LINC01255 combined with BMI1 to regulate Human Mesenchymal Stromal Senescence and Acute myeloid leukemia cell proliferation through repress the transcription of MCP-1

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ABSTRACT:

Background: Long non-coding RNAs (lncRNAs) govern fundamental biochemical and cellular biology processes, for example, participate in chromatin remodeling, imprinting, splicing, transcriptional regulation and translation. Dysregulation of lncRNA expression is act as a feature of various diseases and cancers, including hematopoietic malignancies. However, the clinical relevance of myelodysplastic syndrome (MDS) and acute myeloid leukemia preceded by MDS (MDS-AML) requires further research. Recently, lncRNAs have been demonstrated plays an important role in hematopoiesis, thus, to further finding more functional lncRNA seemed particularly important.

Methods: Western blotting, real-time PCR, RNA-pulldown, RIP, Chromatin immunoprecipitation (ChIP), cellular compartments extraction assays, SA-β-gal staining, lentivirus transfection, cell viability assay and cell proliferation assays were used to examine the relationship between lncRNA LINC01255 and its regulation of p53-p21 pathway in human mesenchymal stromal and acute myeloid leukemia cells.

Results: LncRNA LINC01255 is highly expressed in bone marrow cells of AML patients, CD34+ cells of MDS-AML patients and AML cell lines and the higher expression of LINC01255 is assoicated with poor survival rate of AML patients. LINC01255 can interact with BMI1 and repress the transcription of MCP-1 to active p53-p21 pathway, thus inhibiting the senescence of human mesenchymal stromal and proliferation of acute myeloid leukemia cell.
**Conclusions:** We discovered a novel functional lncRNA LINC01255, which can regulate the senescence of human mesenchymal stromal and the proliferation of acute myeloid leukemia cell through inhibiting the transcription of MCP-1.
Myelodysplastic syndromes (MDS) is a group of ineffective hematopoiesis hemopathies disorders characterized by defects in differentiation of hematopoietic precursor and amplification of the abnormal clones [1-4]. MDS is usually a rare group of “bone marrow failure disorder” that is not recognized and diagnosed. Their hematopoietic stem cells may be immature and accumulate in bone marrow with shortened life span, resulting in fewer mature blood cells in the hematopoietic system than normal people and lower organism healthy. However, although mature cells cannot be produced in bone marrow, MDS is not necessarily a terminal disease. Several MDS patients will have a decrease in blood cells and/or platelets gradually losing the body’s ability to resist infection and control bleeding. In addition, approximately 30% of MDS patients with this type of bone marrow failure syndrome will progress to acute myeloid leukemia (AML) [5]. Recently, some prognostic prediction models have been developed to better stratify the risk of MDS and AML patients, such as the international prognostic scoring system (IPSS) the revised IPSS (IPSS-R) for MDS [6-9] and the European Leukemia Net (ELN) risk classification for AML [10]. Nevertheless, even in the same risk groups, the prognosis of patients may be different [11-13]. Although several approved drug treatment strategies can be used to help treat the symptoms and slow the progression of the disease, they are not appropriate for all MDS patients. Therefore, it is more important to find more functional biomarkers and therapeutic targets for MDS and AML.

In recent years, as an increasingly important method, high-throughput transcriptome analysis uncovers transcription states of genomic. About 75% of genome can be transcribed into RNAs, of which few are transcribed and translated into proteins, and others are transcribed as noncoding RNAs (ncRNAs) [14]. According to the length, ncRNAs can be divided into shorter than 200 bp RNAs, such as microRNAs (miRNAs), housekeeping ncRNAs, PIWI-interacting RNAs (piRNAs), small interfering RNA (siRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs.
(snoRNAs) and longer than 200 bp RNAs, contains the long non-coding RNAs (lncRNAs) [15, 16]. LncRNAs are generally transcribed in genome and govern fundamental biochemical and cellular processes, for example, participate in chromatin remodeling, imprinting, splicing, transcriptional regulation and translation [17]. As well, lncRNAs have been demonstrated plays an important role in hematopoiesis [18, 19]. For example, during myeloid differentiation and maturation, HOTAIRM1 and NEAT1 are highly expressed and play key roles in this progress [18]. Xist located in X chromosome inactivates X chromosome of female cells, and female mice with Xist deleted can develop into a highly aggressive disease mimicking MDS/MPN [20]. MEG3 functions as a scaffold for the transcription regulation complex PRC2, and has been associated with multiple human malignancies, such as MDS and AML [21, 22]. Consistently, up to now, the functional lncRNAs relate to MDS and AML need more deeply research.

