Original Paper

The Effect of LncRNA H19/miR-194-5p Axis on the Epithelial-Mesenchymal Transition of Colorectal Adenocarcinoma

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Key Words

Colorectal adenocarcinoma • LncRNA H19 • miR-194 • FoxM1 • Epithelial mesenchymal transition

Abstract

Background/Aims: Since the combined actions of lncRNAs and miRNAs have been considered to be involved in the occurrence and development of various neoplasms, the main purpose of this study was to discover whether and how lncRNA H19 and miR-194 influenced the epithelial-mesenchymal transition (EMT) process of colorectal adenocarcinoma (CRA).

Methods: Totally 214 pairs of CRA and adjacent normal tissues were collected, and 5 human CRA cell lines (i.e. HCT116, HT-29, RKO SW280 and Lovo) were purchased. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was adopted to quantify the H19 and miR-194-5p expressions in cells and tissues. The expressions of FoxM1, E-cadherin, vimentin, N-cadherin were determined using western blot. On the side, si-H19, si-NC, miR-194-5p mimic, miR-194-5p inhibitor and negative control (NC) were transfected into CRA cell lines. Meanwhile, the invasive, migratory and proliferative conditions of the cells were assessed through transwell, wound healing and colony-forming experiments, with final verification of the relationship between H19 and miR-194-5p employing dual-luciferase reporter gene assay.

Results: Highly-expressed H19, lowly-expressed miR-194-5p, low-grade differentiation and lymph node metastasis appeared as the independent predictors of unfavorable prognosis in CRA patients' (all $P < 0.05$). It indicated that FoxM1 expression displayed positive correlations with H19 expression, yet negative associations with miR-194-5p expression within CRA tissues ($P < 0.05$). In addition, transfection of H19-siRNA and miR-145-5p mimic triggered a conspicuous increase in E-cadherin expression, as well as an evidently down-regulation in vimentin and N-cadherin expressions within HT29 and RKO cells ($P < 0.05$). On the other hand, the invasive and migratory capacities of CRA cells were significantly hindered ($P < 0.05$). Moreover, the luciferase reporter gene assay confirmed that H19 modified miR-194-5p expression through directly targeting at it ($P < 0.05$). Ultimately, FoxM1 could reverse the role

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of miR-194-5p in inhibiting invasion, migration and EMT of CRA cells ($P < 0.05$). **Conclusion:** lncRNA H19/miR-194/FoxM1 axis could serve as a profound target for the diagnosis and treatment of CRA.

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Introduction

Colorectal cancer (CRC) is a globally-observed malignancy that causes up to as high as 0.6 million new deaths per year [1]. The 5-year overall survival (OS) rate of early-stage CRC patients is greater than 90%, while the advanced CRC patients possess a rather low 5-year OS rate at merely 10% [1]. Currently plenty of CRC patients, especially those with colorectal adenocarcinoma (CRA), are identified until the late phase of the disorders, thus the sufferers' prognosis is quite unsatisfactory, and seeking valid diagnostic and treatment schemes for early-stage CRC or CRA becomes extremely critical [2]. Of note, epithelial-mesenchymal transition (EMT) plays a vital role underlying the pathogenesis of CRA, which is embodied in its interfering cell-cell adherence and facilitating tumor metastasis [3-9].

Non-coding RNAs (ncRNAs), mainly divided into long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), counted for much in regulating tumorigenesis [10-12]. For example, over-expressed lncRNA MX1-1 was associated with reduced recurrence-free survival rate of prostate cancer patients [13]. Furthermore, lncRNA H19 could modify progression and EMT of tumor cells (e.g. CRC), and it was located close to the telomeric region of chromosome 11p15.5, where multiple cancer-associated genes were harbored [14-18].

More than that, H19 could modulate EMT-related genes by sponging miRNAs [19]. To be specific, H19 was able to alter proliferation of gallbladder cancer cells via inhibiting miR-194-5p and thereby modulating AKT2 expression [20]. In addition, miR-194 interacting with FoxM1 not only regulated metastasis of CRC cells, but also mediated EMT of non-small cell lung cancer cells [21-23]. However, scholars' opinions were divided regarding the contribution of miR-194 to tumor development. To be specific, miR-194 was documented to inhibit EMT process of endometrial cancer cells via suppressing BMI-1 [24], whereas another investigation asserted that miR-194 promoted EMT within CRC [25]. Therefore, how on earth H19 and miR-194 influenced EMT of CRA remained unclear.

