Small Peptide Targeting ANP32A as a Novel Strategy For Acute Myeloid Leukemia Therapy

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Research

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Abstract

**Background:** Clinic therapy of acute myeloid leukemia (AML) remains unsatisfactory that urges for development of novel strategy. Recent studies identified ANP32A as a novel biomarker of unfavorable outcome of leukemia, which promoted leukemogenesis by increasing H3 acetylation and the expression of lipid metabolism genes. It is of great significance to investigate whether targeting ANP32A is a novel strategy for leukemia therapy.

**Results:** To target ANP32A, we identified a peptide that competed with ANP32A to bind to histone 3 (termed as H3-binding peptide, H3BP). Disrupting ANP32A and H3 interaction by the overexpression of H3BP-GFP fusion protein mimicked the effect of ANP32A knockdown, impaired H3 acetylation on multiple locus of target genes, reduced proliferation, and caused apoptosis in leukemia cells. Furthermore, a synthesized membrane-penetrating peptide TAT-H3BP effectively entered into leukemia cells and reproduced such effect. In vivo, TAT-H3BP showed potent efficacy against leukemia: Intra-tumor injection of TAT-H3BP significantly reduced the volume of subcutaneous tumors in nude mice and recipient mice engrafted with TAT-H3BP-pretreated 6133/MPL W515L cells exhibited ameliorated leukemia burden and prolonged survival. Noticeably, TAT-H3BP efficiently suppressed proliferation and colony-forming unit of human primary AML cells without affecting normal cord blood cells.

**Conclusions:** Intervening the physical interaction of ANP32A with H3 impairs the oncogenicity of ANP32A and may be a promising therapeutic strategy against AML.

**Background**

Significant progress in genomic alterations, gene expression profiling, epigenetic modifications, and metabolic reprogramming help us understand the pathogenesis of acute myeloid leukemia (AML). However, roughly 40-45% overall survival (OS) after 5 years in 18–60 years adult patients and only approximately 10-20% surviving patients above the age of 60 years in current standard chemotherapy is far from satisfaction [1, 2]. Although hematopoietic stem cell transplantation (HSCT) yields a high rate of curability for AML, it is not usually applicable for majority of AML patients [3]. In addition, immunotherapy as a breaking-through therapy in hematopoietic malignancies is only effective in limited subtypes of leukemia [4]. Alternatively, many types of combinatory therapy was developed and currently investigated within clinical trials [5], which offered the promise of effective anti-leukemic activity and alleviating off-target effects in refractory/recurrent AML. In fact, the response to chemotherapy as well as long-term outcome of AML is affected by the heterogeneity of leukemia patients [4, 6]. Hence, massive efforts are required to discover more therapeutic targets and effective agents that pave the way for AML precision medicine in the foreseeable future [4].

The histone modifications, important features of epigenetic inheritance mainly including acetylation, methylation and phosphorylation, are key factors affecting the initiation, occurrence and development, even relapse/refractory of leukemia [7, 8]. Of which, the disorder of histone acetylation patterns is
reported closely associated with drug resistance in leukemic cells [9, 10] and intervention of histone acetylation is a potential therapeutic strategy against AML [11]. However, the role and mechanism of histone acetylation in the occurrence, development and treatment of leukemia have not been fully understood. Further understanding of histone acetylation is sure to desire to define optimal therapeutic strategies in targeting AML. For instance, recent work revealed ANP32A (acidic nuclear phosphoprotein 32 family member A) as an unfavorable prognostic factor in AML patients and ANP32A promotes leukemogenesis by enhancing histone 3 (H3) acetylation and upregulating the expression of lipid metabolism genes [12-14]. These findings attest ANP32A is an unfavorably prognostic factor of AML and it is of great significance to explore whether targeting ANP32A is a novel strategy against AML.