B Lymphoma Mo-MLV Insertion Region 1 (BMI1) is a member of the polycomb group of transcriptional repressor genes, expressed in stem cells, progenitors and many cancer cells, in which it regulates self-renewing proliferation, apoptosis and invasion [23-27]. In recent studies, BMI1 is highly expressed in CD34+ cells of MDS-AML patients and is considered to be a novel molecular biomarker of MDS. In addition, BMI1 can bind to the promoter of MCP-1 to inhibit its expression and reduce the activity of SA-β-gal and p53-p21 signaling pathways in senescence early phase of umbilical cord blood-derived MSCs (UCB-MSCs) proliferating in vitro [28-30].

In this report, we found a novel functional lncRNAs LINC01255, which is highly expressed in AML patients bone marrow cells, MDS-AML patients CD34+ cells and AML cell lines, and can interact with BMI-1 to repress the transcription of MCP-1 thereby activating the activation of p53-p21 pathway. In addition, LINC01255 can inhibit the senescence of human mesenchymal stromal and the proliferation of acute myeloid leukemia cells through inhibiting expression of MCP-1 combined with BMI1.
METHODS

Antibodies and Reagents
The sources of antibodies against the following proteins were: β-actin (A1978, 1:10,000 for WB) from Sigma; BMI1 (A0147, 1:1,000 for WB), Lamin B1 (A11495, 1:1000 for WB), MCP-1 (A7277, 1:1,000 for WB), p-p52-s392 (AP1137, 1:1,000 for WB) and p21 (A1483, 1:1,000 for WB) from ABclonal. RNA polymerase II (ab264350, 1:200 for ChIP), H3K4me3 (ab8580, 1:200 for ChIP) and H2AK119Ub (ab195467, 1:200 for ChIP) from abcam.

Plasmids
lncRNA LINC01255 was carried by pcDNA3.1 vector. FLAG-BMI1 was carried by pLenti-Hygro vector.

Cell Culture
KG1, OCI-AML2, OCI-AML3 and OCI-AML5 were got from the American Type Culture Collection (Manassas, VA) and cultured under the manufacturer’s instructions. UCB-MSC cell was collected from umbilical veins after neonatal delivery under maternal informed consent fails (Supplementary Data 1). UCB was harvests and separated within 24h of collection. Cultures were maintained at 37°C with 21% O2 and 5% CO2 in culture medium with changed twice a week. All of the cultured cells were authenticated by examination of morphology and growth characteristics, and were confirmed to be mycoplasma-free.

Western Blotting
Cells were lysed by RIPA buffer, and re-suspending in 5 × SDS-PAGE loading buffer. The boiled protein samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane incubated with appropriately primary antibodies and secondary antibodies, then imaging under imager WBS2000.
**RNA-pulldown and Silver Staining**

The RNA pull-down assay was modified from previous studies [52]. Substrate RNAs were in vitro transcribed in a 100 μl reaction mix containing 1 μg of pcDNA3.1-LINC01255 DNA detailed protocol as RiboMAX™ Large Scale RNA Production Systems–T7 manufacturer’s instructions (P1300, Promega). In addition, transcribed RNAs produced 3’ End Desthiobiotinylation using Pierce™ RNA 3’ End Desthiobiotinylation Kit (20163, Invitrogen) and incubated with cell lysis for RNA-pulldown assays as Pierce™ Magnetic RNA-Protein Pull-Down Kit (20164, Invitrogen). Then, the eluents were collected and visualized on NuPAGE 4-12% Bis-Tris gel (NP0321BOX, Invitrogen) followed by silver staining with silver staining kit (24600, Invitrogen). The distinct protein bands were retrieved and analyzed by LC-MS/MS.