In response, this investigation was aimed to investigate the function of lncRNA H19/miR-194-5p axis underlying the EMT process of CRA, which might assist in developing novel strategies for upgrading the prognostic status of CRA patients.

Materials and Methods

Collection of CRA tissues and culture of CRA cells

From Jan 2016 to Oct 2017, altogether 214 CRA tissues and corresponding para-carcinoma tissues were collected from Hospital of Jilin Province. The patients hardly received surgery or chemotherapy before, and they were confirmed with CRA by pathological examinations. The CRA subjects would be excluded if they: 1) were with recurrent CRA; 2) had histories of other tumors, genetic diseases and disorders relevant to heart, kidney, liver, endocrine system and central nervous system; 3) were genetically related to each other; and 4) did not offer detailed personal information and clinical data. Furthermore, human CRA cell lines (i.e. HCT116, HT-29, RKO, SW280 and Lovo) and regular colonic epithelial cell line (i.e. CCD-18Co) were purchased from American Standard Biology Collection (ATCC). The CRA cell lines were classified based on Duke grading [26]. They were cultured within DMEM medium (Gibco) or RPMI 1640 medium (Gibco) in 5% CO₂ at 37 °C. The medium was added with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. When cells achieved the logarithmic growth phase, they were digested with 0.25% trypsin and were filtrated into the single-cell suspension.

Cell transfection

Genepharma Inc. (China) synthesized the targeted siRNA for lncRNAH19 (i.e. si-H19) and si-NC. The sequences of si-H19-1 and si-H19-2 were, respectively, 5'-CCAACAUCAAAGACACCAUdTdT-3' and 5'-UAAGUCAUUGCAGUGGUdTdT-3'. The full-length H19 cDNA was sub-cloned into a pCDNA3.1

vector (Gene Pharma, China) to construct pCNA3.1-H19 plasmid. Also, miR-194-5p mimics, miR-194-5p inhibitor, negative control (NC) were all purchased from Qiagen (CA). They were transfected into cells using Lipofectamine® 2000 (Invitrogen) when the cell confluency reached 90%-95%.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted utilizing TRIzol (Ambion), and its concentration was measured with NanoDrop 2000 spectrophotometer (Thermo). After reverse transcription (RT), cDNAs were synthesized with assistance of first-strand cDNA synthesis kit (Biomiga). Then PCR was carried out based on the primers (Biotech Bioengineering, China) shown in Table 1, and GAPDH or U6 was set as the internal reference. The PCR reaction system (20 µl) mainly incorporated cDNA (1 µl), 2× Master Mix (10 µl) and ddH₂O (7 µl). And the PCR reaction conditions were displayed as: 1) pre-denaturation at 94 °C for 2 min; 2) 35 cycles of 94 °C for 30 s, 60°C for 30 s and 72°C for 30 s; and 3) extension at 72 °C for 5 min. Subsequently, 5 µg PCR end-products were handled with 1% agarose gel electrophoresis. Finally, we utilized ChemiDoc XRS gel imaging system of the Bio-Rad company (USA) to conduct grey scanning, and applied Quantity one-4.6.2 system for analyzing the results.

CCK-8 experiment

The cells were inoculated into 96-well plates at the density of 2000/well after digestion and centrifugation. The initial time when cells attached to the wall was set as 0 h, and CCK-8 kit (KaygenBiotech, China) was employed to examine the optical density (OD) values at the time points of 0 h, 24 h, 48 h and 72 h.

Colony formation assay

After culturing the cells for 1-2 weeks, pure methanol was added to fix cells for 15 min, and crystal violet was supplemented to dye cells for 20 min. Then we washed the staining solution away, and the Petri plates were observed under the microscope. A segment was labeled on the plate, and it was set as the scale. At last, we counted the cell colonies whose diameter was $\geq$ scale size.

