RETRACTED ARTICLE: Long non-coding RNA 91H regulates IGF2 expression by interacting with IGF2BP2 and promotes tumorigenesis in colorectal cancer

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ABSTRACT

91H, a long non-coding antisense transcripts located on the position of the H19/IGF2 locus had been suggested to play a critical role in tumour development. However, little study had proved the mechanism in colorectal cancer (CRC). Hence, we performed this study to deeply explore the mechanism of IncRNA 91H in tumour progression. The expression of IncRNA 91H was first detected in CRC tissues and cells which was higher in vitro and in vivo than normal cells or tissues and CRC patients with high IncRNA 91H expression usually had a high risk in tumour metastasis (p < .05). Then, monodansylcadaverine (MDC) staining, scratch wound, migration and invasion assays were conducted which showed to that reduced IncRNA 91H would greatly affect tumour migration, invasion and autophagy. Finally, by RNA pull down and RNA-binding protein immunoprecipitation (RIP) assay, a significant interaction was found between IncRNA 91H and IGF2BP2 which was proved to play an important role in CRC IGF2 expression. All these results suggested IncRNA 91H promotes IGF2 expression by interacting with IGF2BP2 which would provide a new strategy in finding potential CRC diagnostic biomarkers and therapeutic targets.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related morbidity and mortality [1]. Increasing evidence indicates that long non-coding RNAs (lncRNAs) regulate diverse cellular processes, including cell growth, differentiation, apoptosis and cancer progression [2]. Therefore, a better understanding of lncRNAs function in CRC progression would be a new area for finding potential prognostic biomarkers which could further improve CRC diagnosis and therapy.

lncRNA 91H, a 119.392-kb long noncoding antisense transcripts, is located at the position of the H19/IGF2 locus (accession number NC_000011.9). It has been proved to be consistently overexpressed in a number of human cancer tissues, including CRC which is significantly associated with adverse clinical characteristics and plays an important role in tumour development [3–5]. Unfortunately, the exact role of 91H in tumorigenesis is poorly described.

Recently, the aberrant activation of the insulin-like growth factor (IGF) system has been reported to be associated with poor prognosis and therapeutic resistance in several cancers, including CRC [6–8]. Particularly, IGF2, a neighbouring gene expressed from the paternal allele, encodes for a protein of 7.5 kDa involved in embryonic growth, is commonly up-regulated in CRC [9,10]. Early studies had showed that IncRNA 91H could up-regulate IGF2 expression by epigenetic modifications which could further promote breast cancer development [5,11].

In light of these observations, IncRNA 91H might also play a critical role in the development of CRC through IGF2 modification. However, few studies have investigated the potential mechanism between IncRNA 91H and IGF2 expression in CRC. So, we performed this study to deeply explore the biological functions of IncRNA 91H in CRC development and its mechanism in IGF2 expression.

Methods

Clinical samples

CRC tumour tissues and matched adjacent normal tissues were included from 28 patients who underwent surgery at Nanjing First Hospital Affiliated to Nanjing Medical University, between 2011 and 2014 [3]. Particularly, adjacent normal tissues were taken 5–10 cm away from the tumour tissues. Before DNA and RNA extraction, all the specimens were frozen in liquid nitrogen after surgery and stored at −80 °C. Patients received chemotherapy or radiotherapy at pre-operation were excluded. Clinical information of these patients was collected including age, sex, TNM stage, tumour grade and family history of disease.

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Cell line authentication
CRC cell lines (Hct-8, DLD-1, Hct-116) and FHC (normal human intestinal epithelial cell line) were obtained from Shanghai Cell Collection, Chinese Academy of Sciences and maintained in DMEM (Hyclone, Logan, UT) containing 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37°C with 5% CO₂. Short tandem repeat (STR) was performed to confirm the CRC cell lines by in ABI 3500 Genetic Analyzer (USA Life 3500) [3,12].

Real-time PCR
Trizol reagent (Invitrogen, Carlsbad, CA, USA) and E.Z.N.A. Tissue DNA Kit (Omega, Norcross, GA) were used to extract tissues and cell lines RNA and genomic DNA (gDNA). In addition, cDNA was synthesized using the Prime Script RT reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan). 91H expression level was analyzed by quantitative real-time PCR (qRT-PCR) using the SYBR Premix Ex TagTM II (Takara, Kusatsu, China) and ABI 7500 System (Applied Biosystems, Foster City, CA, USA). The primers are: primer A forward, 5'-GCTTGTACAGTAGTGGC-3' and reverse, 5'-CATCCAGTGACCCAGGCTT-3' and primer B forward, 5'-CAGCGGCGTTTCCGGAACAG-3' and reverse, 5'-CCGTGTGTGTACTTTACCTGGC-3'. β-actin was used as an internal control with the following sequences: forward, 5'-CAAGATCATTGCTCCTCCTGA-3' and reverse, 5'-GCCGUUGUCAACGUCACAUTT-3'. 2⁻ΔΔCT method was used to compute the gene expression changes after normalizing for 18S expression [3].

