

Original Article

Long non-coding RNA H19 promotes colorectal cancer metastasis via negative modulation of miR-148b

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Received November 30, 2015; Accepted February 29, 2016; Epub June 15, 2016; Published June 30, 2016

Abstract: Cancer cell invasion and metastasis are the main reason for high mortality (approximately 30%) of colorectal cancer (CRC) patients. Thus, it will be helpful for CRC treatment to understand the potential factors promoting CRC cell metastasis. Long non-coding RNAs (lncRNAs) are newly discovered RNAs which make up 80% of non-coding RNAs, which have been linked in every stage of cell life, including cell proliferation, differentiation, apoptosis and motor. Although H19 has a vital role in cancer, little is known about the mechanism through which H19 exerts its oncogenic activity, and the interaction between H19 and miRNAs remains largely unknown. In the present study, we demonstrated that H19 was upregulated in CRC tissues. H19 upregulated the miRNA-148b target gene cholecystokinin-2 receptor (CCK2R) by competitively 'sponging' miRNA-148b and then promoted the proliferation and invasiveness of CRC cells. Our findings suggest that lncRNA H19 acts as a competing endogenous RNA for miR-148b, and subsequent regulates CCK2R expression. Thus, H19/miR-148b/CCK2R axis may serve as a potential target for CRC therapy.

Keywords: Long non-coding RNAs, microRNA, colorectal cancer, metastasis, H19

Introduction

The number of deaths caused by colorectal cancer (CRC) is ranked the third in the world [1]. Cancer cell invasion and metastasis are the main reasons for high mortality (approximately 30%) of CRC patients [2]. Thus, it will be helpful for CRC treatment to understand the potential factors promoting CRC cell metastasis.

Non-coding RNAs, including microRNA and long non-coding RNAs (lncRNAs), have been reported to be involved in CRC progress [3]. Although microRNAs have been well studied in CRC, lncRNAs, which are newly discovered RNAs making up 80% of non-coding RNAs, are needed to be deeply illustrated [4]. lncRNAs have been linked in every stage of cell life, including cell proliferation, differentiation, apoptosis and motor [5]. And lncRNAs have been identified as oncogene or tumor suppressor, or a predictor of prognosis [6, 7]. High lncRNA-GHET1 levels are correlated with tumor size, tumor invasion and poor survival in gastric cancer, which promotes gastric carcinoma cell proliferation via increasing c-Myc mRNA stability and expres-

sion [8]. The expression of lncRNA-CCAT1 in colorectal tumor tissue is significantly higher than that in normal para-carcinoma tissue, and significantly correlated with local infiltration depth, tumor staging, and vascular invasion [9].

Both of miRNAs and lncRNAs have been well characterized that they can regulate protein-coding genes. Recently, emerging evidence suggests that miRNAs and lncRNAs also can form a well-orchestrated regulatory interaction network: miRNAs and lncRNAs are able to interact with each other, imposing an additional level of posttranscriptional regulation [10]. For example, lncRNA GAS5 can bind miRNA-222 and form a regulatory interaction [11]. It is reported that stable expression of lncRNA H19 significantly promotes EMT progression and accelerates colorectal tumor growth *in vivo* and *in vitro* [12].

However, although H19 has a vital role in cancer, little is known about the mechanism through which H19 exerts its oncogenic activity, and the interaction between H19 and miRNAs remains largely unknown. In the present study,

we demonstrated that H19 was upregulated in CRC tissues. H19 upregulated the miRNA-148b target gene cholecystokinin-2 receptor (CCK2R) by competitively 'sponging' miRNA-148b and then promoted the proliferation and invasiveness of CRC cells. H19 may function as a part of the 'competitive endogenous RNA (ceRNA)' network.

Materials and methods

Tissue samples

A total of 15 colorectal carcinoma tissues and the paired adjacent tissues were collected from the Third Xiangya Hospital of Central South University. Informed consents have been signed by all subjects. All samples were collected and identified by histopathological evaluation, and stored at -80°C until used.

