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Long Noncoding RNA LINC00520 Accelerates the Progression of Colorectal Cancer by Serving as a Competing Endogenous RNA of Microrna-577 to Increase HSP27 Expression

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Abstract

The Long Noncoding RNA (lncRNA) LINC00520 is an important modulator of the oncogenicity of multiple human cancers. However, whether LINC00520 is involved in the malignant characteristics of Colorectal Cancer (CRC) has not been extensively studied until recently. Therefore, the present study aimed to detect LINC00520 expression in CRC and evaluate its clinical significance in patients with CRC. Functional experiments were conducted to test the biological roles and underlying mechanisms of LINC00520 in CRC progression. In this study, high LINC00520 expression was verified in CRC tissues and cell lines, and this high expression was associated with patients' unfavorable clinico-pathological parameters and shorter overall survival and disease-free survival. Functionally, interference of LINC00520 resulted in a significant decrease of CRC cell proliferation, migration, colony formation ability, and invasion. Mechanistically, LINC00520 functioned as a competing endogenous RNA by sponging microRNA-577 (miR-577) and thereby increasing Heat shock protein 27 (HSP27) expressions. Rescue experiments revealed that inhibiting miR-577 or restoring HSP27 could abrogate the effects of LINC00520 silencing on malignant phenotypes of CRC. LINC00520 functioned as an oncogenic lncRNA in CRC, and it facilitated CRC progression by regulating the miR-577/HSP27 axis, suggesting that the LINC00520/miR-577/HSP27 axis is an effective target in anticancer management.

Keywords: LINC00520; Colorectal Cancer; Microrna-577; Heat Shock Protein 27

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Introduction

Colorectal Cancer (CRC) remains one of the most common malignancies globally, with over 1 million cases having been diagnosed in 2014 [1]. Even so, the mechanistic basis for CRC development and progression remains unclear, with many patients exhibiting many associated risk factors contributing to eventual disease onset [2]. CRC also remains the fourth deadliest cancer, although there have been significant reductions in average mortality rates in CRC patients in recent decades owing to diagnostic and therapeutic innovations [3]. CRC patients that go on to suffer from poor outcomes most often do so either as a result of tumor metastasis or the acquisition of a drug-resistant form of disease, with both of these phenotypes in turn arising due to cancer-associated gene dysregulation [2,4-6]. As such, a better understanding of those genes associated with CRC development may be of value, potentially identifying novel diagnostic, therapeutic, or prognostic biomarkers of this disease.

Increasing evidence has revealed that long noncoding RNAs (lncRNAs) comprise a novel group of sequences for studying the mechanisms of several cancer types [7-9]. They belong to a family of linear transcripts longer than 200 nucleotides [10]. Originally, lncRNAs were considered genomic "junk" and "noise" because of their lack of protein-encoding ability [11]. Currently, overwhelming evidence has revealed that lncRNAs is implicated in a wide range of genetic processes and that they

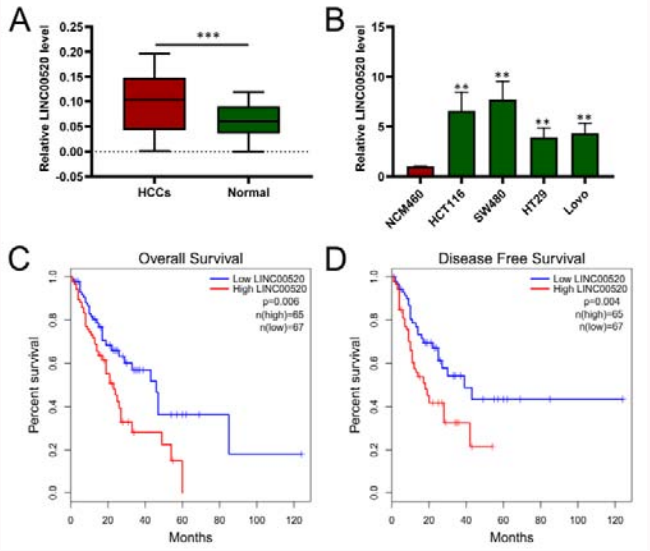

Figure 1: LINC00520 is upregulated in CRC and associated with poor prognosis.
(A) RT-qPCR was performed to measure LINC00520 expression in 132 pairs of CRC tissues and surrounding adjacent normal tissues. *P<0.05 compared with adjacent normal tissues. **(B)** Total RNA was isolated from four CRC cell lines (HCT116, SW480, HT29, and Lovo) and a human normal thyroid cell line (NCM460) and then subjected to RT-qPCR analysis to measure LINC00520 expression. *P<0.05 compared with NCM460. **(C,D)** The Kaplan–Meier method and log-rank test were used to analyze the relationship between LINC00520 and the overall survival and disease-free survival of patients with CRC. *P<0.05; **P<0.01; ***P<0.001; P>0.05.

modulate gene expression at the transcriptional, posttranscriptional, and epigenetic levels [12,13]. The dysfunction of lncRNA is closely associated with multiple human diseases, such as cancer, neurodegeneration, cardiovascular diseases, and endocrine diseases [14]. The aberrant expression of lncRNAs is frequently identified in CRC, and their dysregulation is implicated in the genesis and development of CRC [15-17]. Several studies have highlighted that dysregulated lncRNAs exert tumor-suppressing or tumor-promoting effects and participate in the modulation of several malignant characteristics [18-20]. Accordingly, further research on the detailed roles of lncRNAs in CRC may reveal promising therapeutic targets to improve the prognosis of patients with this disease.

The lncRNA LINC00520 is an important modulator of the oncogenicity of several human cancers [21-24]. Nevertheless, the expression pattern and functional roles of LINC00520 in CRC remain to be investigated and confirmed. Therefore, LINC00520 was selected as the focus of our research. This study may offer novel insights into CRC pathogenesis and provide novel clues for its clinical treatment.

Material and Methods

Patients and tissue specimens

This study was approved by the research ethics committee of T Changhai Hospital, Second Military Medical University and performed in accordance with the Declaration of Helsinki. A total of 132 patients with CRC were recruited in this study, none of whom had been previously treated with preoperative radiotherapy, chemotherapy, or other anticancer therapies. CRC tissues and surrounding adjacent normal tissues were immediately snap-frozen in liquid nitrogen after surgical removal and thereafter transferred to a - 80°C cryogenic refrigerator.

