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Alternative splicing analysis showed the splicing factor polypyrimidine tract-binding protein 1 as a potential target in acute myeloid leukemia therapy

Qiao-Xia ZHANG[1,#], Yu-Ming PAN[1,#], Hong-Li XIAO[2], Na AN[1], Si-Si DENG[1], Xin DU[1,*]

[1]Shenzhen Bone Marrow Transplantation Public Service Platform, Shenzhen Institute of Hematology, Shenzhen Second People's Hospital, First Affiliated Hospital of Shenzhen University, Shenzhen, China; [2]Department of Hematology, People's Hospital of Shenzhen Baoan District, Shenzhen, China

**Correspondence: duxingz@medmail.com.cn*
[#]Contributed equally to this work.

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Alternative splicing (AS) is a universal post-transcriptional regulation process in cells, and increasing evidences have validated its crucial role in tumors. We collected AS event, gene expression, and clinical data of 178 AML patients from The Cancer Genome Atlas (TCGA) project. More than 1,000 AS events were found associated with overall survival (OS), and alternate promoter (AP) events were the most significant. The expression of the KIAA0930 transcript was the most significantly different AS event selected from AP events and significantly correlated with the expression of the splicing factor (SF) polypyrimidine tract-binding protein 1 (PTBP1). Then, the roles of PTBP1 on AS of the KIAA0930 and the proliferation of AML cells were confirmed. KIAA0930 variant 1 (KIAA0930-1) was upregulated and variant 2 (KIAA0930-2) downregulated with knockdown PTBP1 expression of AML cells by specific shRNA. A low level of PTBP1 can decrease the proliferation ability of AML cells. In conclusion, the results showed that PTBP1 might be a potential target for AML therapy.

Key words: alternative splicing, acute myeloid leukemia, polypyrimidine tract binding protein 1

Acute myeloid leukemia is a clonal malignant proliferative disease of myeloid precursors in the hematopoietic system [1]. It is a highly heterogeneous disease group, which can be transformed from hematopoietic progenitor cells at different stages of differentiation and development with diverse genetic abnormalities [2, 3]. In China, approximately 75,300 people are diagnosed with acute myeloid leukemia (AML), and over 53,400 AML-related deaths are reported annually [4]. Systematic studies of the genomic landscape of AML, such as deep sequencing of AML by The Cancer Genome Atlas (TCGA), have generated a catalog of leukemia genes that is increasingly comprehensive [5]. With omics sequencing, AML has emerged as a complex, dynamic disease. There are many leukemia genes, most of which are infrequently mutated or abnormally expressed, and patients typically have more than one driver mutation [6–8]. These discoveries have revealed the biological intricacies of AML.

Alternative splicing (AS) is the primary driving force for generating diverse proteins and is important for functional regulation in eukaryotic cells [9, 10]. Aberrant splicing, including changes in the usage of annotated transcript isoforms, the increased use of aberrant splicing, and somatic mutations impacting splicing changes, has been revealed in many cancers [11–14]. Some studies have used TCGA splicing data to investigate AS events and their clinical value in several cancers, such as bladder cancer [15–17], gastrointestinal neoplasms [18–21], lung cancer [22–24], prostate cancer [25, 26], and ovarian cancer [27, 28]. Recently, two articles published the analysis results of AML splicing events based on TCGA data. Xie et al. reported that low-risk splicing events better predicted patients' survival [29], while Chen et al. considered that the risk scoring model based on all AS event types was the most efficient [30].

In this study, we analyzed AS events of 178 patients obtained from TCGA project again. The results showed that alternate promoter (AP) events were most significantly correlated with AML survival. The splicing network of splicing events and factors revealed 4 interesting interactions, such as the positive correlation between AS events in *KIAA0930* and *PTBP1*, which were verified by experiments of AML cells. The role of *PTBP1* on the viability of AML cells was confirmed.

Materials and methods

Data collection and screening for prognostic AS events. The Percent Spliced In (PSI) value of the tumor samples from AML patients was downloaded from the publicly available TCGA SpliceSeq database (http://bioinformatics.mdanderson.org/TCGASpliceSeq/). PSI refers to the ratio of reads in existing transcripts to the total reads that contain AS events [31]. AS events with a PSI value ≥0.1 standard deviation among samples and ≥0 in more than 75% of samples were considered in our subsequent analysis. The original RNA sequencing level 3 data (HTSeq-count) and clinical data were obtained from TCGA database (https://portal.gdc.cancer.gov/), and the count data were converted into TPM (for gene expression) for the subsequent analysis. Patients with overall survival (OS) time of less than 30 days were eliminated to reduce the deviation.

To obtain AS events that are significantly related to prognosis, the relationship between AS events and OS was analyzed by univariate Cox proportional hazards regression using the coxph function with the survival package in R. AS events with a p-value <0.05 were considered to be associated with prognosis. The UpSetR plot was used to visualize the intersections of the seven AS types and the host genes of prognosis-related AS events. Differences in the hazard ratios (HRs) of the different AS types were assessed using ANOVA and the SNK-q test.

