Luks-PV induce HOXA9 degradation through autophagy in Acute myeloid leukemia with HOXA9 overexpression

Ping Qiang  
Anhui Provincial Hospital

Chao Fang  
Anhui Provincial Hospital

Kaidi Song  
Anhui Provincial Hospital

Lan Shi  
Anhui Provincial Hospital

Yuanyuan Dai  
Anhui Provincial Hospital

Wenjiao Chang  
Anhui Provincial Hospital

Liangfei Xu  
Anhui Provincial Hospital

Xingbing Wang  
Anhui Provincial Hospital

Xin Liu  
Anhui Provincial Hospital

Huilan Liu  
Anhui Provincial Hospital

Zimin Sun  
Anhui Provincial Hospital

Xiaoling Ma  
Anhui Provincial Hospital

Research article

Keywords: autophagy, HOXA9, MLL, Luks-PV, AML

Posted Date: July 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-56347/v2
Abstract

Background: Our previous studies have demonstrated that Luks-PV have good anti-leukemia ability effects and could possibly be a promising therapy for adult acute myeloid leukemia (AML). Aberrant over-expression of HOXA9 is a prominent feature of AML driven by multiple oncogenes, and therapeutic degrading of HOXA9 may be an effective treatment strategy in AML. This paper focused on the Luks-PV-regulating autophagy pathway, aiming to investigate the role of Luks-PV in mixed-lineage leukemia (MLL)-rearranged AML.

Methods: The data of leukemia patients were downloaded from the gene expression profiling of TCGA datasets. Taking primary AML and THP-1 cells as the model system in vitro, Luks-PV-inhibited cell proliferation was determined by CCK-8 and flow cytometry assays. The role of Luks-PV in autophagy regulation was analyzed using immunoblotting, transfection and immunofluorescence.

Results: HOXA9 was over-expressed and associated with a poor prognosis in AML patients bearing MLL rearrangement. After the application of pharmacologic inhibitors of autophagy, Luks-PV induced cytotoxic autophagy in AML cells, as suggested by biochemical and microscopy results. HOXA9 molecules were detectable within autophagosomes after Luks-PV treatment, indicating that autophagy induction accounted for the degradation of HOXA9. Moreover, Luks-PV-induced HOXA9 downregulation inhibited AML cell proliferation, suggesting that HOXA9 could be degraded through Luks-PV-induced autophagy.

Conclusion: Luks-PV suppresses AML cell proliferation by inducing HOXA9 degradation.

Background

AML is characterized by an abnormal increase of myeloblasts in the bone marrow (BM), and generally associated with poor outcomes. One of the tough problems in AML treatment is the occurrence of chemoresistance, which necessitates the development of newer therapies and small molecular targeting drugs for AML patients. Luks-PV was previously shown to be a promising treatment for adult AML due to its anti-leukemia effects [1-2]. HOXA9 is an important transcription factor, which plays a key role in both blood cell development and leukaemia [3]. AML driven by multiple oncogenes is markedly featured by aberrantly over-expressed HOXA9, and may be treated by therapeutic degrading of HOXA9 [3-7].

Autophagy is a multistep lysosomal degradation pathway, which supports metabolic adaptation and nutrient recycling and has implications for the tumor immunity and microenvironment. It is increasingly clear that autophagy induction may restrain the growth of tumors and improve the response to tumor therapeutics[8]. The degradation of leukemia oncoproteins through autophagy has recently paved a new way for therapeutic options in AML.

Previous studies has established the anti-leukemic activity of Luks-PV in AML [2, 9], but not determined the role of autophagy in the degradation of HOXA9. This paper demonstrated that Luks-PV inhibited cell
proliferation by inducing HOXA9 degradation through autophagy in MLL-rearranged AML. The removal of HOXA9 in Luks-PV-treated cells depends upon and relates to the increased autophagic activity.

**Methods**

Recombinant LukS-PV production and purification

Recombinant LukS-PV purification and purification were described previously by Ma et al [10].