ANP32A is characterized by a N-terminal leucine-rich repeats (LRR) domain and a disordered C-terminal acidic tail. ANP32A physically interacted with unmodified N-terminal tail of H3 [15, 16]. The N-terminal LRR domain of ANP32A may involve its interaction with H3 considering the high homology with ANP32B [17]. ANP32A lacking 151-180 amino acids failed to suppress H3 acetylation in vitro [16]. These findings suggest that this small region may be required for ANP32A function and short peptide spanning this region may intervene ANP32A function. Thus we designed a competitive blocking strategy in this study and verified that the amino acid sequence spanning ANP32A 151-180 amino acids bound to H3 (termed as H3-binding peptide, H3BP). Both the overexpress of H3BP-GFP fusion protein or synthesized TAT-H3BP peptide competitively displaced ANP32A off H3. Furthermore, both H3BP-GFP and TAT-H3BP mimicked the effect of ANP32A knockdown in leukemia cells. Most importantly, TAT-H3BP showed potent efficacy against leukemia in human primary AML cells and two mouse models. Our work suggests that ANP32A is a potential target for leukemia therapy and disruption of ANP32A and H3 interaction through small peptide TAT-H3BP may be an effective anti-leukemia strategy.

Results

H3BP-GFP competes with ANP32A to bind to H3 and suppresses leukemia cell proliferation

ANP32A contributes to unfavorable outcomes for AML patients and is associated with significant histone H3 acetylation enrichment and the expression of lipid metabolism genes in leukemogenesis [12, 13]. Besides, sequence spanning 151-180 amino acids of ANP32A is indispensable for its ability to suppress H3 acetylation in vitro [15, 16]. It is possible that ANP32A interaction with H3 mediated by this sequence may be required for ANP32A function in promoting leukemogenesis. To characterize the interaction, we conducted H3 pulldown assay. H3 pulled down GFP fusion with LRR domain of ANP32A (LRR-GFP) or with sequence spanning 151-180 amino acids (termed as H3-binding peptide, H3BP) of ANP32A (H3BP-GFP), but not GFP alone (Figure 1A). Notably, H3BP-GFP overexpression effectively reduced the endogenous ANP32A binding to H3 (Figure 1B). To test the effect of H3BP-GFP on leukemia cell proliferation, we overexpressed GFP or H3BP-GFP under a doxycycline-inducible system. Apparently, doxycycline treatment (Dox+) efficiently induced GFP and H3BP-GFP expression in K562 and HL60 cells compared to doxycycline non-treatment (Dox-) (Figure 1C). In addition, GFP and H3BP-GFP cells exhibited comparable cell proliferation and colony-forming ability without doxycycline treatment (Figures 1D-F). In
contrast, doxycycline-induced H3BP-GFP expression significantly inhibited leukemia cell proliferation and colony formation compared to the doxycycline-treated GFP control cells (Figures 1D-F). Moreover, H3BP-GFP impaired cell cycle and caused apoptosis evidenced by downregulation of CCND1 and BCL2 and upregulation of BAK and BAX proteins in H3BP-GFP cells treated with doxycycline (Figure 1G). These results are consistent with phenotypes of ANP32A knockdown in leukemia cells in our previous study [12]. Our findings also suggest that ANP32A interaction with H3 may be indispensable for ANP32A function in leukemia. Short peptide disrupting the interaction may be an effective way to abolish ANP32A function.

**Synthetic TAT-H3BP impairs ANP32A interaction with H3 and inhibits leukemia cell proliferation in vitro**

To test whether short peptide H3BP interferes ANP32A function, we took advantage of membrane-penetrating peptide TAT derived from HIV and synthesized TAT-H3BP, TAT, and H3BP labeled with FITC. After extensive wash, flow cytometry and confocal experiments confirmed that TAT- and TAT-H3BP-treated K562 cells showed much higher FITC intensity than H3BP-treated cells (Figure 2A-B). K562 cells treated with TAT at 30 μM showed similar FITC intensity to TAT-H3BP treatment at 80 μM (Figure 2A). These results suggest an efficient entry of TAT and TAT-H3BP but not H3BP into K562 cells. Furthermore, the capability of H3 to pull down the exogenous ANP32A overexpressed in 293T cells or endogenous ANP32A in THP1 cells was dampened by TAT-H3BP with increase of TAT-H3BP concentration compared to TAT control (Figures 2C-D). These results suggest that the synthesized short peptide TAT-H3BP mimics H3BP-GFP to compete ANP32A binding to H3.