Establishment of prognostic prediction models based on the AS event signature. Different AS types plus all prognosis-associated AS events were used to build LASSO Cox regression models using the glmnet package in R. The PSI value and OS time were used as input, and the optimal AS signature and lambda value were determined using 10-fold cross-validation during training. In total, eight prediction models were established, and the performances of these models were evaluated by time-dependent receiver operator characteristic (ROC) curves and the area under the curve (AUC) using the time ROC package in R. The risk score of each patient determined by each model was calculated with the following formula:

$$\text{Risk score} = \sum_{i=1}^{n} (\text{PSI}i \times \text{c}i)$$

where c_i represents the risk coefficient of the i-th AS events in the corresponding model, PSI_i represents the PSI value of the i-th AS events in the corresponding patient, and n represents the total number of optimal AS events selected by the model.

For each model, the maximally selected rank statistics method in the R package "survminer" was used to calculate the threshold risk score to group high- and low-risk patients. Patients with a risk score that was higher than the threshold were placed into the high-risk group, and others were placed into the low-risk group. Next, a Kaplan-Meier curve was drawn for each model, and a log-rank test was used to test whether there was a significant difference in survival time (p-value) between the two groups of patients. Additionally, multivariate Cox regression was used to evaluate whether the risk score determined by each prediction model could be an independent prognostic factor together with other clinical factors, including age, sex, and cytogenetic risk category.

Correlation analysis of splicing factors and key AS events in AML progression. We downloaded human splicing factor (SF) genes from the SpliceAid 2 database (www.intronic.it/spliceaid.html) and used the Spearman correlation coefficient to evaluate the correlation between the expression of SF genes and the PSI of key AS events. p-values were corrected for multiple tests using the Benjamini-Hochberg false discovery rate method. An SF-PSI relationship with $|R| \geq 0.4$ and an adjusted p-value <0.05 was considered a potential regulatory relationship. Finally, protein-protein interaction networks were built using proteins translated by transcripts with AS events and proteins coded by known AML driver genes in the high- and low-risk groups. Cytoscape software was used to visualize the network, hub genes were identified by the degree algorithm in the cytoHubba plugin, and submodules of the network were identified using the MCODE module of Cytoscape.

Cell culture. K562, OCI-AML2, and MOLM-13 cell lines were purchased from the Shanghai Institute of Life Sciences (Shanghai, China). The cells were maintained in Iscove's Modified Dulbecco's Medium (Gibco, CA, USA) (K562 and OCI-AML2 cells) or Roswell Park Memorial Institute medium 1640 (Gibco) (MOLM-13 cells) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin in 25 cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2. Primary AML cells were derived from the bone marrow mononuclear cells (BMMNCs) of AML patients and cultured in CellGenix® GMP SCGM medium supplemented with IL-3 (60 ng/ml, StemCell Technologies, Canada), TPO (100 ng/ml, Novoprotein, China), SCF (300 ng/ml, StemCell Technologies), and Flt3-L (300 ng/ml, Peprotech, USA). The culture conditions were 95% air and 5% CO_2, 37 °C, and saturated humidity. Informed consent was obtained from patients. This study was approved by the medical ethics committee of Shenzhen Second People's Hospital. The clinical characteristics of AML patients are listed in Table 1.

Lentiviral transfection. To knock down *PTBP1*, shRNA oligonucleotides (PTBP1-shRNA: CAACGTCAAGTACAACAAT, Ctrl-shRNA: TTCTCCGAACGTGTCACGT) were designed and linked to lentivirus vector GV112 (GENECHEM, China). For the packaged lentivirus, the 293T cells were transfected with mixed PTBP1-shRNA or Ctrl-shRNA vector and the packaging plasmid. After 48 h, the supernatant was collected and filtrated through a 0.45 μm filter. Virus infection was performed according to the manufacturer's instructions. Virus-infected cells were screened with 2 μg/ml puromycin (InvivoGen, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using RNAiso Plus (TaKaRa) and reverse transcribed into cDNA using NovoScript™ Plus cDNA Synthesis SuperMix (Novoprotein, China), according to the manufacturer's protocol. PCR was carried out with EmeraldAmp™ PCR Master Mix (TaKaRa), and the products were detected by agarose gel electrophoresis. The primer sequences were: *KIAA0930-1*, 5'-CTCTCAGGCT-GCTGCTGAG -3', and 5'-TCAGGCAGACGCTCTCCTC-3'; *KIAA0930-2*, 5'-TCTTAGCCTGCGCCGCGA GGT-3', and 5'-CGATGTCAGGGTCTCCCAGGC-3'; *GAPDH* (internal control), upstream 5'-GATATTGTTGCCATCAATGAC-3', and 5'-TTGATTTTGGAGGGAT CTCG-3'.

Cell viability assay. Cell viability was evaluated using Cell Counting Kit (CCK, TransGen Biotech, China). Treated cells in 96-well plates were incubated with 10 μl CCK reagent in each well at 37 °C for 2 h, and the absorbance was measured at 450 nm.

Cell cycle assay. The cells were harvested and washed twice with ice-cold PBS, fixed in 70% ethanol, and stored at –20 °C for a minimum of 1 h. Subsequently, cells were washed again with ice-cold PBS and resuspended in 500 μl PI/RNase Staining Buffer (BD Biosciences, USA). The cell cycle was evaluated with a FACScan using an excitation laser set at 480 nm and a detection wavelength of 575 nm. A minimum of 100,000 cells was analyzed.