Cell culture

The human colorectal cancer cell lines, Lovo, HCT-116 and Caco-2, and the human colonic epithelial cells, HCoEpiC, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All the cells were cultured in RPMI1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen Life Technologies) at 37°C in a humidified 5% CO₂ incubator.

Transfection

The wild type H19 (Wt-H19) and mutated H19 (Mut-H19) expressed vector were designed and constructed in Fulengen (Guangzhou, China). And the empty pcDNA3.1 plasmid was used as a negative control. To overexpress the Wt-H19 or the Mut-H19, Lovo and HCT-116 cells were transfected with empty vector, Wt-H19 or Mut-H19 for 48 h by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). The expression of H19 was detected by real time PCR, and the transfected cells were used for further analysis.

To knock down the expression of H19, Lovo and HCT-116 cells were transfected with scramble sequences or H19-siRNA for 48 h by using Lipofectamine 2000 (Life Technologies). The siRNA sequences of H19 were designed and purchased from Ribobio Co. Ltd. (cat no. Q000283120-1-B, Guangzhou, China).

Ectopic expression of miR-148b was achieved by transfecting Lovo and HCT-116 cells with miR-148b mimics or inhibitors (cat no. miR10000759-1-5 and miR20000759-1-5, RiboBio, Guangzhou, China) through using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Real time quantitative PCR (qPCR) analysis

Trizol reagent (Invitrogen, USA) was used to extract total RNA from the indicated cells and tissues according to the manufacturer's instructions. One-Step Real-Time RT-PCR Master Mixes (Thermo Fisher Scientific, Grand Island, NY, USA) was used for real time PCR to detect the expression of H19 and CCK2R. The primers of H19, CCK2R and β -actin are as follows: H19, forward, CGCTTTTGAACCAGCAGGG, reverse, TTCCCGAGGCTTTGGTGTG; CCK2R, forward, ATCTGTCCAGCCACGAATCA, reverse, ATTAGCACCTCCATCCAGCA. β -actin, forward, AGGGGCCGGACTCGTCATACT, reverse, GGCGGCACCACCATGTACCCT. The expression of H19 and CCK2R was normalized by β -actin. The MiScript Reverse Transcription kit (Qiagen, Hilden, Germany) was used to reversely transcribe RNA into cDNA, and the MiScript SYBR-Green PCR Kit (Qiagen, Hilden, Germany) was used for real-time PCR to detect the expression of miR-148b. Specific primer sets for miRNA-148b (cat no. HmiRQP0205) and U6 (cat no. HmiRQP9001) were purchased from Fulengen (Guangzhou, China). U6 expression was used as an endogenous control. All the qPCR data were processed using 2^{- $\Delta\Delta$ CT} method.

Dual luciferase reporter assay

Wild type (WT) and mutant (MUT) forms of the 3'UTR of H19 were inserted downstream of the dual luciferase reporter vector. For the luciferase assay, 5×10⁴ HCT-116 cells were cultured in 96-well plates to reach approximately 80% confluence. The fibroblasts were co-transfected with 50 nM miR-148b mimics or miR-148b inhibitors and 30 ng of the WT/MUT 3'UTR of H19 dual luciferase reporter vector using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). After 48 h of transfection, a Dual-Luciferase-Reporter Assay System (Promega, Madison, WI) was used to detect luciferase activity using a GloMax[®]-Multi+Luminometer (Promega). Luciferase activity was normalized to Renilla luciferase activity.