Transwell experiment

Matrigels (BD, USA) were diluted at the ratio of 1: 5 and were then spread evenly within the upper chamber (Corning, China) at 37 °C for 30 min. About 8×10^4 cells were digested, centrifuged and counted. They were added to the upper chamber, and 200 µl FBS-free RPMI-1640 medium (HyClone, USA) was also supplemented. Extra 600 µl RPMI-1640 medium was added to the lower chamber. After 24-hour cultivation, cells were fixed with methanol/acetic acid (v/v: 3:1) for 15 min, and were then stained with Giemsa for 10 min. The inverted microscope was utilized to count cells within 5 views, and the average value of the counted cells was considered as the number of invasive cells. Besides, the migratory ability of cells was evaluated nearly in the same way as measuring the invasive ability, except that Matrigel was not layered.

Scratch experiment

The transfected cells were seeded in 6-well plates at the density of 8×10^4 . A sterile tip was employed to draw lines in the hole, and hardly any cells grew within the crossed places. We utilized a microscope to observe the widths between the scribed cells, and cell migration ability was determined according to the following formula: healing degree =

$$\left(1 - \frac{\text{scratch width at some certain point}}{\text{original scratch width}}\right) \times 100\%$$

Western Blotting

We applied RIPA lysate for extraction of total protein, whose concentration was measured with Anthos 2010 microplate reader. Then we adjusted the protein loading volume to 120 µg/well, according to the result of protein concentration. The sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under 100 V for 2 hours, and films were transferred under 300 mA for 1 hour. Next, the PVDF membranes were fetched before the required bands were sealed with 5% BSA at room

Table 1. The primers for lncRNA H19, GAPDH, miR-194-5p and U6 involved in this study

DNAs	Primers
lncRNA H19	Forward: 5'-ATCGGTGCCTCAGCGTTCGG-3' Reverse : 5'-CTGCTCTCGCCGTACACCG-3'
GAPDH	Forward:5'-GTCAACGGATTGGTCTGTATT-3' Reverse : 5'-AGTCTTCTGGGTGGCAGTGAT-3'
miR-194-5p	Forward:5'-GCCGCTGTAACAGCAACTCCAT-3' Reverse : 5'-GTGCAGGGTCCGAGGT-3'
U6	Forward: 5'-GCUUCGGCAGCACAUUACUA AAAU-3' Reverse : 5'-CGCUUCACGAUUUGCGU GUCAU-3'

temperature for 2 hours. Subsequently, primary antibodies for E-cadherin (mouse anti-human, 1: 500, Santa Cruz Biotechnology, USA), N-cadherin (mouse anti-human, 1: 300, Santa Cruz Biotechnology, USA), vimentin (mouse anti-human, 1: 300, Santa Cruz Biotechnology, USA) and β -actin (mouse anti-human, 1: 300, Santa Cruz Biotechnology, USA) were mixed with the cultured cells at 4 °C for overnight. On top of that, we added goat anti-mouse/goat anti-rabbit secondary antibodies (1: 2000) that were labeled with horseradish peroxidase (HRP). The samples' membrane surfaces were coated in a dark room, according to the instructions of chemiluminescent kit (Beyotime, China). Finally, films were exposed to X-ray, and the protein bands were developed, fixed, scanned and quantified using Quality One 4.5 software.

Double luciferase reporter gene assay

We took the advantage of PCR to amplify H19 cDNA fragments that included specific miR-194-5p binding sites, and then cloned them into the luciferase reporter vector (i.e. psiCHECK2) to produce H19 Wt. Through a similar way, H19 Mut that contained mutant sequences at the miR-194-5p binding site was constructed. Subsequently, miR-194-5p mimic and miR-NC were, respectively, transfected into HT29 and RKO cells that have been transfected with H19 Wt, H19 Mut and psiCHECK2. After 48-h transfection, we added 100 μ l PLB buffer to each well and collected 20 μ l cell lysates after 15-min shake at room temperature. The cell lysates were then applied to the luminous panel, and the background values were scanned for 2 seconds with GloMax bioluminescence detector. The experimental values were also scanned for 2 seconds after addition of 100 μ l LAR II solution to each sample. Finally, we added 100 μ l Stop&Glo® Reagent into each sample, and examined the results with the luminescent detector.