To further verify the inhibitory effect of the intracellular TAT-H3BP on leukemia cell proliferation, we pretreated K562, HL60, THP1 and 6133/MPL W515L leukemia cells with different concentrations of TAT-H3BP and calculated the half maximal inhibitory concentration (IC50). TAT-H3BP showed potent efficacy against leukemia cells with IC50 at micromole concentration and THP1 was the most sensitive cell line with IC50 around 40 μM (Figure 3A). Indeed, TAT-H3BP pretreatment significantly suppressed THP1 cell proliferation at 30 μM and K562, HL60 and 6133/MPL W515L cell proliferation at 50 μM over time whereas TAT or H3BP showed no significant effect (Figure 3B). Furthermore, TAT-H3BP pretreatment of leukemia cells reduced the colony formation in soft agar (Figures 3C-D). These cells showed reduced expression of CCND1 and BCL2 and increased expression of BAX and BAK in THP1 cells (Figure 3E). These observations demonstrate that the synthesized TAT-H3BP potently disrupts ANP32A interaction with H3, suppresses leukemia cell proliferation, and causes cell apoptosis in vitro.

**H3BP alters H3 acetylation and gene expression that mimics ANP32A knockdown in leukemia cells**

ANP32A has been shown to promote leukemogenesis by increasing H3 acetylation that subsequently alters the expression of lipid metabolism genes [12]. We further verified that H3BP intervened ANP32A-mediated H3 acetylation and altered gene expression. Doxycycline-induced H3BP-GFP expression caused a decrease of global H3 acetylation (Acetyl-H3) level (Figure 4A). Particularly, ChIP-qPCR showed that the enrichment of H3 acetylation on the promoters of lipid metabolism genes including APOC1, P2RX1, PCSK9, and LPPR3 was decreased (Figure 4B). These genes as key downstream targets of ANP32A
showed relatively high expression levels in leukemia patients compared with that in healthy donors [12, 18]. Consistently, the expression of these genes was downregulated upon H3BP-GFP expression in comparison with the GFP control expression (Figure 4C) while no alteration of these genes was observed without doxycycline treatment (Supplementary Figure 1). As expected, TAT-H3BP rather than TAT peptide exerted suppressive effect on the expression of these lipid metabolism genes in leukemia cells (Figure 4D). These results demonstrate that H3BP alters H3 acetylation and gene expression that mimics ANP32A downregulation in leukemia cells.

**TAT-H3BP exhibits potent efficacy against leukemia in vivo**

To test the in vivo function of H3BP against AML, we used two mouse models. Xenograft mouse model was established by injection of THP1 cells into BALB/c nude mice subcutaneously. When tumor formation was visible (around two weeks), tumor-bearing mice were randomly separated into three groups. Intra-tumor injection of PBS (Vehicle), TAT, or TAT-H3BP was performed as indicated (Figure 5A). TAT-H3BP injection resulted in a significant decrease in tumor volume compared with Vehicle or TAT (Figures 5B-C). TAT-H3BP injection also augmented apoptosis and impaired cell proliferation as evidenced by increased pyknotic nuclei (shrunken and dark) and reduced Ki-67 (Figures 5D-E). The apoptosis was also confirmed by Western blot that showed increase of cleaved Caspase 3 (C-Caspase 3), Bax, and decrease of Bcl2 (Figure 5F).

The in vivo function of H3BP was further tested in an AMKL mouse model. 6133/MPL W515L cells have been used to induce acute megakaryocyte leukemia (AMKL) in C57BL mice [19, 20]. We pretreated 6133/MPL W515L cells with PBS (Vehicle), TAT or TAT-H3BP for 12 hours and intravenously injected same number of live cells into sub-lethally irradiated C57BL recipient mice (Figure 6A). All three groups of mice showed splenomegaly and pale livers compared to irradiated control mice without injection, typical symptoms of leukemia (Supplementary Figure 2A). Among them, TAT-H3BP pretreatment group exhibited a reduced leukemia burden. Notably, TAT-H3BP pretreatment significantly delayed the onset time of AMKL and improved survival rate compared to Vehicle and TAT groups (Figure 6B). Complete blood count (CBC) test showed a tendency of reduction in red blood cells (RBC), hemoglobin (HGB), and platelets (PLT) from Vehicle and TAT groups compared to that from TAT-H3BP group (Supplementary Figure 2B). Consistently, the percentage of CD41+ cells in bone marrow (BM) and spleen from TAT-H3BP group were decreased compared to Vehicle and TAT groups (Figures 6C-D) while control mice showed low percentage as expected (Supplementary Figure 2C). Moreover, the pathology analysis revealed that TAT-H3BP pretreatment significantly reduced 6133/MPL W515L cell infiltration into liver and spleen, ameliorated agglomeration and myelofibrosis compared with Vehicle and TAT group (Figure 6E). Wright’s Giemsa staining showed multiplied karyocytes in peripheral blood (PB) harvested from Vehicle and TAT groups compared with TAT-H3BP group (Figure 6F). In general, phenotypes of TAT-H3BP group mice were relatively mild in comparison to irradiated control mice (Supplementary Figures 2D and E). Taken together, these two mouse models confirm the in vivo function of H3BP against leukemia.