Interaction of H19 and miR-148b in CRC

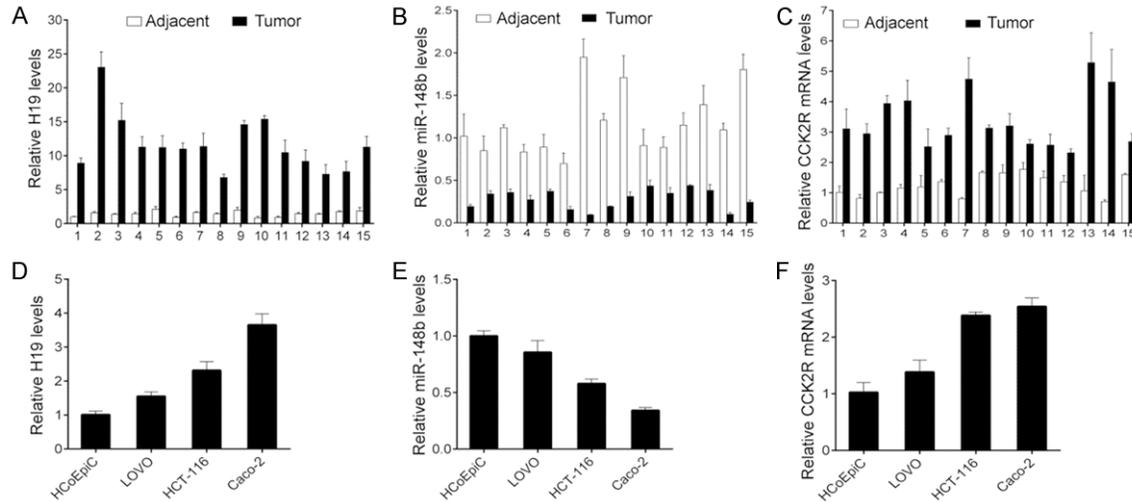


Figure 1. Expression of H19, miR-148b and CCK2R in CRC cancer tissues and CRC cell lines. Real-time PCR analysis for H19 (A), miR-148b (B) and CCK2R (C) in 15 paired CRC adjacent and tumor tissues. Real-time PCR analysis for H19 (D), miR-148b (E) and CCK2R (F) in CRC cell lines. Data are presented as mean \pm s.d.

CCK-8 cell proliferation assay

Cell growth was measured by CCK-8 assay. 1000/well Lovo or HCT-116 cells transfected with Wt-H19 or H19-siRNA were seeded in each 96-well plate for 12 h, and further incubated for 0h, 24 h, 48 h and 72 h, respectively. 1 hour before the ending of incubation, 10 μ l CCK-8 reagents (Dojindo, Japan) were added to each well. OD value at 570 nm in each well was determined by an enzyme immunoassay analyzer.

Transwell assay

The indicated cells were starved for 24 h, and then resuspended in serum-free medium and added to the upper chamber. The lower chamber was filled with medium containing 10% FBS. Following 48 hours culture, cells attached to the bottom were fixed and stained with crystal violet for 45 min and dried in air. The optical density (OD) at 570 nm of crystal violet dissolved by 10% acetic acid was detected by an enzyme immunoassay analyzer (SynergyTM Mx; BioTek, Winooski, VT, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA) and the data are presented as the mean \pm standard deviation (SD). An unpaired two-tailed Student's *t*-test or one way analysis of variance (ANOVA) with

Bonferroni *t* post-test was used to analyze the data depending on conditions. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

H19 and CCK2R are significantly upregulated, but miR-148b is reduced in CRC cancer tissues and cell lines

To investigate the role of H19 in CRC, we firstly analyzed the expression of H19 in CRC cancer tissues. We found that H19 expression was significantly increased in the CRC samples compared with the adjacent tissues (**Figure 1A**). Furthermore, we found that H19 is a putative target of miR-148b through the informatics tool, start base (<http://www.lncrnablog.com/tag/starbase-v2-0/>). Thus, we detected the expression of miR-148b and its target CCK2R in the cancer tissues and their matched adjacent tissues by using real time qPCR. All of the cancer tissues had lower miR-148b levels and higher CCK2R levels than their matched adjacent tissues (**Figure 1B, 1C**). In addition, the similar results were observed in the CRC cell lines (**Figure 1D-F**). These results indicate that H19 and miR-148b/CCK2R signaling may contribute to CRC tumor development.

