ORIGINAL RESEARCH

Role and mechanism of PCBP1-AS1 in Hodgkin lymphoma for patients with Hodgkin lymphoma

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Abstract

Background

Hodgkin lymphoma (HL) is a malignant tumour of the lymphoid system with complex pathogenesis. Increasing evidence suggests that long non-coding RNAs (lncRNAs) participate in HL progression, yet the role of poly(C)-binding protein 1 antisense RNA 1 (PCBP1-AS1) remains unclear.

Methods

Human HL cell lines (KM-H2, L1236, L428 and L540) and germinal-centre B cells were analysed for PCBP1-AS1, miR-516b-5p and TRIM2 expression by RT-qPCR and western blotting. Loss- and gain-of-function assays, including CCK-8, colony-formation and Transwell assays, were used to assess cell proliferation, migration and invasion. RNA pull-down, luciferase reporter and RNA immunoprecipitation assays verified molecular interactions among PCBP1-AS1, miR-516b-5p and TRIM2.

Results

PCBP1-AS1 was markedly up-regulated in HL cells compared with germinal-centre B cells. Silencing PCBP1-AS1 significantly reduced HL-cell proliferation, colony formation, migration and invasion. Mechanistically, PCBP1-AS1 directly bound to miR-516b-5p, which was down-regulated in HL cells, thereby relieving the inhibitory effect of miR-516b-5p on its downstream target TRIM2. Inhibition of miR-516b-5p reversed the suppressive effects of PCBP1-AS1 knockdown, while TRIM2 overexpression abolished the inhibitory effects of miR-516b-5p up-regulation on HL-cell malignant phenotypes.

Conclusion

PCBP1-AS1 promotes HL progression by acting as a competing endogenous RNA that sponges miR-516b-5p to up-regulate TRIM2. The PCBP1-AS1/miR-516b-5p/TRIM2 axis may represent a promising molecular target for HL therapy.

Keywords: Hodgkin lymphoma, PCBP1-AS1, miR-516b-5p, TRIM2

Introduction

Lymphomas are malignant tumors that originate from the lymphoid hematopoietic system[1]. Based on the pathological features, lymphomas are classified into Hodgkin lymphoma (HL) and non-Hodgkin lymphomas (NHLs)[2]. HL is featured by the existence of the mononuclear Hodgkin as well as multi-nuclear Reed-Sternberg (HRS) cells[3]. HL is identified as one of the most survivable and curable tumors in humans through multi-drug chemotherapy, local radiotherapy and hematopoietic stem cell transplantation[4]. However, due to the adverse events of treatment, which can result in cardiovascular events, an elevated risk of secondary malignant diseases, as well as severe endocrine long-term impact unique to children, many cured cases can't achieve life expectancy or have poor quality of life[5]. Therefore, the potential mechanism of HL still need to be further explored.

As a kind of non-coding RNA with over two hundred nucleotides in length[6], long non-coding RNA (lncRNA) was initially recognized to be the "noise" of genomic transcription, a byproduct that has no biological potential[7]. Recent researches have pointed out lncRNAs play vital roles in various kinds of biological process, containing cell proliferation, apoptosis as well as migration[8]. More and more reports have validated that lncRNA is implicated in affecting the progression of various tumors, and HL is also included[9,10]. Poly(C)-binding protein 1-antisense RNA 1 antisense RNA 1 (PCBP1-AS1) belongs to a lncRNA located in the upstream of PCBP1 with a distance of 462bp[11]. Previous reports have confirmed PCBP1-AS1 is closely linked to cancer progression[12]. For instance, PCBP1-AS1 inhibits lung adenocarcinoma metastasis[13]. PCBP1-AS1 aggravates hepatocellular carcinoma progression by the AKT pathway[14]. However, the role along with mechanism of PCBP1-AS1 in HL is uncharted.

MicroRNAs (miRNAs) are also considered to be crucial regulators in cancers[15]. miRNAs belong to a kind of small non-coding RNA molecules comprising approximate twenty-two nucleotides in length, and are capable to negatively modulate their target mRNAs expression by specifically combining with their target mRNAs, thus repressing mRNA translation[16]. The discovery of miRNA has revealed new aspects of gene expression regulation that influence many physiological and pathological processes of life[17]. Many abnormal miRNA expression patterns have been discovered in a variety of human cancers, which function by oncogenes or tumor repressors[18]. Certain miRNAs have been discovered to characterize various subtypes of HL and play crucial roles in lymphomagenesis[19]. Previous literatures have proved the important involvement of miR-516b-5p in diverse cancers, containing lung cancer, glioma along with hepatocellular

carcinoma[20-22]. Nevertheless, miR-516b-5p's expression and role in HL is not clear.

In this paper, the potential of PCBP1-AS1 in HL was assessed, as well as the interplay between PCBP1-AS1 and miR-516b-5p in HL progression was explored, which may supply a hopeful target for HL therapy.

Material and methods

Cell Culture

American Type Culture Collection (ATCC) supplied human HL cell lines KM-H2, L1236, L428 and L540. The first two types of cells were cultivated in Dulbecco's modified Eagle's medium (DMEM). The latter two kinds of cells were cultivated in RPMI-1640 medium. Mediums were added with 10% fetal bovine serum (FBS) together with 1% penicillin/streptomycin at 37 °C containing 5% CO_2.