**Mass spectrometry (MS) analysis.**

IncRNA LINC01255 interacted proteins performed LC-MS/MS analyzed by Thermo Finnigan LTQ linear ion trap mass spectrometer (Thermo Fisher Corporation, San Jose, CA) in line with a Thermo Finnigan Surveyor MS Pump Plus HPLC system. The mass spectrometry analysis was carried out in a data-dependent mode with an automatic switch between a full MS and an MS/MS scan in the obitrap. Peptide sequences were searched using trypsin specificity and allowing a maximum of two missed cleavages. Sequest was searched with a peptide tolerance of 3.0 Da and a fragment ion tolerance of 1.0 Da. The results of peptide sequences information of LINC01255 interact proteins were offered in Supplemental Table 1.

**RNA immunoprecipitation (RIP).**

For KG1 cells, 1 × 10^7 cells were harvested and crosslinked with 0.3% formaldehyde for 10 min at RT and quenched with 0.125 M glycine for 5 min. Nuclei were extracted and lysed in RIP Cross-Linked Lysis Buffer and product protocol supplied by Magna Nuclear RIP™ (Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation
Kit (17-10520, Millipore). In addition, RNAs were extracted with TRIzol and detected by qRT-PCR.

**Chromatin immunoprecipitation (ChIP)**

ChIP experiments were performed according to the procedure described previously [31, 32]. About $1 \times 10^7$ cells were cross-linked and quenched with 1% formaldehyde and 125mM glycine. The fixed cells were resuspended in SDS lysis buffer and sonicated to generate chromatin fragments of 250-500bp. Then the sonicated lysis were immuneprecipitated with indicated antibodies, and analyzed with qRT-PCR.

**qRT-PCR**

Total cellular RNAs were isolated by TRIzol reagent (Invitrogen) and transcribed by the Reverse Transcription System (Roche). Quantitation of all gene transcripts was done by qPCR using a Power SYBR Green PCR Master Mix (Roche) and Q5 detection system (Thermo) with the expression of ACTB as the internal control. The primers used were listed in Supplementary Data 2.

**RNA Interference**

In our studies, the short hairpin RNAs (shRNAs) against LINC01255, BMI1 or MCP-1 were expressed as lentiviral, purchased from GenePharma, transfected into appropriate cells. The sequences of shRNAs are provided in Supplementary Data 3.

**SA-β-gal staining**

SA-β-gal staining was used as a biomarker of senescence in UCB-MSCs. SA-β-gal activity was qualitatively assessed with a senescence cells histochemical staining kit (CS0030; Sigma) according to the manufacturer’s instructions.

**Cell Viability Assay**

Indicated cells were cultured in appropriate media for 24h in 96-well plates, add 10 μl CCK-8 regents ahead of 2-4 hours for test OD value of 450nm. The detail protocols
were supplied by Cell Counting Kit-8 (HY-K0301-500T, MedChem Express).

Statistical Analysis
All of experimental data from biological triplicate experiments are presented with error bar as mean ± S.D. and two-tailed unpaired Student’s t-test was used for comparing two groups of data. Analysis of variance (ANOVA) with Bonferroni’s correction was used to compare multiple groups of data. A P value of less than 0.05 was considered significant and higher than 0.05 was considered no specific differences. All of the statistical testing results were determined by SPSS 22.0 software diagrams were conducted by GraphPad prism 8.0. Before statistical analysis, variation within each group of data and the assumptions of the tests were checked.

Data Availability
All relevant data are available from the authors on request.
RESULTS

LncRNA LINC01255 is highly-expressed in AML patients bone marrow cells, MDS-AML patients CD34+ cells and AML cell lines.