RNA interference
RNA interference was carried out by using synthetic siRNA duplexes. As our previous studies, two synthetic siRNA duplexes (si-IncRNA 91H1 and si-IncRNA 91H2) corresponding to the IncRNA 91H RNA sequences 5'-GCCGUACAUUCUGAUUGGGACTT-3' and 5'-UCAGAGGACGUUAAAGUCCUTT-3' were used [12]. By the way, IGF2BP2 siRNA was used as 5'-GCCGGUUGUACGUACAAUTT-3' and 5'-CGUUGGGGUUGAUGCUGAU-3' was purchased as a negative control.

Scratch wound assay
HCT-8 cells were separated into three groups: siRNA-91H, siRNA-NC and blank control (cells treated with nothing) and cultured in six-well plates for 48 h. Then, a 20 μL pipette tip was used to create wounds in cells. Free-floating cells and debris were removed using PBS. Then, the cells were observed at 0 and 48 h. Each experiment was repeated in triplicate. The results were recorded by wound healing percentage (ImageJ, Bethesda, MD, USA) [12]. Wound healing percentage=(original area–area after 48 h)/original area.

Migration and invasion assays
Migration and invasion assays were performed with Transwell chambers [3,12]. HCT-8 cells were separated into three groups: siRNA-91H, siRNA-NC and blank control. Then, cells (1 x 10⁵) were cultured in the upper chamber of transwell plates with serum-free DMEM (2 µg/µL) or the upper transwell chambers coated with Matrigel for invasion assay, in a 24-well format with 6mm diameters. Six hundred microlitres DMEM with 5% FBS were added to the bottom chamber. Culturing for 24 h, cells were fixed with methanol and stained with Wright’s stain. The numbers of migrating cells were counted in five random areas under a light microscope and the average value of five fields was recorded.

Monodansylcadaverine (MDC) staining of autophagic vacuoles
Cells were treated with 1 M of TAM dissolved in Me2SO/EtOH (1:1, v/v) or incubated for four days with 20 M PDMP in the absence of TAM for four days. Then, 100 mM Myrco dissolved in methanol was added with TAM in the first group. An MDC stock solution (0.1 M in Me2SO) was applied to cells for 30 min at 37°C by diluting 1:1000 in DMEM. Washing with phosphate-buffered saline, the cell autophagy particles were finally dyed green fluorescent and examined under fluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany).

Immunofluorescence (IF)
Cells (5 x 10⁵) treated with or without siRNAs were seeded on sterile coverslips in fully supplemented keratinocyte media and allowed to adhere for approximately 4 h. After washing with PBS twice, cells were fixed with mixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Then, fixed cells were incubated with antibodies IGF2 (1:50; R&D Systems Inc., Minneapolis, MN), FITC or Rhodamine B-conjugated anti-rabbit secondary antibody (1:100; Sigma, Oakville, Canada) and cellular nuclei were stained with DAPI [13].

5-aza-CdR treatment
The demethylating agent 5-aza-CdR (Sigma, St. Louis, MO, USA) was diluted in DMSO at 2.5 mM, 5 mM and 10 mM and kept frozen at –20 °C as our previous study [13]. Twenty-four hours later, Hct-8 cells (5 x 10⁵) seeded in six-well plates were incubated with 5-aza-CdR and replaced with fresh medium with 5-aza-CdR every 24 h for a period of 48 h.

Western blot analysis
Cells were lysed using a lysis buffer. Then, a BCA protein assay kit (Beyotime, Shanghai, China) was applied to quantify the protein concentration [3,12]. Western blotting was performed using anti-IGF2 (ab9574, Abcam, Cambridge, UK), anti-IGF2BP2 (ab124930, Abcam, Cambridge, UK), anti-mTOR (ab2732, Abcam, Cambridge, UK) and anti-β-actin (ab15020, ab8227, Abcam, Cambridge, UK). Secondary antibodies were goat anti-rabbit HRP secondary antibodies (System Biosciences, Mountain View, CA, USA).
RNA-binding protein immunoprecipitation

RNA-binding protein immunoprecipitation (RIP) was performed with the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Following the protocols, CRC cells were treated with RIP lysis buffer. Next, clear supernatant and IGF2BP2 antibody (or immunoglobulin G (IgG)/SNRNP70 control) were mixed to perform the immunoprecipitation. After washing, RNAs binding to IGF2BP2 were eluted and quantified. RT-PCR and real-time PCR were performed to examine whether certain RNAs were co-immunoprecipitated with the IGF2BP2 antibody.

