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Original Article

LINC00520 promotes colorectal cancer progression through miRNA-195-3p / NAT2 axis

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Abstract

In this study, we investigated the role of LINC00520 in colorectal cancer (CRC) progression. We analyzed LINC00520 expression in 15 pairs of CRC tissues and adjacent tissues using qRT-PCR, revealing significantly elevated levels in CRC tissues and cell lines. Lentivirus-mediated up/down-regulation of LINC00520 in CRC cell lines demonstrated that increased LINC00520 expression enhanced cell invasiveness, as confirmed by transwell and wound healing assays. Bioinformatics analysis identified a regulatory axis involving LINC00520, microRNA-195-3p, and NAT2. Luciferase assays confirmed direct binding between LINC00520 and microRNA-195-3p, as well as microRNA-195-3p and NAT2. Overexpression of NAT2 reversed the inhibitory effects on invasion and migration induced by LINC00520 silencing. This suggests that LINC00520, highly expressed in CRC tissues, may modulate tumor biological functions through the microRNA-195-3p/NAT2 axis. Our findings provide insights into the mechanism underlying CRC progression, highlighting the potential of LINC00520 as a therapeutic target.

Keywords: LINC00520; microRNA-195-3p / NAT2 axis; Colorectal cancer (CRC); Metastasis

1. Introduction

Colorectal cancer (CRC) is one of the cancers of the digestive tract with a high incidence, which has become a serious disease endangering human health [1-3]. With the rapid development of economy and the gradual improvement of quality of life, people's diet structure and living conditions have also undergone great changes, which have led to an increase in the number of CRC patients year by year [4,5]. Moreover, the increased number of younger CRC patients makes this cancer more concern [1,3]. Worldwide, it is reported that 1.2 million new cases of CRC are diagnosed annually, and over 600,000 patients die of CRC [2,3]. Early screening and diagnosis are particularly difficult since the early symptoms of CRC are not obvious; therefore, most of patients have been in a late stage and thus miss the optimal treatment time [6,7]. Currently, the comprehensive treatment of CRC is still based on surgery. Despite the rapid development of CRC surgery technology, the 5-year survival rate of patients has not been remarkably improved, and postoperative local recurrence and distant organ metastasis are still the main causes of death of CRC patients [8,9]. Chemoradiotherapy is an important treatment method for various malignant tumors; however, it cannot effectively control the distant metastasis and local recurrence of the low sensitivity of CRC to chemoradiotherapy [10]. Therefore, early diagnosis and effective treatment of CRC are still difficult issues waiting to be solved. It is of great clinical significance to further explore the pathogenesis and mechanism of CRC and look for more effective and specific treatment methods for this cancer [11,12].

With the deepening of studies on CRC, researchers have found that some epigenetic genes are also involved in the occurrence and evolution of CRC[12]. In particular, many non-coding RNAs (ncRNAs), which do not translate into proteins but regulate protein-coding mRNA or regulate the translation of specific target mRNA, have been discovered in recent decades [13,14]. NcRNAs can be divided into short and long non-coding RNAs (lncRNAs) according to their length. Previous studies mainly focused on the function of miRNAs, one kind of short ncRNAs, and have discovered many tumor-related miRNAs [14-16]. However, in recent years, lncRNA, involved in various physiological processes of cells, has also attracted much attention from scientists [17,18]. Dysregulation of lncRNA expression will lead to occurrence of many diseases and be engaged in the formation, proliferation, invasion and metastasis of malignant tumors [14,16,18].

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So far, LINC00520 has been shown to be expressed anomalously in a variety of tumor tissues, however, few studies have reported LINC00520' role in CRC [19-21]. In this study, we detected LINC00520 expression in CRC and studied its effect on the biological behavior of tumor cells to further reveal its molecular regulatory mechanism and bring new ideas for the diagnosis and treatment of CRC.

