b

The AC005592.2 expression was higher in CRC cell lines than in the immortalized colon epithelial cell line FHC. c AC005592.2 is located on chr5:141,704,858-141,843,619 with a length of 591bp using the UCSC Genome Browser database. d AC005592.2 was predicted to be non-coding RNA by CPC, CPAT and PhyloCSF. e The subcellular location of AC005592.2 expression is mostly in nucleus. U6, nucleus control; GAPDH, cytoplasm control. CPAT: Coding Potential Assessment Tool; CPC: Coding Potential Calculator. C/CN: Coding or noncoding. ** P-value < 0.01

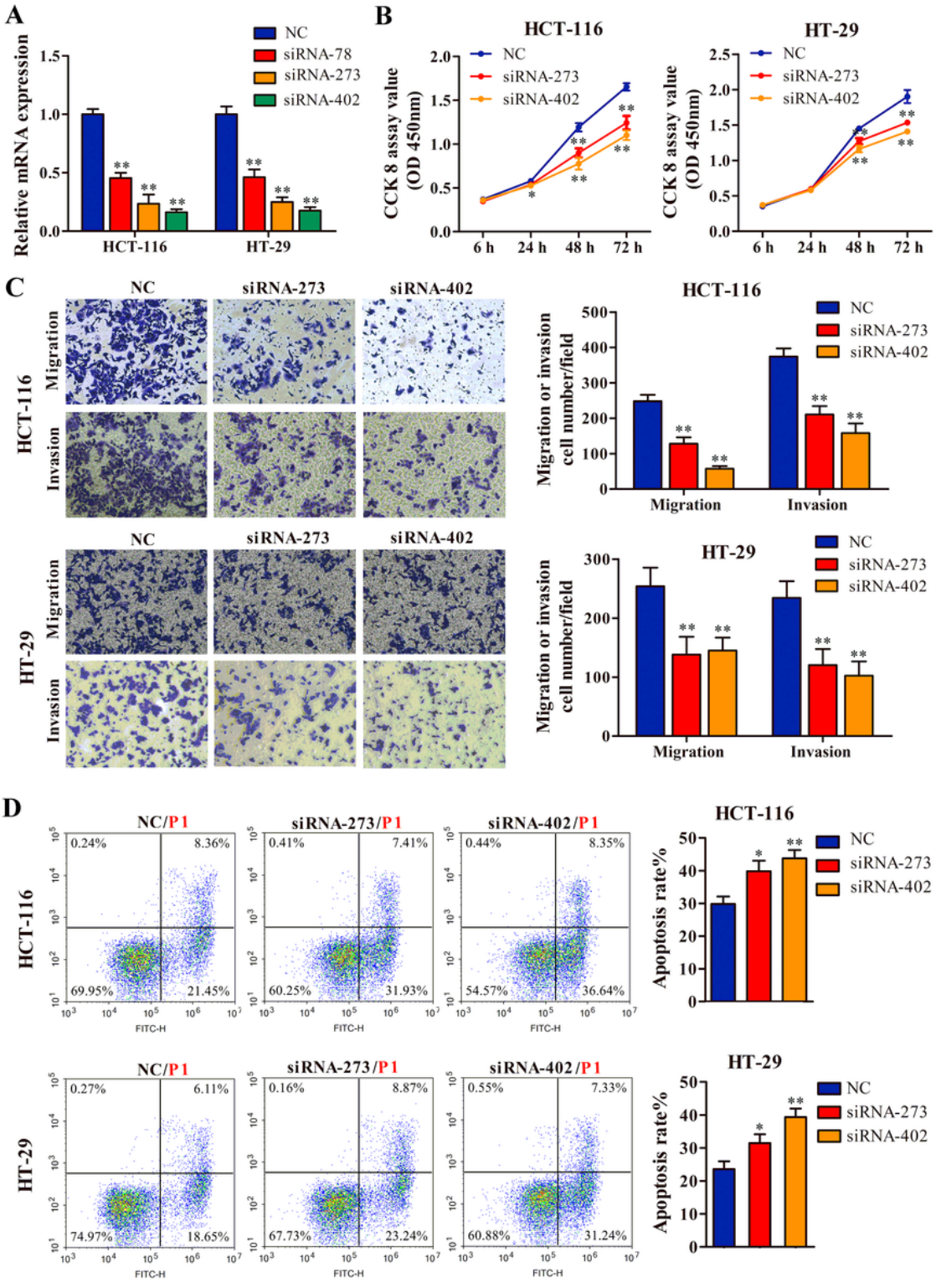


Figure 3

Knockdown of AC005592.2 inhibited CRC cell proliferation, invasion, and migration, induced apoptosis. a Relative expression of AC005592.2 in HCT-116 and HT-29 cells after transfection with AC005592.2 siRNAs. b CCK8 assays revealed that AC005592.2 knockdown significantly inhibited HCT-116 and HT-29 cells proliferation. c Transwell assays revealed that AC005592.2 knockdown significantly inhibited migration and invasion ability in HCT-116 and HT-29. d Apoptosis assays by flow cytometry indicated that AC005592.2 knockdown increased apoptosis rate in HCT-116 and HT-29 cells. * P-value < 0.05, ** P-value < 0.01