Germinal center (GC)-B cells were isolated from tonsil tissue samples from 3 HL donors with the age of 3-10 years. Based on CD20+lgD-CD38+ expression, 2 of the 3 GC-B cells were purified >98% from human tonsil tissues as described (23). According to IgD-CD138-CD3-CD10+ expression, the third sample was purified by magnetic activated cell sorting >95%. All children's parents signed written informed consent for the use of tonsil samples.

Cell transfection

GenePharma (Shanghai, China) offered two shRNAs targeting PCBP1-AS1 (sh-PCBP1-AS1#1 and sh-PCBP1-AS1#2) along with the negative control (sh-NC), the miR-516b-5p mimics, miR-516b-5p inhibitor along with corresponding negative control (NC mimics and NC inhibitor), and TRIM2 overexpression vector (pcDNA3.1-TRIM2) along with the empty vector (pcDNA3.1). The transfections were conducted by means of Lipofectamine 3000 (Invitrogen, USA).

RT-qPCR

Utilizing the TRIzol reagent (Invitrogen, USA), total RNA was isolated from HL cells. The cDNA was synthesized by means of PrimeScript RT Master Mix kit (TaKaRa, Japan). RT-qPCR was implemented by means of the SYBR-Green kit (Takara, Japan) with specific primers (Table 1).The $2^{-\Delta\Delta Ct}$ method was adopted for calculating the relative gene expression. The miRNA expression was normalized to U6. The lncRNA and mRNA expression was normalized to GAPDH.

Western blot

Utilizing RIPA lysis buffer (Beyotime, China), proteins were isolated from cells. Proteins were isolated on 10% SDS-PAGE and then shifted to PVDF membranes (Millipore, USA). The membranes were subjected to block with 5% BSA for 60 min, followed by cultivating with the primary antibodies (anti-TRIM2 and anti-GAPDH) at 4 °C for one night. The next day, the membranes were cultivated with secondary antibodies for 2 h at 25 °C. The protein bands were developed by means of an ECL chemiluminescent substrate kit (Biosharp Life Sciences, China).

CCK-8

Cells following transfections were planted in 96-well plates and cultivated for 0, 12, 24, 48 and 72 hr, respectively. Afterwards, 10 μl CCK-8 reagent (Beyotime, China) was subjected to add into the plates for 2 h of cultivation. The absorbance of cells at 450 nm was detected utilizing a microplate reader (BioTeke, USA).

Colony formation

HL cells were planted in 6-well plates (500 cells/well) and maintained for 14 days. Then, cells were subjected to fix with methanol for 10 min, followed by dying with crystal violet for 10 min. Colonies (≥ 50 cells) were counted manually.

Transwell

To assess cell migration, the transfected cells (1×105 cells/well) were planted in the upper chambers. The medium which contained 20% FBS was placed into the lower chambers. Following cultivation at 37 °C for one night, the migratory cells were fixed, followed by staining and counting. Matrigel (BD Biosciences, USA) was added to the well in advance for the invasion experiment, and other procedures were the same as for the migration experiment.

RNA pull-down assay

The biotinylated PCBP1-AS1/miR-516b-5p probe and the corresponding control (Bio-probe NC) were pre-cultivated with Streptavidin-Dyna beads M-280 (Invitrogen, USA) for 2 h at 25 °C. Then, the cell lysate was cultivated with the beads at 4 °C for one night. Following washing the beads, RNA complexes bound to the beads were eluted and extracted, followed by RT-qPCR.

Luciferase reporter assays

Wild-type (WT) or mutant (MUT) PCBP1-AS1 or TRIM2 3'UTR sequences including miR-516b-5p predicted binding sites were sub-cloned into pmirGLO vectors (Promega, USA). After the luciferase reporter plasmids were co-transfected with miR-516b-5p mimics or NC mimics in HL cells for 2 days, the relative activity of luciferase was measured by dual luciferase reporter assay kit (Hanbio Biotechnology, China).

RNA immunoprecipitation (RIP) assay

Utilizing the Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, USA), this assay was conducted. The HL cell lysates were cultivated with 5 μg Ago2 antibody or a control IgG antibody coated beads at 4 °C for one night under rotation. Afterwards, total RNA was gathered for RT-qPCR analysis.

Statistical analysis

All experiments were conducted at least 3 times. Data was exhibited as mean with SD. The statistical analysis was evaluated by the SPSS 18.0 version. Students t test or one way ANOVA and Tukeys post hoc test were recommended to analyze 2 or multiple groups. $P<0.05$ meant the difference was statistically significant.