Statistical analyses

The whole statistical analyses were performed via SPSS 17.0 statistical software. The measurement data were expressed as mean $\pm$ standard deviation (SD), and the enumeration data were presented as percentage (%). The multi-group comparisons were processed with one-way analysis of variance (ANOVA), and inter-group comparisons were managed with Bonferroni method. A *P* values less than 0.50 was deemed as statistically significant.

Results

H19 and miR-194-5p expressions within CRA tissues and cells

According to the TCGA Data Portal of starBASE v2.0, we found that H19 expressions were significantly distinct between cancer tissues and normal tissues ($P < 0.05$) (Fig. 1A), and H19 expression within CRC tissues was remarkably beyond that within adjacent normal tissues ($P < 0.05$) (Fig. 1B). Also a negative correlation between H19 expression and miR-194-5p expression was found among the 299 CRC samples ($P < 0.05$) (Fig. 1C). Analogously, it was drawn from the tissues we collected that H19 expression within CRA tissues was sharply elevated, yet miR-194-5p expression was substantially decreased in comparison to adjacent normal tissues ($P < 0.05$) (Fig. 2A). What's more, H19 expression within four CRA cell lines (i.e. HCT116, HT29, RKO, SW480 and Lovo) exceeded that within normal colonic epithelial cell line (i.e. CCD-18Co) ($P < 0.05$), whereas miR-194-5p expression within HCT116, HT29, RKO, SW480 and Lovo were significantly lower than that within CCD-18Co ($P < 0.05$) (Fig. 2B). H19 and miR-194-5p expressions were also negatively inter-related within the gathered CRA tissues ($P < 0.05$) (Fig. 2C).

Correlation between lncRNA H19 or miR-194-5p expression and overall survival (OS) of CRA patients

According to the expression performance of lncRNA H19 and miR-194-5p, the obtained CRA tissues were categorized into highly-expressed H19 group ($>$ median H19 expression) and lowly-expressed H19 group ($\leq$ median H19 expression). The identical population was also divided into highly-expressed miR-194-5p group ($>$ median miR-194-5p expression) and lowly-expressed miR-194-5p group ($\leq$ median miR-194-5p expression). The results indicated that H19 and miR-194-5p expressions were both markedly correlated with family

Fig. 1. lncRNA H19 and miR-194-5p expressions within colorectal adenocarcinoma (CRA) tissues and cells. (A) H19 expressions were compared between cancer tissues and normal tissues according to the TCGA Data Portal of starBASE v2.0. (B) H19 expression was compared between CRA tissues and normal tissues in light of the TCGA Data Portal of starBASE v2.0. (C) H19 expression was negatively correlated with miR-194-5p expression within CRA in line with the TCGA Data Portal of starBASE v2.0.

Fig. 2. (A) H19 and miR-194-5p expressions were compared between CRA tissues and adjacent normal tissues. *: $P < 0.05$ when compared with adjacent normal tissues. (B) H19 and miR-194-5p expressions were compared between CRA cell lines (i.e. HCT116, HT29, RKO and Lovo) and normal colonic epithelial cell line (i.e. CCD-18Co). *: $P < 0.05$ when compared with CCD-18Co. (C) A negative correlation between H19 expression and miR-194-5p expression was observed among the included CRA subjects. (D) The lowly-expressed H19 was associated with higher overall survival (OS) rate of CRA patients. (E) The highly-expressed miR-194-5p was found to be with more desirable OS rate.

history, differentiation degree, serum CEA content, AJCC stage and metastatic stage of CRA patients ($P < 0.05$) (Table 2). However, neither H19 expression nor miR-194-5p expression showed any significant correlation with age, sex or tumor location ($P > 0.05$). In addition, single-factor regression analysis claimed that higher H19 expression, lower miR-194-5p expression, lower differentiation grade, preoperative high serum CEA content, AJCC_{III+IV} stage and metastatic conditions (i.e. M_{1a} and M_{1b}) were significantly associated with poor prognosis of CRA patients ($P < 0.05$) (Table 3). The results of multivariate regression analysis pointed out that merely higher H19 expression, lower miR-194-5p expression, lower grade of differentiation, late AJCC stage, lymph node metastasis and distal metastasis served as the independent determinants for poor prognosis of CRA patients ($P < 0.05$). Simultaneously, the survival status of patients with lower H19 expression or higher miR-194-5p expression appeared to be much more desirable than ones with higher H19 expression or lower miR-194-5p expression ($P < 0.05$) (Fig. 2D and 2E).