Wild type H19 regulates miR-148b/CCK2R signaling

To confirm whether miR-148b directly target to H19, we performed the dual luciferase assay,

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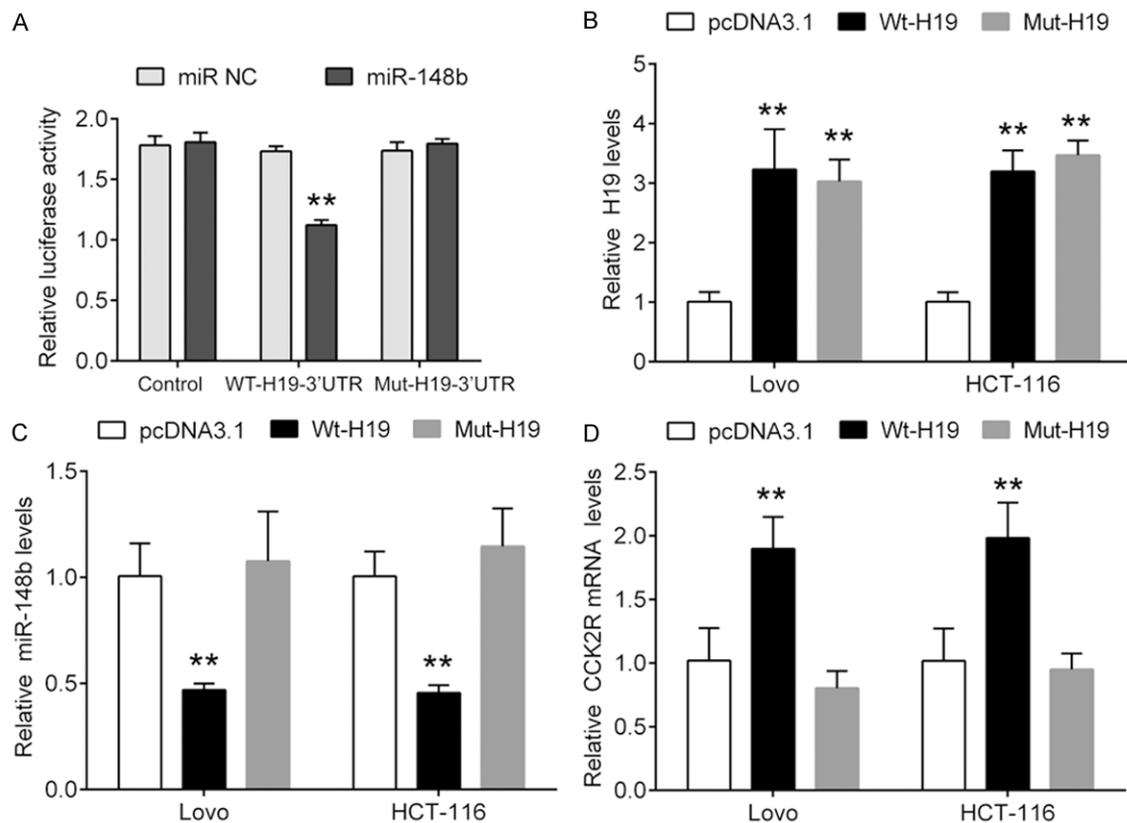


Figure 2. Overexpression of wild type H19 reduced miR-148b expression in Lovo and HCT-116 cells. (A) Dual luciferase analysis was performed in HCT-116 cells co-transfected with Wt-H19 3'UTR or Mut-H19 3'UTR and miR-148b. Real-time PCR analysis for H19 (B), miR-148b (C) and CCK2R (D) in Lovo and HCT-116 cells transfected with pcDNA3.1 plasmid, Wt-H19 expressed vector or Mut-H19 expressed vector. Data are presented as mean \pm s.d. Experiments are independently repeated three times. **P<0.01.