2. Material and Methods

2.1. Patients and CRC samples

Tumor tissue specimens and adjacent ones of 15 CRC patients undergoing radical surgery were collected. All subjects had not received any radiotherapy or chemotherapy before surgery. CRC pathological classification and staging criteria are implemented in accordance with the International Union Against Cancer (UICC) CRC staging criteria. Patients and their families signed informed consent. This study complies with the Helsinki Declaration Clinical Practice Guidelines. This study was approved by the Ethics Committee of China-Japan Friendship Hospital. Signed written informed consent were obtained from all participants before the study.

2.2. Cell culture

Human colon cancer cell lines (HT-29, HCT-116, HCT-8, Caco-2, SW620) and normal human intestinal epithelial cell line (FHC) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). HT-29 and SW620 were cultured with dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 ug/mL), while HCT-116, HCT-8 and Caco-2 were cultured with Roswell Park Memorial Institute 1640 (RPMI 1640) medium (HyClone, South Logan, UT, USA). All cells were cultured in a 37°C cell incubator with 5% CO_2.

2.3. Transfection

Lentivirus transfection was performed with LINC00520 overexpression vector (LINC00520) or knockdown vector (Anti-LINC00520) (GenePharma, Shanghai, China) when cell density reached 30%-60%. Cells were collected 48 hours later for cell function experiments.

2.4. Transwell assay

Cell migration capacity was tested using a 24-well plate cell pre-coated or not coated with matrix gel according to the manufacturer's instructions.

2.5. Cell wound Healing Test

Cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5×10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. Afterwards, cells were rinsed gently with phosphate-buffered saline (PBS) 2-3 times and observed again after incubation in low-concentration serum medium for 24 h.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells to extract total RNA from the tissue. Real-time PCR was performed according to the instructions of SYBR® Premix Ex Taq ™ kit (TaKaRa, Tokyo, Japan) on StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA), with β-actin and U6 as internal reference. Primers used in the qPCR reaction: LINC00520: forward: 5'-GGGAGTAAGAGGTGTGGCAA-3', reverse: 5'-CCATGGCCATTTTGCAAGGA-3'; NAT2: forward: 5'-TTGCTGGCCAAAGGGATCAT-3', reverse: 5'-TTCTCAAAGGGAACAGCCCG-3'; glyceraldheyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'; microRNA-195-3p: forward: 5'-CCAATATTGGCTGTGCTGCTCC-3', reverse: 5'-CCACAGCAGCAGAAACT-3'; U6: forward: 5'-GCGCGTCGTGAAGCGTTC-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'.

2.7. Western blot

Cells were lysed, shaken on ice for 30 minutes, and centrifuged at 4°C, 14000 × g for 15 minutes. Total protein concentration was calculated by CRCA protein assay kit (Beyotime, Shanghai, China). The extracted proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was carried out based on standard procedures.

2.8. Luciferase assay

The transcription factor expression plasmid to be tested was co-transfected with the reporter plasmid into the CRC cell lines. The luciferase activity was measured using a luciferase reporter kit (Promega, Madison, WI, USA).

2.9. Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data were compared using t-test, and categorical variables were analyzed via χ^2 test or Fisher's exact probability method. Kaplan-Meier method was used for survival analysis. Data are expressed as X±SD (standard deviation), and p less than 0.05 was considered statistically significant.

3. Results

3.1. LINC00520 was highly expressed in CRC

We first detected LINC00520 levels in CRC and adjacent control tissues by qRT-PCR. Figure 1A shows a significantly elevated expression of LINC00520 in CRC tissue samples. Consistently, in comparison to FHC, LINC00520 was also increased in CRC cell lines (Figure 1B). LINC00520 may serve as a new biological index for predicting the prognosis of CRC.

