The Identification of Long Non-coding RNA H19 Target and Its Function in Chronic Myeloid Leukemia

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H19 is a long non-coding RNA which was lowly expressed in chronic myeloid leukemia (CML). Here, we found that the over-expression of H19 significantly inhibited cell viability and colony formation and prolongs survival in CML cell lines and three xenografted mouse models. The H19 target proteins and microRNAs (miRNAs) were identified using a combination of computational prediction and RNA pull-down, including PCBP1, FUS protein, and miR-19a-3p and miR-106b-5p. Targeting PCBP1, FUS protein, miR-19a-3p, and miR-106b-5p significantly inhibits the cell growth and colony formation of CML cell lines. Co-overexpression of H19 and PCBP1, FUS, miR-19a-3p, and miR-106b-5p decreases the inhibitory effect of H19 in CML. These findings might provide a novel molecular insight into CML.

RESULTS

Overexpressing H19 Inhibits the Proliferation of K562 Cells and Prolongs the Survival of the CML Model Mouse

The H19 gene is a highly conserved and maternally expressed imprint gene located on human chromosome 11p15.5. It encodes a non-coding RNA that is 2.3 kb in size and plays an imperative role in mammalian and embryonal development.9–12 Furthermore, recent studies have shown that H19 is closely associated with cell proliferation and metastasis in a variety of cancers, such as bladder cancer,13 colorectal cancer,14 gastric cancer,15 esophageal cancer,16 and so on.

miRNAs are non-coding single-strand RNAs consisting of approximately 22 nucleotides in length.18 These miRNAs regulate the expression of protein-coding genes by binding with the 3′ UTRs (3′ untranslated regions) of the targeted mRNAs. A majority of miRNAs share only partial complementarity with their mRNA targets, nevertheless 7–8 nt in the 5′ end of miRNAs, known as the seed sequence, bind to the mRNA in fact.19 In our study, we used locked nucleic acids’ (LNAs’) modified seed sequences to knockdown miRNA.
A

B

C

D

E

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cells with the H19-expressing vector and selecting positive cells using puromycin. CML patients’ bone marrow mononuclear cells were over-expressed with H19 lentivirus. Colony-forming assays were performed to measure the colony-forming ability of K562 cells. Overexpression of H19 in CML patients’ bone marrow mononuclear cells significantly inhibited the cell growth (Figure 1B). As shown in Figure 1C, overexpression of H19 significantly decreased the number of colonies in K562 cells transfected with H19, compared to the empty vector transfection. The results indicate that overexpression of H19 significantly decreased the colony-forming ability in K562 cells. The tumor size and tumor volume were recorded of each group and the overall survival was monitored by Kaplan-Meier analysis. The results showed that overexpression of H19 prolonged the overall survival of recipient mice (Figures 1D and 1E).

**IncRNA H19 Was Obtained by In Vitro Transcription, and RNA-Protein-Binding Compounds Were Produced by RNA Pull-Down**

To identify the molecular mechanism and binding partners of IncRNA H19 in K562 cells, the DNA template with T7 promoter was transcribed into IncRNA H19 that was verified by agarose gel electrophoresis (Figure 2B). Biotinylated IncRNA H19 was mixed with cell lysis buffer followed by incubation with washed streptavidin magnetic beads. Then the complexes were eluted and IncRNA H19 protein-binding compounds were separated by 10% SDS-PAGE, visualized by Coomassie blue staining (Figure 2C).

**Identification of the Targets of IncRNA H19**

To identify the targeted proteins and miRNAs of IncRNA H19, we performed mass spectrometry and RNA sequencing (RNA-seq) followed by RNA pull-down assay. The catRAPID and starBase v. 2.0 predicted the targeted proteins of IncRNA H19. PCBP1 and FUS protein prediction can be detected by mass spectrometry (MS) (Figure 3A). Then PCBP1 and FUS from RNA pull-down products were tested by western blot (Figure 3B). There are five targeted miRNAs of IncRNA H19 predicted by the starBase v 2.0 (Figure 3C). The five miRNAs can also be monitored by RNA-seq. Two miRNAs of miR-19a-3p and miR-106b-5p are confirmed by real-time PCR (Figure 3D).