Cell culture

A human normal colonic epithelial cell line (NCM460) and four CRC cell lines (HCT116, SW480, HT29, and Lovo) were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco; Thermo Fisher Scientific, Inc.). All aforementioned cell lines were grown at 37°C in a humidified atmosphere comprising 5% CO_2.

Cell transfection

For loss-of-function study, Small Interfering RNA (siRNA) Specifically Targeting LINC00520 (si-LINC00520) and Nontargeting Control siRNA (si-NC) were obtained from Guangzhou RIBOBIO Co., Ltd (Guangzhou, China). miR-577 agomir (agomir-577) was designed and generated by GenePharma Co., Ltd (Shanghai, China), and Negative Control Agomir (agomir-NC) was used as a control. miR-577 antagomir (antagomir-577) was used to silence endogenous miR-577 expression, and antagomir-NC was used as the control for antagomir-577. The HSP27 overexpression vector pcDNA3.1-HSP27 (pc-HSP27) and empty pcDNA3.1 vector was also synthesized by GenePharma. Cells were seeded into 6-well plates 1 day before transfection. The aforementioned molecular products were transiently transfected into cells using Lipofectamine 2000™ reagent (Invitrogen; Thermo Fisher Scientific). Reverse transcription quantitative polymerase chain reaction (RT-qPCR).

After the isolation of total RNA using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), its concentration and quality were determined using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). To detect miR-577 expression, reverse transcription was conducted using a Mir-X™ miRNA First-Strand Synthesis Kit (TaKaRa Biotechnology, Co., Ltd., Dalian, China), and the synthesized complementary DNA (cDNA) was subjected to quantitative PCR using a Mir-X™ miRNA qRT-PCR TB Green® Kit (TaKaRa Biotechnology). U6 small nuclear RNA functioned as an endogenous control for normalizing miR-577 expression. To quantify LINC00520 and HSP27 mRNA expressions, total RNA was converted into cDNA using a PrimeScript™ RT Reagent Kit (TaKaRa Biotechnology). Subsequently, cDNA products were amplified using a SYBR-Green PCR Master Mix (TaKaRa Biotechnology). The amounts of LINC00520 and HSP27 mRNA were normalized to GAPDH expression. The expressions of all the genes were analyzed using the 2-ΔΔCq method.

Cell counting Kit-8 (CCK-8) assay: CCK-8 assay was employed to investigate cellular proliferation. After 24 h of incubation, transfected cells were treated with trypsin, collected, and seeded into 96-well plates with a density of 2×10^3 cells/well. CCK-8 assay was performed by adding 10 µl of CCK-8 solution (Shanghai Haling Biotechnology, Co., Ltd., Shanghai, China) into each well, after which the cells were incubated at 37°C for 2 h. The absorbance of the mixture in each well was read at 450 nm in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transwell assays: Forty-eight hours after transfection, the cells were digested using trypsin, and the harvested cells were washed twice with PBS. For invasion assays, 5×10^4 transfected cells re-suspended in 200 µl of FBS-free DMEM were added into each of the upper compartments of a Transwell insert (8 µm pore; Corning, Inc.,

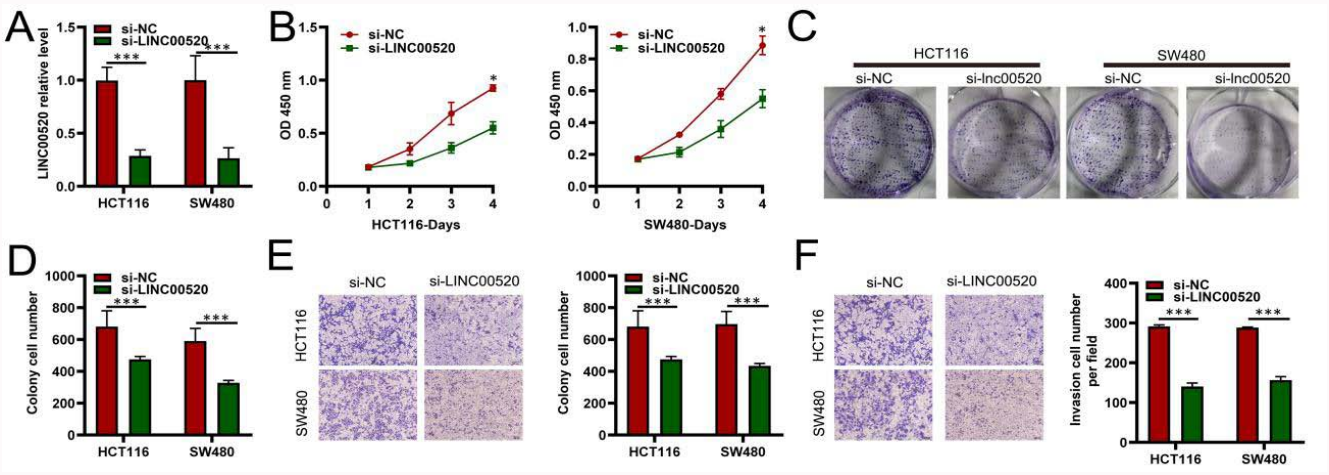

Figure 2: A decrease in LINC00520 expression suppresses HCT116 and SW480 cell proliferation, migration, invasion, and induces cell apoptosis *in vitro*. **(A)** RT-qPCR analysis of LINC00520 expression in HCT116 and SW480 cells after the transfection of si-LINC00520 or si-NC. **(B)** The proliferation of LINC00520 deficient-HCT116 and SW480 cell was detected using the Cell Counting Kit-8 assay. **(C,D)** Colony form assays were performed in HCT116 and SW480 cells after si-LINC00520 or si-NC transfection. **(E,F)** Transwell assays were performed to assess the migratory and invasive abilities of HCT116 and SW480 cells following si-LINC00520 or si-NC transfection. *P<0.05; **P<0.01; ***P<0.001; P>0.05.