Nursing interventions

Nurses explained the cause of HL, chemotherapy methods and medication precautions to patients; Nurses observed the occurrence of adverse reactions of patients after chemotherapy, and treat patients according to the doctor's advice if necessary; Nurses evaluated the nutritional status of patients, and provided dietary and exercise guidance; Nurses gave discharge guidance to patients and informed patients to develop good living habits; The patient was asked to return to the hospital for reexamination regularly and was followed up by telephone once a month.

https://dx.doi.org/10.4314/mmj.v37i5.12

Results

PCBP1-AS1 is high-expressed in HL cells and accelerates HL cell malignant behaviors

As reported previously, PCBP1-AS1 is involved in HL progression (24), but its specific expression and role in HL presents unclear. Therefore, our study was intended to explore PCBP1-AS1's expression and role in HL. Firstly, RT-qPCR results demonstrated that compared to GC-B cell, PCBP1-AS1 showed a significant up-regulation in HL cells (Figure 1A). Since both L1236 and L428 cells harbored higher PCBP1-AS1 expression than other HL cells, L1236 and L428 cells were selected for subsequent function assays. The selected HL cells transfected with two specific shRNAs targeting PCBP1-AS1 to silence PCBP1-AS1 expression, and RT-qPCR analysis verified that after transfection of sh-PCBP1-AS1#1/2, PCBP1-AS1 expression was significantly diminished in HL cells (Figure 1B). CCK-8 results manifested that PCBP1-AS1 silencing obviously reduced the viability (Figure 1C). Likewise, the quantity of colonies was also cut down in HL cells following sh-PCBP1-AS1#1/2 transfection (Figure 1D). Transwell assays displayed the migratory and invasive properties of HL cells were weakened after PCBP1-AS1 reduction (Figure 1E-1F).

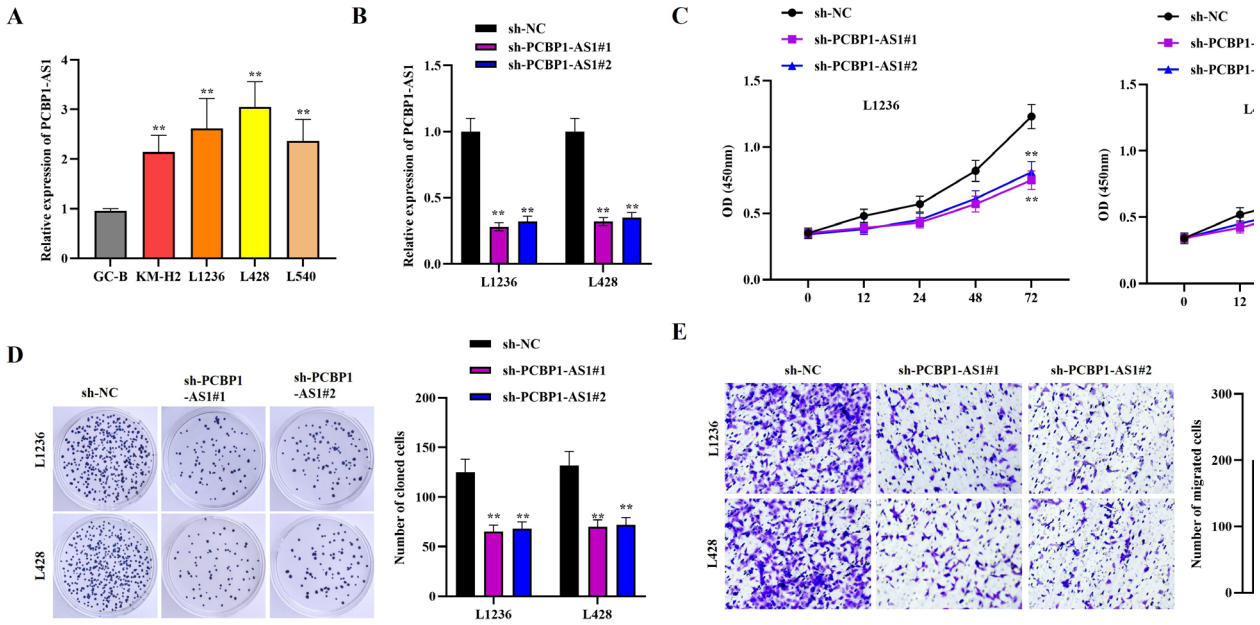

Figure 1 PCBP1-AS1 is high-expressed in HL cells and accelerates HL cell proliferation, migration and invasion. (A) RT-qPCR analysis of PCBP1-AS1 expression in HL cells and GC-B cell. (B) RT-qPCR proved the transfection efficiency of shRNAs targeting PCBP1-AS1 in HL cells. (C) CCK-8 assay assessed the viability of HL cells following transfection of shRNAs targeting PCBP1-AS1. (D) Colony formation assay examined the number of colonies in HL cells following transfection of shRNAs targeting PCBP1-AS1. (E-F) Transwell assays measured the migration and invasion of HL cells following transfection of shRNAs targeting PCBP1-AS1. **P<0.01.