Western blot analysis. Total protein was extracted using RIPA lysis buffer (Thermo Fisher, USA) containing protease inhibitor cocktail (Roche Applied Science). Protein concentration was measured with a BCA protein assay kit (Thermo Fisher, USA). Protein samples (20 mg/lane) were resolved on a 10% bis-Tris gel (SDS-PAGE) and transferred onto 0.22 μm PVDF membranes (Millipore, USA). After blotting, the membranes were blocked with 3% BSA in TBS-Tween for 2 h and incubated with anti-PTBP1 (Abcam, ab134950), or anti-β-Actin (Cell Signaling Technology, USA, #4967) antibodies overnight at 4 °C. The membranes were washed three times with TBS supplemented with 0.1% Tween 20, hybridized with HRP-labelled anti-rabbit IgG (Cell Signaling Technology, #7074S), and then visualized with a chemiluminescence detection reagent (Thermo Fisher, USA).

Table 1. Clinical characteristics of AML patients.

#Patient Identifier	Patient-1	Patient-2
Cancer type	AML	AML
Sex	Female	Male
FAB	M5	M5
Diagnosis age	35	34
Bone marrow blast percentage	89%	85%
Cytogenetics	45, XX-7[20]	47, XY, inv(5) (q14q35),+8[19]/46,XY[1]
Mutations	Mutant for PTPN11 and KRAS	FLT3 ITD+; Mutant for WT1

Results

Identification of overall survival-related AS events in AML. In general, we collected AS event, gene expression, and clinical data of 178 AML patients from TCGA project. After excluding patients with OS time of less than 30 days, 169 patients and 99,968 AS events were used to analyze OS-related AS events using univariate Cox regression. A total of 1,023 AS events from 720 genes, including 55 Alternate Acceptors (AAs) in 54 genes, 51 Alternate Donors (ADs) in 50 genes, 382 Alternate Promoters (APs) in 230 genes, 215 Alternate Terminators (ATs) in 117 genes, 235 Exon Skips (ESs) in 218 genes, 5 Mutually Exclusive Exons (MEs) in 5 genes and 86 Retained Introns (RIs) in 85 genes, were found to be significantly associated with OS ($p<0.05$) (Figure 1A). The most frequent AS type was APs, while the rarest AS type was MEs. The intersection visualization plot involving seven types of OS-related AS events and their host genes was generated using the UpSetR package. Most genes carry one AS event, and there were 32 genes with two different AS events, and one gene with three (Figure 1B). Cox regression analysis showed that the HRs of OS-related AS events were significantly different between the 7 types (Figure 1C, $p<0.001$, ME events were too few to participate in the comparison). Furthermore, the HRs were compared between the pairwise AS types, and we found that the HRs of APs and ATs were significantly different from those of AAs, ESs, and RIs (SNK-q test, $p<0.001$, Figure 1C).

AP events were most significantly correlated with AML survival. Based on the 7 types of AS events and all AS events, the LASSO Cox expression was used to find those most significantly associated with survival. The optimal AS signature was obtained by 10 times cross-validation. The risk score of each AML patient was obtained according to the optimal AS signature, and then patients were divided into high-risk and low-risk groups based on the calculated threshold value (see Methods).

We subsequently plotted the Kaplan-Meier curves of the high- and low-risk patients and performed the log-rank test to compare their survival rates. The survival rate in the high-risk group was significantly lower than that in the low-risk one based on the 7 types of AS events and all AS events (p-value <0.001) (Figures 2A–2H). The time-dependent ROC curves of the models showed that the AUC values of all types of AS events except for the ME type (AUC=0.693) were higher than 0.8, among which the AP model showed the best predictive performance for AML prognosis (AUC=0.965) (Figure 2I).

To evaluate whether the risk score determined by the AS signature-based analysis was an independent prognostic factor for AML, univariate Cox regression was used to calculate the HRs of the risk scores and other clinical features (such as age and sex) and to explore their correlation with OS. Interestingly, the risk scores of the models were all significantly correlated with OS, as shown in Table 2. In addition,