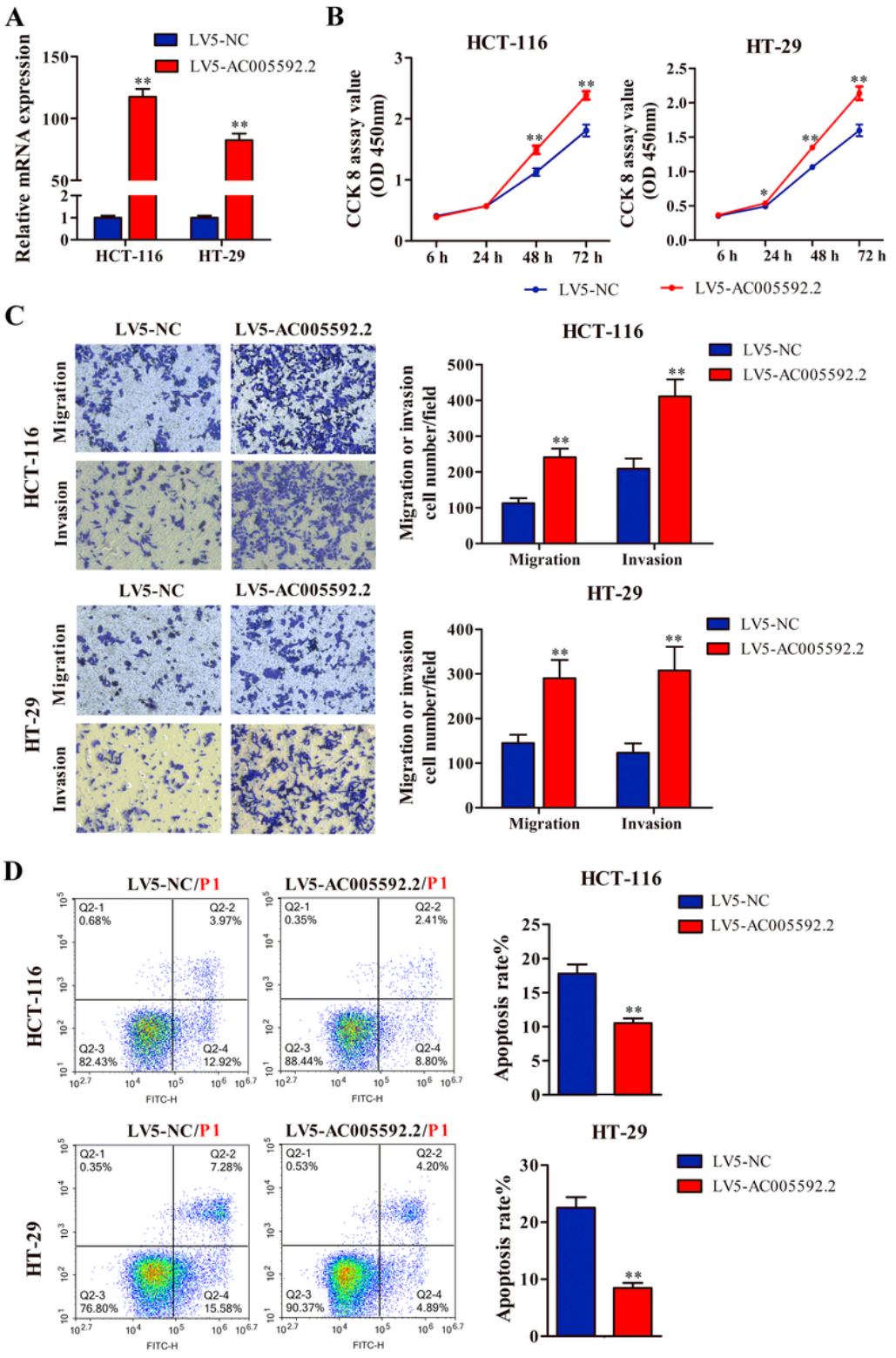


Figure 4

Overexpression of AC005592.2 promoted CRC cell proliferation, invasion, and migration, inhibited apoptosis. a Relative expression levels of AC005592.2 in HCT-116 and HT-29 cells following LV5-NC and LV5-AC005592.2 infection. b CCK8 assays revealed that AC005592.2 overexpression significantly promoted HCT-116 and HT-29 cells proliferation. c Transwell assays revealed that AC005592.2 overexpression significantly promoted migration and invasion ability in HCT-116 and HT-29. d Apoptosis assays by flow cytometry indicated that AC005592.2 overexpression decreased apoptosis rate in HCT-116 and HT-29 cells. * $p < 0.05$, ** $p < 0.01$