**The Targeted Inhibition of PCBP1, FUS, miR-19a-3p, and miR-106b-5p Could Reduce the Malignant Progression of K562 Cells**

Next, we studied whether inhibiting PCBP1 and FUS by PCBP1 (small interfering RNA) siRNA and FUS siRNA could attenuate CML malignant behavior. As indicted in Figure 4A, either PCBP1 or FUS significantly increased K562 cell sensitivity on imatinib (IM) treatment; PCBP1 or FUS siRNA inhibited the cell growth of CML patients’ bone marrow mononuclear cell (MNCs). Colony-formation assay revealed that the colony-forming ability of
K562 cells transfected with PCPB1 and FUS siRNA was significantly reduced compared to cells transfected with Scramble RNA (SCR) (p < 0.01) (Figure 4B). Coexpression of H19 and FUS or PCPB1 plasmid in K562 cells reverses the inhibitory effects of H19 (Figures 4C and 4D). Taken all together, these statistics demonstrate that targeted inhibition of PCPB1 and FUS attenuate CML malignant proliferation.

The Targeted Inhibition of miR-19a-3p and miR-106b-5p Could Decrease the Malignant Progression of K562 Cells
To confirm the effect of miR-19a-3p and miR-106b-5p on cell viability, the t-anti-miR-19a-3p and t-anti-miR-106b-5p were transfected into K562 cells for 48 h, and the cell viability and colony-formation assay were performed. As shown in Figure 5A, the t-anti-miR-19a-3p and t-anti-miR-106b-5p can significantly reduce cell viability with SCR controls. The colony-forming capability of K562 cells transfected with t-anti-miR-19a-3p and t-anti-miR-106b-5p was notably reduced compared to cells transfected with SCR (Figure 5B). Coexpression of H19 and miR-19a-3p or miR-106b-5p mimics in K562 cells reverse the inhibition of cell growth of H19 (Figures 5C and 5D). Taken all together, these statistics demonstrate that H19 sponges miR-19a-3p and miR-106b-5p attenuate CML malignant proliferation.

Overexpressing H19 Expands the Survival of Patient-Derived Xenografts in the CML Model Mouse
To identify the effect of overexpression of H19 in patient-derived xenografts in a CML model mouse, we constructed the H19 lentiviral transduction in CML patients, bone marrow cells. As shown in Figure 6, these results showed that overexpression of H19 prolonged the overall survival of recipient mice (Figures 6A and 6B).

DISCUSSION
Studies have shown that H19 differentially methylated region/imprinting control region (DMR/ICR) was hypomethylated and associated with H19 expression in CML patients. Furthermore, demethylation of H19 DMR/ICR reactivated H19 expression in K562 cells. A reduction of H19 expression was observed in all leukemia samples, including chronic myelomonocytic leukemia (CMML; n = 43), CML (n = 40), and acute myelogenous leukemia (AML; n = 32) cases.
Figure 4. The Targeted Inhibition of PCBP1 and FUS Could Reduce the Malignant Progression of CML Cells

(A) K562 cells and CML patient bone marrow mononuclear cells transfected with SCR, PCBP1 siRNA, and FUS siRNA were treated with different concentrations of imatinib (0–0.25 μM) for 48 h, and the viability of cells was determined by MTT assay (**p < 0.01, *p < 0.05, PCBP1-siRNA and FUS-siRNA transfected groups versus K562 group and SCR transfected group). (B) Inhibition of PCBP1 and FUS reduces colony formation of K562 cells. A total of 1,000 K562 cells transfected with SCR, PCBP1, and FUS were mixed with RPMI-1640 medium containing 0.9% methylcellulose solution and 20% FBS and seeded into 24-well plates. Colony numbers were counted after 1 week. Histogram and statistics indicating the relative number of colonies per 1,000 plated cells are shown. Statistical significance was assessed by one-way ANOVA (**p < 0.01). (C) K562 cells were co-transfected with H19 lentivirus and FUS plasmid, and the viability of cells was determined by MTT assay. (D) K562 cells were co-transfected with H19 lentivirus and PCBP1 plasmid, and the viability of cells was determined by MTT assay.
compared to the healthy controls including peripheral blood samples from normal (n = 98). H19 was initially identified as a tumor suppressor. Indeed, its overexpression in some tumor cells was associated with inhibition of proliferation, morphological changes, decrease of clonogenicity in soft agar, and tumorigenicity in nude mice. In our study, the expression of H19 was tested in blood samples from normal people (n = 4), in CML patients’ peripheral blood mononuclear cells (n = 3), K562 cells, KCL-22 cells, BV173 cells, 8266 cells, U266 cells, Raji cells, H1299 cells, and A2780 cells. A decrease of H19 expression was viewed in all cancer cells compared to the healthy controls (Figure 1A), but another research has the different results it need further detection. Next, we studied the effect of H19 overexpression on K562 cells and CML patients’ bone mononuclear cells by colony-formation assays and 3-(4, 5-dimenthyl-thiazol-2-yl)-2, 4-diphenyl-tetrazolium bromide (MTT) assays. The results show that overexpression of H19 inhibited the cell growth of CML patients’ bone mononuclear cells and inhibited the colony-formation ability of K562 cells (Figures 1B and 1C). To further research the effect of H19 on a xenograft mouse model of human CML, the P210 cells and K562 cells overexpressing H19 were constructed. The P210 cells transfected with H19 or empty vector were injected into sublethally irradiated (3 Gy) BALB/c recipient mice subcutaneously and the H19 transfected K562 cells were injected into 5-week-old, sublethally irradiated (3 Gy) NOD/SCID recipient mice randomly via tail vein. The survival of the xenograft mouse model of human CML was observed, and the overall survival was prolonged (Figures 1D and 1E).