Table 2. The correlation between lncRNA H19/miR-194-5p expressions and the colorectal cancer patients' baseline clinical characteristics

Characteristics	lncRNAH19 expression			miR-194-5p expression		
	Low	High	P	Low	High	P
Age						
≤50	43	78		63	58	
>50	38	55	0.426	53	40	0.474
Sex						
Male	47	65		59	53	
Female	34	68	0.194	57	45	0.639
Family history						
Positive	20	51		46	25	
Negative	61	82	0.04	70	73	0.029
Tumor Location						
Colon	46	72		58	60	
Rectum	35	61	0.705	58	38	0.1
Differentiation						
Well-→Moderate-differentiation	60	75		63	72	
Poor differentiation	21	58	0.009	53	26	0.004
Preoperative serum CEA (ng/mL)						
≤6	66	88		76	78	
>6	15	45	0.016	40	20	0.022
AJCC stage						
I + II	32	13		10	35	
III	21	30	0.003	24	27	0.011
IV	28	90	<0.001	82	36	<0.001
Metastasis						
M ₀	53	43		34	62	
M _{1a}	24	58	0.001	52	30	<0.001
M _{1b}	4	32	<0.001	30	6	<0.001

Comparison of proliferation, EMT and viability among SW480 (i.e. without metastasis), HT-29 (i.e. with LN metastasis) and HCT-116 (i.e. with distant metastasis) cell lines

It was drawn from Fig. 3A that the distantly metastatic tissues displayed the maximum correlation coefficient (i.e. r_s) between H19 expression and miR-194-5p expression ($r_s = -0.811$; $P < 0.001$), and the r_s value of LN-metastatic CRA tissues was ranked as the 2nd largest ($r_s = -0.684$; $P < 0.001$). Besides, HCT-116 cell line showed the most pronounced proliferative ability and viability, whereas SW280 cell line was associated with the least proliferation and viability (all $P < 0.05$) (Fig. 3B and 3C). At the meantime, HCT-116 cell line was determined with the lowest E-cadherin expression, as well as the highest expressions of N-cadherin and vimentin ($P < 0.05$) (Fig. 3D).

H19 and miR-194-5p regulated EMT, invasion and migration of CRA cells

H19 expression and miR-194-5p expression were, respectively, largely depressed within si-H19 group and miR-194-5p inhibitor group, when compared with NC group ($P < 0.05$) (Fig. 4A). In contrast, H19 and miR-194-5p expressions were, separately, greatly promoted when CRA cells were treated with pcDNA3.1-H19 and miR-194-5p mimic ($P < 0.05$). Furthermore, si-H19 and miR-145-5p mimic groups possessed significantly up-regulated E-cadherin expression, as well as down-regulated vimentin and N-cadherin expressions ($P < 0.05$). The pcDNA3.1-H19 and miR-194-5p inhibitor groups were reflected with an opposite trend regarding the above protein expressions ($P < 0.05$) (Fig. 4B and 4C). More than that, less

Table 3. The relationship between clinical characteristics and the colorectal cancer patients' overall survival