AML patient samples and cell lines

THP-1, NB4, HL60 and OCL-AML3 cell lines were purchased from the Shanghai Institute for Biological Sciences (SIBS, Shanghai, PR, China). Twenty untreated and newly diagnosed adult AML patients were recruited from January 2015 through January 2020. BM specimens were obtained before chemotherapy. The cells were separated by using the lymphocyte separation medium (TBD, China), and then purified by anti-CD33 and anti-CD34 magnetic-activated cell sorting (MACS) separation columns (Miltenyi Biotec, Bisley, United Kingdom). Cells with >95% viability detected by trypan blue staining were used.

Antibodies and reagents

LC3B (D11, # 3868S), HOXA9 (# ab140631), anti-HOXA9/AF 350 (# bs-6667R-AF350), 3-Methyladenin (3-MA, #M9281) and Balomycin A1 (Baf-A1, #S1413) were obtained from Cell Signaling Technology, Abcam, Bioss, Sigma and Selleck, respectively.

RNA extraction, real-time PCR and HOXA9 mRNA expression analysis

Total RNA from cell lines and human BM specimens were extracted using the Trizol (Invitrogen). Reverse transcription and quantitative real-time PCR for determination of HOXA9 and GAPDH mRNA expression were performed using the SYBR Green PCR Kit (GenePharma, Shanghai, PR China). The primer sequences of HOXA9 was as follows: HOXA9 forward: 5′-CACCAGACGAACAGTGAGGA-3′; reverse (5′-3′): TGGTCAGTAGGCCTTGAGGT. Analysis of relative mRNA expression was using 2−△△CT method.

mRFP-GFP-LC3 transfection and immunofluorescence staining

The adenoviral vector adenovirus encoding both mRFP-GFP and LC3 proteins was obtained from HanBio Technology (Shanghai, China). THP-1 and primary AML cells were first seeded into 96-well plates at a final density of 5×10^4 cells per well, followed by transfection according to the manufacturers' instructions.

Western blotting

Proteins were resolved using 10% SDS-polyacrylamide gels and electro-transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocked in Tris-buffered saline 0.1% Tween 20% to 10% milk, membranes were probed with an appropriate amount of primary antibodies and
horseradish peroxidase-conjugated secondary antibodies, and then visualized with an enhanced chemoluminescence detection system.

EDU assay

Cell proliferation was assessed by 5-ethynyl-2’-deoxyuridine (EdU) assay (KeyGen BioTECH, JiangSu, China) according to the manufacturers' instructions. Cells were analyzed by fluorescence activated cell sorter Calibur flow cytometer (BD Pharmingen, San Diego, CA).

Cell counting kit-8 assay

Cell viability was detected by cell counting kit-8 (CCK-8, Dojindo) following the manufacturers' instructions. Cells were first grown in 96-well plates at a final density of 5000 cells (200μL) per well, and then incubated with different reagents in different conditions. Afterward, 20μl of CCK-8 solution was added to each well to culture the cells for 1-2 hours, and the absorbance at 450 nm (OD450nm) in a microplate reader was recorded subsequently. All of the CCK-8 data were performed in triplicate.

Statistical analysis

Data of different groups were analyzed by the unpaired two-tailed Student’s t test with the help of SPSS (version 24), R language, and GraphPad Prism (version 7) software. A log-rank test was used for survival analysis and Kaplan-Meier survival curve was applied to the assessment of overall survival (OS) in AML patients.

Results

1. HOXA9 is overexpressed in MLL-rearranged AML and associated with patients outcomes

The expression of HOXA9 in THP-1, NB4, HL60, OCL-AML3 and primary AML cells was first investigated. The results showed that HOXA9 was highly expressed in THP-1, OCL-AML3 and primary AML cells, especially in THP-1 cells (Fig 1 A), which mimicked several clinical features of AML blast cells with MLL/AF9 oncprotein. Besides, HOXA9 expression in 8 healthy controls and 18 newly diagnosed adult AML patients was compared. It was revealed that the expression of HOXA9 was significantly higher in AML patients than in controls (P<0.0001, Figure 1 C). Moreover, compared with AML without MLL rearranged, the expression of HOXA9 was significantly increased in AML patients bearing MLL rearrangement (P<0.01, Figure 1 D). Finally, according to the analysis of the clinical and RNA sequencing information of 135 AML samples obtained from the TCGA database, AML patients with higher HOXA9 expression presented with worse OS (P<0.05, Fig 1B).