and found that miR-148b significantly repressed the luciferase activity of wt-H19 3'UTR, but did not alter the luciferase activity of Mut-H19 3'UTR, indicating that miR-148b directly bound to 3'UTR of H19 (Figure 2A). Furthermore, we investigated whether H19 could regulate the expression of miR-148b. The wild type and mutated type H19 expressed vector were transfected into Lovo and HCT-116 cells (Figure 2B). Then, the expression of miR-148b was analyzed. We found that miR-148b expression was significantly decreased by wild type H19, and subsequently resulting in upregulation of CCK2R. However, the mutated type of H19 could not change the expression of miR-148b and CCK2R. In contrast, we knocked down the expression of H19 by using siRNA transfection, and found that knockdown of H19 was able to upregulate the expression of miR-148b, and subsequent reduction of CCK2R (Figure 3A-C). In the other hand, we further investigated

whether miR-148b could regulate H19 expression because of their interaction. We knocked down or overexpressed miR-148b in Lovo and HCT-116 cells (Figure 3D), and found that miR-148b negatively regulated the expression of CCK2R, but not alter the expression of H19. These findings suggest that H19 regulates miR-148b/CCK2R signaling through its interaction with miR-148b.

Knockdown of H19 inhibits cell proliferation and invasion in CRC cells

In order to investigate the role of H19 in CRC cells, we transfected the Lovo and HCT-116 cells with H19-siRNA or H19 expressed vector. CCK-8 was used to detect the Lovo and HCT-116 cells proliferation. We found that Knockdown of H19 inhibited cell proliferation, whereas Wt-H19 transfection significantly induced cell proliferation (Figure 4A and 4B).

Interaction of H19 and miR-148b in CRC

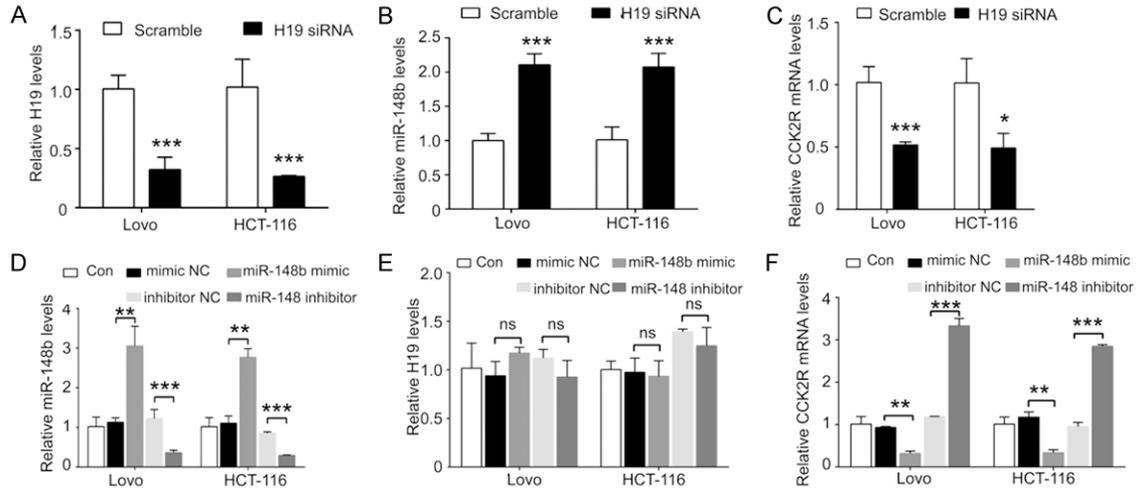


Figure 3. H19 regulated miR-148b/CCK2R signaling. Real-time PCR analysis for H19 (A), miR-148b (B) and CCK2R (C) in LoVo and HCT-116 cells transfected with scramble sequences or H19 siRNA sequences. Real-time PCR analysis for miR-148b (D), H19 (E) and CCK2R (F) in LoVo and HCT-116 cells transfected with miR-148b mimic, or miR-148b inhibitor or their negative control. Data are presented as mean \pm SD. Experiments are independently repeated three times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, no significance.