RNA pulldown and mass spectrometry analysis

As reported in our previous study [3,12], LncRNA 91H RNAs in vitro were transcribed using the Biotin RNA Labelling Mix (Roche, Basel, Switzerland) and SP6 RNA polymerase (Ambion, Austin, TX, USA) and purified by RNA Clean &Concentrator™. Magnetic Beads (Bioclone, San Diego, CA) were prepared and immediately subjected to RNA (20 μg) capture in RNA capture buffer for 25 min at room temperature with agitation. Lately, the suspension was washed with NT2 buffer and incubated with 30 mg cell lysates diluted in NT2 buffer supplemented with 50 U/mL RNase OUT™, 50 U/mL SuperaseINTM, 2 mM dithiothreitol, 30 mM EDTA and heparin 0.02 mg/ml at 4°C for 2 h with rotation. Afterward, a scratch wound assay was first performed to evaluate the effect of LncRNA 91H on cell motility. Cells treated with siRNA-91H resulted in significant decreased motility and wound recovery than cells transfected with negative control (Figure 1(A)). Moreover, the expression of LncRNA 91H in patients tumour tissues (1.41 ± 1.34) was significantly higher than adjacent normal tissues (0.94 ± 0.60, p < .05). Furthermore, the results were compared with patients’ clinicopathological features which are shown in Table 1. Significant differences were not found in the comparison with age, sex, TNM, grade and family history. But we found that patients with high LncRNA 91H expression usually had a high risk in tumour metastasis.

Biological functions of LncRNA 91H in cells

The expression level of LncRNA 91H was detected higher in HCT-8 cells than the other cells (Supplemental Figure 1). Hence, HCT-8 cells were chosen to explore the potential biological functions of LncRNA 91H. After transfection with siRNA-91H or siRNA-NC for 48 h, LncRNA 91H was effectively silenced in HCT-8 cells (p < .05, Supplemental Figure 2). Afterward, a scratch wound assay was first performed to evaluate the effect of LncRNA 91H on cell motility. Cells treated with siRNA-91H resulted in significant decreased motility and wound recovery than cells transfected with negative control (Figure 1(A)). Then, migration and invasion assays were performed to further study the effect of LncRNA 91H on CRC cells. The results demonstrated that the migration and the invasiveness were both significantly decreased in transfected cells with siRNA-91H, compared with negative control cells (p < .05, Figure 1(B)). These findings both indicated that LncRNA 91H might be closely correlated with proliferation, migration, and invasion of CRC cell lines.

Moreover, MDC was used to detect the autophagy in CRC cells. The results showed that the autophagy was significantly increased in transfected cells with siRNA-91H, compared with negative control cells (p < .05, Figure 2(A)). Moreover, the expression of mTOR was also tested in the study which further demonstrated cells with LncRNA 91H silence usually had a higher mTOR expression than the other groups (p < .05, Figure 2(B)).

Table 1. The association between LncRNA 91H expression and clinicopathological parameters on CRC patients.

| Risk factors          | Category | Expression | p    |
|-----------------------|----------|------------|------|
| Sex                   | Male     | 1.54 ± 1.54| .285 |
|                       | Female   | 1.18 ± 0.91| .182 |
| Age (years)           | <65      | 0.76 ± 0.70| .256 |
|                       | ≥65      | 1.59 ± 1.43| .233 |
| TNM                   | I-II     | 0.77 ± 0.72| .256 |
|                       | III-IV   | 1.59 ± 1.43| .233 |
| Grade                 | G1       | 0.90 ± 0.78| .256 |
|                       | G2-3     | 1.52 ± 1.42| .233 |
| Metastasis            | YES      | 2.05 ± 1.31| .038 |
|                       | NO       | 0.43 ± 0.26| .233 |

Results

The expression of LncRNA 91H in CRC

By performing qRT-PCR, the expression of LncRNA 91H in cell lines HCT-8, DLD-1 and HCT-116 was significantly higher than FHC with p < .05 (Supplemental Figure 1). Then, LncRNA 91H expression of 28 tumour tissues and matched adjacent normal tissues was also compared which showed that the expression of LncRNA 91H in patients tumour tissues (1.41 ± 1.34) was significantly higher than adjacent normal tissues (0.94 ± 0.60, p < .05). Furthermore, the results were compared with patients’ clinicopathological features which are shown in Table 1. Significant differences were not found in the comparison with age, sex, TNM, grade and family history. But we found that patients with high LncRNA 91H expression usually had a high risk in tumour metastasis.

