The Long Non-coding RNA HOTTIP Is Highly Expressed in Colorectal Cancer and Enhances Cell Proliferation and Invasion

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Long non-coding RNAs (lncRNAs) are associated with a spectrum of biological processes such as gene regulation on transcriptional and post-transcriptional levels. The HOXA transcript at the distal tip (HOTTIP) IncRNA plays an important role in carcinogenesis; however, the underlying role of HOTTIP in colorectal carcinoma (CRC) remains unknown. The aim of the present study was to evaluate the expression and function of HOTTIP in CRC. In the present study, we analyzed HOTTIP expression levels of CRC patients in tumor and adjacent normal tissue by real-time quantitative PCR. Knockdown of HOTTIP by RNA interference was performed to explore its roles in cell proliferation, migration, and invasion. Our results found that HOTTIP was upregulated in human primary CRC tissues. Knockdown of HOTTIP inhibited CRC cell proliferation, migration, and invasion. Above all, knockdown of HOTTIP could represent a rational therapeutic strategy for CRC.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and the cause of cancer-related death, and it is more prevalent in developed countries.1 The accumulation of genetic and epigenetic alterations mediates CRC formation and progression by deregulating key signaling pathways in cancer cells.2 Several molecular biomarkers and signaling pathways have been implicated in colorectal oncogenesis.3 However, its exact mechanism has not been fully characterized. Therefore, a better understanding of the molecular mechanisms underlying CRC carcinogenesis will contribute to the development of novel therapeutic strategies.

RESULTS

IncrNA HOTTIP Is Highly Expressed in CRC Samples

To identify the expression of HOTTIP in CRC, we used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to evaluate the levels of HOTTIP in 58 CRC tumors and paired noncancerous tissues. As shown in Figure 1A, the HOTTIP expression level was significantly increased in 60.34% (35/58) of CRC tissue samples when compared with normal tissues. Furthermore, a correlation analysis of HOTTIP expression with clinicopathological parameters revealed that the HOTTIP expression level was predominantly increased in late-stage tumor tissues and positively correlated with tumor size (Table 1).

HOTTIP Is Overexpressed in Human CRC Cell Lines

We also evaluated the expression of HOTTIP in a panel of human CRC cell lines by qRT-PCR. The expression of HOTTIP was observed to be higher in CRC cell lines compared with HCOEpiC cells. As

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shown in Figure 1B, qRT-PCR results revealed that LoVo and SW480 cells showed higher expression of HOTTIP; however, RKO cells showed lower expression of HOTTIP. Thus, we used LoVo, SW480, and RKO cells as a model to investigate the effects of HOTTIP.

**HOTTIP Regulates Cell Proliferation and Cell Cycle in CRC**

*In Vitro*

To further examine whether HOTTIP is involved in CRC progression, *in vitro* functional analyses were performed. We investigated the function role of HOTTIP in CRC progression. We employed small interfering RNA (siRNA) and expressing plasmid to enhance efficiency of HOTTIP knockdown and overexpression in CRC cell lines (Figures 2A–2C). The results of cell proliferation assays showed that knockdown of HOTTIP by RNA interference (RNAi) significantly decreased proliferation of SW480 and LoVo cells (Figures 3A and 3B), and overexpression of HOTTIP increased proliferation of RKO cells (Figure 3C).

To assess whether the pro-proliferative effects of HOTTIP on the CRC cells are mediated by promoting cell cycle progression, we examined cell cycling in CRC cells by flow cytometry. As shown by flow cytometry analysis, HOTTIP knockdown led to an arrest in the G1 phase of SW480 and LoVo cells (p < 0.01; Figures 4A and 4B). Correspondingly, overexpression of HOTTIP decreased G0/G1 phase percentage in RKO cells (p < 0.01; Figure 4C). Therefore, these data suggested that HOTTIP promotes cell proliferation of CRC by facilitating DNA synthesis.