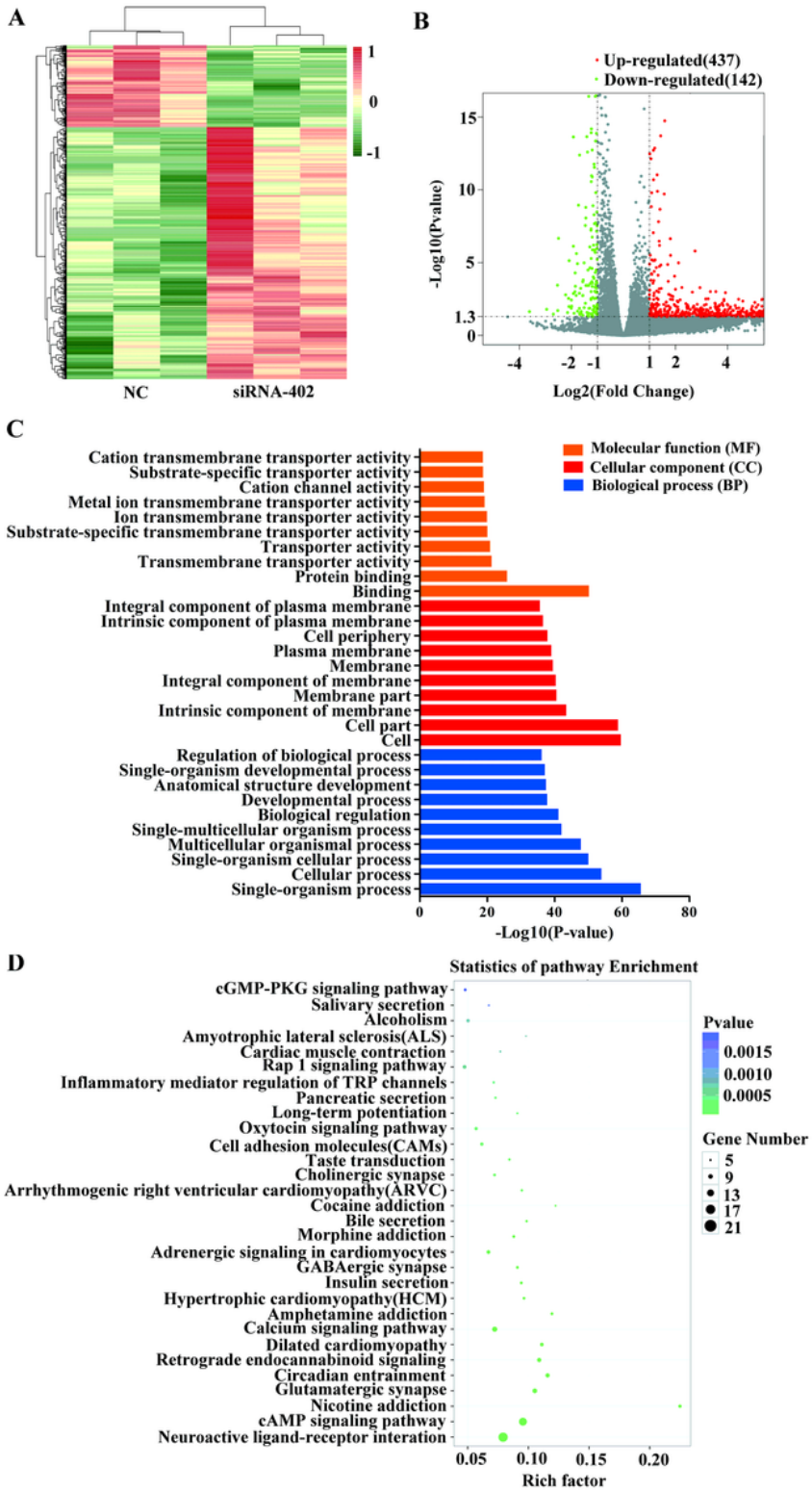


Figure 5

RNA-seq assay revealed potential downstream signaling of AC005592.2. a Hierarchical cluster analysis of dysregulated genes (fold change > 2, P-value < 0.05) in siRNA treated HT-29 cells. b Volcano plots visualizing the dysregulated genes. c Gene ontology (GO) analysis of the dysregulated genes were involved in biological process (BP), cellular component (CC) and molecular function (MF). d The top 30 enrichment KEGG pathways of the dysregulated genes.