To elucidate the molecular mechanism of H19 in CML, the targeted proteins and miRNAs were identified by performing MS and RNA-seq followed by RNA pull-down. 586 proteins were detected by MS from RNA pull-down products. Two proteins predicted by catRAPID also existed in MS. Thus, we take the PCPB1 and FUS proteins as the candidates of lncRNA H19 (Figure 3A). PCBP1 and FUS proteins can be detected by immunoblotting in RNA pull-down products (Figure 3B). 1,129 small RNA were detected by RNA-seq from RNA pull-down products, and 35 miRNAs were predicted by starBase v. 2.0. Five miRNAs existed not only in RNA-seq but in predictions. miR-19a-3p and miR-106b-5p were tested by qRT-PCR from RNA pull-down products (Figures 3C and 3D).

Many lncRNAs, including H19, participate in molecular regulation pathways through their interactions with proteins and modulation of their activities. Combined with the results of RNA pull-down and bioinformatics prediction, PCBP1 and FUS may be the target proteins of H19. PCBP1 is an essential regulator in some cancers. Several findings revealed that PCBP1 might play an important role in preventing the process of epithelial-mesenchymal transition (EMT) in non-small-cell lung cancer, so it might be a promising therapeutic target to inhibit non-small-cell lung cancer (NSCLC).
miR-19a-3p and t-anti-miR-106b-5p decrease the cell viability and targeted inhibition of miR-19a-3p and miR-106b-5p using t-anti-linc-MD1, governs the time of muscle differentiation by acting as a target genes. A recent report showed that a muscle-specific lncRNA H19 can also act as a sponge to interact with miR-106a-5p and the Declaration of Helsinki principles. CML bone marrow cells and human peripheral blood mononuclear cells were cultured in 100 ng/mL stem cell factor (SCF), 100 ng/mL granulocyte-colony stimulating factor (G-CSF), 20 ng/mL FMS-like tyrosine kinase 3 (FLT3), 20 ng/mL interleukin (IL)-3, and 20 ng/mL IL-6.

It has been shown that lncRNAs may act as endogenous sponge RNAs to interact with miRNAs and influence the expression of miRNA target genes. A recent report showed that a muscle-specific lncRNA, linc-MD1, governs the time of muscle differentiation by acting as a competing endogenous RNA in mouse and human myoblasts. lncRNA H19 can also act as a sponge to interact with miR-106a-5p in melanoma cells. The miRNA therapeutics, including inhibition of oncogenic miRNAs or acceleration of tumor-suppressive miRNAs, has been suggested in a large number of studies. For instance, antisense miRNAs can sequester the miRNA competing with target miRNAs by leading to functional inhibition of the certain miRNAs. Antisense-mediated miRNA inhibition can increase binding affinity and improve efficiency in vivo by optimizing the oligonucleotides. A variety of chemical modifications achieve this. In our study, we used LNA in which the furanose ring in the sugar-phosphate backbone is chemically locked in an RNA mimicking N-type (C3'-endo) conformation by a 2'-O, 4'-C methylene bridge, into an antisense sequence significantly improve its affinity for the target. The targeted inhibition of miR-19a-3p and miR-106b-5p using t-anti-miR-19a-3p and t-anti-miR-106b-5p decrease the cell viability and colony-forming ability of K562 cells and CML patients' bone marrow mononuclear cells (Figures 5A and 5B). Co-expression of H19 and miR19a-3p (miR106b-5p) in K562 cells reverses the inhibition effect of H19; it reveals the results that the effect of H19 by sponging miR19a-3p and miR106b-5p.