LncRNA 91H increased IGF2 expression correlated with IGF2BP2

CRC cells was first treated with 5-aza-CdR as our previous study [13]. However, LncRNA 91H and IGF2 expression did not show any significant differences in cells treated with different 5-aza-CdR concentration which showed that IGF2 ICR methylation might not play an important role in LncRNA 91H and
IGF2 expression (p > 0.05, Supplemental Figure 3). Then, we conducted lncRNA 91H interference in Hct-8 and DLD-1 cell lines. By Western blot, we found that the IGF2 expression both significantly decreased in siRNA-91H groups compared with siRNA-NC and blank groups (p < 0.05, Figure 3(A)). Similarly, the results of IF also showed that Hct-8 and DLD-1 cells treated with siRNA-91H both had a lower IGF2 expression than the other groups which confirmed the Western blot results before (Figure 3(B)). Then, IGF2BP2 was also silenced in Hct-8 and DLD-1 cell lines which demonstrated that though lncRNA 91H expression was not changed in all the groups, IGF2 expression was significantly lower in siRNA-IGF2BP2 groups than siRNA-NC and blank groups by Western blot (p < 0.05, Figure 4(A)). By the way, IF was also performed which declared that cells treated with siRNA-IGF2BP2 had a lower IGF2 expression than the others (Figure 4(B)). Furthermore, scratch wound, migration and invasion assays were also performed in cells treated with IGF2BP2 siRNA which showed that cells transfected with IGF2BP2 siRNA had a decline in cell motility, migration and invasion compared with negative controls (p < 0.05, Supplementary Figure 4). In addition, the siRNA IGF2 was used to guarantee the test effect. As shown in Supplementary Figure 6, we found that IGF2 expression significantly decreased after treating with siRNA IGF2 which was consistent with the results of Figures 2 and 3.

lncRNA 91H interacted with IGF2BP2 in vitro

Then, RNA pull down mass spectrometry analysis was used to screen the potential factors involved in lncRNA 91H regulation which demonstrated that IGF2BP2 protein highly combined with lncRNA 91H in CRC cells (Figure 5(A)). Then, RIP was performed later to further determine the interaction...
Figure 2. The affection of lncRNA 91H in CRC autophagy. (A) MDC staining method was used to test the autophagy in the groups of Hct-8 cells treated with: siRNA-91H (+), siRNA-NC (−) and blank which demonstrated that siRNA-91H group showed a significant decreased autophagy compared with other groups. (B) mTOR expression was significantly higher in cells treated with siRNA-91H than other groups ($p < .05$).

Figure 3. Association between LncRNA 91H and IGF2BP2 in IGF2 expression by LncRNA 91H interference. (A) Western blot results of Hct-8 and DLD-1 cells treated with siRNA-91H, siRNA-NC and blank (cells without any treatment). Though the IGF2BP2 expression was nearly the same in each group, the expression of IGF2 was significantly lower in cells treated with siRNA-91H than the other groups ($p < .05$). (B) IF results of Hct-8 and DLD-1 cells treated with siRNA-91H (+) and siRNA-NC (−).
Figure 4. Association between LncRNA 91H and IGF2BP2 in IGF2 expression by IGF2BP2 mRNA interference. (A) Western blot results of Hct-8 and DLD-1 cells treated with siRNA-91H, siRNA-NC and blank. The expression of IGF2 was significantly lower in cells treated with siRNA-IGF2BP2 than the other groups ($p < .05$) meanwhile significant difference was not found in lncRNA 91H expression ($p > .05$). (B) IF results of Hct-8 and DLD-1 cells treated with siRNA-IGF2BP2 (+) and siRNA-NC (−).