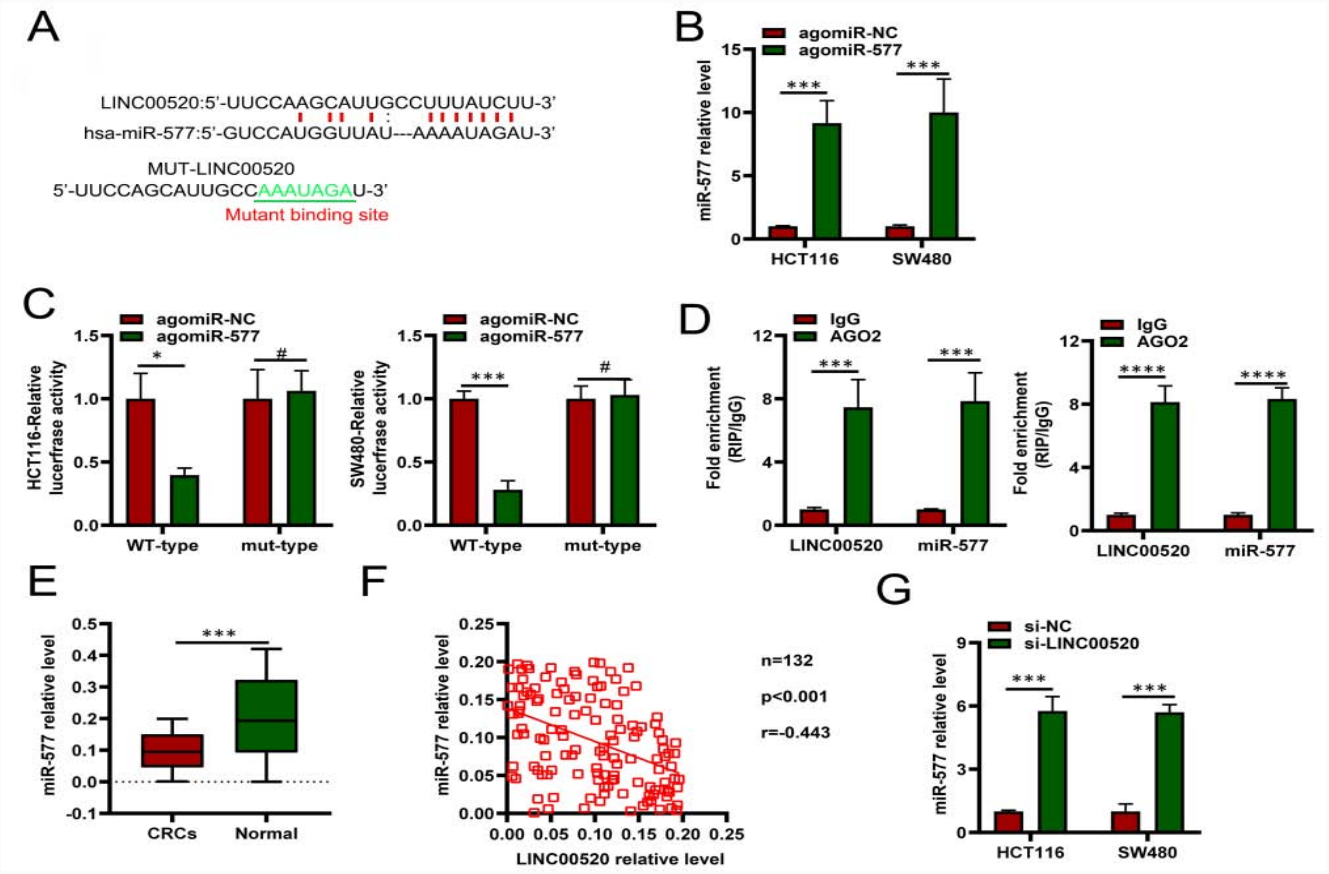

Figure 3: LINC00520 directly interacts with miR-577 and sponges its expression in CRC cells. **(A)** Bioinformatics analysis revealed the bindings sites of wild-type (WT) and mutant (MUT) miR-577 within LINC00520. **(B)** miR-577 expression analysis in HCT116 and SW480 cells transfected with agomir-577 or agomir-NC. **(C)** WT-LINC00520 or MUT-LINC00520 were co-transfected with agomir-577 or agomir-NC into HCT116 and SW480 cells. Luciferase activity was detected 48 h after co-transfection. **(D)** LINC00520 and miR-577 were enriched in Ago2-containing immunoprecipitated compared with the IgG control. **(E)** miR-577 expression in 132 pairs of CRC tissues and surrounding adjacent normal tissues was measured using RT-qPCR. **(F)** The correlation between INC00520 and miR-577 expressions was validated in the same CRC tissues via Spearman's correlation analysis. **(G)** miR-577 expression in LINC00520-silenced HCT116 and SW480 cells was determined by RT-qPCR. *P<0.05; **P<0.01; ***P<0.001; P>0.05.

Corning, NY, USA), the membranes of which had been precoated with Matrigel® (BD Biosciences, Franklin Lakes, NJ, USA). The bottom compartments were filled with 600 µl of culture medium comprising 10% FBS. The Transwell inserts were maintained at 37°C

in a 5% CO_2 humidified atmosphere for 24 h. Subsequently, the noninvasive cells remaining on the top side of the insert membrane were wiped away with a cotton swab, whereas the invasive cells were subjected to 100% methanol fixation and crystal violet staining. Cell migration assays were performed in a similar manner except that the membranes were not precoated with Matrigel®. The migrating and invasive cells were imaged using an Olympus microscope (Olympus Corporation, Tokyo, Japan). The capacities of migration and invasion were assessed by respectively counting the number of migrating and invasive cells in five randomly selected fields.

Bioinformatics analysis: Starbase 3.0 (http://starbase.sysu.edu.cn/) was used to predict the potential targets of LINC00520.

RNA immunoprecipitation (RIP) assay: A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) was used to perform the RIP assay to evaluate the interaction between LINC00520 and mir-577 in CRC cells. Cells were treated with lysis buffer, and cell lysates were further incubated with magnetic beads conjugated with human AGO2 antibody (Millipore) or negative control igG (Millipore) at 4°C overnight. Subsequently, the magnetic beads were collected and subjected to the isolation of immunoprecipitated RNA. Finally, the enrichment of LINC00520 and mir-577 was determined *via* RT-qPCR analysis.

Luciferase reporter assay: The fragments of LINC00520 comprising Wild-Type (WT) and Mutant (MUT) mir-577 binding sites were designed and synthesized by Shanghai Genepharma. The fragments were inserted into the pmirGLO Dual-luciferase Target Expression Vector (Promega Corporation, Madison, WI, USA) to obtain the WT-LINC00520 and MUT-LINC00520 reporter plasmids. The WT-HSP27 and MUT-HSP27 reporter plasmids were generated in a similar manner. One night before transfection, the cells were seeded into 24-well plates at 60% to 70% confluence. Either WT or MUT reporter plasmids were introduced into the cells in the presence of agomir-577 or agomir-NC. Forty-eight hours after transfection, the cells were collected, and their luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega Corporation). Renilla luciferase activity was used to normalize the data.