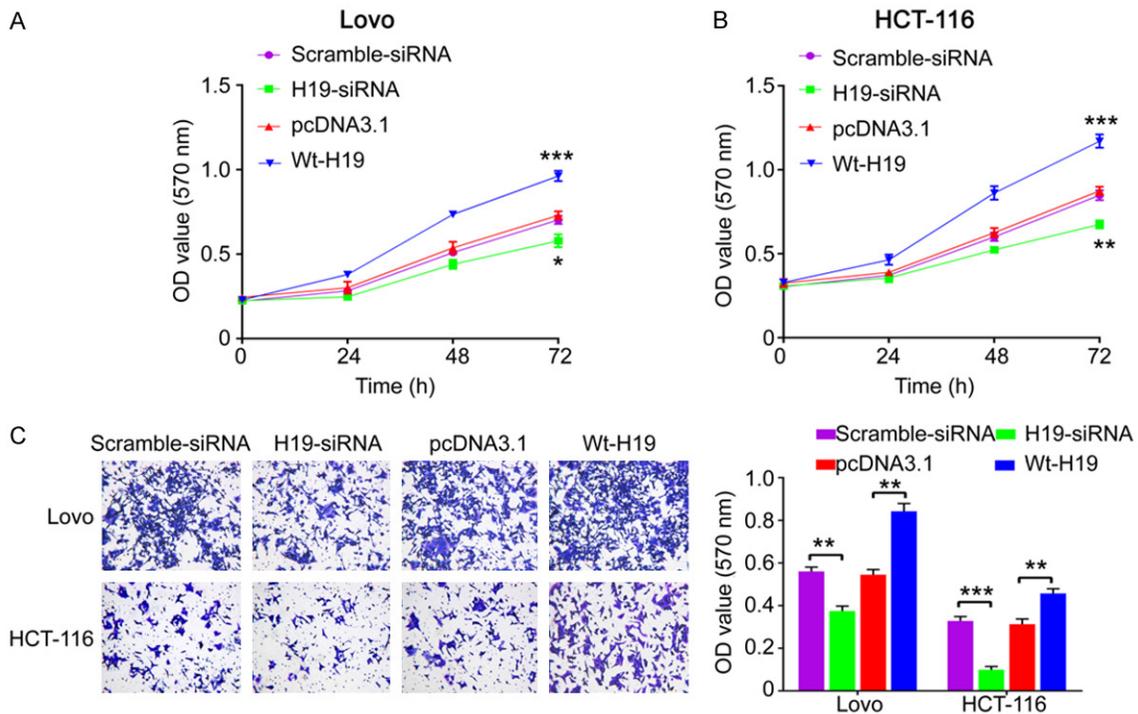


Figure 4. H19 promotes cell proliferation and invasion in LoVo and HCT-116 cells. CCK-8 assay in LoVo (A) and HCT-116 (B) cells transfected with H-19 expressed vector or H19 siRNA sequences or their negative control. (C) Transwell assay in LoVo and HCT-116 cells transfected with H-19 expressed vector or H19 siRNA sequences or their negative control. Data are presented as mean \pm s.d. Experiments are independently repeated three times. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Transwell assay showed that the invasive ability was significantly decreased in H19 siRNA group compared with the negative control

group, while the invasive ability was enhanced in Wt-H19 group than the pcDNA3.1 control group (Figure 4C). These results indicate that

knockdown of H19 is able to repress CRC cell growth and invasion.