Characteristics	Univariate analysis			Multivariate analysis		
	Hazard Ratio	95% CI	P value	Hazard Ratio	95% CI	P value
LncRNAH19 expression						
High vs. Low	2.78	1.91-4.03	<0.001	3.968	2.58-6.10	<0.001
miR-194-5p expression						
High vs. Low	0.315	0.22-0.45	<0.001	0.232	0.16-0.35	<0.001
Sex						
Female vs. Male	1.23	0.89-1.69	0.215	0.986	0.70-1.39	0.934
Age						
>50 vs. ≤50	0.92	0.66-1.28	0.615	0.91	0.65-1.28	0.585
Family history						
Negative vs. Positive	0.853	0.61-1.19	0.353	1.238	0.86-1.78	0.244
Tumor Location						
Rectum vs. Colon	1.536	1.11-2.12	0.009	1.371	0.98-1.91	0.063
Differentiation						
Poor differentiation vs. Well-/Moderate-differentiation	1.923	1.39-2.66	<0.001	1.495	1.07-2.09	0.019
Preoperative serum CEA (ng/mL)						
>6 vs. ≤6	1.785	1.27-2.51	0.001	1.367	0.93-2.00	0.108
AJCC stage						
III vs. I + II	0.15	0.06-0.37	<0.001	0.21	0.06-0.72	0.013
IV vs. I+II	0.07	0.03-0.15	<0.001	0.17	0.05-0.62	0.007
Metastasis						
M _{1a} vs. M ₀	0.18	0.09-0.37	<0.001	0.22	0.08-0.60	0.003
Distant metastasis vs. Negative	0.03	0.01-0.19	<0.001	0.1	0.01-0.99	0.049

Fig. 3. The correlation between H19 and miR-194-5p (A) were determined within SW480, HCT-116 and HT-29 cell lines, and the cell lines' proliferation (B), viability (C) and epithelial-mesenchymal transition (EMT)-specific proteins (i.e. E-cadherin, N-cadherin and vimentin) (D) were also compared.

Fig. 4. Effects of H19 and miR-194-5p on EMT process of colorectal adenocarcinoma (CRA) cells. (A) H19 expression was detected after transfection with si-H19 and pcDNA3.1-H19, and miR-194-5p expression was detected after transfection with miR-194-5p mimic and miR-194-5p inhibitor. (B) The expressions of endothelial-mesenchymal transition (EMT)-related proteins (i.e. E-cadherin, N-cadherin and Vimentin) were determined among pcDNA3.1 H19, si-H19, miR-194-5p mimics and miR-194-5p inhibitor groups within HT29 (B) and RKO (C) cell lines. *: $P < 0.05$ when compared with pcDNA; #: $P < 0.05$ when compared with si-NC; @: $P < 0.05$ when compared with miR-NC.

trans-membrane cells were observed within si-H19 and miR-194-5p mimic groups ($P < 0.05$), and addition of pcDNA3.1-H19 or miR-194-5p inhibitor, to a great extent, facilitated the cells to cross membranes ($P < 0.05$) (Fig. 5). Similarly, the results of scratch assays and CCK-8 experiments presented that si-H19 and miR-194-5p mimic groups were associated with conspicuously restrained cell motility ($P < 0.05$) (Fig. 6). On the contrary, treatments with pcDNA3.1-H19 and miR-194-5p inhibitor accelerated cell motility ($P < 0.05$).

H19 targeted miR-194-5p to inhibit its expression

We predicted the targeting sites between H19 and miR-194-5p via Starbase 2.0 software (<http://starbase.sysu.edu.cn/seedTargetInfo.php?type=lncRNA&database=hg19&name=hsa-miR-194-5p&geneName=H19&autoId=1100&orgTable=mirLncRNAInteractionsA11>) (Fig. 7A). Furthermore, consistent with the prediction results, the luciferase activity of H19 Wt+miR-194-5p mimics group was manifested to be weaker than that of control group ($P < 0.05$), nonetheless, the luciferase activity of H19 Mut+miR-194-5p mimics group and psiCHECK2 group failed to show any differences from that of H19 Wt+miR-NC group (Fig. 7B). Besides, lowly-expressed H19 greatly increased miR-194-5p expression ($P < 0.05$), while regulated miR-194-5p expressions were unable to alter the expression of H19 ($P < 0.05$) (Fig. 7C and 7D).

Fig. 5. The migratory (A-B) and invasive (C-D) abilities of colorectal adenocarcinoma (CRA) cells were observed by Transwell assay among pcDNA3.1 H19, si-H19, miR-194-5p mimics and miR-194-5p inhibitor groups within HT29 and RKO cell lines. *: $P < 0.05$ when compared with pcDNA; #: $P < 0.05$ when compared with si-NC; @: $P < 0.05$ when compared with miR-NC.