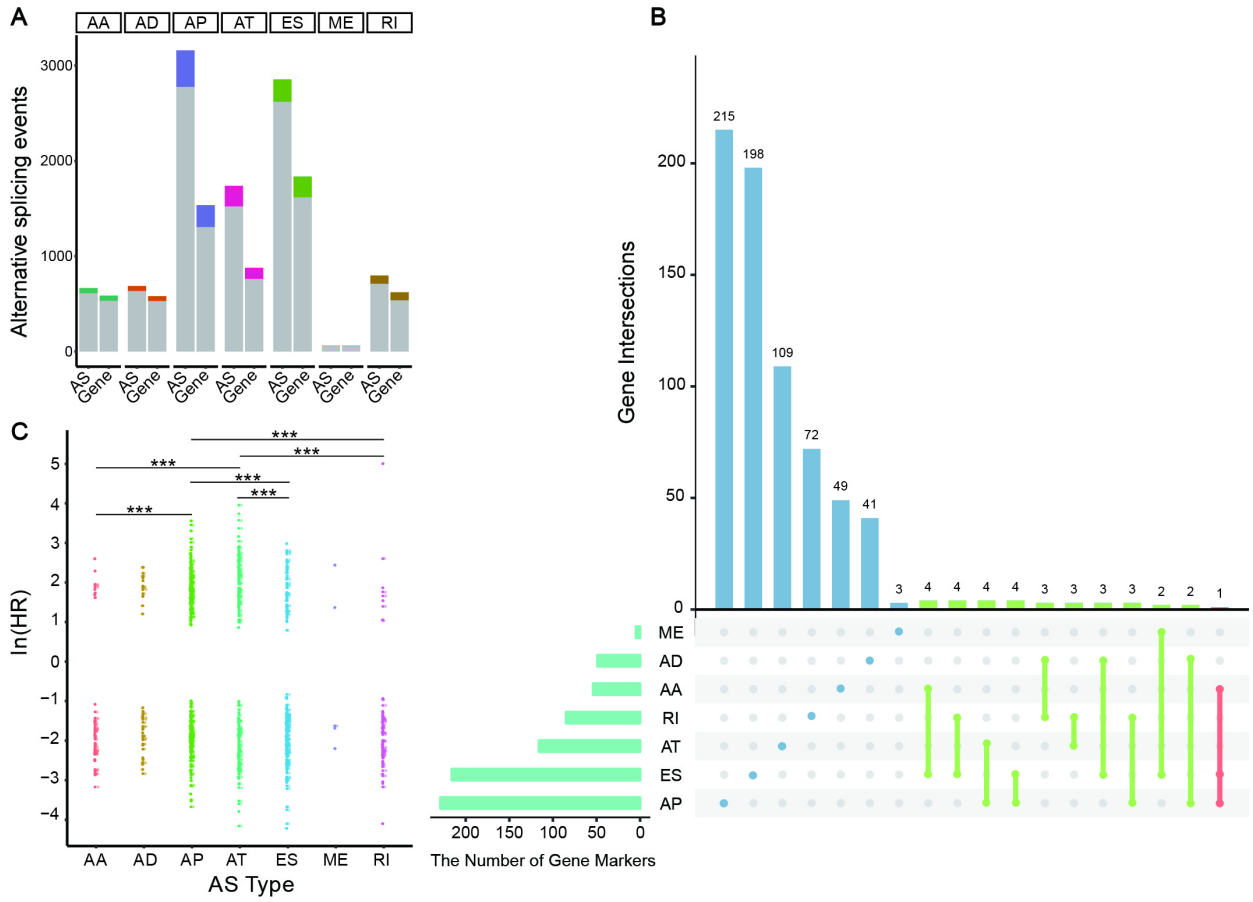

Figure 1. Overall survival (OS)-related alternative splicing (AS) events in AML. A) Overview of the seven types of OS-related AS events and AS host genes in this study. The grey bars represent the AS events or AS host genes that are not related to OS, and other color bars represent the OS-related AS events or AS host genes filtered by univariate Cox regression. B) UpSetR plot displaying gene interactions between the seven types of OS-related AS events in AML. Genes with different numbers of types of OS-related AS events are marked with different colors: blue dots represent genes with only one type of AS event, and green and red dots represent genes with two and three types of AS events, respectively. C) Differences in the HRs of OS-related AS events between different AS types. Multiple comparisons were conducted using ANOVA ($p<0.05$), and pairwise comparisons were conducted using the SNK-q test. Each point represents an AS event. ***$p<0.001$

age, neoadjuvant treatment history, and bone marrow baseline percentage were also found to be significantly correlated with OS ($p<0.05$). When the above three features were used as covariates for further multivariate Cox regression analysis, all model risk scores remained independent prognostic factors for AML patients, as shown in Table 3.

Regulatory network of key AS events in high- and low-risk patients. We also downloaded the mRNA expression data of SF genes from TCGA and analyzed their correlation with the signature PSI of the eight prognostic models. According to $|R|\geq 0.4$ and an adjusted p-value <0.05, a total of 33 significant SF-AS relationship pairs were identified. Specifically, the scatter diagram shows that in the AP model, the PSIs of three key AS events and the expression of their SFs, namely *ATF7IP-RBM4*, *KIAA0930-PCBP1*, and *KIAA0930-PTBP1*, exhibited a positive correlation with SF gene expression, while one, namely, *FBXO34-TRA2B*, exhibited a negative correlation (Figures 3A–3D). These results revealed the regulatory relationship between SFs and corresponding AS events. To screen the SF-AS regulatory relationships with clinical value, we studied differences in the PSIs of the above-mentioned AS events between the high- and low-risk patients and then obtained the prognosis-associated AS events. The results indicated that AS events in *KIAA0930* had a significantly higher PSI in the high-risk patients than in the low-risk patients ($p<0.001$, paired t-test, Figure 3E). The PSI of *FBXO34* AS events was also significantly higher in the high-risk patients ($p<0.01$) but with a weak statistical effect compared to that of *KIAA0930*. No significant difference in the PSI of the *ATF71P* AS event was found between the two groups. Therefore, the *KIAA0930* AS event regulated by PCBP1 and PTBP1 was considered prognostic SF-AS relationships for further study.

To confirm the effect of the *PTBP1* gene on AS events of *KIAA0930*, leukemia cells were transfected with lentivirus containing PTBP1-shRNA or Ctrl-shRNA. Compared with

Table 2. Analysis of prognostic factors, including risk scores, determined by eight models using univariate Cox regression.