**TAT-H3BP dampens proliferation and survival of human primary AML cells**
We further tested the efficacy of TAT-H3BP against leukemia in human primary AML cells. To exclude the non-specific effect and determine the optimal dose, we first treated mononuclear cells (MNCs) isolated from human cord blood (CB-MNC) with different concentrations of TAT, H3BP, or TAT-H3BP and perform colony-forming unit (CFU) assay. Apparently, TAT, H3BP, or TAT-H3BP did not affect CFU up to 50 μM. At 80 μM concentration, all three peptides impaired CFU indicating a non-specific effect (Supplementary Figure 3A-B). Thus, we used 50 μM of peptides to treat MNCs isolated from AML patient PB (PBMC) or bone marrow (BM) for proliferation and CFU assay. We confirmed the overexpression of ANP32A in many of human primary AML samples compared with normal control cells (Figure 7A). As expected, TAT-H3BP potently impaired cell proliferation and reduced CFU (Figures 7B-D and Supplementary Figures 4A-C). Noticeably, TAT-H3BP-pretreated cells produced relatively smaller colonies and give rise to less cells than TAT-pretreated cells (Figure 7E and Supplementary Figures 4B-D). Consistently, TAT-H3BP treatment effectively suppressed the expression of ANP32A target genes including APOC1, P2RX1, PCSK9 and LPPR3 (Figure 7F and Supplementary Figure 5). TAT-H3BP treatment also decreased the expression of survival genes (Figure 7G). In total, we tested the efficacy of TAT-H3BP in 11 cases of AML samples (3 BM and 8 PBMC samples) (Supplementary Table 1). Of which, cell proliferation was successfully performed in 6 cases (1 BM and 5 PBMC samples) and CFU assay in 4 cases (1 BM and 3 PBMC samples) with similar results. These observations demonstrate a potent efficacy of TAT-H3BP to interfere the function of ANP32A that is required for the survival of human primary AML cells.

**Discussion**

High expression of ANP32A has been reported to contribute to AML by altering H3 acetylation on key biological processes including lipid metabolism [12]. A recent report performing multivariable analysis to evaluate the prognostic significance of ANP32A expression verified ANP32A as an unfavorable prognostic biomarker in AML risk stratification and a potential therapeutic target for AML patients [13]. In this study, we presented evidence that damaging ANP32A function by disrupting the interaction of ANP32A and H3 via H3BP was an effective strategy against AML. Both H3BP-GFP fusion and synthesized TAT-H3BP effectively interfere the interaction of ANP32A and H3 (Figures 1A-B and 2C-D) in vitro and functionally mimicked ANP32A deficiency: H3BP-GFP fusion protein and synthesized TAT-H3BP impaired proliferation, reduced colony-forming ability, attenuated survival, decreased H3 acetylation, and inhibited the expression of multiple target genes in leukemia cells (Figures 1, 3 and 4). Furthermore, TAT-H3BP exhibited potent efficacy against leukemia in vivo: Intratumor injection of TAT-H3BP peptide suppressed the subcutaneous tumor formation of THP1 cells in BALB/c nude mice and TAT-H3BP pretreatment dampened the oncogenicity of 6133/MPL W515L cells to induce AMKL in C57BL mice (Figures 5 and 6). Most importantly, micromole concentration of TAT-H3BP peptide efficiently inhibited the proliferation and CFU of human primary AML cells (Figure 7 and Supplementary Figures 4-5). Our study demonstrates that intervention of ANP32A by disrupting physical interaction of ANP32A and H3 via H3BP may be an effective strategy for anti-leukemia therapy.