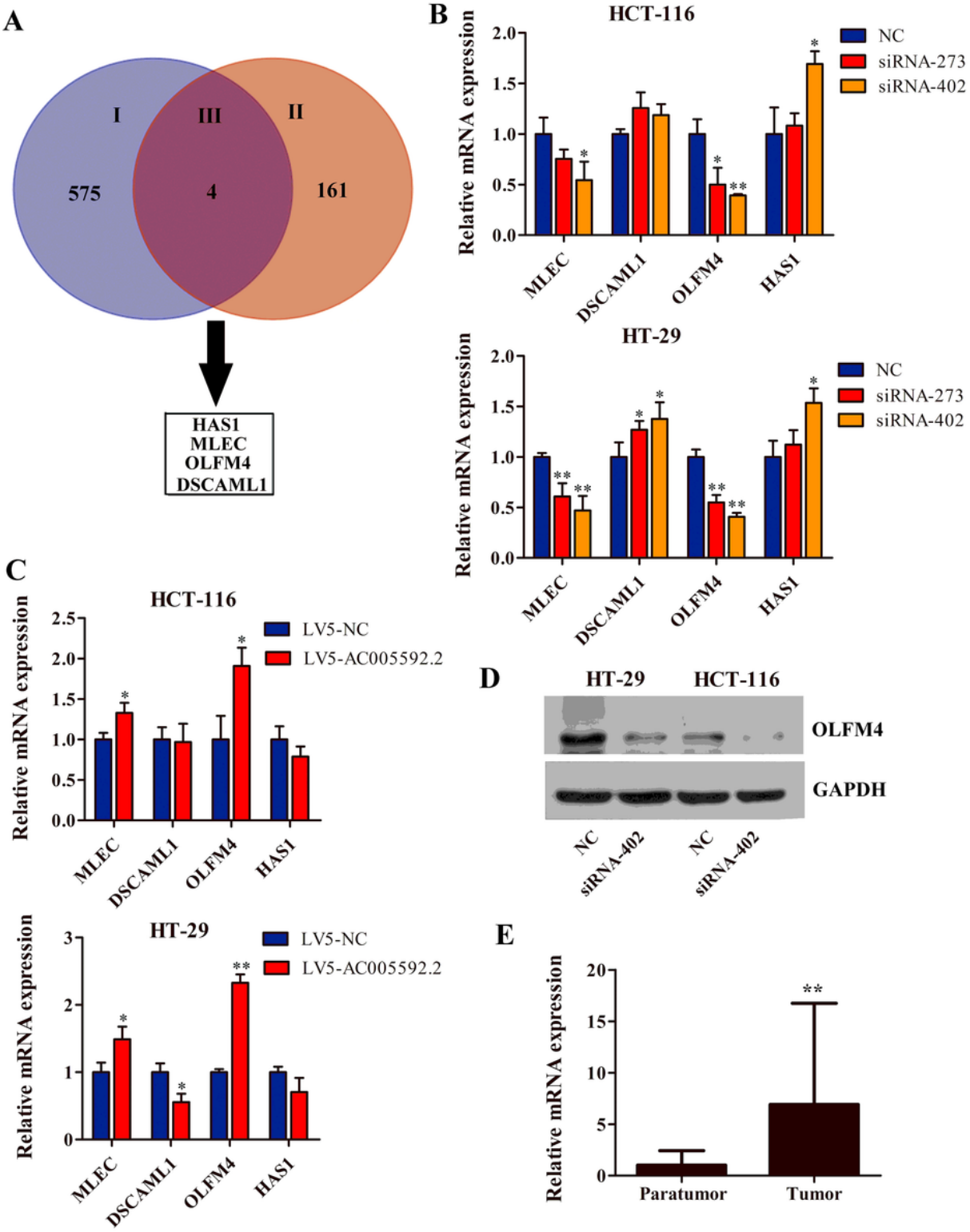


Figure 6

AC005592.2 directly regulates OLFM4 expression in CRC cells. a Four candidate genes (MLEC, DSCAML1, OLFM4, HAS1) were obtained in the study. Ⅰ: 579 dysregulated genes obtained from RNA-seq assay; Ⅱ: 165 differentially expressed mRNA identified from SE-lncRNA microarray. Ⅲ: 4 candidate genes (MLEC, DSCAML1, OLFM4, HAS1). b Four candidate genes were confirmed by qRT-PCR in AC005592.2 knockdown HCT-116 and HT-29 cells. c Four candidate genes were confirmed by qRT-PCR in AC005592.2

overexpression HCT-116 and HT-29 cells. d OLMF4 protein levels in AC005592.2 knockdown HCT-116 and HT-29 cells. e The OLMF4 expression in CRC tissues was significantly higher than peritumoral tissues. * $p < 0.05$, ** $p < 0.01$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalfileofWB.tiff
- SupplementaryTableS1.docx