Fig. 6. The motility of HT29 and RKO cell lines were detected with scratch assay (A-B) and CCK-8 experiment (C-D) among pcDNA3.1 H19, si-H19, miR-194-5p mimics and miR-194-5p inhibitor groups at the time points of 12 h, 24 h, 36 h and 48 h. *: $P < 0.05$ when compared with pcDNA; #: $P < 0.05$ when compared with si-NC; @: $P < 0.05$ when compared with miR-NC.

Fig. 7. The relationships among lncRNA H19, miR-194-5p and FoxM1. (A) H19 targeted miR-194-5p in specific sites. (B) The luciferase activities of H19 Wt+miR-194-5p mimic and H19 Mut+miR-194-5p mimic groups were evaluated within HT29 and RKO cell lines. *: $P < 0.05$ when compared with miR-NC. (C) The effects of miR-194-5p mimic and miR-194-5p inhibitor on H19 expression were assessed within HT29 and RKO cell lines. (D) The effects of down- and up-regulated H19 on miR-194-5p expression were appraised within HT29 and RKO cell lines. *: $P < 0.05$ when compared with si-NC; #: $P < 0.05$ when compared with pcDNA. (E) The H19 expressions were positively correlated with FoxM1 expressions within included CRA tissues. (F) The miR-194-5p expressions were negatively correlated with FoxM1 expressions within included CRA tissues.

H19 and miR-194-5p modified EMT, invasion and migration of CRA cells through acting on downstream FoxM1

It was clinically indicated that FoxM1 expression was positively correlated with H19 expression, yet negatively correlated with miR-194-5p expression within CRA tissues ($P < 0.05$) (Fig. 7E and 7F). Besides, FoxM1 expression was significantly decreased after transfection of si-H19 and miR-194-5p mimics ($P < 0.05$) (Fig. 8A and 8B). The result above indicated that the performance of FoxM1 was controlled and adjusted by H19 and miR-194-5p. We also observed that miR-194-5p+FoxM1 group displayed over-expressed vimentin and N-cadherin, along with down-expressed E-cadherin, when compared with miR-194-5p group ($P < 0.05$) (Fig. 8C and 8D). It was insinuated that FoxM1 could reverse the reduced EMT process caused by miR-194-5p within CRA cells. In addition, the number of transmembrane cells within miR-194-5p+FoxM1 group far surpassed that within miR-194-5p group ($P < 0.05$) (Fig. 9), highlighting the role of FoxM1 in promoting miR-194-5p-suppressed migration and invasion of CRA cells. Analogously, the cell motility of miR-194-5p+FoxM1 group was encouraged in comparison to miR-194-5p group ($P < 0.05$) (Fig. 10).

Fig. 8. The FoxM1 expressions were detected among pcDNA3.1 H19, si-H19, miR-194-5p mimics and miR-194-5p inhibitor groups within HT29 (A) and RKO (B) cell lines, and the expressions of endothelial-mesenchymal transition (EMT)-related proteins (i.e. E-cadherin, N-cadherin and Vimentin) were determined among miR-194-5p, miR-194-5p mimics and miR-194-5p+FoxM1 groups within HT29 (C) and RKO (D) cell lines. A-B: *, $P < 0.05$ when compared with pcDNA; #: $P < 0.05$ when compared with si-NC; @: $P < 0.05$ when compared with miR-NC. C-D: *, $P < 0.05$ when compared with miR-194-5p.

Fig. 9. The migratory (A-B) and invasive (C-D) abilities of colorectal adenocarcinoma (CRA) cells were observed by Transwell assay among miR-194-5p mimics, miR-194-5p and miR-194-5p+FoxM1 groups. *: $P < 0.05$ when compared with miR-194-5p.

Fig. 10. The motility of HT29 and RKO cell lines were detected with scratch assay (A-B) and CCK-8 experiment (C-D) among miR-194-5p mimics, miR-194-5p and miR-194-5p+FoxM1 groups at the time points of 12 h, 24 h, 36 h and 48 h. *: $P < 0.05$ when compared with miR-194-5p.