Our finding revealed that disruption of physical interaction of ANP32A and H3 by small molecule peptide TAT-H3BP mimics the effect of ANP32A on reducing H3 acetylation (Figure 8). It is worth mentioning that
the disorder of histone acetylation pattern is associated with drug resistance in leukemic cells [9, 21] and plays an important role in the occurrence and development of leukemia [22, 23]. Although both acetylation and deacetylation have been proposed as good strategies for leukemia intervention [11], the development and clinical application of histone deacetylases inhibitors are relatively successful. For example, the pan-HDAC inhibitor Pracinostat combined with DNA methyltransferase inhibitor 5-azacytidine improved efficacy in elderly patients with AML in a phase II study (NCT01912274) [24]. In contrast, the development of histone acetyltransferase (HAT) inhibitors is impeded for lack of cell permeability or specificity, including Bisubstrate inhibitor and some natural product HAT inhibitors [25]. Alternatively, small molecule inhibitors (OTX015 [26, 27], CPI-0610 and Molibresib, etc.) targeting histone acetylation readers like Bromodomain and Extra-Terminal (BET) have been tested [11]. However, their toxic effects, the mechanism of action, and true therapeutic scope remains unaddressed. Here, we present a novel strategy for intervention of histone acetylation by targeting ANP32A in AML. Although ANP32A has been reported to be an acetyltransferase inhibitor in vitro, our previous study has shown ANP32A function to promote histone acetylation in AML [12, 16]. The function of ANP32A in histone acetylation may be very dynamic and cell context depending on the cofactors recruited by ANP32A [16, 28, 29] (Figure 8). The intervention of histone acetylation through H3BP may spare global acetyltransferase activity and restrict its effect on ANP32A target genes, which potentially reduce the toxicity of acetylation inhibition. Thus, our study provides a novel way to targeting histone acetylation for anti-leukemia therapy.

We also found that the effect of H3BP on cell proliferation appears to correlate to the malignancy of cells. TAT-H3BP exhibited a significantly inhibitory effect on CFU of human primary AML cells. However, TAT-H3BP had little effect on CFU of MNCs isolated from normal cord blood at the same micromole concentration. The addiction of leukemia cell to ANP32A may provide a therapeutic window and rationale for the clinical application of H3BP. Apart from leukemia, researches also reported that ANP32A overexpression promoted cell proliferation in various solid tumors and correlated with the progression and metastasis of cancers including colorectal cancer (CRC), hepatocellular carcinoma (HCC), oral squamous cell carcinoma (OSCC), etc. [30-32]. Our preliminary investigations also found the indispensable requirement of ANP32A in many types of cancer cells (data not shown). It is worth testing whether H3BP may exhibit anti-tumor activity in these cancers.

Our study also uncovered that the effect of TAT-H3BP seems to correlate the expression level of ANP32A. Remarkable response of PBMCs to TAT-H3BP was observed in AML 60 patient with relatively high expression of ANP32A whereas relatively mild sensitivity was observed in AML 84 patient with relatively low expression of ANP32A (Figure 7 and Supplementary Figure 4). Noticeably, TAT-H3BP showed a significant inhibition in samples from relapsed or refractory elderly patients (Figure 7 and Supplementary Table 1) apart from newly diagnosed AML patients. Moreover, THP1 cells shows great sensitivity to H3BP treatment (Figure 3) in consistent to that ANP32A is required for mouse bone marrow cell immortalization by MLL-AF9 [12]. Furthermore, one study indicated an overexpression of ANP32A in MLL-translocation subgroups in AML patients than normal BM [13]. Consequently, it would be very interesting that exploration of the promising therapeutic potential of TAT-H3BP combined with other therapeutic agents
for relapsed and refractory leukemia and identifying the subtypes of AML sensitive to TAT-H3BP therapeutic strategy accurately in further investigations.

Conclusions

Our findings demonstrate that targeting ANP32A as an unfavorable outcome biomarker competitively through small molecule peptide H3BP is an effective strategy against AML. It may provide scientific basis for improvement of AML targeted therapy.

Materials And Methods

Cell cultures and animals

The leukemia cell lines (K562, HL60, THP1, 6133/MPL W515L) and HEK293T cells were cultured in complete 1640 RPMI medium (Gibco BRL, Grand Island, NY, USA) or Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 1% streptomycin and penicillin. All animal studies were approved by the Animal Care and Use Committees of College of Life Sciences of Wuhan University.

Human primary cells isolation and culture

Bone marrow (BM) or peripheral blood (PB) were obtained from leukemia patients or healthy donors. Isolation details of Mononuclear cells (MNCs) from samples are listed in Supplementary information.

Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) analysis was performed according to our previous report [33]. The primers used for the qRT-PCR analysis are listed in Supplementary information.