In conclusion, this study identifies H19 downexpression in CML. Overexpression of H19 in K562 cells and CML patients' bone marrow mononuclear cells significantly inhibits the cell growth and colony-formation ability. PCBP1, FUS, miRNA-106b-5p, and miRNA-19a-3p are included both in the bioinformatics prediction and RNA pull-down products, which may be the target of H19. Inhibition of the expression of PCBP1 and FUS by siRNA and LNA-modified anti-miRNA-106b-5p and anti-miRNA-19a-3p inhibits cell growth and colony-formation ability. H19 might provide a new treatment strategy for CML.

MATERIALS AND METHODS

Patient Samples, Human Cells, and Cell Lines

Healthy peripheral blood or CML bone marrow samples were obtained from healthy adult donors in Guangdong Provincial Emergency Hospital/the Guangdong Second Provincial General Hospital after written informed consent was obtained according to the institutional guidelines and the Declaration of Helsinki principles. CML bone marrow cells and human peripheral blood mononuclear cells were cultured in 100 ng/mL stem cell factor (SCF), 100 ng/mL granulocyte-colony stimulating factor (G-CSF), 20 ng/mL FMS-like tyrosine kinase 3 (FLT3), 20 ng/mL interleukin (IL)-3, and 20 ng/mL IL-6.

The K562 cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO2. The medium was changed every 2 days; all cellular assays were conducted in the exponential phase of growth. The K562 cell was purchased from the Institute of Shanghai Cell Biology (Chinese Academy of Sciences, Shanghai, China).

Cell Transfection

The full-length human H19 sequence was synthesized and subcloned into the pEZ-Lv201 vector (GeneCopeia, Guangzhou, China). K562 cells stably overexpressing H19 or control were established by
transfecting K562 cells with H19-expressing vector (H19 pEZ-Lv201) or empty vector (pEZ-Lv201). Transfection of cells was performed using Lipofectamine 3000 reagent (Invitrogen, USA) following the manufacturer’s protocol. The cells were selected in medium containing puromycin (1 mg/mL). The expression of H19 was monitored by real-time PCR assays.

**Lentiviral Transduction in CML Patients’ Bone Marrow Cells**

For the packaging of lentivirus, H19 overexpression plasmids were transfected into HEK293T cells along with the envelope plasmid pCMV-SVG and packaging plasmid pCMV-dR8.2 using Lipofectamine 2000 (Life Technologies, USA). At 48 h post-infection, the culture media containing lentivirus were collected and CML patients’ bone marrow cells (1 × 10^6 cells/mL) were infected by spinoculation (1,500 × g, 90 min, 32°C) with virus-containing supernatants twice. Cells were harvested 48 h later.

**Colony-Forming Assays**

The colony assays for dispersed single cells were performed to measure the capacity of cell-colony formation. After the harvest of transfected cells, cells (1 × 10^7) were mixed completely with RPMI-1640 medium containing 0.9% methylcellulose solution, 20% fetal bovine serum (FBS), 2 mM L-glutamine, and 5 μM 2-mercaptoethanol and seeded onto 24-well plates. Single cells were randomly and evenly distributed throughout the wells. Colonies were formed and counted 2 weeks later using an inverted microscope (Olympus, Japan). The number of colonies containing more than 50 cells was counted. All analysis were performed in triplicate.

**Xenograft Mouse Model of Human CML**

BALB/c nude mice were used for leukemogenesis experiments and maintained in a temperature and humidity-controlled environment. A total of 500,000 P210 cells transfected with H19 or empty vector were injected into sublethally irradiated (3 Gy) BALB/c recipient mice subcutaneously (seven mice per group). Tumor burden of mice after cell injection was shown in vivo imaging systems.

**CML Progression in NOD/SCID Mice**

In the CML progression assay, the H19-transfected K562 cells were injected into 5-week-old, sublethally irradiated (3 Gy) BALB/c recipient mice subcutaneously (seven mice per group). Tumor burden of mice after cell injection was shown by in vivo imaging systems.

**Establishment of Patient-Derived Xenografts**

CML patients’ bone marrow cells were transfected with H19 overexpression lentivirus, and then cells (1 × 10^6 cells/mouse) were transplanted by tail vein injection into sublethally irradiated (300 cGy) 8-week-old NOD/SCID mice. The live time of mice (seven mice per group) were measured.