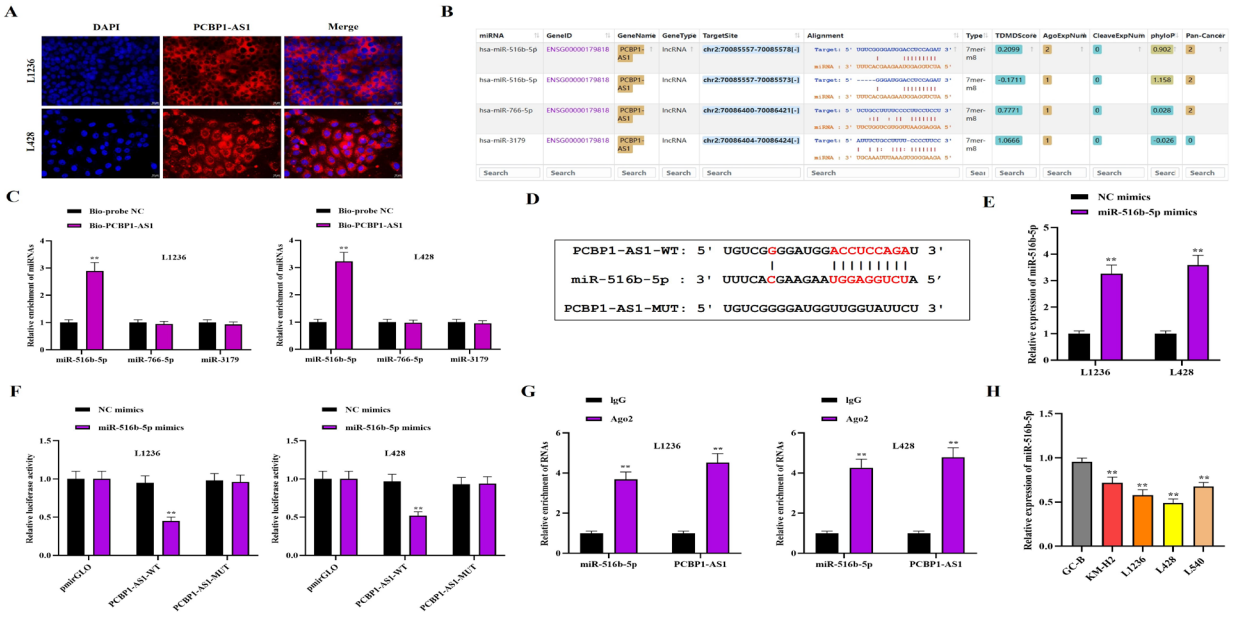

Figure 2 PCBP1-AS1 functions as a sponge of miR-516b-5p in HL cells. (A) FISH assay detected the subcellular localization of PCBP1-AS1 in HL cells. (B) Starbase predicted the miRNAs interacted with PCBP1-AS1. (C) RNA pull-down assay assessed the binding relationship between miRNAs and PCBP1-AS1. (D) Binding sites between PCBP1-AS1 and miR-516b-5p. (E) RT-qPCR proved the transfection efficiency of miR-516b-5p mimics in HL cells. (F) Luciferase reporter assay examined the impacts of miR-516b-5p overexpression on the luciferase activity of PCBP1-AS1 wild-type and mutant in HL cells. (G) Ago2-RIP assay assessed the binding relationship between PCBP1-AS1 and miR-516b-5p. (H) RT-qPCR analysis of miR-516b-5p expression in HL cells and GC-B cell. **P<0.01.