Western blotting and antibodies

Western blotting was performed according to a standard protocol [34]. Antibodies used in this study are listed in the Supplementary information.

Pulldown assay

HIS-tag pulldown assay was performed as previously described [35]. Briefly, whole cell lysates from THP1 cells, GFP or H3BP-GFPoverexpression 293T cells were used as the source of GFP, H3BP, or ANP32A protein. 6×HIS-tagged H3 was immobilized on Ni-NTA-Sepharose padding and then incubated with cell lysates and/or synthesized TAT orTAT-H3BP peptides. Then the beads were washed and the bound proteins were eluted and size-fractioned by SDS-PAGE for immunoblotting.

Colony-forming unit assay
Human primary cells were pretreated by peptides and colony-forming unit-granulocyte macrophage (CFU-GM) assay was performed using Methocult H4535 medium (STEMCELL, Technologies), following manufacturer’s instructions. PB (2´10^5) or BM/CB (5×10^4) mononuclear cells were seeded in Methocult medium supplemented with 10% FBS, 2% penicillin-streptomycin solution (PS), IL-3, SCF, and GM-CSF (PeproTech, USA). The cell colonies were counted under microscope after 14-21 days.

**Cell proliferation and colony formation in soft agar**

Cell proliferation and colony formation in soft agar was performed as previously described [14]. Briefly, cell proliferation was measured by counting cell numbers for 3-7 days. For colony formation analysis, 4×10^3 cells were mixed with pre-warmed soft agar suspension (0.35%) and the mixtures were plated on the top of an agar (1.0%) underlay agar. Cells were cultured for 14-21 days and the colony numbers were counted under microscope.

**Subcutaneous tumor formation assay**

For the subcutaneous tumor formation assay, THP1 cells were subcutaneously injected into BALB/c nude mice as previous description [20] and intra-tumor injection of PBS, TAT, or TAT-H3BP fusion protein. Details were listed in the Supplementary information.

**Confocal laser scanning fluorescence microscope assay**

TAT, H3BP or TAT-H3BP was added to exponentially growing cells for 12 hours [36]. Cells were fixed in methanol, permeabilized in 0.05 % Triton, incubated with primary antibody then secondary antibody. Cell nuclei was stained with DAPI. The glass slides were observed with confocal laser scanning microscopy.

**Chromatin immunoprecipitation (ChIP)-qPCR assay**

ChIP-qPCR assay was performed according to previously described [37]. Details and primer sequences in regions of target genes were listed in the Supplementary information.

**AMKL mouse model**

AMKL mouse experiment was performed as previously described [19, 38]. Briefly, C57BL mouse were sub-lethally irradiated (400 rad) and received 6133/MPL W515L cells (1×10^6 live cells/mouse) through tail intravenous injection. Details were listed in the Supplementary information.

**Statistical analysis**

All statistical analyses are reported as the means ± S.D. The significance of comparison analysis between two groups was evaluated using student's t test (unpaired, two-tail). * p < 0.05, ** p < 0.01, *** p < 0.001, NS indicates no significant difference. Statistic results of cell proliferation assay, qRT-PCR and flow cytometry are derived from the representative experiment with triplicates of at least three independent
experiments with similar results. Statistical results of immunoblotting are derived from the densitometric analysis of one representative blot of at least three independent experiments with similar results.

Detailed Materials and Methods were provided in the Supplementary information.

List Of Abbreviations

Acute myeloid leukemia (AML), H3-binding peptide (H3BP), Colony-forming unit (CFU), Overall survival (OS), Hematopoietic stem cell transplantation (HSCT), Histone 3 (H3), Leucine-rich repeats (LRR), H3 acetylation (Acetyl-H3), Acute megakaryocyte leukemia (AMKL), Complete blood count (CBC), Red blood cells (RBC), Hemoglobin (HGB), Platelets (PLT), Mononuclear cells (MNCs), Cord blood (CB), Peripheral Blood (PB), MNCs isolated from AML patient PB (PBMC), Bone marrow (BM), Colorectal cancer (CRC), Hepatocellular carcinoma (HCC), Oral squamous cell carcinoma (OSCC).

Declarations

Ethics approval and consent to participate

All experiments involving in human samples were approved by the Medical Ethics Committees of Union Hospital of Huazhong University of Science and Renmin Hospital of Wuhan University. Consent form was obtained from each AML patient and healthy donor. This study complies with the requirements of medical ethics.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflicts of interest in this article.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon request.