LncRNAs regulate basic biochemical and cellular biology processes and play important role in regulating development, cell differentiation, proliferation and apoptosis of hematopoiesis and cancer cells [33]. In recent report, we compared the expression profile of IncRNA by comparing 6 patients with AML and 6 healthy controls. The analysis of the volcano graph with 2 fold change cut-off found that there were 1011 IncRNA specific expression disorders, including 562 upregulated IncRNAs and 449 downregulated IncRNAs, respectively [34]. After further analysis, we found that LINC01255 is one of highly expressed IncRNAs in AML patients. In order to further validate this, we detected the expression of LINC01255 in bone marrow cells of AML patients and compared it with healthy control group. As shown in Figure 1A, IncRNA LINC01255 is highly-expressed in bone marrow cells of AML patient and has a positive correlation with the poor prognosis of AML patients (Figure 1B). For the reason of the disease of MDS patients may develop into AML, we detected the expression of LINC01255 in CD34+ cells of MDS patients. The results show that LINC01255 is highly expressing in CD34+ cells of MDS patients (Figure 1C). To further explore the function of LINC01255, we detected the expression levels in AML cell lines and normal bone marrow cells, such as KG1, OCI-AML2, OCI-AML3 and OCI-AML5. The results show that LINC01255 is highly expressed in AML cell lines (Figure 1D), these results suggest that LINC01255 may play a role in AML cell.

LINC01255 is physically associated with BMI1

To better understand the mechanistic role of IncRNA LINC01255 in AML cells, we employed RNA pull-down assays through incubate LINC01255, transcribed in vitro, together with KG1 cell lysates, then, conduct mass spectrometry (MS) to analysis potential LINC01255 associated proteins. After analysis we identified BMI1 as a possible LINC01255-interacting protein (Figure 2A). Furthermore, we performed
RNA pull-down assays and followed by western blotting assays, and immunoblotting with BMI1 antibody in KG1 cells (Figure 2B), the results showing that LINC01255 could interact with BMI1. In another way, we employed an RNA immunoprecipitation (RIP) assays with anti-BMI1 antibody and followed by qRT-PCR assays (Figure 2C), these results further validated the interaction between IncRNA LINC01255 and BMI1.

To confirm the interaction between IncRNA LINC01255 and BMI1, we employed another RNA pull-down assay and RIP assays in OCI-AML3 cells, which indicates that the interaction between LINC01255 and BMI1 is prevalent in AML cell lines (Figure 2D). In addition, we performed RIP assays to detect the interaction between LINC01255 and BMI1 in different cellular compartments of KG1 cells, the results indicated that BMI1 could interact with LINC01255 in nuclear but not cytosolic (Figure 2E), which is more likely to depend on the nuclear location of LINC01255 (Figure 2F). In conclusion, LINC01255 could interact with BMI1 and the nuclear location characteristics of LINC01255 indicate that it has a transcription functions combined with BMI1.

**LINC01255 associated with BMI1 to repress the transcription of MCP-1**

As a transcriptional repressor, BMI1 could prevent the premature activation of senescence associated genes, including INK4a, ARF and MCP-1 during senescence process [35, 36]. In order to further explore the function of LINC01255, we examined the expression of LINC01255 in early and late stage of UCB-MSC senescence, the results showing that the expression of LINC01255 is significantly decreased in senescent late stage of UCB-MSC cells (Figure 3A). In previous studies, BMI1 could bind to the promoter of MCP-1 to repress the expression of MCP-1, we detected the binding of BMI1 to the MCP-1 locus in early stage of UCB-MSC senescence in the absence of RNAse or not. The result indicated that RNAse could significantly decrease the binding of BMI1 to MCP-1 locus in senescence early stage of UCB-MSC cells and KG1 cells (Figure 3B) analyzed by qChIP assays. In addition, we transfected shRNA plasmids targeting BMI1 into KG1 cells, and the expression of
BMI1 is reduced, as shown in Figure 3C. In addition, we performed qChIP analysis and the results showed that knockdown BMI1 significantly decreased the monoubiquitination modification at lysine 119 of histone2A (H2AK119Ub) (Figure 3D) and increased the trimethylation modification at lysine 4 of histone 3 (H3K4me3) and localization of polymerase II at the promoter regions of MCP-1 (Figure 3E). In thus, the above results indicate that BMI1 binding to the promoter regions of MCP-1 may be regulated by functional RNAs.