Discussion

Even though lncRNAs were initially deemed as biologically non-functional, they were later found to play a role in regulating occurrence, development, metastasis and EMT of neoplasms [27, 28]. For instance, applying lncRNA chip to study the mammary epithelial cell MCF10A derived that about 99 types of lncRNAs might participate in the Twist-induced EMT process [29]. In addition, lncRNAs HOTAIR, MALAT-1, H19 and BANC1 could accelerate tumors' metastasis through activating EMT process [30-33]. In the middle of the process, such interstitial marker proteins as N-cadherin and vimentin were up-regulated to enhance migration and invasion of malignancies, which were originated from epithelial cells [34].

Intriguingly, the carcino-embryonic antigen gene (i.e. H19) was controversial in how it developed its cancer-promoting or cancer-suppressing function [32]. In particular, it was documented that H19 might motivate metastasis of pancreatic cancer cells via let-7/HMGA2 signaling [35], and H19 specifically combined with histone methyltransferase (HMT) could activate Wnt- β -catenin signaling to induce EMT of bladder cancer cells [36]. Nonetheless, Matouk et al. pointed out that there existed a positive feedback cycle between H19 and slug within hepatocellular carcinoma cells, and the two molecules jointly modified the EMT process through regulating E-cadherin expression [37]. In this investigation, we presented that H19 expression was elevated within CRA cells than within para-carcinoma tissues, and this tendency has been confirmed through in-vitro studies (Fig. 1 and 2). As for whether H19

cooperated with other molecules to form a feedback loop, we still have to investigate more in the future.

Based on Starbase database (<http://starbase.sysu.edu.cn/pcMirLncRNAInfo.php?database=hg19&name=hsa-miR-194-5p&targetGene=H19&autold=471>), H19 was manifested to directly target miR-194 within various cancers. In fact, miR-194 was aberrantly expressed within diversified malignant neoplasms [38, 39]. More specifically, miR-194 could suppress invasion of gastric cancer and liver cancer cells, and miR-194's interfering with the EMT process of tumor cells also strengthened this effect [21, 40]. Another investigation found that miR-194 might motivate formation of colonic neoplasms by targeting AKT2, and this effect was mediated by PI3K/Akt/mTOR signaling pathway [41]. It was also deemed that miR-194 could act on MAP4K4/c-Jun/MDM2 signaling to curb CRC progression [42], and miR-194 also served as a regulatory molecule for the EMT process of CRC [25]. The consequences of this investigation testified that miR-194 was subject to the modulation of H19, and both H19 and miR-194 could synthetically encourage EMT, proliferation and viability of CRA cells (Fig. 4-6).

Interestingly, the effect exerted by miR-194 on FoxM1 was suggested to be involved in the EMT process of neoplasms [21], and several microarray assays revealed that FoxM1 was highly expressed within > 20 solid neoplasms, including hepatocellular carcinoma, pancreatic adenocarcinoma, mastocarcinoma, ovarian cancer, lung cancer and CRC [43-47]. Besides, FoxM1 was over-expressed within actively-proliferating tumor cells, and it functioned to facilitate cell multiplication mainly by regulating cell cycle-specific genes [48]. For example, up-regulated FoxM1b expression could accelerate tumor cells' transition from G2 stage into S stage [49]. Furthermore, over-expressed FoxM1 not merely urged shaping of EMT-related phenotypes, but also boosted expressional alterations of epithelial or mesenchymal biomarkers [50]. The serial actions have been specifically verified within hepatocellular carcinoma, pancreatic cancer and lung cancer [51-53]. Consistently, this study demonstrated that FoxM1, which was regulated by H19 and miR-194, acted as an oncogenic parameter for CRA (Fig. 8).

Generally speaking, the current study revealed that H19/miR-194/FoxM1 signaling pathway was essential for the development and aggravation of CRA, providing clues for developing diagnostic biomarkers and treatment targets for CRA. Nevertheless, several shortcomings should not be ignored. First of all, this investigation did not adopt animal models to verify the influences of H19, miR-194 and FoxM1 on CRA development. Secondly, additional intermediary molecules between miR-194 and FoxM1 failed to be investigated, which could more directly act on CRA pathogenesis. Finally, tissues of other ethnicities should also be investigated due to the different results arising from genetic distinction. To sum up, additional researches were recommended to remedy the above-mentioned gaps.

Disclosure Statement

The authors declare to have no competing interests.

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