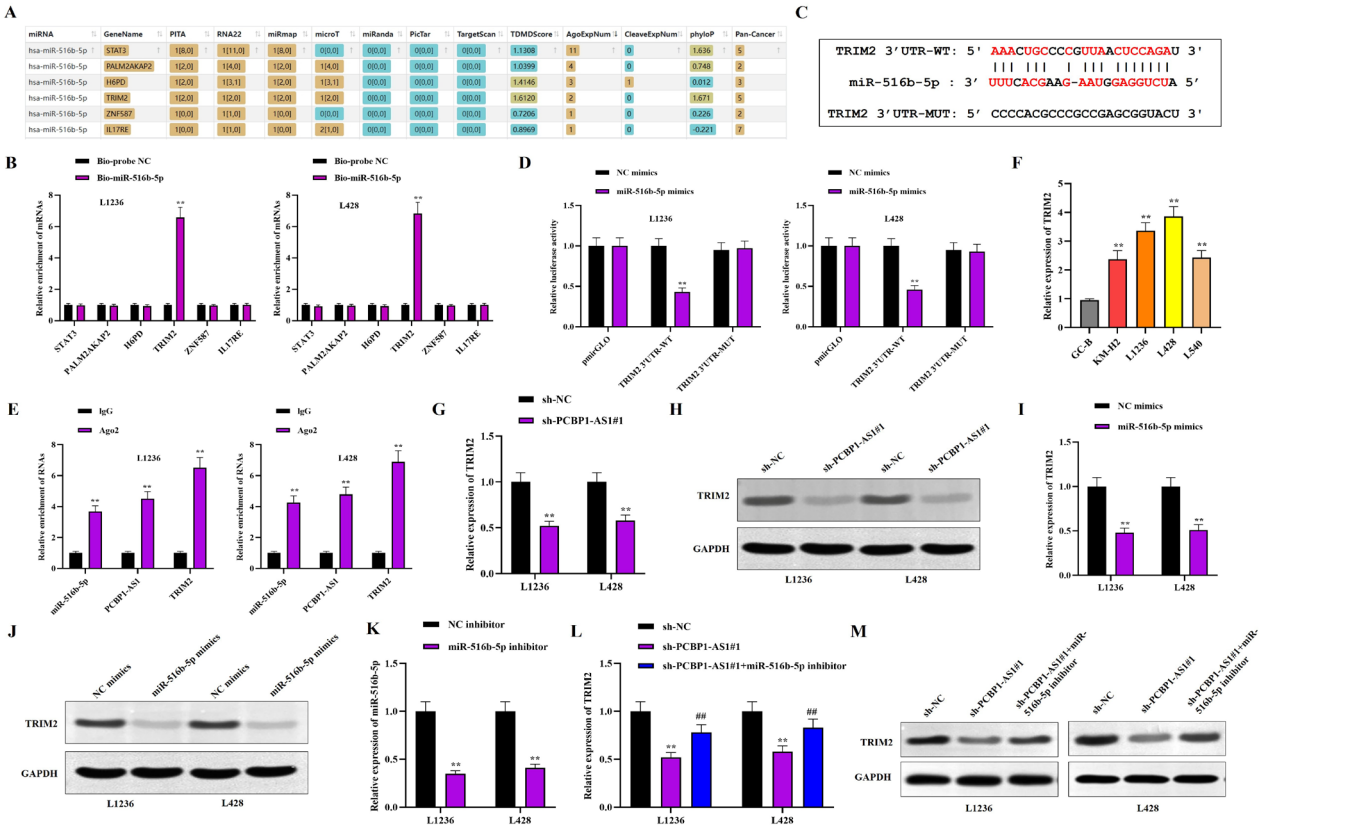

Figure 3 PCBP1-AS1 sponges miR-516b-5p to regulate TRIM2 expression in HL cells. (A) Starbase predicted the mRNAs targeted by miR-516b-5p. (B) RNA pull-down assay assessed the binding relationship between mRNAs and miR-516b-5p. (C) Binding sites between miR-516b-5p and TRIM2 3'UTR. (D) Luciferase reporter assay examined the impacts of miR-516b-5p overexpression on the luciferase activity of TRIM2 3'UTR wild-type and mutant in HL cells. (E) Ago2-RIP assay assessed the binding relationship among PCBP1-AS1, miR-516b-5p and TRIM2. (F) RT-qPCR analysis of miR-516b-5p expression in HL cells and GC-B cell. (G-J) RT-qPCR and western blot examined the mRNA and protein levels of TRIM2 in HL cells following transfection of sh-PCBP1-AS1#1 or miR-516b-5p mimics. (K) RT-qPCR proved the transfection efficiency of miR-516b-5p inhibitor in HL cells. (L-M) RT-qPCR and western blot examined the mRNA and protein levels of TRIM2 in HL cells following transfection of sh-PCBP1-AS1#1 or sh-PCBP1-AS1#1+miR-516b-5p inhibitor. **P<0.01, ##P<0.01.