Variable	p-value	Coefficient	HR	90% CI (HR)
Age	0.007	0.630	1.878	1.190–2.964
Sex	0.882	–0.031	0.969	0.643–1.462
Stage:	0.383	0.056	1.058	0.932–1.201
M0-M7				
Cytogenetic risk category:	0.950	0.009	1.009	0.766–1.329
Favorable Intermediate/Normal Poor				
Neoadjuvant treatment history	0.027	0.528	1.695	1.063–2.702
Abnormal lymphocyte percent	0.628	–0.010	0.990	0.953–1.030
Blast cell outcome percent	0.501	0.004	1.004	0.993–1.015
Bone marrow band cell percent	0.659	0.020	1.020	0.935–1.113
Bone marrow basophil percent	0.036	0.146	1.157	1.010–1.326
Bone marrow eosinophil percent	0.868	–0.010	0.990	0.884–1.109
Bone marrow metamyelocyte value	0.411	0.088	1.091	0.886–1.345
Bone marrow myelocyte percent	0.632	–0.032	0.968	0.848–1.105
Bone marrow neutrophil percent	0.330	–0.012	0.989	0.966–1.012
Bone marrow prolymphocyte percent	0.255	–0.595	0.552	0.198–1.535
Bone marrow promyelocyte percent	0.886	–0.006	0.994	0.919–1.075
Hemoglobin specified value	0.092	–0.051	0.950	0.895–1.008
Leukocyte unspecified value	0.591	0.003	1.003	0.993–1.013
Monocyte percent	0.501	0.005	1.005	0.990–1.021
Platelet count	0.381	0.003	1.003	0.996–1.011
Molecular abnormality test:	0.053	–0.123	0.885	0.781–1.001
NPM1 positive IDH positive PML-RARA positive				
AA	<0.001	1.631	5.108	3.797–6.871
AD	<0.001	1.852	6.374	4.466–9.098
All	<0.001	3.870	47.935	23.064–99.628
AP	<0.001	2.463	11.739	7.476–18.434
AT	<0.001	1.822	6.186	4.512–8.482
ES	<0.001	5.369	214.725	59.828–770.645
ME	<0.001	1.309	3.703	2.091–6.555
RI	<0.001	2.538	12.655	7.365–21.745

Table 3. Prognostic factor analysis for AML patients using multivariate Cox regression.

Variable	p-value	Coefficient	HR	90% CI (HR)
AA	<0.001	1.645	5.181	3.799–7.065
AD	<0.001	1.860	6.427	4.438–9.307
All	<0.001	3.822	45.706	21.664–96.426
AP	<0.001	2.454	11.638	7.377–18.360
AT	<0.001	1.805	6.080	4.404–8.393
ES	<0.001	5.292	198.835	54.384–726.966
ME	<0.001	1.527	4.605	2.500–8.483
RI	<0.001	2.564	12.985	7.393–22.807

Ctrl-shRNA, PTBP1-shRNA was highly efficient at reducing *PTBP1* expression in K562, OCI-AML2, and MOLM-13 cells. In cells with PTBP1-shRNA, the expression of a splicing variant of the *KIAA0930* gene, *KIAA0930-1*, was markedly increased, while another variant *KIAA0930-2* expression was remarkably reduced (Figure 3F).

Decreased *PTBP1* expression inhibits the proliferation of leukemia cells. *PTBP1* is reported that promotes the proliferation of various cancer, such as lung carcinoma [32],

colorectal cancer [33], glioblastoma [34]. However, it has also been reported to have anti-tumor activity [35, 36]. Through our analysis, PTBP1 is possible to be involved in the abnormal splicing events of *KIAA0930,* which is associated with the prognosis of AML patients. But *PTBP1* expression level was not correlated with the survival of AML patients based on the same data from TCGA. To determine the role of the *PTBP1* gene, we then analyzed its effect on proliferation and the cell