2. Luks-PV activates autophagy

In order to assess autophagy-promoting activity of Luks-PV in MLL-rearranged AML cells, the microtubule-associated protein 1 light chain 3 (LC3) in THP-1 and primary AML cells was studied by
western blotting. During autophagy, cytosolic LC3 (LC3-I, upper band) was converted to autophagic membranes (LC3-II, lower band). The LC3-II level was specifically associated with autophagosomes, and a good evaluation method for autophagic activity [11]. As can be seen in Figure 2A-B, Luks-PV increased the LC3-II levels in THP-1 and primary AML samples in a concentration-dependent manner, indicating that Luks-PV activated autophagy. Moreover, the addition of the lysosomal protease inhibitor bafilomycin A1 (BafA1) to Luks-PV treatment further increased the amount of LC3-II (Figure 2C). Since cytoplasmic contents of autophagosomes are surrounded with double-membrane vacuolar structures, high-resolution Transmission Electron Microscopy (TEM) was used to identify autophagosomes. As indicated by Figure 2D, autophagic vacuoles can be found in Luks-PV-treated THP-1 cells but not in phosphate buffer saline (PBS) control cells. Furthermore, an increased number of LC3-containing cytoplasmic structures elevated the level of autophagy. The effect of Luks-PV on the sub-cellular distribution of LC3 protein in THP-1 and primary AML cells was examined by immunofluorescence microscopy, which demonstrated an accumulation of LC3-positive structures in THP-1 and primary AML cells exposed to Luks-PV, indicating more autophagosomes were formed (Figure 3E). Taken together, Luks-PV induced autophagy of THP-1 and primary AML cells.

3. Luks-PV inhibits AML cell proliferation by inducing HOXA9 down-regulation

Down-regulated HOXA9 in MLL-rearranged acute leukemia cells was previously proven to be related to the immediate reduction of cell proliferative capacity [3]. To explore whether Luks-PV inhibited AML cell proliferation through down-regulating HOXA9, we first assessed its impact on HOXA9 expression, and found that Luks-PV remarkably down-regulated the expression of HOXA9 protein and mRNA in THP-1 and primary AML cells (Figure 3A-D). Edu assays revealed that the percentage of Edu-positive cells decreased after treated with 2µM Luks-PV for different time, indicating that Luks-PV inhibited cell proliferation in a time-dependent manner (Figure 3E). CCK assays suggested that Luks-PV treatment lowered the metabolic rate, inhibited the proliferation and reduced the viability of THP-1 cells (Fig. 3F). Kinetic experiments were carried out to measure both cell proliferation and HOXA9 protein level of THP-1 cells exposed to 2µM Luks-PV for 8 to 24 hours before cell lysis. The experimental results showed that HOXA9 protein expression was positively correlated with the percentage of Edu-positive cells. The above analysis confirmed that Luks-PV induced HOXA9 down-regulation, which initiated AML cell proliferation.

4. Luks-PV induces HoxA9 degradation is autophagy-dependent

Because autophagy was a multistep lysosomal degradation pathway, we investigated the role of autophagy in the degradation of HOXA9 after Luks-PV treatment. It can be seen from Figure 4A-B that the addition of the lysosomal protease inhibitor 3-Methyladenine partially restored HOXA9 expression in Luks-PV-treated cells. According to immunofluorescence analysis, HOXA9 molecules colocalized with LC3 positive structures in THP-1 and primary AML cells treated with 2µM Luks-PV (Figure 4C), indicating that HOXA9 could be degraded through Luks-PV-induced autophagy.