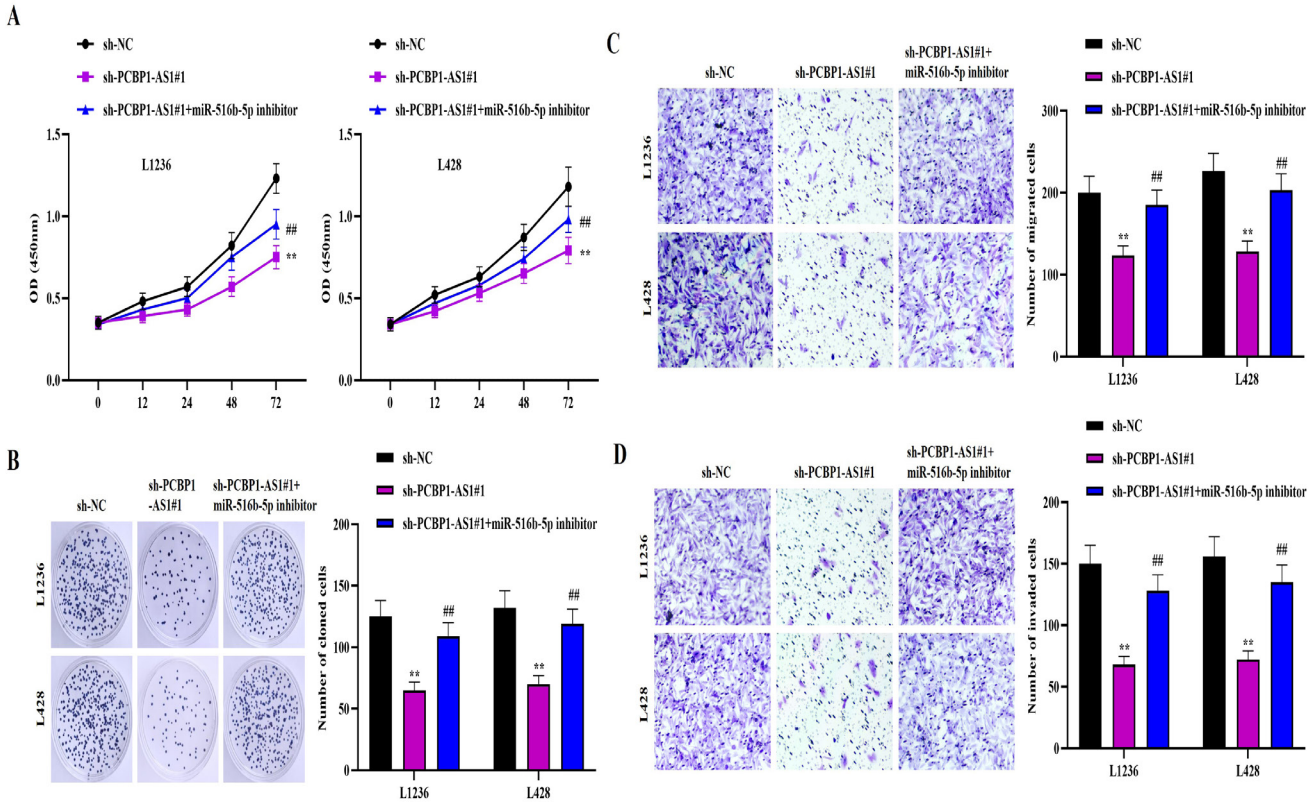

Figure 4 PCBP1-AS1 accelerates HL progression via sponging miR-516b-5p. (A) CCK-8 assay assessed the viability of HL cells following transfection of sh-PCBP1-AS1#1 or sh-PCBP1-AS1#1+miR-516b-5p inhibitor. (B) Colony formation assay examined the number of colonies in HL cells following transfection of sh-PCBP1-AS1#1 or sh-PCBP1-AS1#1+miR-516b-5p inhibitor. (C-D) Transwell assays measured the migration and invasion of HL cells following transfection of sh-PCBP1-AS1#1 or sh-PCBP1-AS1#1+miR-516b-5p inhibitor. **P<0.01, ##P<0.01

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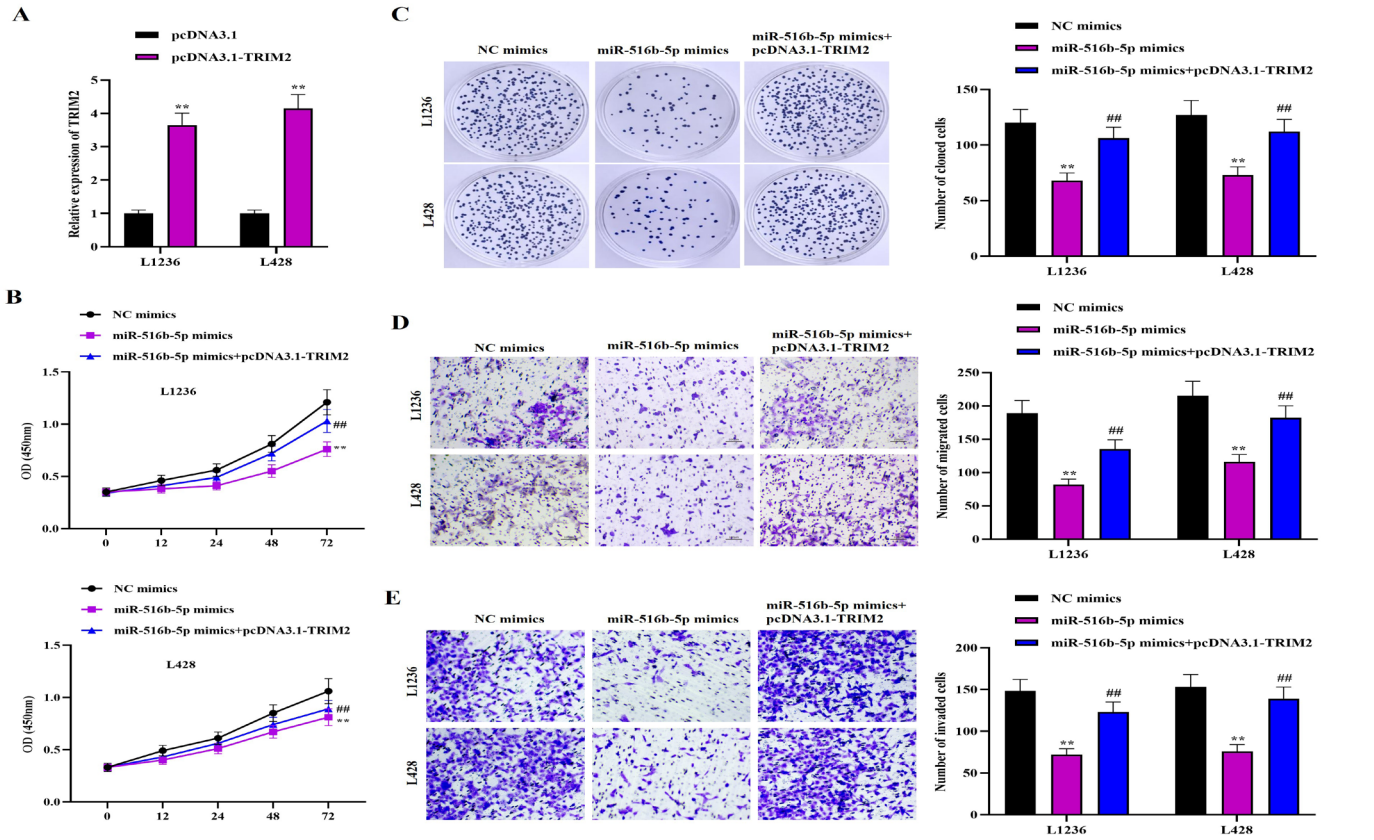