Discussion

Non-coding RNAs play a vital role in carcinogenesis, cancer development and cancer metastasis [13]. The studies on non-coding RNA provide new insights into the biology of cancers. Over the past decade, miRNAs have been well demonstrated in almost all the cancers [14, 15]. It would facilitate the development of lncRNA-directed therapy that understanding the precise molecular mechanism by which lncRNAs function. Emerging evidence also shows that lncRNAs contribute to the development of cancers in every stage, including CRC [16]. However, the role of lncRNAs in the carcinogenesis of CRC remains largely unknown. In this study, we provide evidence that H19 exhibits oncogenic activities partly through modulation of miRNA-148b and then its target CCK2R.

Recent evidence suggests that there is an interaction network involving competitive endogenous RNA (ceRNA), where lncRNAs could regulate miRNA by binding and removing them from miRNAs' binding sites on protein coding messengers [17, 18]. It was reported that lncRNA HULC was highly upregulated in liver cancer, and HULC exhibited an inhibitory effects on the expression and activity of miRNA-372 [19]. And lncRNA MALAT1 acted as an oncogene in bladder cancer, its expression was suppressed by miR-125b [20]. There also was an interaction on loc285194 and miR-211 in colon cancer [21]. The interaction between lncRNA-miRNA in CRC cancer remains to be explored. Kallen et al. demonstrated that H19 could interact with let-7 and modulate let-7 availability, and inhibits muscle differentiation through antagonizing let-7 [22].

The imprinted oncofetal lncRNA H19 is expressed in the embryo, down-regulated at birth and then reappears in tumors [23]. Higher expression of H19 was positively correlated with advanced tumor-node-metastasis stage and tumor size in non-small-cell lung cancer [24]. Recent studies suggest that lncRNAs may exert functions through targeting miRNAs. H19 enhanced the aggressive phenotype of breast cancer cells through targeting miR-675 and then decreasing both c-Cbl and Cbl-b expres-

sion [25]. Tsang WP et al. demonstrated that there was an inverse relationship between the expressions of RB and H19/miR-675 in human CRC tissues and colon cancer cell lines. These findings revealed that H19-derived miR-675 regulated the CRC development through down-regulation of its target RB and thus may serve as a potential target for CRC therapy [26]. Liang WC found that H19 was characterized as a competing endogenous RNA (ceRNA) for miR-138 and miR-200a and acted as a novel regulator of epithelial to mesenchymal transition (EMT) in CRC [27]. And H19 was highly expressed in mesenchymal-like cancer cells and primary CRC cancer tissues [27, 28]. Herein, we found that H19 interacted with miR-148b in CRC cancer cells. There were significant changes in the expression of miR-148b in response to wild type H19 knockdown or over-expression. And ectopic expression of H19 also induced the upregulation of CCK2R, a target of miR-148b. However, miR-148b was unable to regulate the expression of H19 in CRC cancer cells. MiR-148b have been identified as a tumor suppressor in CRC, overexpression of miR-148b can inhibit cell proliferation and arrest cell cycle progression in CRC cell lines and decrease tumor growth *in vivo*. Furthermore, p53 expression is associated with miR-148b expression, and p53 directly activates the transcription of miR-148b by binding to its promoter [29]. The expression of miR-148b is significantly downregulated in human colorectal cancer tissues and three cell lines compared with non-tumor adjacent tissues, which is associated with tumor size in colorectal cancer patients. Moreover, overexpression of miR-148b in HCT-116 cells can inhibit cell proliferation *in vitro* and suppress tumorigenicity *in vivo* by targeting cholecystokinin-2 receptor gene (CCK2R) [30]. The present study revealed that H19 was shown to promote cell proliferation and invasion in CRC cell lines. These findings suggest that H19 may promote tumor development through 'sponging' miRNA-148b, and then regulating CCK2R. However, the precise downstream mechanism requires further investigation in the future.

In conclusion, lncRNA H19 acts as a competing endogenous RNA for miR-148b, and subsequent regulates CCK2R expression. Thus, H19/miR-148b/CCK2R axis may serve as a potential target for CRC therapy.

Disclosure of conflict of interest

None.

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