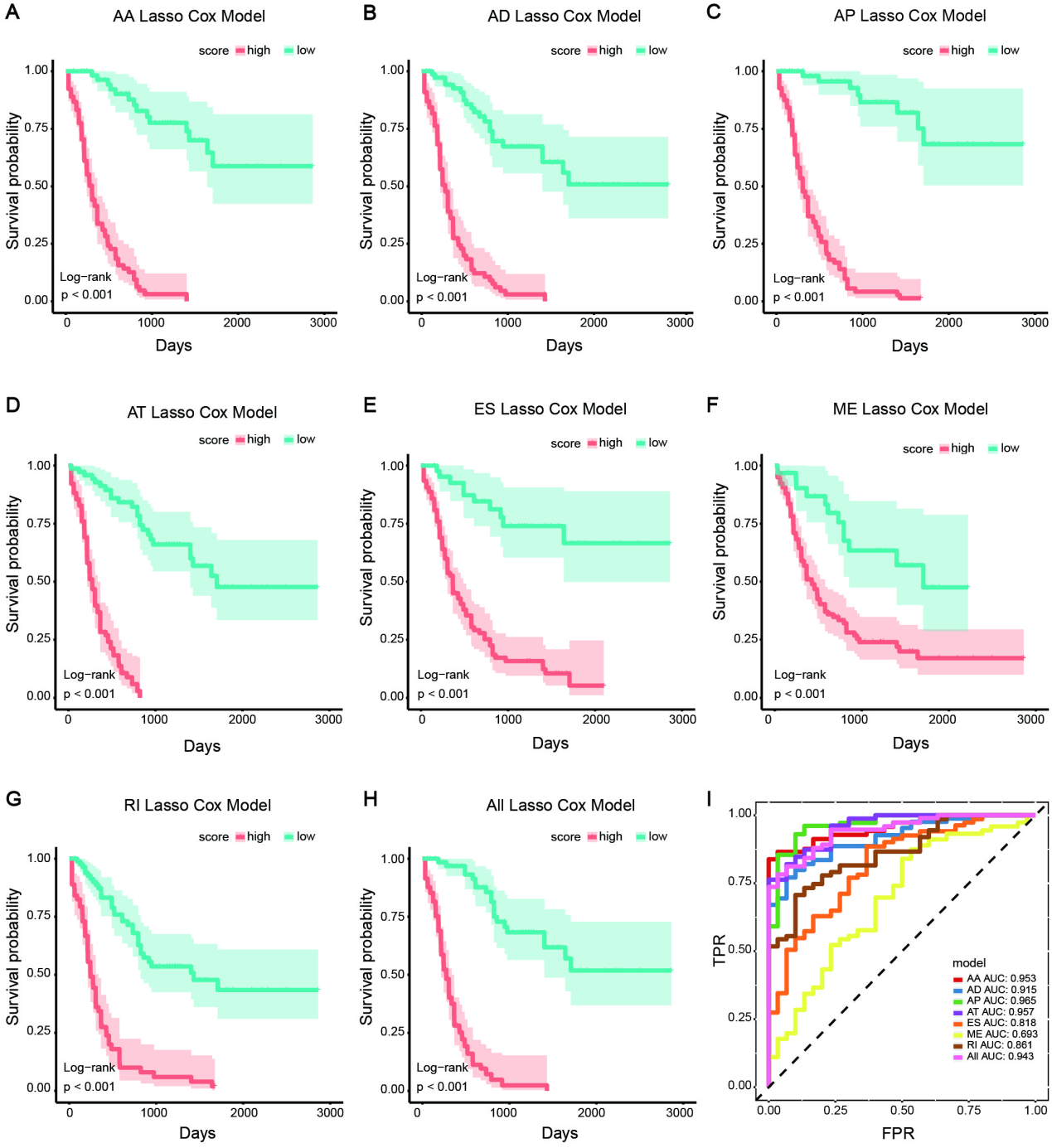

Figure 2. PSI prognostic model of AML patients based on the prognosis-related alternative splicing (AS) signature. Patients were divided into low- and high-risk groups according to the risk score thresholds. A–H) Kaplan-Meier plots of patients in the high- and low-risk groups determined by the prognosis-related AS events of eight models: A) Alternate Acceptor site (AA); B) Alternate Donor site (AD); C) Alternate Promoter (AP); D) Alternate Terminator (AT); E) Exon Skip (ES); F) Mutually Exclusive Exon (ME); G) Retained Intron (RI); and H) All. I) ROC curves of all 8 prognosis-related AS signature-based prediction models for AML patients. AML, acute myeloid leukemia. Abbreviation: ROC-receiver operating characteristic