3.2. LINC00520 promoted cell migration of CRC cell lines

To specify the impact of LINC00520 on CRC cell functions, we constructed LINC00520 overexpression/knockdown models in CRC cell lines HCT-8/SW620 and verified the transfection efficiency by qRT-PCR (Figure 2A,B). Subsequently, the results of transwell and cell wound healing experiments demonstrated that upregulation of LINC00520 significantly increased the number of CRC cells perforating in the transwell chamber and en-

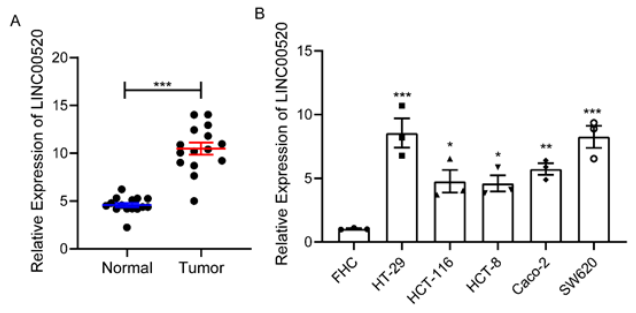

Fig. 1. LINC00520 is highly expressed in colorectal cancer tissues and cell lines. (A) qRT-PCR was used to detect the expression of LINC00520 in colorectal cancer tumor tissues and adjacent tissues; (B) qRT-PCR was used to detect the expression level of LINC00520 in colorectal cancer cell lines; Data are average ± SD, * P<0.05, ** P<0.01, *** P<0.001.

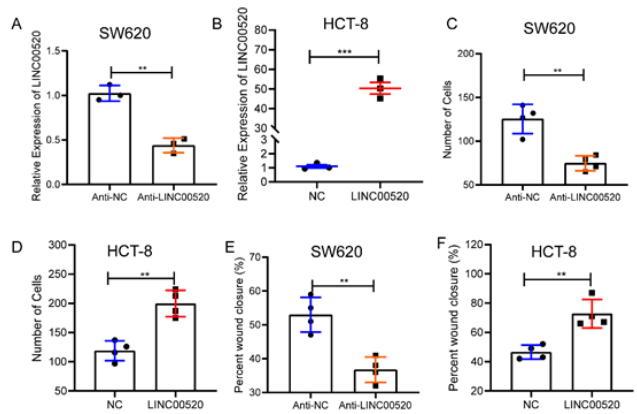

Fig. 2. LINC00520 promotes invasion and migration of colorectal cancer cells. (A and B) qRT-PCR verified the transfection efficiency of LINC00520 after transfection of LINC00520 overexpression or knockdown vector in colorectal cancer cell lines SW620 and HCT-8; (C and D) Transwell migration assay was used to detect the number of CRC cells perforating in the transwell chamber after transfection of LINC00520 overexpression or knockdown vector in colorectal cancer cell lines SW620 and HCT-8; (E and F) Cell wound healing test was used to detect the crawling ability of colorectal cancer cells after transfection of LINC00520 overexpression or knockdown vector in colorectal cancer cell lines SW620 and HCT-8. Data are average ± SD, ** P<0.01, *** P<0.001.

hanced their crawling ability (Figure 2D,F); meanwhile, the opposite results were observed in LINC00520 downregulation group (Figure 2C,E). The above results suggest that LINC00520 is able to improve the migration capacity and invasiveness of CRC cells.

3.3. LINC00520 regulated microRNA-195-3p / NAT2 axis

Bioinformatics research found that there may exist a mutual regulation between LINC00520 and microRNA-195-3p (Figure 3A) Hence, we performed luciferase assay and verified that LINC00520 can indeed bind to a specific sequence of microRNA-195-3p (Figure 3B); meanwhile, NAT2 can be paired by microRNA-195-3p on its 3'UTR (Figure 3C,D). Subsequently, qRT-PCR detection of CRC tissue specimens revealed that microRNA-195-3p levels were remarkably reduced in CRC tumor tissues (Figure 3E). In addition, in vitro cell experiments revealed that knockdown of LINC00520 enhanced the level of microRNA-195-3p (Figure 3F), while overexpression of NAT2 reduced microRNA-195-3p expression (Figure 3G).