Western blot analysis: Cell lysates were prepared by extracting proteins from cultured cells using peer-cooled radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China). A bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) was used to detect protein concentrations. Equivalent amounts of proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After 2 h of blocking at room temperature using a 5% solution of defatted milk powder, the membranes were incubated with primary antibodies overnight at 4°C, followed by 2 h of incubation with a Horseradish Peroxidase (HRP)-conjugated secondary antibody (1:5,000; cat. No. Ab205718; Abcam). Subsequently, protein signals were detected using an Immobilon Western Chemiluminescent HRP Substrate kit (EMD Millipore). The primary antibodies included a mouse antihuman IGF-1R antibody (cat. No. Ab182408; Abcam) and a mouse antihuman GAPDH antibody (cat. No. Ab128915; Abcam). All primary antibodies were used at 1:1000.

Statistical analysis

All results are expressed as the mean ± SD of three independent experiments. The χ^2 test was applied to determine the correlations between LINC00520 expression and clinical parameters in patients with CRC. Comparisons between two groups were performed using the Student's t-test, whereas one-way analysis of variance followed by Tukey's post hoc test was performed to compare differences among multiple groups. The overall survival rate of patients with CRC was analyzed using the Kaplan–Meier method and compared using the log-rank test. The correlation of expression between two genes in CRC tissues was tested using Spearman's correlation analysis. A P value of <0.05 was considered statistically significant.

Results

LINC00520 is increased in CRC

To test the role of LINC00520 in CRC, we first analyzed its expression profile in 132 pairs of CRC tissues and surrounding adjacent normal tissues via RT-qPCR. The data revealed that LINC00520 expression was upregulated in CRC tissues than that in the surrounding adjacent normal tissues (Figure 1A, P<0.05). To further confirm the aforementioned observation, LINC00520 expression in four CRC cell lines (HCT116, SW480, HT29, and Lovo) and a human normal colonic epithelial cell line (NCM460) was detected using RT-qPCR. LINC00520 expression was higher in CRC cell lines than in NCM460 cells (Figure 1B, P<0.05). After verifying the aberrant upregulation of LINC00520, we subsequently examined the clinical significance of LINC00520 in CRC. For this, the median value of LINC00520 expression in CRC tissues was defined as the cutoff value, and all the patients with CRC recruited into this study were subdivided into low LINC00520 or high LINC00520 expression group. As illustrated in (Table 1), increased LINC00520 expression was significantly associated with larger tumor size (P=0.019), present of lymph node metastasis (P=0.003) and advantaged TNM stage (P=0.005) among patients with CRC. In addition, patients with CRC featuring high LINC00520 expression exhibited shorter overall survival and Disease-free survival than those with low LINC00520 expression (Figure 1C and D, OS: P=0.006, DFS P=0.004). These findings suggested that LINC00520 may play crucial roles in CRC malignancy.

Silencing of LINC00520 inhibits the malignant phenotypes of CRC cells *in vitro*

LINC00520 expression was higher in HCT116 and SW480 cells than in HT29 and Lovo cells accordingly; HCT116 and SW480 cells were selected for subsequent analysis. To explore the role of LINC00520 in CRC progression, we depleted LINC00520 in HCT116 and SW480 cells via transfection with si-LINC00520 (Figure 2A, P<0.05). The cells transfected with si-NC served as a control. As determined using the CCK-8 assay, the proliferative ability of both HCT116 and SW480 cells transfected with si-LINC00520 was obviously hindered compared with that in cells transfected with si-NC (Figure 2B, P<0.05). Subsequently, the colony formation ability of LINC00520 deficient HCT116 and SW480 cells greatly inhibited in HCT116 and SW480 cells transfected with si-LINC00520 (Figures 2C and 2D, P<0.05). Further, LINC00520-silenced HCT116 and SW480 cells exhibited substantial decreases in migration (Figure 2E, P<0.05) and invasion (Figure 2F, P<0.05) compared with those observed in si-NC-transfected cells. These data strongly suggest that LINC00520 is a cancer-promoting lncRNA in CRC.

LINC00520 directly interacts with miR-577 in CRC cells as a molecular miRNA sponge

Emerging evidence has implicated lncRNAs in several molecular

Table 1: Association between LINC00520 expression and clinicopathological features of human.

Clinical features	Total	LINC00520 expression		*p*-value
		High (N=65)	Low (N=67)	
Age (years)				
< 60	38	16	22	0.297
≥ 60	94	49	45	
Gender				
Male	73	39	34	0.285
Female	59	26	33	
Tumor location				
Rectum	59	25	34	0.156
Colon	73	40	33	
Tumor size (cm)				
< 5	52	19	33	0.019
≥ 5	80	46	34	
Differentiation grade				
Well	44	21	23	0.806
Moderate + Poor	88	44	44	
TNM stage				
I + II	82	38	44	0.005
III	50	27	23	
Depth of invasion				
T1 + T2	61	25	36	0.079
T3 + T4	71	40	31	
Lymph node metastasis				
No	82	32	50	0.003
Yes	50	33	17	
Distant metastasis				
No	101	49	52	0.763
Yes	31	16	15	
Adjuvant chemotherapy				
No	62	29	33	0.593
Yes	70	36	34	
CA19-9, kU/L				
< 40	99	52	47	0.191
≥ 40	33	13	20	

CA19-9: Carbohydrate Antigen 19-9; Pearson chi-square test was used for comparison between subgroups

biological events through acting as competing endogenous RNA (ceRNA) for miRNAs. We then used the publicly available algorithm starBase 3.0 to predict the directly interacting miRNAs of LINC00520. MiR-577 (Figure 3A) was found to share complementary binding sites with LINC00520, and it was selected for further verification because this miRNA was reported to act as a tumor suppressor during CRC progression [25]. To confirm this prediction, luciferase reporter assay was performed to assess the binding between miR-577 and LINC00520 in CRC cells. WT-LINC00520 or MUT-LINC00520 was transfected into HCT116 and SW480 cells in the presence of agomir-577 or agomir-NC. MiR-577 expression was significantly increased in HCT116 and SW480 cells after transfection