Figure 5 miR-516b-5p hinders HL progression via targeting TRIM2. (A) RT-qPCR proved the transfection efficiency of pcDNA3.1-TRIM2 in HL cells. (B) CCK-8 assay assessed the viability of HL cells following transfection of miR-516b-5p mimics or miR-516b-5p mimics+pcDNA3.1-TRIM2. (C) Colony formation assay examined the number of colonies in HL cells following transfection of miR-516b-5p mimics or miR-516b-5p mimics+pcDNA3.1-TRIM2. (D-E) Transwell assays measured the migration and invasion of HL cells following transfection of miR-516b-5p mimics or miR-516b-5p mimics+pcDNA3.1-TRIM2. **P<0.01, ##P<0.01.

Table 1 Primer sequences for RT-qPCR

Primers	Sequences
PCBP1-AS1	F 5'-GACGATAGAGATCAGTGGGCTTTG-3'
	R 5'-TGCTGTCTGAGAATGCTGATGG-3'
miR-516b-5p	F 5'-GCGCGATCTGGAGGTAAGAAG-3'
	R 5'-AGTGCAGGGTCCGAGGTATT-3'
TRIM2	F 5'-CAGCATCCCAAGTCCCGTGGT-3'
	R 5'-TCTCGCAGAAAGTGTGCAGACAG-3'
GAPDH	F 5'-TGCACCACCAACTGCTTAGC-3'
	R 5'-GGCATGGACTGTGGTCATGAG-3'
U6	F 5'-CTCGCTTCGGCAGCACA-3'
	R 5'-AACGCTTCACGAATTTGCGT-3'

PCBP1-AS1 functions as a miR-516b-5p sponge in HL cells

The action mechanism of PCBP1-AS1 in HL cells was explored. Through FISH results, PCBP1-AS1 was majorly expressed in the cytoplasm of HL cells (Figure 2A), mirroring the potential of PCBP1-AS1 as a competing endogenous RNA (ceRNA) to affect HL cell biological behavior by sponging miRNA. The possible miRNAs were predicted through starBase (https://rnasysu.com/encori/) website. There were 3 miRNAs that could interact with PCBP1-AS1, as displayed in Figure 2B. To determine which miRNA was sponged by PCBP1-AS1, a RNA pull-down experiment was arranged. The outcomes manifested only miR-516b-5p was abundant in the Bio-PCBP1-AS1 group (Figure 2C). The binding sequences of miR-516b-5p and PCBP1-AS1 was manifested in Figure 2D. To certify the binding potential between miR-516b-5p and PCBP1-AS1, miR-516b-5p expression was enhanced in HL cells (Figure 2E), followed by performing a luciferase reporter assay. As a result, miR-516b-5p elevation observably diminished the luciferase activity of the PCBP1-AS1 wild-type vector, whereas did not affect that of the mutant vector (Figure 2F). The RIP assay manifested that the Ago2 antibody was capable to pull down both PCBP1-AS1 and miR-516b-5p (Figure 2G). In addition, miR-516b-5p expression was apparently down-regulated in HL cells (Figure 2H).

PCBP1-AS1 sponges miR-516b-5p to affect TRIM2 expression in HL cells

The target mRNAs of miR-516b-5p were also explored utilizing starBase website. Through prediction of PITA, RNA22 and miRmap database, six mRNAs were predicted to potentially bind to miR-516b-5p (Figure 3A). Subsequent RNA pull-down assay manifested that relative to Bio-probe NC group, only TRIM2 was significantly enriched in Bio-miR-516b-5p group (Figure 3B). The binding sequences between miR-516b-5p and TRIM2 3'UTR were manifested in Figure 3C. The outcomes of luciferase reporter assay manifested miR-516b-5p up-regulation apparently lessened the luciferase activity of the TRIM2 3'UTR wild-type vector, whereas didn't influence the mutant vector (Figure 3D). The RIP assay made clear that PCBP1-AS1, miR-516b-5p and TRIM2 were highly abundant in Ago2 precipitates (Figure 3E). In addition, TRIM2 expression was apparently up-regulated in HL cells relative to GC-B cell (Figure 3F). Besides, after silencing PCBP1-AS1 or overexpressing miR-516b-5p, TRIM2 levels were markedly declined in HL cells (Figure 3G-3J). miR-516b-5p inhibitor was administrated

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into HL cells to inhibit miR-516b-5p level (Figure 3K). More importantly, TRIM2 levels were apparently declined in HL cells following sh-PCBP1-AS1#1 transfection, but miR-516b-5p inhibitor co-transfection rescued this effect (Figure 3L-3M).