3.4. LINC00520 modulated microRNA-195-3p / NAT2 axis in CRC

In order to confirm the LINC00520/miR-195-3p/NAT2 axis in CRC, the expression levels of NAT2 in CRC and adjacent pairs were detected by qRT-PCR, and the result showed that NAT2 was up-regulated in CRC tissues (Figure 4A). Then, we simultaneously transfected LINC00520 knockdown and NAT2 overexpression vector, or LINC00520 overexpression and NAT2 knockdown vector in CRC cell lines HCT-8 and SW620 to further explore the interrelationship between LINC00520 and microRNA-195-3p / NAT2 axis, and how they affect the malignant progress of CRC. Overexpression of NAT2 reversed the reduced expression of NAT2 induced by knockdown of LINC00520, while downregulation of NAT2 enhanced that induced by overexpression of LINC00520, measured by Western blot (Figure 4B). Besides, overexpression of NAT2 could down-regulated the level of miR-195-3p, while knockdown of NAT2 exhibited the opposite results (Figure 4C, 4D). In addition, overexpression of NAT2 also reversed the inhibitory effect of knocking down LINC00520 on the migration and invasive capacities of CRC cells; and knockdown of NAT2 led to opposite results (Figure 4E, 4F).

4. Discussion

Colorectal cancer is one of the most common mali-

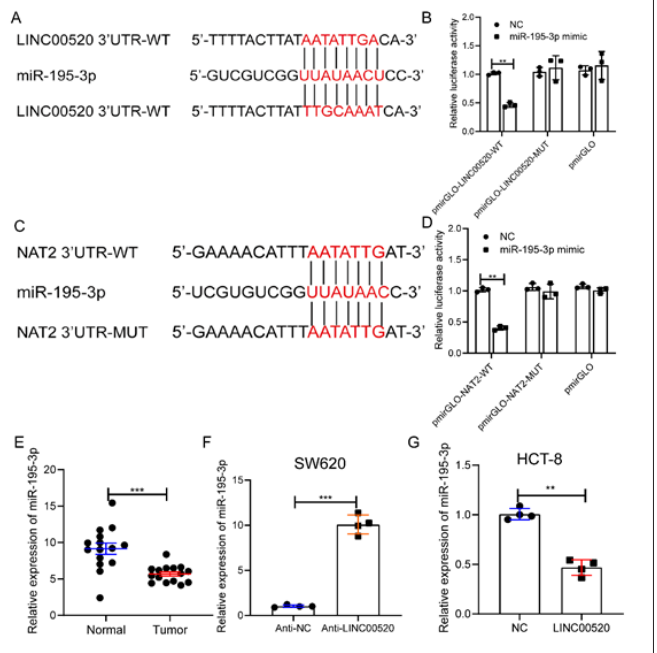

Fig. 3. LINC00520 regulates the miR-195-3p/NAT2 axis. (A and B) The luciferase reporter experiment showed that LINC00520 can bind to the specific sequence of miR-195-3p; (C and D) The luciferase reporter experiment showed that miR-195-3p can pair with the 3'UTR of NAT2; (E) qRT-PCR was used to detect the expression of miR-195-3p in colorectal cancer tumor tissues and adjacent tissues; (F and G) qRT-PCR verified the expression level of miR-195-3p after transfection of LINC00520 knockdown vector in colorectal cancer cell lines SW620 and HCT-8; The data are presented as average ± SD, *P<0.05, *** P<0.001.