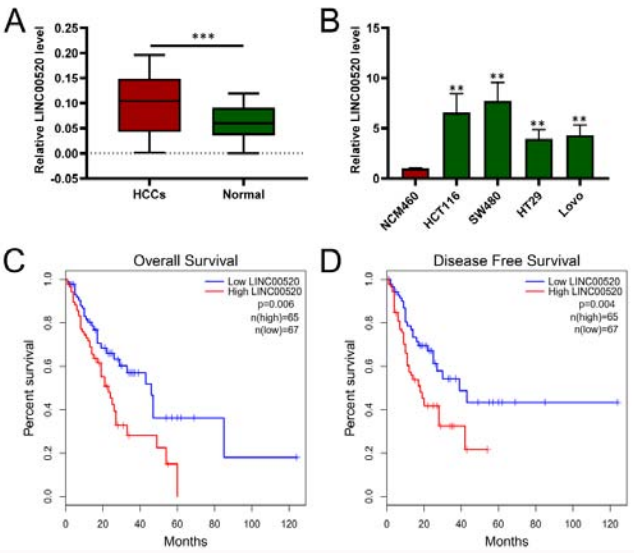

Figure 4: LINC00520 functions as a ceRNA to sponge miR-577 and thereby regulate HSP27 expression.
(A,B) si-LINC00520 or si-NC was introduced into HCT116 and SW480 cells, and the mRNA and protein expressions of HSP27 were quantified by RT-qPCR and Western blotting, respectively. **(C)** HSP27 mRNA expression was analyzed in 132 pairs of CRC tissues and surrounding adjacent normal tissues using RT-qPCR. **(D)** The correlation between LINC00520 and HSP27 mRNA expressions in 132 CRC tissues was identified *via* Spearman's correlation analysis. **(E)** miR-577 expression was detected in HCT116 and SW480 cells transfected with antagomir-577 or antagomir-NC using RT-qPCR. **(F,G)** HCT116 and SW480 cells were transfected with antagomir-577 or antagomir-NC in the presence of si-LINC00520. After transfection, RT-qPCR and Western blotting were performed to detect HSP27 mRNA and protein expressions, respectively. *P<0.05; **P<0.01; ***P<0.001; P>0.05.

with agomir-577 (Figure 3B, P<0.05). The luciferase activity of WT-LINC00520 cells was drastically decreased after transfection with agomir-577 in HCT116 and SW480 cells (P<0.05); however, no changes in the activity of MUT-LINC00520 were detected in the presence of miR-577 upregulation (Figure 3C). The interaction between miR-577 and LINC00520 was further determined using RIP assay, and the data revealed that LINC00520 and miR-577 were enriched in Ago2-containing immunoprecipitates compared with that observed in the IgG control (Figure 3D, P<0.05). To further support these findings, we conducted RT-qPCR to measure miR-577 expression in 132 pairs of CRC tissues and surrounding adjacent normal tissues. miR-577 was weakly expressed in CRC tissues in comparison with that in surrounding adjacent normal tissues (Figure 3E, P<0.05). In addition, an inverse expression relationship was observed between LINC00520 and miR-577 in the 132 CRC tissues (Figure 3F; Spearman r= -0.443, P<0.001), as demonstrated by Spearman's correlation analysis. Furthermore, we attempted to test whether LINC00520 modulated miR-577 expression in CRC cells. The results indicated that miR-577 expression was substantially elevated by LINC00520 silencing in HCT116 and SW480 cells (Figure 3G, P<0.05). Collectively, these results suggested that LINC00520 acts as a molecular sponge for miR-577 in CRC cells.

LINC00520 positively modulates HSP27 expression in CRC cells via miR-577 sponging

A previous study has indicated that HSP27 is a direct target gene of miR-577 in CRC cells [26]. After finding that miR-577 is sponged by LINC00520, we examined whether LINC00520 participates in the regulation of HSP27 in CRC cells. To this end, si-LINC00520 or si-NC

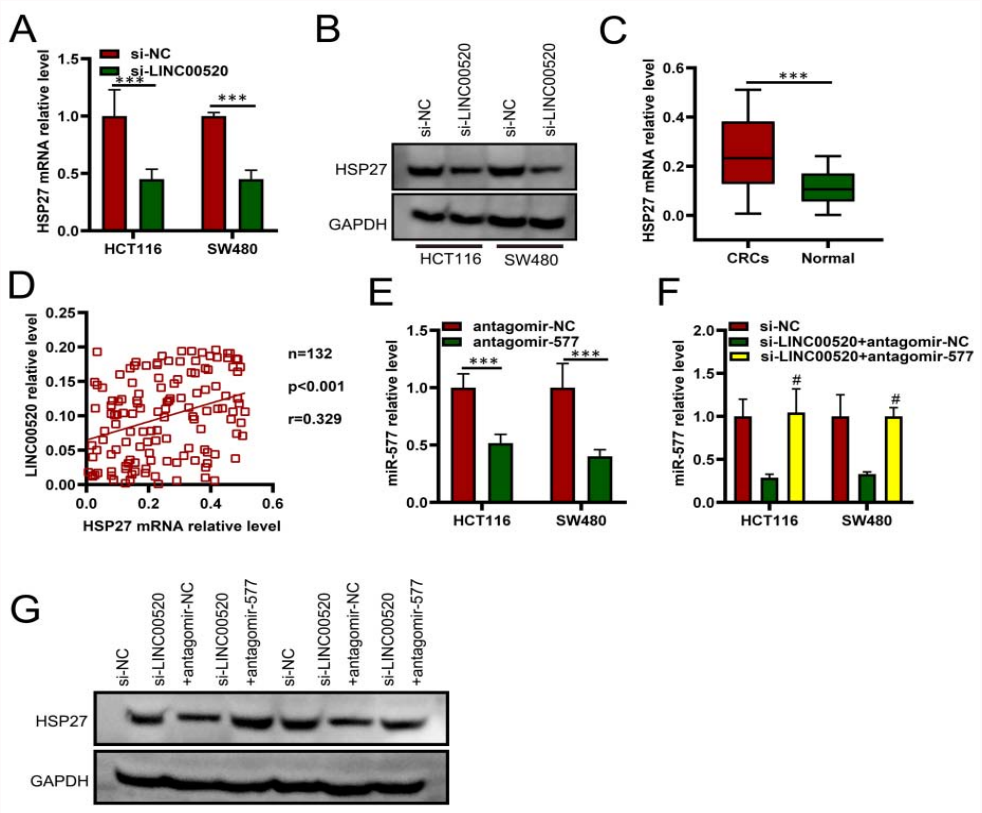

Figure 5: Inhibition of miR-577 partially rescues the effects of LINC00520 silencing in HCT116 and SW480 cells.
HCT116 and SW480 cells were co-transfected with si-LINC00520 and antagomir-577 or antagomir-NC and used in the experimental research. **(A,B)** Cell proliferation and apoptosis were analyzed using the CCK-8 assay and flow cytometry analysis, respectively. **(C,D)** Transwell assays of the migratory and invasive abilities of treated HCT116 and SW480 cells. *P<0.05 compared with si-NC. *P<0.05; **P<0.01; ***P<0.001; P>0.05.