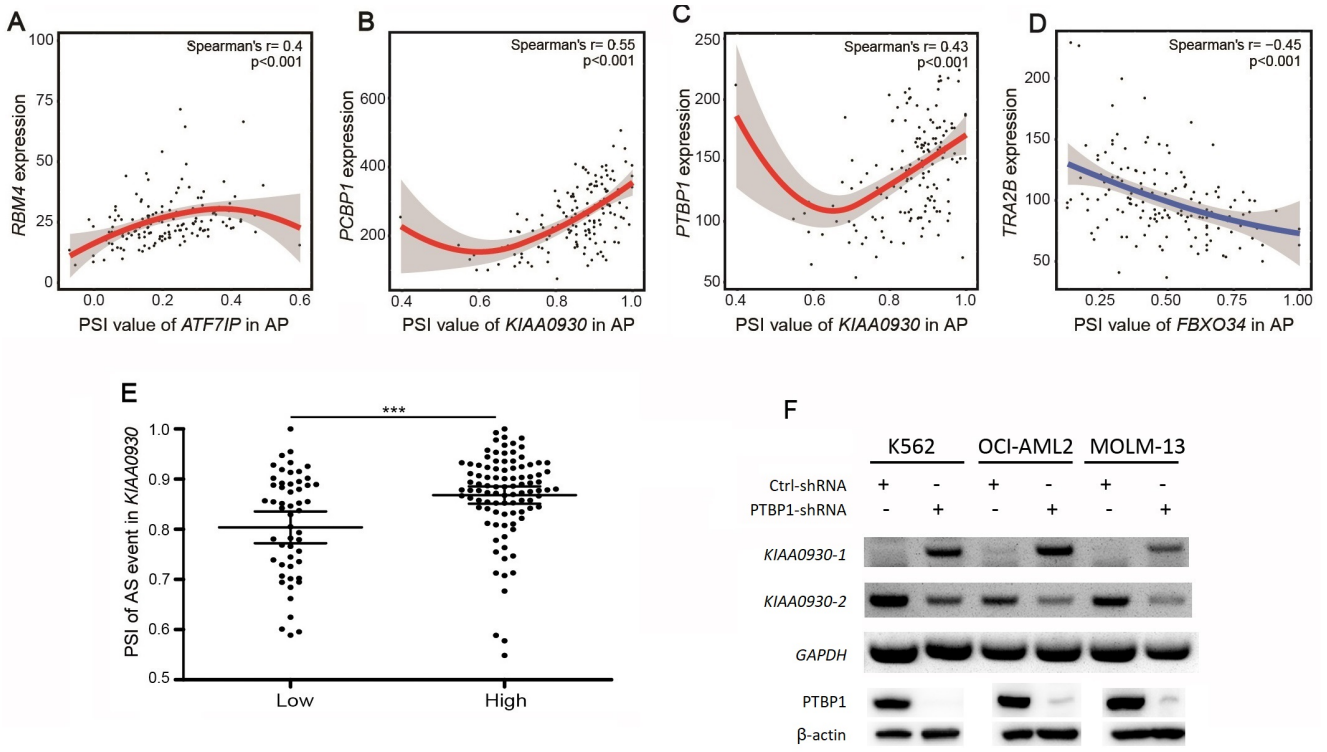

Figure 3. A representative Splicing Factor-Percent Spliced In (SF-PSI) relationship in the Alternate Promoter (AP) model. A–D) Representative correlation curves between SF gene expression and the PSI value of alternative splicing (AS) events in the AP model. Strong correlations of SFs and AS events were revealed by a correlation coefficient |R|≥0.4 and a Benjamini-Hochberg-adjusted p-value <0.05. R>0 indicates a positive correlation; R<0 indicates a negative correlation. (A) *RBM4* showed a positive correlation with *ATF7IP*. B, C) *PCBP1* (B) and *PTBP1* (C) showed a positive correlation with *KIAA0930*. D) *TRA2B* showed a negative correlation with *FBXO34*. E) The PSI of AS events in *KIAA0930* was significantly different between low- and high-risk patients as determined by the AP model. F) The AS of *KIAA0930* was affected by *PTBP1* knockdown.

cycle in AML cells. The proliferation of K562, OCI-AML2, and MOLM-13 cells was inhibited by *PTBP1* knockdown (Figures 4A–4C). Flow cytometry analysis showed that G1/S phase arrest occurred in these cells (Figures 4D–4F). Two cases of bone marrow mononuclear cells from AML patients were cultured *in vitro* and treated with lentivirus Ctrl-shRNA or PTBP1-shRNA. The cellularity of primary AML cells decreased significantly 6 days after being infected with lentivirus PTBP1-shRNA (Figure 4G). Similar to those cell lines, *PTBP1* knockdown also caused G1/S phase arrest of primary AML cells from patient-1 (Figure 4I).

Discussion

Abnormal regulation and modification during the processing of mRNA are important drivers of tumorigenesis [37]. As a post-transcriptional modification of most eukaryotic organisms, AS dysregulation is believed to play an important role in oncogenesis and tumor progression [38–40]. With the development of high-throughput RNA-seq technology and the advantage of genomic studies in large-scale studies, researchers can now study all AS events of cancer and explore their clinical value, which promotes our understanding of the roles and clinical value of AS in tumors.

By analyzing AS events and their PSI levels of 178 AML tumor samples from TCGA SpliceSeq database [41], which integrates potential alternative splicing events across 33 types of cancer using RNA-seq data [31], we obtained nearly ten thousand AS events, and more than one thousand AS events in 720 genes were found to be significantly associated with survival, indicating that alternative splicing is a common process and closely linked to prognosis in AML. The AP type was the most common and prognostic of the seven AS types, which differs from that in solid cancers, such as liver, colon, and breast cancers, in which the most common AS type is ES [15, 42, 43]. APs usually alter the translation efficiency of their host genes, while ESs can delete a portion of the pre-mature mRNA to affect protein diversity by altering the amino acid sequences of the translated products. This finding indicates that the regulation of AS differs between blood and solid cancers, although more evidence in other blood cancers, such as acute lymphocytic leukemia, is needed.

Due to the complex interaction network and heterogeneity of AML tumorigenesis, using a single molecular marker to build a prognostic prediction model is usually less