Authorship Contributions
Zan Huang and Manman Wang designed the research and drafted the manuscript. Manman Wang, Hao Guo, and Tan Zhang performed the experiments. Min Zhang and Min Peng provided patient samples. Xuechun Zhang, Xiyang Wang, and Hu Tao performed the statistical analysis. Zan Huang supervised the research. All authors approved the final manuscript.

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**Figures**
Figure 1

H3BP-GFP competes with ANP32A to bind to H3 and suppresses leukemia cell proliferation (A) Pulldown assays were performed to verify the binding capacity of LRR or H3BP to 6*HIS-tagged histone H3 (6*HIS-H3). Cell lysates from HEK293T cells transfected with GFP, LRR-GFP or H3BP-GFP expressing vector were used as prey and 6*HIS-H3 recombinant protein was used as bait. Immunoblotting was performed using antibodies detecting GFP or HIS-tag. Whole cell lysates were used as input. Independent experiments
were repeated three times. (B) Pulldown assays showed that the overexpression of H3BP-GFP competitively suppressed the endogenous ANP32A binding to 6*HIS-H3 but not GFP. Whole cell lysates were used as input. Independent experiments were repeated three times. (C-F) A Tet-On system expressing GFP or H3BP-GFP under doxycycline control was introduced into K562 and HL60 cell lines. GFP or H3BP-GFP expression was confirmed by Western blot in the absence (Dox-) or presence (Dox+) of doxycycline (C). HSC70 is used as a loading control. Cell proliferation (D) and colony formation in soft agar (E-F) were performed. (G) Cell cycle and apoptosis protein levels of CCND1, BCL2, BAX, BAK and HSC70 (loading control) were detected by Western blot. The data was representative results of three independent experiments with triplicates and presented as means ± SD. *, p < 0.05; **, p < 0.01 versus GFP.
Figure 2

Synthesized TAT-H3BP penetrates into leukemia cells and impairs ANP32A interaction with H3 (A-B) K562 cells were incubated with PBS (Vehicle) or FITC-labeled TAT (30 μM), H3BP, or -TAT-H3BP (80 μM) peptides for 12 hours followed by extensive wash and subjected to immunofluorescence with DAPI staining. Flow cytometry (A) analysis indicates similar fluorescent intensity of cells treated with TAT or TAT-H3BP as indicated but very low intensity for H3BP. (B) FITC and DAPI were detected by confocal laser
scanning fluorescence microscopy. Original magnification: 40×. (C) Pulldown assays were performed to verify the suppression of exogenous FLAG-ANP32A binding to 6×HIS-H3 by TAT-H3BP peptides but not TAT. (D) TAT-H3BP also suppressed the endogenous ANP32A binding to 6×HIS-H3 in THP1 cells. Whole cell lysates were used as input and the presence of proteins were detected by Western blot.
TAT-H3BP inhibits leukemia cell proliferation and induces apoptosis (A) Half maximal inhibitory concentration (IC50) test of TAT-H3BP peptide in K562, HL60, THP1, and 6133/MPL W515L cells. (B-D) THP1 cell were treated by PBS (Vehicle), TAT, H3BP or TAT-H3BP (30 μM) and the rest of AML cells were incubated with PBS (Vehicle), TAT, H3BP or TAT-H3BP (50 μM) for 12 hours followed by extensive wash and reseeded in fresh medium. The cell numbers were counted every day for cell proliferation assay (B). The treated cells also used for colony formation assay in soft agar (C) and the colony numbers were quantified (D). The data was representative results from three independent experiments with triplicates and presented as means ± SD. *, p < 0.05; **, p < 0.01, ***, p < 0.001 versus TAT. Original magnification: 4*. (E) THP1 cells treated with PBS (Vehicle), TAT, or TAT-H3BP (30 μM) were collected for western blot to measure protein levels of CCND1, BCL2, BAX, BAK and HSC70 (loading control).
Figure 4