was transfected into HCT116 and SW480 cells, and HSP27 expression was detected. Silencing of LINC00520 considerably decreased HSP27 expression at both the mRNA (Figure 4A, P<0.05) and protein levels (Figure 4B, P<0.05) in HCT116 and SW480 cells. HSP27 expression in 132 pairs of CRC tissues and surrounding adjacent normal tissues was detected using RT-qPCR. HSP27 expression was obviously overexpressed in CRC tissues in comparison with that in adjacent normal tissues (Figure 4C, P<0.05). Additionally, HSP27 mRNA expression was positively correlated with LINC00520 expression in the 132 CRC tissues, as confirmed *via* Spearman's correlation analysis (Figure 4D; Spearman r=0.329, P<0.001). To explore whether LINC00520 regulates HSP27 expression through sponging miR-577, si-LINC00520 plus antagomir-577 or antagomir-NC was introduced into HCT116 and SW480 cells. First, the efficiency of antagomir-577 transfection was verified using RT-qPCR. The data revealed that transfection with antagomir-577 resulted in a significant decrease in miR-577 expression in HCT116 and SW480 cells (Figure 4E, P<0.05). Furthermore, the downregulation of HSP27 mRNA (Figure 4F, P<0.05) and protein expression (Figure 4G, P<0.05) caused by LINC00520 was reversed in HCT116 and SW480 cells through antagomir-577 re-introduction. Taken together, these results suggested that LINC00520 positively regulated HSP27 expression in CRC cells and this was achieved through sponging miR-577.

Inhibition of miR-577 partially rescues the effects of LINC00520 silencing in CRC

Rescue experiments were used to further elucidate whether the activities of LINC00520 in CRC cells were mediated by the miR-577/HSP27 axis. First, si-LINC00520 was co-transfected with antagomir-577 or antagomir-NC into HCT116 and SW480 cells, and cell proliferation, apoptosis, migration, and invasion were examined. The silencing of LINC00520 inhibited HCT116 and SW480 cell proliferation (Figure 5A, P<0.05), promoted cell apoptosis (Figure 5B, P<0.05), and impaired cell migration (Figure 5C, P<0.05) and invasion (Figure 5D, P<0.05). Meanwhile, miR-577 inhibition partially neutralized the effects of LINC00520 silencing in these cells.

The effects of LINC00520 silencing on the malignant phenotypes of CRC cells were abolished by restoring HSP27 expression

In addition, HCT116 and SW480 cells were co-transfected with si-LINC00520 and pc-HSP27 or the empty pcDNA3.1 vector. Western blotting was used to assess the transfection efficiency of pc-HSP27. The results revealed that HSP27 protein expression (Figure 6A, P<0.05) was notably increased in HCT116 and SW480 cells after pc-HSP27 injection. As revealed using the CCK-8 assay, co-transfection of pc-HSP27 abolished the decrease in the proliferation (Figure 6B, P<0.05) of HCT116 and SW480 cells induced by LINC00520 silencing. In addition, the rate of apoptosis in HCT116 and SW480 cells was increased after LINC00520 silencing, whereas this effect was reversed after HSP27 upregulation (Figure 6C, P<0.05). The migration (Figure 6D, P<0.05) and invasion (Figure 6E, P<0.05) of HCT116 and SW480 cells displayed similar trends as the aforementioned results. Altogether, these observations indicated that LINC00520 exerted its pro-oncogenic effects in CRC progression through functioning as a ceRNA for miR-577 and thereby increasing HSP27 expression.

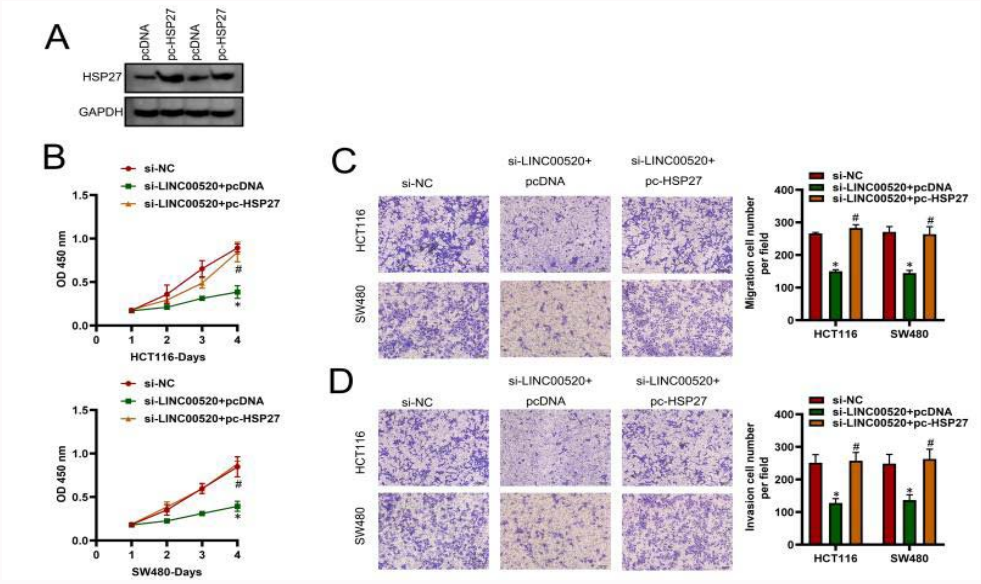

Figure 6: The effects of LINC00520 silencing on the malignant phenotypes of HCT116 and SW480 cells were abolished by restoring HSP27 expression. **(A)** Western blotting was used to measure HSP27 protein expression in HCT116 and SW480 cells transfected with pc-HSP27 or empty pcDNA3.1 plasmid. **(B-E)** HCT116 and SW480 cells were co-transfected with si-LINC00520 plus either pc-HSP27 or pcDNA3.1. After transfection, cellular proliferation, apoptosis, migration, and invasion were measured using the CCK-8 assay, flow cytometry, and Transwell assays. *P<0.05; **P<0.01; ***P<0.001; P>0.05.