PCBP1-AS1 accelerates HL progression via sponging miR-516b-5p

Whether PCBP1-AS1 regulated HL progression via miR-516b-5p was explored by performing rescue assays. Knockdown of PCBP1-AS1 weakened the viability together with number of colonies in HL cells, whereas miR-516b-5p inhibitor co-transfection counteracted this appearance (Figure 4A-4B). Besides, miR-516b-5p repression abolished PCBP1-AS1 down-regulation-mediated repressive effects on HL cell migration as well as invasion (Figure 4C-4D).

miR-516b-5p hinders HL progression via targeting TRIM2

Whether miR-516b-5p regulated HL progression via TRIM2 was also explored by performing rescue assays. We firstly enhanced TRIM2 expression in HL cells by transfecting pcDNA3.1-TRIM2 (Figure 5A). Then, we discovered miR-516b-5p elevation diminished the viability along with number of colonies in HL cells, while co-transfection of pcDNA3.1-TRIM2 reversed this phenomenon (Figure 5B-5C). Besides, TRIM2 up-regulation counteracted the suppressing impacts of miR-516b-5p elevation on HL cell migration as well as invasion (Figure 5D-5E).

Discussion

In this paper, we made clear that PCBP1-AS1 promoted HL cells proliferation, migration along with invasion of via interacting with miR-516b-5p to elevate TRIM2 expression. Besides, miR-516b-5p repression offset PCBP1-AS1 silencing-repressed proliferation, migration along with invasion in HL cells. TRIM2 overexpression abrogated miR-516b-5p augmentation-repressed proliferation, migration, as well as invasion in HL cells.

Numerous reports have manifested PCBP1-AS1 is abnormally expressed in many tumors, and it serves as oncogene or suppressor gene depending on tumor types[25,26]. But it is regrettable that the expression and role of PCBP1-AS1 in HL is still obscure. Previous research unveiled PCBP1-AS1 suppressed oral squamous cell carcinoma cell growth[27]. On the contrary, our work discovered a high expression of PCBP1-AS1 in HL cells and silencing of PCBP1-AS1 hindered HL cell proliferation, migration along with invasion, suggested that PCBP1-AS1 played a role in HL progression of HL by endowing cancer cells with an aggressive phenotype. Consistently, PCBP1-AS1 accelerates the hepatocellular carcinoma progression via modulating the PCBP1/PRL-3/AKT pathway[14].

Abundant researches have manifested lncRNAs play role of ceRNAs to influence tumor progression[28]. LncRNAs bind to miRNAs through the same miRNA response elements, thus modulating target mRNAs expression and finally influencing cancer progression[29]. In this paper, we manifested that PCBP1-AS1 could bind to miR-516b-5p in HL cells. Consistently, PCBP1-AS1 has been proved to sponge miR-126-5p in osteoporosis[30]. miR-516b-5p plays a role of tumor repressor in many cancers, containing thyroid cancer along with ovarian cancer[31,32]. In line with these reports, our study also suggested miR-516b-5p was underexpressed in HL cells. Overexpression of miR-516b-5p impeded HL progression. Moreover, we proved that miR-516b-5p repression could reverse PCBP1-AS1 silencing-mediated inhibitory impacts on HL cell malignant behaviors.

Another important finding of this paper was that we continued to excavate miR-516b-5p's target mRNA. Our work pointed out that TRIM2 was miR-516b-5p's target mRNA. TRIM2 is considered to be a part of the TRIM protein family and is a ring finger E3 ubiquitin[33]. Recent literatures have manifested that TRIM2 is high-expressed in numerous cancers, containing pancreatic cancer, colorectal cancer, as well as lung adenocarcinoma[34-36]. Similarly, our research made clear TRIM2 was up-regulated in HL cells. In addition, this paper proved that PCBP1-AS1 elevated TRIM2 expression by interacting with miR-516b-5p in HL cells. More importantly, this paper validated TRIM2 elevation neutralized miR-516b-5p up-regulation-mediated suppressing impacts on HL cell malignant behaviors. Consistently, Liu et al. suggested that up-regulated TRIM2 abolished the suppressive effect of miR-493 elevation on neuroblastoma progression[37].

Our study has some limitations. First, the correlation between PCBP1-AS1/miR-516b-5p/TRIM2 and HL patients' clinical characteristics was explored. Second, we only studied the potential of PCBP1-AS1/miR-516b-5p/TRIM2 axis in vitro cells and did not conduct in vivo experiments to prove the potential of PCBP1-AS1/miR-516b-5p/TRIM2 in HL. Therefore, more in-depth studies will be arranged in the future.

In short, our work demonstrates that PCBP1-AS1 accelerates HL cell malignant behaviors through the miR-516b-5p/TRIM2 axis, which may supply a hopeful therapeutic method for HL.

Declarations

Ethics approval and consent to participate

The study involving human materials was approved by the Ethics Committee of The Second People's Hospital of Changzhou, the Third Affiliated Hospital of Nanjing Medical University, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents or legal guardians of all paediatric donors of tonsil tissue.

Consent for publication

Not applicable.

Availability of data and materials

All data supporting the findings of this study are included within the article and its materials; additional information is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

No specific funding was received for this work.

Authors' contributions

BX performed experiments, acquired and analysed data, and drafted the manuscript. MC conceived and designed the study, supervised the work, verified the underlying data, and critically revised the manuscript. Both authors read and

approved the final manuscript and agree to be accountable for all aspects of the work.

Acknowledgements

We thank the clinical staff of the Departments of Hematology and Geriatrics, The Second People's Hospital of Changzhou, for assistance with sample handling and patient care.

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