H3BP alters H3 acetylation and gene expression that mimics ANP32A knockdown in leukemia cells (A-C). The total protein levels of acetyl-H3 and H3 in K562 or HL60 cells were determined by Western blot in the absence (-) or presence (+) of doxycycline treatment. HSC70 serves as a loading control (A). Doxycycline-induced cells (GFP+Dox or H3BP-GFP+Dox) were processed for Chromatin immunoprecipitation (ChIP) with antibody for the acetyl-H3 epitope. The enrichment of DNA of promoter regions of lipid metabolism...
genes (APOC1, P2RX1, PCSK9, and LPPR3) relative to the total input chromatin was assessed by qPCR (B). The relative mRNA levels of lipid metabolism genes were detected by qRT-PCR (C). (D) Leukemia cells were treated with TAT or TAT-H3BP for 12 hours followed by wash and culture in fresh medium for 48 hours. The relative mRNA level of lipid metabolism genes was detected by qRT-PCR. The data was representative results from three independent experiments with triplicates and presented as means ± SD. **, p < 0.01, ***, p < 0.001 versus GFP+Dox or TAT.

Figure 5
TAT-H3BP impairs THP1 cell subcutaneous tumor formation in nude mice (A) Flow chart illustrating subcutaneous tumor formation of THP1 cells in nude mice and treatment with intra-tumor injection of TAT-H3BP peptide. Intra-tumor injection of PBS (Vehicle), TAT, or TAT-H3BP (2 mg/mL in 0.1 mL) was performed on day 0, 3, 6 as indicated. (B) Tumor volume was recorded every 3 days. (C) Tumors were removed on day 10. (D-E) Tumor sections were stained with hematoxylin and eosin (H&E) (D) and Ki-67 (E). Original magnification: 10* and 40*. (F) Tumor lysates were used to measure Bcl2, C-Caspase3, and Bax. β-Actin was used as loading control.
Figure 6

TAT-H3BP attenuated the oncogenicity of 6133/MPL W515L cells in mice (A) The flow diagram of AMKL mouse model. 6133/MPL W515L cells were incubated with PBS (Vehicle), TAT, or TAT-H3BP (50 μM) for 12 hours. 1×10⁶ live cells/mouse were injected into sub-lethally irradiated recipients to establish mouse AMKL model (n=7). (B) Kaplan-Meier analysis of the survival rate of AMKL mice. NS: no significance when Vehicle vs TAT; p=0.0155 when TAT-H3BP vs TAT. (C-D) The bone marrow and spleen cells were collected for CD41 staining. The percentage of CD41+ cells (C) was analyzed by flow cytometry. (D) Statistical analysis of the expression of CD41 in BM and spleen cells (n=3). The data are presented as the means ± S.D. *, p < 0.05; **, p < 0.01 versus TAT. (E) The bone marrow (BM), liver and spleen were stained with hematoxylin-eosin (H&E). The arrows indicate the leukemia cells infiltrated and agglomerated in the liver. (F) Wright's Giemsa-stained of peripheral blood (PB). Original magnification: 40*, 20*, 10*, and 4* as indicated.
Figure 7

TAT-H3BP dampens proliferation and survival of human primary AML cells. (A) The relative mRNA level of ANP32A in MNCs isolated from bone marrow (BM) (AML 19, 20 and 33, n=3) or peripheral blood (PBMCs) (AML, n=8) of AML patients or healthy volunteer donors (Normal, n=6) was detected by qRT-PCR. The data are presented as the means ± SD of triplicates. (B-E) MNCs isolated from AML patient PB or BM pretreated with PBS (Vehicle), TAT, or TAT-H3BP (50 μM) were reseeded in fresh medium. The cell...
numbers were counted on each day for cell proliferation assay (B). The pretreated cells were also used for CFU (C). The colony numbers (D) and the final total number of cells (E) were quantified. Original magnification: 4* and 10*. (F-G) The relative mRNA level of lipid metabolism genes (F) including APOC1, P2RX1, and LPPR3 or survival genes (G) (BCL2, BCL-XL, BCL-W, MCL1, and BFL) were detected by qRT-PCR in TAT or TAT-H3BP treated PBMCs. The data was presented as means ± S.D. from triplicates. *, p < 0.05; **, p < 0.01, ***, p < 0.001 versus TAT.

Figure 8
Schematic illustration of H3BP targeting ANP32A as a novel strategy for leukemia therapy. The abnormally high expression of ANP32A induces disordered H3 acetylation and gene expression on lipid metabolism genes and promotes leukemia (left). Disruption of physical interaction of ANP32A and H3 TAT-H3BP alleviates H3 acetylation and the expression of lipid metabolism genes and results in apoptosis or death of leukemia cell (right).

Supplementary Files
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