Discussion

Recently, lncRNAs have generated huge interests owing to their crucial roles in cancer progression and their potential clinical applications [27,28]. The altered expression of lncRNAs has been widely reported in CRC, and their aberrant expression contributes to CRC onset and progression [29]. Therefore, studying the roles of lncRNAs in the tumorigenesis of CRC may reveal promising therapeutic targets for managing patients with this disease. As mentioned above, this study aimed to detect LINC00520 expression in CRC and assess its clinical significance in patients with CRC. The specific roles of LINC00520 in the malignancy of CRC cells were investigated. Furthermore, we elucidated the detailed mechanisms responsible for the pro-oncogenic roles of LINC00520 in CRC progression.

LINC00520 is upregulated in laryngeal squamous cell carcinoma, and it exhibits a significant correlation with lymph node metastasis [21]. Increased LINC00520 expression has also been reported in nasopharyngeal carcinoma [22] and breast cancer [23]. Increased LINC00520 expression results in a worse survival rate in patients with nasopharyngeal carcinoma [23]. Conversely, LINC00520 is weakly expressed in cutaneous squamous cell carcinoma [24]. These conflicting observations attracted our attention to determine the expression profile of LINC00520 in CRC. Our results indicated that LINC00520 was highly expressed in both CRC tissues and cell lines. The high LINC00520 expression was obviously correlated with larger tumor size (P=0.019), present of lymph node metastasis (P=0.003) and advantaged TNM stage (P=0.005) in patients with CRC. In addition, patients with CRC and high LINC00520 expression had shorter overall survival and disease-free survival than those with low LINC00520 expression. In terms of function, LINC00520 plays an oncogenic role in nasopharyngeal carcinoma and promotes cell growth *in vitro* and *in vivo* [22]. In breast cancer, LINC00520 silencing attenuates cell migration and invasion in 3D [23]. Conversely, LINC00520 has been identified as a tumor-suppressing lncRNA in cutaneous squamous cell carcinoma, and its overexpression reportedly results in the suppression of cell growth, migration, and adhesion [24]. However, whether LINC00520 participates in the oncogenicity of CRC has not been extensively studied until recently. In this study, the functional experiments revealed that silencing of LINC00520 restricts cell proliferation, colony formation ability, migration, and invasion.

LncRNAs perform important roles in carcinogenesis and cancer progression through a sophisticated mechanism. Currently, the ceRNA regulatory mechanism by which lncRNAs competitively interact with miRNAs and thus upregulate specific miRNA target genes is predominant [30]. Subsequent to demonstrating the tumor-promoting effects of LINC00520 in CRC, we attempted to elucidate the mechanisms by which lncRNA is implicated in its aggressive behavior. First, miR-577 was predicted to share a complementary binding site for LINC00520, and the interaction and binding between LINC00520 and miR-577 were further confirmed using luciferase reporter and RIP assays. Second, miR-577 was weakly expressed in CRC tissues, and this expression was negatively correlated with LINC00577 expression. Third, LINC00520 downregulation increased miR-577 expression and thereby resulted in a decrease in HSP27 expression. Fourth, inhibiting miR-577 or restoring HSP27 could abrogate the effects of LINC00520 silencing on malignant phenotypes of CRC. Taken together, our findings revealed a ceRNA model including LINC00520, miR-577, and HSP27 in CRC cells.

Mechanistically, Heat Shock Protein 27 (HSP27) is a member of the small heat shock protein family, and over-expression of HSP27 is associated with promoting drug resistance, aggressive cancers, metastasis, and poor patient outcomes [31]. Currently, there are a number of related reports of HSP27 concerning CRC proliferation and metastasis [25,32-35]. In the present study, the results identified a novel upstream mechanism controlling the miR-577/HSP27 axis in CRC cells both *in vitro*. LINC00520, which harbors a miR-577 binding site, functioned as a molecular ceRNA and sponged miR-577 expression in CRC cells, thereby increasing HSP27 expression.

Identification of the LINC00520/miR-577/HSP27 regulatory network may help to fully clarify the pathogenesis of CRC and provide potential targets for therapeutic regimens for patients with this disease.

The present study identified a novel mechanism by which LINC00520 participates in the tumorigenesis of CRC. LINC00520 silencing hindered the malignant phenotypes of CRC cells. LINC00520 positively regulated HSP27 expression by sponging miR-577 in CRC cells, and the validated ceRNA model was responsible for the cancer-promoting effects of LINC00520 during CRC progression. The LINC00520/miR-577/HSP27 pathway might be a promising target for the diagnosis, prognosis, prevention, and treatment of CRC.

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Authors Contribution

Ming Chen conceived the study and designed the experiments. Xi-Han Jin and Yong-Gang Hong provided the experimental materials. Peng Li performed the experiments. Li-Qiang Hao performed the data analysis. Xi-Han Jin wrote the manuscript. All authors contributed to the interpretation and discussion of the results and reviewed the manuscript.