Furthermore, in order to character the function of LINC01255 binding to BMI1, we transfected shRNAs plasmids target LINC01255 into KG1 cells to reduce its expression (Figure 3F). Then, we detect the binding of BMI1 to the promoter of MCP-1, and the results showed that reducing the expression of LINC01255 significantly decreased the enrichment of BMI1 at the MCP-1 locus (Figure 3G), simultaneously, the expression of MCP-1 was increased in senescent early stage of UCB-MSC cells and KG1 cells.(Figure 3H). In conclusion, LINC01255 may repress the transcription of MCP-1 combined with BMI1 in UBC-MSC and AML cell lines.

**Down-regulation of LINC01255 active the p53-p21 signaling pathway through enhancing MCP-1 expression**

In previous studies, MCP-1 is a ‘trigger factor’ of ROS-p38-MAPK-p53/p21 signaling cascade. MCP-1, as a component of senescence-associated secretory phenotype (SASP) during expansion of UCB-MSCs, can activate the p53-p21 signaling cascade and increase the protein level of Ser392 phosphorylated p53 and the total expression of p21 [37]. To better understand the relationship between LINC01255 and p53-p21 signaling pathway, we detected the expression of p53 phosphorylated at Ser392 and the total expression of p21 under knockdown of LINC01255 in senescent early stage of UCB-MSC cells, and the result showed that knocking down LINC01255 can increase the activity of p53-p21 pathway by increasing the expression of p53 phosphorylation at Ser392 and the total expression of p21 (Figure 4A). In addition, knockdown of LINC01255 could also increase the activity of SA-β-gal (Figure 4B).
Also, knockdown of LINC01255 could increase the activity of p53-p21 pathway in KG1 cells (Figure 4A). While, knocking down BMI1 could also increase the activity of p53-p21 pathway in KG1 cells (Figure 4C). Interestingly, the increased activity of p53-p21 pathway due to knockdown of LINC01255 could be reversed by overexpression of BMI1 (Figure 4D). Overexpression BMI1 could inhibit the transcription of MCP-1 through binding to the promoter of MCP-1 and this inhibition could rescue by knockdown LINC01255 in some extent analyzed by qChIP against H3K4me3 and polymerase II antibodies (Figure 4E). Under these results, we detected that the increased activity of p53-p21 pathway under down-regulation of LINC01255 may be related to the increased expression of MCP-1. To prove this hypothesis, we examined the expression of p53 phosphorylated at Ser392 and the total expression of p21 under knockdown LINC01255, the increased activity of p53-p21 signaling could obliterated by knockdown of MCP-1 through special shRNAs (Figure 4F).

**BMI1/LINC01255 axis regulate senescence of MSC and proliferation of AML cells**

In a recent report, MCP-1 was identified as a crucial component of the SASP in human UCB-MSC senescence process, the secretion of MCP-1 was inhibited by BMI1 in the early stage, and increased along with senescence. During this process, the expression of BMI1 was decreased [37]. According to our previous results, the expression of LINC01255 is decreased in late stage of UCB-MSC senescence (Figure 3A). Then, we asked whether downregulated LINC01255 could affect the senescence of human mesenchymal stromal cells (MSC). Therefore, we transfected the shRNAs target for LINC01255 into UCB-MSC cells and the result showed that knocking down LINC01255 increased SA-β-gal activity (Figure 5A) and accelerated the senescence of UCB-MSC (Figure 5B). To further exploring the function of LINC01255 in AML cells, we detected the cell growth ability of KG1 cells by CCK8 assays (Figure 5C) and growth examination experiments (Figure 5D) and the results indicated that down-regulation of LINC01255 could accelerate the proliferation of KG1 cells, but this acceleration will be braked by overexpressing BMI1.
DISCUSSION

In this study, we discovered a new functional lncRNA LINC01255, which is highly expressed in AML patient bone marrow cells, MDS-AML patient CD34+ cells and AML cell lines, and combined with BMI1 to regulate the senescence of UCB-MSCs and proliferation of KG1 cells through affecting p53-p21 signaling activity related to MCP-1.