**HOTTIP Regulates CRC Cell Invasion and EMT**

We next investigated the effect of HOTTIP on invasiveness of CRC cells. We found that HOTTIP knockdown dramatically decreased their migration and invasion capabilities. Quantification of invading cells revealed a significant decrease in the number of invading cells for both cell lines after HOTTIP knockdown (Figures 5A and 5B). Conversely, the migration activity of HOTTIP-overexpressing cells was significantly increased (Figure 5C). Because epithelial-mesenchymal transition (EMT) is vital for cell invasion, we next examined whether silencing HOTTIP expression inhibited mesenchymal features. As expected, HOTTIP knockdown decreased the expression of Vimentin and N-cadherin, and increased E-cadherin expression (Figures 6A and 6B). Therefore, inhibition of HOTTIP in CRC cells changed the cell morphology from a mesenchymal to a more epithelial phenotype.

**HOTTIP Knockdown Inhibits the Growth of CRC Cells In Vivo**

Finally, an *in vivo* tumorigenicity assay was conducted to analyze the role of HOTTIP in tumor growth of CRC cells. The tumor xenografts in the HOTTIP-specific short hairpin RNA (shRNA) (sh-HOTTIP) group developed smaller tumor volumes (Figures 7A) and lighter tumor weights (Figure 7B) relative to those in the negative control shRNA (sh-NC) group. After removing the tumor xenografts, real-time quantitative PCR analysis was performed to detect HOTTIP expressions. Lower HOTTIP levels were observed in the tumor xenografts derived from sh-HOTTIP-transfected SW480 cells (Figure 7C). The expression level of Ki67 was examined by immunohistochemistry (IHC). The results showed that, compared to negative controls, expression of Ki67 significantly decreased (Figure 7D). These results demonstrate that HOTTIP is significantly associated with tumor growth of CRC cells.

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**Table 1. Correlation between HOTTIP Expression and Clinicopathologic Characteristics of CRC Patients**

| Characteristic          | Overall (n = 58) | High (n = 35) | Low (n = 23) | p    |
|-------------------------|------------------|---------------|--------------|------|
| Age (Years)             |                  |               |              |      |
| More than median        | 27               | 15            | 12           | 0.337|
| Sex                     |                  |               |              |      |
| Male                    | 32               | 15            | 17           | 0.298|
| Tumor Stage             |                  |               |              |      |
| T3 and T4               | 24               | 20            | 4            | 0.001|
| Metastatic Status       |                  |               |              |      |
| Present                 | 10               | 9             | 1            | 0.001|
| Neoadjuvant Therapy     |                  |               |              |      |
| Yes                     | 17               | 9             | 8            | 0.478|
| Grade                   |                  |               |              |      |
| 1 and 2                 | 49               | 28            | 21           | 0.263|
DISCUSSION
The prognosis of CRC is extremely poor, so it is urgent to understand the mechanisms underlying CRC progression.\textsuperscript{10} IncRNAs have been shown to aberrantly express in various cancers and to be involved in carcinogenesis.\textsuperscript{11–13} In CRC, expression of IncRNAs has only been analyzed in a few studies.\textsuperscript{14–16} Besides, the functions and molecular mechanisms of most IncRNAs have not been well characterized. Increased HOTTIP expression has been reported in lung cancer, pancreatic cancer, and hepatocellular carcinoma.\textsuperscript{9,17} In these tumors, HOTTIP may serve as a potential oncogene, and HOTTIP overexpression was associated with enhanced cell proliferation, reduced apoptosis, and increased cell migration.\textsuperscript{18} However, the functions of HOTTIP in CRC were previously unknown. In this study, we provided the evidence that lncRNA HOTTIP was significantly upregulated in CRC tissues compared with adjacent normal tissues, and overexpression of HOTTIP in CRC patients was associated with an increased tumor size and advanced tumor/node/metastasis (TNM) stage. Additionally, HOTTIP expression was markedly increased in CRC cell lines compared with normal colonic epithelial cells, suggesting that high HOTTIP expression was closely associated with CRC carcinogenesis.

In addition, knockdown of HOTTIP also inhibited migratory ability of CRC cells and significantly abrogated lung metastasis in a mouse xenograft mode. A similar trend was seen in our study. We also found that knockdown of HOTTIP also inhibited migratory ability of CRC cells.