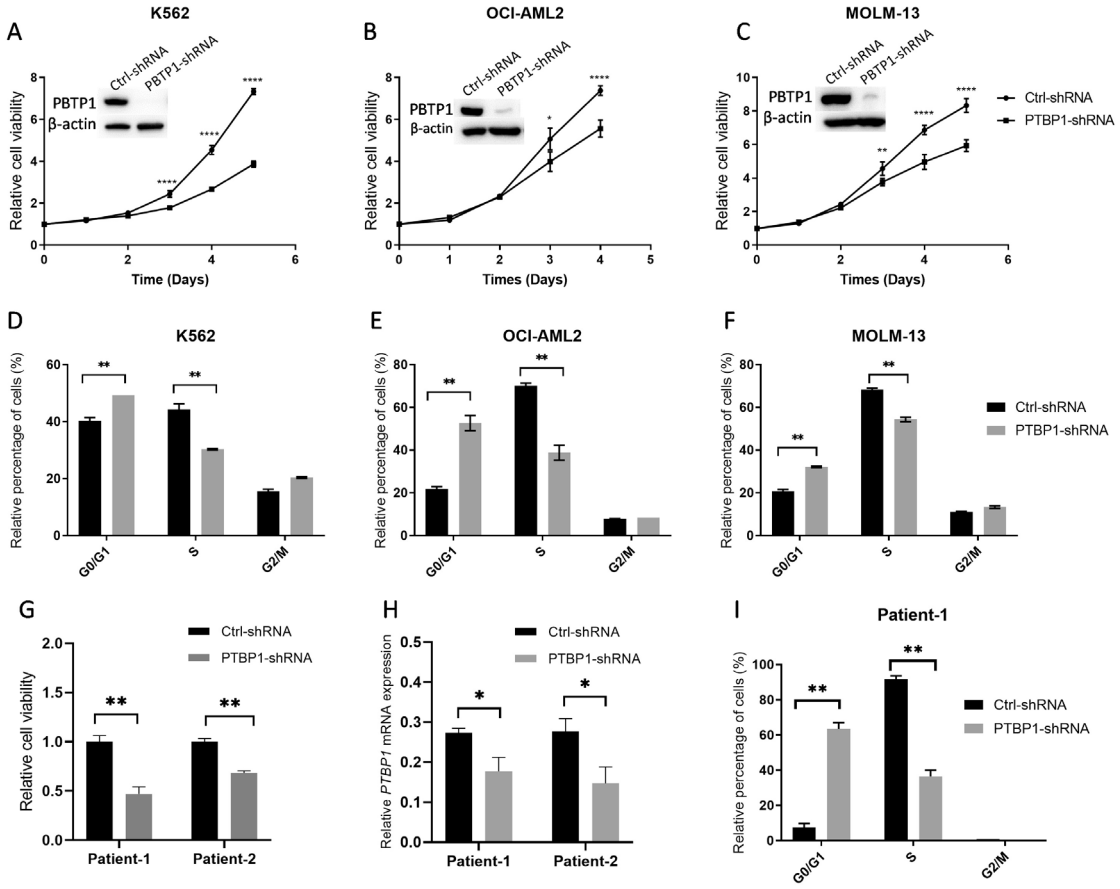

Figure 4. Reduced *PTBP1* expression inhibited cell viability and cell cycle progression in leukemia cells. A–C) The line graph shows the relative cell viability 7 days after transfected lentivirus Ctrl-shRNA or PTBP1-shRNA. Western blot shows the expression levels of PTBP1 and β-Actin in the designated cells. (D–F) The distribution of cells during the cell cycle. G, H) The relative cell viability (G) and *PTBP1* mRNA level (H) of the BMMCs from 2 AML patients 6 days after transfected lentivirus Ctrl-shRNA or PTBP1-shRNA. (I) The distribution of BMMCs from Patient-1 during the cell cycle. The experiment was performed independently three times. Data are presented as the mean ± s.d. *p<0.05, **p<0.01, ****p<0.0001, as determined by unpaired two-tailed Student's t-test.

efficient than integrating multiple biomarkers into an aggregated model [44]. Chen et al. reported that the risk scoring model based on all AS event types was the most efficient in identifying the prognosis for AML patients, with the area under the curve 0.852, 0.935, 0.955 at 1-, 3-, 5-year, respectively. However, in another paper, Xie et al. reported that the predictive efficacy of the prognostic model based on AT type events was best with the area under the curve at 0.781. In this study, our multiple AS event prognostic model based on AP type events can achieve robust and good performance, with the area under the curve at 0.965, which indicates high sensitivity and specificity in prognosis monitoring and the important roles of AP type events in cancer progression. The performance of the AP type model was more efficient than any other model, even when all AS events were used ("All" type), further explaining the important function of APs and the effect of changes in protein abundance on the prognosis of AML. Additionally, using the LASSO method in Cox regression analysis significantly optimized the composition of the AS signature, which made their application easier, and subsequent mechanistic research can be more targeted.

The splicing network of splicing events and factors revealed some interesting interactions, such as the positive correlation between *PTBP1* and AP events in *KIAA0930*. There are various reports and even contradictory results about the effect of *PTBP1* on the occurrence and development of various tumors [45]. High expression of the *PTBP1* gene in colon cancer and lung cancer can enhance tumor migration and invasion [32, 46, 47]. PTBP1 interacts with PKM2 and promotes the oncogenesis of anaplastic large cell lymphoma [48], bladder cancer [49], and NPM1-mutated AML [50]. In multiple myeloma, PTBP1 promotes cell proliferation by upregulating the expression of *c-Myc* [51]. The PTBP1-dependent regulation of *USP5* alternative RNA splicing plays a role in glioblastoma tumorigenesis [52]. However, *PTBP1* has also been reported to exert anti-tumor effects. The overexpression of *PTBP1* attenuates the invasion of the colorectal cancer cell line HT29 induced by mesenchymal stem cells [53]. As a

direct regulator of *BCL-X* AS, the overexpression of *PTBP1* promotes the generation of the pro-apoptotic *BCL-Xs* splice variant, and the depletion of *PTBP1* enhances the splicing of the anti-apoptotic *BCL-XL* variant [36].

There was little research on the function of *PTBP1* in AML. In addition to the above-mentioned NPM1-mutated AML, there was another study that reported that a circRNA, circMYBL2, regulates *FLT3* translation by recruiting PTBP1 to promote FLT3-ITD AML progression [54]. However, our results showed not only the proliferation of FLT3-ITD$^+$ cells MOLM-13 but also that of FLT3-ITD$^-$ cells K562, were inhibited by *PTBP1* expression knockdown. This suggested that there were other ways of *PTBP1* affecting AML cells, besides *FLT3* translation.

Our results found that *PTBP1* could determine the alternative promoter of *KIAA0930*, which was closely related to the prognosis of AML patients. These suggested that the function of *PTBP1* in promoting AML cell proliferation might be related to its regulation of *KIAA0930* variants. Different splicing subtypes of *KIAA0930* may have different biological functions, similar to the *Bcl-x* gene. Of course, this needs to be supported by direct evidence.

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