In previous studies, BMI1 is a transcriptional repressor and plays an essential roles in the maintenance of appropriate gene expression, while, BMI1 deletion could repress the proliferative and self-renewal of HSC through INK4A/ARF pathway [38-40]. In addition, the expansion and self-renewal of primary leukemic AML CD34+ cells dependent on BMI1 repress cell cycle regulators, p16 and p19/p14. However, whether LINC01255 is participated in the regulation of p16 and p19/14 combined with BMI1 in leukemic AML CD34+ is not clear. While, our data show that LINC01255 is highly expressed in AML patient CD34+ and can interact with BMI1 to regulate the proliferation of AML cell lines, therefore, LINC01255 may have the function of regulating the self-renewal of leukemic AML CD34+. In MDS and MDS-AML patients, BMI1 is required to regulate the self-renewal in CD34+ cells and it might be a novel biomarker for disease progression and prognosis in these patients, and highly expressed of LINC01255 might provide a new diagnostic approaches [27].

The monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes. It is also expressed in tumor cells, B-ALL, AML cells and tumor stroma cells, which are mainly secreted by inflammatory cells and endothelial cells [41-44]. MCP-1 is associated with a poor prognosis in patient with certain cancers, such as breast cancer. Also, MCP-1 can promote lung metastasis or glioblastoma and tumor growth [41]. In addition, MCP-1 is associated with senescence of UCB-MSC through activating p53/p21 signaling pathway. In our results, lncRNA LINC01255 could interact with BMI1 and repress the
expression of MCP-1 in UCB-MSC and AML cell lines. However, in some case, highly secreted MCP-1 could not promote the proliferation of KG1 and it was not originate from AML cells of MDS-AML patients, it might be secreted by inflammatory or endothelial cells [45, 46].

CONCLUSIONS
In our study, we found a new functional lncRNA LINC01255, which is highly expressed in UCB-MSC, MDS-AML patients CD34+ cells and AML cells. Interestingly, LINC01255 can interact with BMI1 to repress the expression of MCP-1 and active p53-p21 signaling pathway in UCB-MSC and AML cells. In thus, this novel functional lncRNA may provide a new therapeutic approach for MDS-AML or related diseases.

ABBREVIATIONS
MDS: Myelodysplastic syndromes; AML: Acute myeloid leukemia; IPSS : International prognostic scoring system; IPSS-R: international prognostic scoring system (IPSS) the revised IPSS; ELN : European Leukemia Net; ncRNAs : noncoding RNAs; miRNAs: microRNAs; piRNAs : PIWI-interacting RNAs; siRNAs : small interfering RNA; snRNAs : small nuclear RNAs; snoRNAs : small nucleolar RNAs; lncRNAs : long non-coding RNAs; BMI1: B Lymphoma Mo-MLV Insertion Region 1; UCB-MSCs : Umbilical cord blood-derived MSCs; RIP : RNA immunoprecipitation; SASP : Senescence-associated secretory phenotype; MSC : Human mesenchymal stromal cells.

DECLARATIONS
Ethics approval and consent to participate
Ethics approval and consent files were offered in Ethics approval file.

Availability of data and materials
Not applicable
Author contributions
Hong Zhang conceived this project; Qinghua Liu, Hong Zhang, Jing Dong, Jie Li, Yanchao Duan, Keqiang Wang and Qiuhong Kong conducted experiments; Qinghua Liu and Hong Zhang acquired and analysed data; Hong Zhang wrote the manuscript.

Funding
This study was supported by Medical and Health Science and Technology Development Project of Shandong (2018WS122), Science and Technology Development Plan (guidance plan) of Taian (2018NS0123), 2019-2020 Chinese Medicine Science and Technology Development Project of Shandong (2019-0343).

Acknowledgements
Not applicable.

Consent for publication
Not applicable.

Conflict of interest
The authors have declared that no conflict of interest exists.
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