EMT has been recognized as an important process that is associated with the progression and metastasis of CRC. Whether HOTTIP plays a role in EMT has not been reported until now. In our study, HOTTIP knockdown decreased the expression of Vimentin and Snai1 and increased E-cadherin expression, at both the mRNA and protein levels. These findings revealed that HOTTIP might be involved in CRC progression and contribute to molecular-targeted therapy.

Figure 2. The Efficiency of HOTTIP Knockdown and Overexpression in CRC Cell Lines
(A) The siRNA decreased the expression of HOTTIP in Lovo cells. (B) The siRNA decreased the expression of HOTTIP in SW480 cells. (C) The expressing plasmid increased the expression of HOTTIP in RKO cells.

Figure 3. HOTTIP Regulates Cell Proliferation of CRC In Vitro
(A) MTS assay showing that knockdown of HOTTIP inhibited cell proliferation of Lovo cells. (B) MTS assay showing that knockdown of HOTTIP inhibited cell proliferation of SW480 cells. (C) MTS assay showing that overexpression of HOTTIP promoted cell proliferation of RKO cells.
In conclusion, our findings indicated that the expression level of HOTTIP has the potential to be an oncogene for CRC. Our results provide new insights into the function of lncRNAs in the development of CRC and suggest that HOTTIP represents a potential therapeutic target and prognostic biomarker for CRC.

MATERIALS AND METHODS

Patient Samples and Cell Lines

CRC tissues and paired adjacent noncancerous tissues were obtained from surgical resection at The First Hospital of Jilin University. Both tumors and noncancerous tissues were subjected to histological analysis for diagnostic confirmation. The pathological type of each cancer was identified as adenocarcinoma. After resection, all samples were immersed immediately in RNAlater solution overnight, then stored at −80°C in order to avoid degradation of RNA. Prior to the use of these clinical materials for research purposes, written consent from all patients and approval of the Hospital Ethics Review Committees were obtained.

Six CRC cell lines (HCT116, LoVo, RKO, SW620, SW480, and HT29) were purchased from ATCC (Rockville, MD, USA) and from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The human colonic epithelial cell line HCoEpiC was obtained from American Type Culture Collection (Manassas, VA, USA). They were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) in humidified air at 37°C with 5% CO2. All media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Shanghai, China).

RNA Isolation and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using a reverse transcription kit (Takara, Dalian, China). HOTTIP expression levels were measured with qRT-PCR using an ABI 7500 system and SYBR Green PCR master mix (Takara). GAPDH was used as an internal control. The primer sequences for HOTTIP were 5’-GGGGGCCCAGACCCGC-3’ (forward) and 5’-CTCAGACACATCGGCCGGAAC-3’ (reverse). Each assay was performed in triplicate, and relative HOTTIP expression was normalized to GAPDH using the 2^(-ΔΔCt) method. The fold change of HOTTIP in CRC relative to the matched paired adjacent noncancerous tissues was determined by the 2^(-ΔΔCt) method, where ΔΔcycle threshold (Ct) = (CtHOTTIP - CtGAPDH) (in GC samples) - (CtHOTTIP - CtGAPDH) (in NATs).

Transfection

siRNA and nonspecific control siRNA were synthesized (Invitrogen, Carlsbad, CA, USA) and transfected into cells using Lipofectamine 2000 (Invitrogen, USA). To overexpress HOTTIP, the full-length coding sequence for HOTTIP was amplified and subcloned into the pcDNA 3.1(+) vector (Invitrogen) according to the manufacturer’s instructions. Cells were transfected with a negative control vector or the HOTTIP-expressing plasmid according to the manufacturer’s protocol. Cells were harvested after 48 h for qRT-PCR analyses.

Cell Proliferation Assay

Cell proliferation was determined using the WST colorimetric assay (EZ-CyTox, Dael Lab Service, Seoul, Korea). In 96-well plates, 5 × 10^3 cells/well were seeded and incubated for 24 h. After addition of 10 μL of WST reagent to each well, the plate was incubated for 2 h, and absorbance was detected at a wavelength of 450 nm. The assay was performed in triplicate. Cell numbers were determined using the EVE automatic cell counter (NanoEntek, Seoul, Korea).