**Polymerase Chain Reaction Assays**

The DNA fragment of H19 was PCR-amplified using a T7-containing primer (Sangon Biotech, Shanghai, China) and the high-fidelity DNA polymerase (Thermo Fisher Scientific, USA) on a Bio-Rad C1000 thermal cycler. The following PCR primers were used: H19 + T7 F, 5’-TAA TAC GAC TCA CTA TAG GGA GAG GAC CAT GGC CCC G-3’; R, 5’-TG TCT TAA CAG TGT TTA TTG ATG ATG AGT CCA GGG CTC C-3’. The purification of DNA was performed by agarose gel electrophoresis followed by gel extraction using a gel extraction kit (GBCBIO Technologies, Guangzhou, China).

**Transcription In Vitro and RNA Pull-Down Assays**

The purified DNA was transcribed in vitro into RNA by a MEGAscript kit (Thermo Scientific, USA). The 3’ terminus of the RNA strand was attached to a single biotinylated nucleotide by T4 RNA ligase (Pierce RNA 3’ end dethiobiotination kit; Thermo Fisher Scientific, USA). Biotinylated RNA (50 pmol) was mixed with washed streptavidin magnetic beads and incubated at room temperature for 30 min with agitation. Cell lysis buffer (protein concentration was greater than 2 mg/mL) was added to the RNA-bound beads, and further incubated for 60 min at 4°C with agitation. The elution buffer was added to the washed RNA-binding protein complexes and mixed well by vortexing. The complexes were incubated for 30 min at 37°C with agitation.

**MS**

The lncRNA protein-binding complexes from RNA pull-down were detected by MS followed by electrophoresis and Coomassie brilliant blue staining. The catRAPID and starBase v. 2.0 predicted the targeted proteins of lncRNA H19. Specific proteins from RNA pull-down products both existed in MS and prediction were tested by western blot.

**Western Blot Analysis**

Cells were collected and washed twice with PBS; the Radioimmuno-precipitation assay buffer (RIPA buffer) in the presence of proteinase inhibitor (Selleck Chemicals, Houston, TX, USA) was added. The crude lysates were transfized to pre-chilled Eppendorf tubes and centrifuged at 12,000 × g for 15 min at 4°C. The whole-cell lysates were resolved on a 10% SDS-polyacrylamide gel and electrophoretically transferred to PVDF (polyvinilindene difluoride) membrane (Millipore). The membrane was incubated with antibody against PCBP1 (1:1,000, ab168377, Abcam, USA) and β-actin (1:2,000, Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C overnight. After being washed, these membranes were followed by HRP (horseradish peroxidase)-labeled goat anti-rabbit immunoglobulin G (IgG) (1:2,000, Santa Cruz Biotechnology, USA) at room temperature for 2 h. The signals were visualized with enhanced chemiluminescence (ECL; Beyotime Company) and analyzed using a BI-2000 system. To quantify the protein band intensities, the films were analyzed using NIH ImageJ software.

**miRNA First-Strand Synthesis and qRT-PCR Assay**

To validate the miR-19a-3p and miR-106b-5p detected by RNA-seq from RNA pull-down products, cDNA was synthesized using poly(A) polymerase (Takara, Japan), and the expression level of miR-19a-3p and miR-106b-5p was determined using SYBR qRT-PCR (Takara, Japan).
miR-19a-3p was amplified with the primer 5′-CTGTGCAA ATCTATGCAAACCTG-3′. miR-106b-5p was amplified with the primer 5′-TAAAGTGTGACAGTGCAGAT-3′.

**siRNAs or t-Anti-miRNA LNAs and Their Transfection**

The sequences used in this study were as follows: PCBP1 siRNA (5′-AGGCGGGGTGTAAGTCA-3′) and FUS-siRNA (5′-GGAC AGCAGCAAA GCTATA-3′). The RNA duplexes were synthesized and purified by the Guangzhou RiboBio. The sequence of t-anti-miR-19a-3p and t-anti-miR-106b-5p LNAs were designed according to the principle of sequences complementary to seeds of mature miR-19a-3p and miR-106b-5p. The LNA-modified nucleotide sequences used in this study are listed as follows: t-anti-miR-19a-3p (5′-TGAATCTG-3′); t-anti-miR-19a-3p (5′-TGTCG AAA-3′). The LNA-modified nucleotides were chemically synthesized and modified with LNA by Shanghai Sangon Bio-engineering Technology Program of Guangzhou City (no. 201604021040), the Fundamental Research Funds for the Central Universities (no. 21617461), and the Sanming Project of Medicine in Shenzhen (SZSM201601062).

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