Figure 5. Interaction between LncRNA 91H and IGF2BP2. (A) Pull down results of LncRNA 91H by Silver staining which IGF2BP2 was marked in black box. (B) RIP results of IGF2BP2. IGF2BP2 expression was significantly higher in treated group than input group by Western blot. LncRNA 91H expression was higher in anti-IGF2BP2 group than anti-IgG group.
between IncRNA 91H and IGF2BP2 which showed that IncRNA 91H also highly combined with IGF2BP2 protein compared with IgG negative control and SNRP70, a positive control (Figure 5(B)).

Discussion

Though overexpressed IncRNA 91H had been reported in lots of tumours. Few studies have described the potential mechanism of IncRNA91H in cancer. In this study, we demonstrated for the first time, that up-regulated IncRNA 91H usually leads to a high IGF2 expression by interacting with IGF2BP2 which would promote CRC progression and cause a high risk in tumour metastasis.

Nowadays, IncRNAs had become a hot topic based on their emergence as novel regulators in the cancer paradigm. Emerging investigations indicated that most IncRNAs might regulate cancer progression by completely combining with miRNA to produce ceRNA or promote protein stabilization at the post-transcript level [14]. However, recent studies reported that some IncRNAs could directly interact with functional domains of signalling proteins to suppress cancer metastasis [15]. Consistent with the view, our results also found that IncRNA 91H could participate in tumorigenesis by directly regulating IGF2 protein expression through interacting with IGF2BP2 protein which might be a new way in CRC IncRNA study. Our early study found that IGF2 ICR methylation might play an important role in IGF2 and IncRNA 91H expression in oesophageal squamous cell carcinoma [13]. However, in this study, the correlation was not found in CRC cells which might be due to their different pathological type.

The IGF2 mRNA binding protein (IGF2BP or IMP) family is comprised of three related members: IGF2BP1, IGF2BP2 and IGF2BP3 [16]. As the foetal transcripts of IGF2, IGF2BP family is normally expressed in adults’ tissues but become reactive in cancer [17]. The unregulated expression of them had been demonstrated in many studies, especially IGF2BP2 which had been pointed to a tumorigenic role in individual cancer types, often with a poor prognosis [18–20]. In this study, we similarly found that cells with IGF2BP2 silence usually caused a low IGF2 expression which confirmed the association that IGF2BP2 played an important role in IGF2 expression. Then by RNA pull down and RIP, a strong interaction had been found between IncRNA 91H and IGF2BP2. Furthermore, RNA interference was conducted which also proved that IncRNA 91H and IGF2BP2 both participated in IGF2 protein expression.

By the way, early study had proved that IGF2 could bind to IGF1R which would further activate the PI3K/AKT and mTOR pathways so that to mediate diverse cellular functions, such as growth, proliferation, apoptosis, differentiation, cell adhesion and motility [21]. In this study, cells treated with siRNA-91H usually had a decreased proliferation, migration and invasion which might be due to the decreased regulation of IncRNA 91H on IGF2 expression that lead to the inactivation of PI3K/AKT and mTOR pathways.

In this study, we fortunately found IncRNA 91H high expression was significantly related to CRC metastasis and CRC cells’ motility, migration and invasion. The epithelial-mesenchymal transition (EMT) in cancer had been reported to play an essential role in metastasis [22]. Early studies had demonstrated that EMT and autophagy signalling, an evolutionarily conserved lysosome-dependent catabolic process which degraded cells’ components in order to recycle substrates to exert optimally and adapt to tough circumstances, were linked to one another [23,24]. Early studies had reported that through IGF1R, IGF2 could enhance tumour autophagy which played an important role in tumour progression [25]. As a result, we also observed a significantly decreased autophagy since IncRNA 91H was silenced in CRC cells and also found a association between IncRNA 91H and mTOR expression, a autophagy inhibiting protein, which suggested that IncRNA 91H might also affect tumour autophagy by regulating IGF2 and mTOR expression.

In early study, HOTS, an antisense transcript to H19, had been reported by Onyango and Feinberg [26]. To figure out whether HOTS affected IGF2 expression, as previous study [11], the siRNA used against 91H was out of the HOTS sequence. Fortunately, significant down-regulate IGF2 expression was observed which might indicate the observed sh91H-induced phenotype was HOTS-independent.

Conclusions

In summary, IncRNA 91H was highly expressed in CRC cells and tumour tissues. Patients with high IncRNA 91H expression usually had a high risk in tumour metastasis which might be due to its effect on tumour proliferation, migration, invasion and autophagy. Moreover, our results suggested that IncRNA 91H promotes IGF2 expression by interacting with IGF2BP2 which would provide a new strategy in finding potential CRC diagnostic biomarkers and therapeutic targets.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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