Detection of Cell Cycle by Flow Cytometry

Cells for cell cycle analysis were stained with propidium oxide by the Cycletest Plus DNA reagent kit (BD Biosciences) following the protocol and analyzed by FACScan. The percentage of the cells in G0-G1, S, and G2-M phase were counted and compared.

Matrigel Invasion Assay

Cells were seeded (2.5 × 10^4 cells in 0.5 mL of media lacking FBS) into 24-well Matrigel invasion chambers (Corning, NY, USA) with polyethylene terephthalate membrane containing 8.0-μm pores. As a chemotaxis factor, FBS (10%) was added to the lower compartment of the chambers. After incubation for 24 h at 37°C, all cells that did not enter the filter were removed by gently scraping the upper side of the filter with a wet cotton swab. Cells that migrated to the bottom
filter surface were fixed by soaking the insert in MeOH for 3 min, stained with hematoxylin and eosin, and air-dried. Filters cut from inserts were mounted upside-down on glass slides. Invading cells were counted under a light microscope. The assay was performed in triplicate.

**Western Blotting Assay**

Cells were lysed in the cell lysates (Thermo Scientific) supplemented with protease inhibitors PMSF and cocktail (Roche). Proteins were separated in 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (0.22 mm, Whatman). Membranes were blocked with blocking buffer (LI-COR Biosciences), and then sequentially incubated in primary antibodies and secondary antibody. The primary antibodies included rabbit anti E-cadherin, anti-N-cadherin, anti-Vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-human GAPDH (CST). The secondary antibody was goat Anti-rabbit IgG (Invitrogen). Protein levels were measured by gray value with Quantity One software.

**In Vivo Tumorigenicity Assay**

sh-HOTTIP and sh-NC constructed by GenePharma were used for the in vivo tumorigenicity assay. The sh-HOTTIP and sh-NC were incorporated into a pLKO vector to produce the pLKO-sh-HOTTIP and pLKO-sh-NC plasmids. A lentivirus carrying either pLKO-sh-HOTTIP or pLKO-sh-NC was introduced into cells. To obtain the stable knockdown cell line, the transfected cells were selected with 2 g/mL puromycin.

5-week-old male BALB/c nude mice were raised in specific pathogen-free conditions and manipulated in line with protocols authorized by the animal center of Jilin University. All nude mice were randomly classified into either the sh-HOTTIP or sh-NC group. Mice in the sh-HOTTIP group were subcutaneously injected with SW480 cells stably transfected with pLKO-sh-HOTTIP, whereas those in the sh-NC group were subcutaneously injected with cells stably transfected with pLKO-sh-NC. Following successful transplantation, tumor width and length were measured every 5 days. All nude mice were sacrificed on day 30, and the tumor xenografts were resected and weighed. The volume of tumor xenografts was calculated using the following formula: (length × width²)/2. All experimental steps were approved by the Ethics Committee for Animal Research of Jilin University.

**IHC**

IHC analysis was performed under the manufacturer’s instructions. Briefly, the slides were incubated with primary antibodies overnight at 4°C and then incubated with secondary antibodies at room temperature.
temperature for 2 h. The expression was evaluated using a composite score obtained by multiplying the values of staining intensities (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and the percentage of positive cells (0, 0%; 1, <10%; 2, 10%–50%; 3, >50%).

Statistical Analysis
The data are presented as the mean ± SEM from triplicate experiments and additional replicates as indicated. Significance was assessed using two-way ANOVA (p < 0.0001) followed by two-tailed Student’s t tests. Survival analysis was performed using the Kaplan-Meier method, and the curves were compared using the log-rank test. A p value of <0.05 was considered statistically significant.

AUTHOR CONTRIBUTIONS
S.L. and T.L. performed primers design and experiments. H.W. and H.Y. contributed flow cytometry assay and animal experiments. H.Y. collected and classified the human tissue samples. M.B. contributed to RT-PCR and qRT-PCR. Z.Y. and S.H. analyzed the data. T.L. wrote the paper. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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