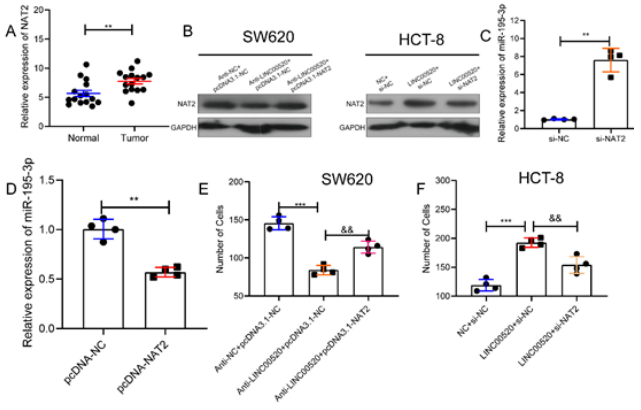

Fig. 4. LINC00520 regulates miR-195-3p / NAT2 axis in colorectal cancer cell lines. (A) qRT-PCR was used to detect the expression of NAT2 in colorectal cancer tumor tissues and adjacent tissues; (B) Western blot and qRT-PCR experiments detected the expression levels of NAT2 after co-transfection of LINC00520 knockdown vector and NAT2 overexpression vector in colorectal cancer cell lines SW620 and HCT-8; (C and D) qRT-PCR verified the expression of miR-195-3p after transfection of NAT2 overexpression vector in colorectal cancer cell lines SW620 and HCT-8. (E and F) Transwell migration assay was used to detect the number of CRC cells perforating in the transwell chamber after transfection of LINC00520 knockdown vector and NAT2 overexpression vector in colorectal cancer cell lines SW620 and HCT-8. (Data are average ± SD, * P<0.05, ** P<0.01, *** P<0.001, && P<0.01.

gnant tumors, with its morbidity and mortality accounting for the top three among malignant tumors globally [1-3]. In China, especially in cities, its incidence rate is increasing rapidly and the age of CRC onset has become younger [22]. In addition, the lack of early monitoring and effective treatment for different tumor stages has led to a high CRC mortality [4-6]. Meanwhile, the high cost of targeted drugs limits its use in improving postoperative recurrence and metastasis of patients with advanced CRC [6,7]. Therefore, the selection of treatment options, prognosis evaluation and chemotherapy efficacy for patients with advanced CRC are faced with great challenges [7]. As the current research on the pathogenesis of CRC has reached gene level, the key to improving the survival rate and quality of life of patients with intermediate and advanced CRC is to carry out CRC-related genomics research, find biomarkers that can effectively assess the prognosis of patients, choose individualized diagnosis and treatment programs based on the patient's genetic background, and find targets for improving chemotherapy resistance [10-12].

In the past decades, great achievements have been made in the research on ncRNAs, but the research mainly focuses on miRNA, with relatively few studies on lncRNAs. Moreover, the regulation of gene expression by lncRNAs is more complex than that of miRNAs, which can modulate related protein-coding genes at different levels in a variety of ways [13-15]. In this study, LINC00520 was found up-regulated in CRC tumor tissues. Further, transwell assay and cell wound healing experiments revealed that LINC00520 could promote the migration and crawling ability of CRC cells. However, the specific molecular mechanism still remains to be clearly determined.

The regulatory mechanism of lncRNAs is extremely complicated, involving various biological processes on epigenetic, transcriptional and post-transcriptional levels [15-17]. To clarify the biological function of LINC00520, it is necessary to further search for its target gene and explore the influence of its interaction with miRNA and mRNA on the progression of CRC. In our study, we proved a binding relationship between LINC00520 and microRNA-195-3p, as well as microRNA-195-3p and NAT2. It was found that microRNA-195-3p showed a reduction while NAT2 showed an increase in CRC tissues. Therefore, we suspected that LINC00520 may promote the malignant progression of CRC through microRNA-195-3p / NAT2 axis. Subsequently, *in vitro* cell transfection experiments indicated that overexpression of NAT2 can reverse the influence of LINC00520 knockdown on metastasis rate of CRC cells, and vice versa. Therefore, it was concluded that LINC00520 may accelerate the malignant progression of CRC via regulating microRNA-195-3p /NAT2 axis.

5. Conclusion

In summary, this study demonstrates that LINC00520, remarkably increased in CRC tissues. LINC00520 regulates the biological functions of CRC cells through the modulation of microRNA-195-3p /NAT2 axis and thus plays a regulatory role as an oncogene. Therefore, LINC00520/ microRNA-195-3p /NAT2 axis may become a potential therapeutic target of CRC.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

This study was approved by the ethics committee of China-Japan Friendship Hospital.

Informed Consent

Signed written informed consents were obtained from the patients and/or guardians.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Haibin Liu and Xin Song designed the study and performed the experiments, Guochao Zhang collected the data, Chaofeng Li analyzed the data, Haibin Liu and Xin Song prepared the manuscript. All authors read and approved the final manuscript.

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