Discussion
The effect of Luks-PV in activating autophagy and inhibiting proliferation of AML cells suggests a crosstalk between proliferation and autophagy. Luks-PV regulates the expression of HOXA9 at the protein level through induction of autophagy, and inhibits AML cell proliferation. Based on the THP-1 proliferative interference effect of Luks-PV determined by previous studies [9], our study uncovers the regulative role of HOXA9 oncprotein and provides more details as to why MLL-rearranged AML cells are particularly sensitive to Luks-PV. What’s more, it was established by previous research that inhibiting HOXA9 expression in MLL-rearranged acute leukemia cells by a lentiviral-based shRNA approach rapidly reduced the proliferative capacity of the cells [3]. Hence, RNA interference is not adopted to inhibit the expression of HOXA9 in THP-1 and primary AML cells in this paper.

Bacterial toxins play an important role in either altering the tumor cellular biological processes or inducing disease regression by directly killing tumor cells [12-13]. Several bacterial toxins have been approved by the Food and Drug Administration (FDA) as cancer therapies on account of their safety and good anticancer effects [14]. For example, diphtheria toxin-related interleukin 2 fusion protein, also called Denileukin Diiftitox, is the first bacterial immunotoxin approved by FDA for the treatment of adult T-cell leukemia/lymphoma (ATL) [15]. Panton-Valentine leukocidin (PVL), composed of Luks-PV and Lukf-PV, is a pore forming cytotoxin secreted by methicillin-resistant Staphylococcus aureus (MRSA) [16]. PVL activates caspase-9 and caspase-3, and induces human neutrophil apoptosis by the Bax-independent mitochondrial pathway [17]. The human complement receptor C5aR is the target of Luks-PV, mediating both immunotoxins and cytotoxicity. Its expression is lower in non-myeloid cells than in myeloid cells [18]. Luks-PV can binds specifically to human neutrophils and monocytes and induce C5aR-mediated pore formation [19]. It shows an anti-leukemia performance [1], and may serve as a potential C5aR-mediated small-molecule targeting drug.

MLL is an extremely aggressive malignant hematological disease with unique biological and clinical characteristics, and sometimes it is lethal due to the development of resistance and relapse with established therapies including hematopoietic stem cell transplantation (HSCT) [20]. HOXA9 has been shown to be over-expressed in approximately 50% of AML patients and is associated with an unfavorable prognosis [7, 21]. Autophagy is one of the two major clearance mechanisms that govern the regulation of intracellular proteolysis. It has been demonstrated previously that autophagy is suppressed in leukemia cells. For example, Bortezomib kills AML cells by inducing the degradation of FLT3-ITD oncprotein through an autophagy-dependent mechanism [11]. ATO and ATRA induce autophagy via the mTOR pathway in PML/RARa-rearranged APL cells, and autophagic degradation contributes significantly to the therapy-induced proteolysis of PML/RARA oncprotein [22]. The results of this study demonstrate that increased autophagic activities of THP-1 and primary AML cells due to the Luks-PV treatment result in HOXA9 degradation, which elucidates the molecular mechanisms by which Luks-PV triggers autophagy-mediated HOXA9 degradation in MLL-rearranged AML cells. Moreover, further studies are required to clarify which molecular players are involved in this Luks-PV-induced-autophagy switch in the context of MLL-rearranged AML cells.
Conclusion

This study provides a mechanism-based rationale for the study of Luks-PV in MLL-rearranged AML. Luks-PV is expected to be a potential C5aR-mediated small-molecule targeting drug for MLL-rearranged AML.

Abbreviations


Declarations

Ethics approval and consent to participate

Ethical approval to undertake this study was examined from the Research Ethics Committee, Anhui Medical University (No.20150150). A written informed consent for publication was obtained from the patient.

Consent for publication

Not applicable.

Availability of data and materials

All of the data are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 81572065). The funding enabled the purchase of all reagents and materials used in the study.