References

1. Siegel R, Desantis C, Jemal A. Colorectal Cancer Statistics, 2014. CA Cancer J Clin. 2014;64(2):104-17.
2. Brenner H, Kloor M, Pox CP. Colorectal Cancer. Lancet. 2014;383(9927):1490-502.
3. Welch HG, Robertson DJ. Colorectal cancer on the decline--why screening can't explain it all. N Engl J Med. 2016;374(17):1605-7.
4. Glunde K, Bhujwalla ZM, Ronen SM. Choline metabolism in malignant transformation. Nat Rev Cancer. 2011;11(12):835-48.
5. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global Cancer Statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
6. Guo P, Huang ZL, Yu P, Li K. Trends in cancer mortality in China: An update. Ann Oncol. 2012;23(10):2755-62.
7. Yinghao LV, Huang S. Role of Non-Coding RNA in Pancreatic Cancer. Oncol Lett. 2019;18(4):3963-73.
8. Chi Y, Wang D, Wang J, Yu W, Yang J. Long Non-Coding RNA in the Pathogenesis of Cancers. Cells. 2019;8(9).
9. Jiang MC, Ni JJ, Cui WY, Wang BY, Zhuo W. Emerging Roles of LncRNAs in Cancer and Therapeutic Opportunities. Am J Cancer Res. 2019;9(7):1354-66.
10. Batista PJ, Chang HY. Long Noncoding RNAs: Cellular address codes in development and disease. Cell. 2013;152(6):1298-307.
11. Ponting CP, Oliver PL, Reik W. Evolution and functions of long noncoding RNAs. Cell. 2009;136(4):629-41.
12. Fernandes JCR, Acuña SM, Aoki JI, Floeter-Winter LM, Muxel SM. Long Non-Coding RNAs in the Regulation of Gene Expression: Physiology and Disease. Noncoding RNA. 2019;5(1).
13. Hauptman N, Glavač D. Long Non-Coding RNA in Cancer. Int J Mol Sci. 2013;14(3):4655-69.
14. Bao Z, Yang Z, Huang Z, Zhou Y, Cui Q, Dong D. LncRNADisease 2.0: An Updated Database of Long Non-Coding RNA-associated Diseases. Nucleic Acids Res. 2019;47(D1):D1034-D7.
15. Liu W, Wang Z, Wang C, Zhilong A. Long non-coding RNA MIAT promotes papillary thyroid cancer progression through upregulating LASP1. Cancer Cell Int. 2019;19:194.
16. Zhuang X, Tong H, Ding Y, Wu L, Cai J, Si Y, et al. Long noncoding RNA ABHD11-AS1 functions as a competing endogenous RNA to regulate papillary thyroid cancer progression by miR-199a-5p/SLC1A5 Axis. Cell Death Dis. 2019;10(8):620.
17. Chen ZB, Cao WL, Su K, Mao M, Zeng XY, Li JH. MIR22HG inhibits cell growth, migration and invasion through regulating the miR-24-3p/p27kip1 axis in thyroid papillary carcinomas. Eur Rev Med Pharmacol Sci. 2019:23(13):5851-62.
18. Xia F, Chen Y, Jiang B, Du X, Peng Y, Wang W, et al. Long noncoding RNA HOXA-AS2 promotes papillary thyroid cancer progression by regulating miR-520c-3p/S100A4 pathway. Cell Physiol Biochem. 2018;50(5):1659-72.
19. Wu DM, Wang S, Wen X, Han XR, Wang YJ, Shen M, et al. LncRNA SNHG15 Acts as a ceRNA to Regulate YAP1-Hippo signaling pathway by sponging miR-200a-3p in papillary thyroid carcinoma. Cell Death Dis. 2018;9(10):947.
20. Xu Y, Wang J, Wang J. Long noncoding RNA XIST promotes proliferation and invasion by targeting miR-141 in papillary thyroid carcinoma. Onco Targets Ther. 2018;11:5035-43.
21. Wu YY, Gao W, Zhang YL, Niu M, Cui JJ, Xiang CX, et al. [Expression and Clinical Significance of Long Non-Coding RNA LINC00520 in Laryngeal Squamous Cell Carcinoma]. Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi. 2018;32(2):91-5.
22. Xie T, Pi G, Yang B, Ren H, Yu J, Ren Q, et al. Long non-coding RNA 520 is a negative prognostic biomarker and exhibits pro-oncogenic function in nasopharyngeal carcinoma carcinogenesis through Regulation of miR-26b-3p/USP39 Axis. Gene. 2019;707:44-52.
23. Henry WS, Hendrickson DG, Beca F, Glass B, Lindahl-Allen M, He L, et al. LINC00520 is induced by Src, STAT3, and PI3k and plays a functional role in breast cancer. Oncotarget. 2016;7(50):81981-94.
24. Mei XL, Zhong S. Long noncoding RNA LINC00520 prevents the progression of cutaneous squamous cell carcinoma through the inactivation of the PI3K/Akt signaling pathway by downregulating EGFR. Chin Med J. 2019;132(4):454-65.
25. Jiang H, Ju H, Zhang L, Lu H, Jie K. microRNA-577 suppresses tumor growth and enhances chemosensitivity in colorectal cancer. J Biochem Mol Toxicol. 2017;31(6).
26. Wang Y, Lu Z, Wang N, Feng J, Zhang J, Luan L, et al. Long noncoding RNA DANCR promotes colorectal cancer proliferation and metastasis *via* miR-577 Sponging. Exp Mol Med. 2018;50(5):57.
27. Chu F, Xue L, Miao H. Long noncoding RNA TP73-AS1 in human cancers. Clin Chim Acta. 2020;500:104-8.
28. Gourvest M, Brousset P, Bousquet M. Long noncoding RNAs in acute myeloid leukemia: Functional characterization and clinical relevance. Cancers (Basel). 2019;11(11):1638.
29. Ma S, Long T, WJM H. Noncoding RNAs in inflammation and colorectal cancer. RNA Biol.2019;1-8.
30. Abdollahzadeh R, Daraei A, Mansoori Y, Sepahvand M, Amoli MM, Tavakkoly-Bazzaz J. Competing endogenous RNA (ceRNA) cross talk and language in ceRNA regulatory networks: A new look at hallmarks of breast cancer. J cell physiol. 2019;234(7):10080-100.
31. McConnell JR, McAlpine SR. Heat Shock Proteins 27, 40, and 70 as Combinational and Dual Therapeutic Cancer Targets. Bioorg Med Chem Lett. 2013;23(7):1923-8.
32. Yuan W, Sui C, Liu Q, Tang W, An H, Ma J. Up-regulation of

microRNA-145 associates with Lymph Node Metastasis in Colorectal Cancer. PLoS One. 2014;9(7):e102017.

33. Hung CS, Huang CY, Lee CH, Chen WY, Huang MT, Wei PL, et al. IGFBP2 Plays an Important Role in Heat Shock Protein 27-Mediated Cancer Progression and Metastasis. Oncotarget. 2017;8(33):54978-92.

34. Konda JD, Olivero M, Musiani D, Lamba S, Di Renzo MF. Heat-Shock protein 27 (HSP27, HSPB1) is synthetic lethal to cells with oncogenic activation of MET, EGFR and BRAF. Mol Oncol. 2017;11(6):599-611.

35. Zhao L, Li ZG, Ding YQ. [Expression of HSP27 in colorectal carcinoma and its relationship with lymphatic metastasis]. Nan Fang Yi Ke Da Xue Xue Bao. 2008;28(1):41-4.