Authors' contributions

PQ, CF, KS, LS, YD WC, and LX performed all of the experiments. XW, XL, HL, ZS, and XM designed the study. PQ analysed the data, and wrote the paper. XM provided financial support and final approval of the manuscript. All authors approved and commented on the manuscript.
Acknowledgements

All of the authors would like to thank the patients and their families, as well as the nurses and doctors in our department.

References


**Figures**

**Figure 1**
HOXA9 expression in AML cells.

(A) HOXA9 expression in primary AML cells and a series of AML cell lines. (B) The Kaplan-Meier analysis of the OS of AML patients. The HOXA9 RNA-sequencing data and survival information were obtained from the TCGA database. High (n=67) or low (n= 68) HOXA9 mRNA expression. (C) The mean expression of HOXA9 was higher in primary AML patients (P<0.0001). (D) The mean expression of HOXA9 was higher in primary AML patients bearing MLL rearrangement (P<0.01).

Figure 2

Activation of autophagy by Luks-PV in AML cells.

(A-B) LC3-II accumulation after Luks-PV exposure. THP-1 was treated 2 hour with Luks-PV of different concentrations. Cells were treated with 2μM of Luks-PV for 2 hours. Actin and LC3 antibodies were used as reagents in WB analysis. (C) Luks-PV induced an active autophagic flux. THP-1 and primary AML cells were incubated 16 hours with 2μM of Luks-PV alone or its combination with 100nM BafA1. Anti-tubulin and anti-LC3 antibodies were adopted for Western blotting analysis. (D) TEM of autophagosomes. THP-1 was treated with or without 2μM of Luks-PV for 2 hours. (E) Immunofluorescence microscopy was used to analyze the active autophagic flux. THP-1 and primary AML cells were incubated with adenovirus
encoding both mRFP-GFP and LC3 protein for 24h and then with 2μM of Luks-PV for 16h. The Zeiss Apotome microscope was used to analyze the picture. At least 100 cells were counted for quantification of LC3-positive dots under the condition of no less than 3 independent experiments.

**Figure 3**

HOXA9 expression is down-regulated by Luks-PV and inhibits cell proliferation.
(A) THP-1 cell was treated with Luks-PV of different concentrations. Actin and HOXA9 antibodies were used for Western blotting analysis were used. (B) THP-1 cells were treated with 2μM of Luks-PV for different time and then analyzed by Western blotting. (C) The primary AML cells were incubated 16 hours with 2μM of Luks-PV and then analyzed by Western blotting. (D) HOXA9 mRNA expression in THP-1 and primary AML cells incubated 16 hours with 2μM of Luks-PV. (E) Edu assays of THP-1 cells treated with 2μM of Luks-PV for different time. 10000 cells were incubated 30 min in 1ml of reaction mixture. (F) HOXA9 down-regulation inhibited cell proliferation. THP-1 cells were treated with 2μM of Luks-PV for 8 to 24 hours. Actin and HOXA9 antibodies were employed in WB analysis. Cell proliferation was evaluated by Edu. Meanwhile, HOXA9 and actin protein expression levels were determined by densitometry Image J software. (G) CCK-8 assays of THP-1 cells treated with 2μM of Luks-PV or PBS. 5000 cells were seeded in 96-well plates. The absorbance at 3 to 24 hours was analyzed.

**Figure 4**

Luks-PV induces autophagy to reduce HOXA9 expression in AML cells.

(A) THP-1 cells were incubated 16 hours with 2μM of Luks-PV alone or its combination with 5nM of 3-MA. Anti-actin and anti-HOXA9 were applied in Western blotting analysis. (B) The primary AML samples were treated 16 hours with 2μM of Luks-PV alone or its combination with 5nM of 3-MA, followed by Western blotting analysis. (C) Colocalization of HOXA9 and LC3-positive structures. THP-1 and primary AML cells were incubated first with adenovirus encoding both mRFP-GFP and LC3 protein for 24h and then with 2μM of Luks-PV or PBS for 16h. The cells were analyzed by immunofluorescence with HOXA9 antibodies. The Zeiss Apotome microscope was used to analyze the picture.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- originalfulllengthgelandblotimages.docx