Original Article

Acidic leucine-rich nuclear phosphoprotein-32A expression contributes to adverse outcome in acute myeloid leukemia

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Background: Acidic leucine-rich nuclear phosphoprotein-32A (*ANP32A*) is a novel regulator of histone H3 acetylation and promotes leukemogenesis in acute myeloid leukemia (AML). However, its prognostic value in AML remains unclear.

Methods: In this study, we evaluated the prognostic significance of *ANP32A* expression using two independent large cohorts of cytogenetically normal AML (CN-AML) patients. Multivariable analysis in CN-AML group was also presented. Based on the *ANP32A* expression, its related genes, dysregulation of pathways, interaction network analysis between microRNAs and target genes, as well as methylation analysis were performed to unveil the complex functions behind *ANP32A*.

Results: Here we demonstrated overexpression of *ANP32A* was notably associated with unfavorable outcome in two independent cohorts of CN-AML patients (OS: P=0.012, EFS: P=0.005, n=185; OS: P=0.041, n=232), as well as in European Leukemia Net (ELN) Intermediate-I group (OS: P=0.018, EFS: P=0.045, n=115), National Comprehensive Cancer Network (NCCN) Intermediate Risk AML group (OS: P=0.048, EFS: P=0.039, n=225), and non-M3 AML group (OS: P=0.034, EFS: P=0.011, n=435). Multivariable analysis further validated *ANP32A* as a high-risk factor in CN-AML group. Multi-omics analysis presented overexpression of *ANP32A* was associated with aberrant expression of oncogenes and tumor suppressor, up/down-regulation of metabolic and immune-related pathways, dysregulation of microRNAs, and hypomethylation on CpG island and 1st Exon regions.

Conclusions: We proved *ANP32A* as a novel, potential unfavorable prognosticator and therapeutic target for AML.

Keywords: Acidic leucine-rich nuclear phosphoprotein-32A (*ANP32A*); expression; prognostic biomarker; cytogenetically normal acute myeloid leukemia (CN-AML)

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Introduction

Acute myeloid leukemia (AML) is a clinically disparate hematopoietic malignancy, characterized by predominant fatality and high heterogeneity (1). It is the most common type of adult acute leukemia with an annual incidence of 3–8/10^5 (2). To date, cytogenetic analysis has been one of the most useful prognostic methods to classify AML

patients into different prognostic risk groups (favorable, intermediate, unfavorable) (3). The cytogenetically normal AML (CN-AML), representing 40–50% AML patients, is the largest subset of de-novo AML cases (4,5). Although CN-AML constitutes the main body of the intermediate-risk group, yet different molecular subtypes of CN-AML diverged in transcription and DNA methylation profiles (6). Moreover, due to the wide variety of clinical outcome, the prognosis of CN-AML cannot be predicted solely by cytogenetics (7). Intriguingly, without any microscopically detectable chromosome abnormalities in its leukemic blast, CN-AML harbour aberrantly expressed genes and microRNAs, mutations, epigenetic changes that can be used as potential prognostic markers (8). It is now recognized that *WT1*, *ASXL1*, *DNMT3A*, *FLT3-ITD* and *TET2* mutations presence in CN-AML represent a subgroup of patients with unfavorable prognosis, while *NPM1* and *CEBPA* are associated with favorable prognosis (9-15). Furthermore, unfavorable prognostic factors include high expression of *CPT1A*, *ATP1B1*, *RUNX1*, *MAPKBP1*, *MAP7*, *ERG*, *DNMT3B*, miR-3151, miR-155, etc. (16,17). As leukemogenic mechanisms of CN-AML still remains unclear, it is vital to continue identifying new molecular biomarkers of clinical significance.

Acidic leucine-rich nuclear phosphoprotein-32A (*ANP32A*), also known as PP32, is an acidic leucine-rich nuclear phosphoprotein which has shown to be divergently expressed in normal tissues as well as in certain types of tumor cells (18). On one hand, *ANP32A* correlates with cancer progression and metastasis, demonstrated to be a candidate biomarker in patients with pancreatic tumor, colorectal cancer, hepatocellular carcinoma, oral squamous cell carcinoma, etc. (19,20). On the other hand, *ANP32A* is a tumor suppressor in prostate cancer, non-small cell lung cancer and breast cancer by stimulating apoptosis (18,21,22). Recent study indicates that *ANP32A* is a novel regulator of histone H3 acetylation and promotes leukemogenesis in AML, suggesting the expression of *ANP32A* might be related to the prognosis of CN-AML patients (23). Therefore, the focus on the exact functions of *ANP32A* in hematopoietic malignancy might lead us to the identification of *ANP32A* as a potential molecular marker of clinical significance in CN-AML.

Here, we presented *ANP32A*^{high} as an unfavorable prognostic biomarker for CN-AML. We also explored *ANP32A*-associated genomic and epigenomic patterns to further elucidate its function. Our study represented direct evidences for *ANP32A* being a prognostic biomarker in AML risk stratification and a potential therapeutic target for patients with AML.

Methods

Patients and treatment

The study was approved by the local institutional review boards. In accordance with the Declaration of Helsinki, all patients provided written informed consent. The treatment of all patients was uniformly under the protocols of Dutch-Belgian Cooperative Trial Group of Hematology-Oncology (details see in http://www.hovon.nl).

The first cohort of this study was derived from a whole AML cohort, containing 185 primarily untreated CN-AML patients, all diagnosed at Erasmus University Medical Center in Rotterdam from 1990 to 2008. The cohort age ranged from 16 to 60 years old and the median age was 47 years old. All samples were collected at the time of diagnosis and contained more than 80% blast cells after thawing (24). At least 20 metaphases from bone marrow (BM) samples were examined by conventional cytogenetic methods to diagnose normal karyotype. The presence of *FLT3-ITD*, *FLT3-TKD* and the mutations of *IDH1*, *IDH2*, *NPM1*, *CEBPA*, *K-RAS*, *N-RAS* were all assessed by reverse transcription-polymerase chain reaction (RT-PCR) assays. The clinical, cytogenetic, molecular and gene expression data of these AML cases can be downloaded from the Gene Expression Omnibus (accession number: GSE6891, details see in http://www.ncbi.nlm.nih.gov/geo) (25).

The second cohort of this study contained 162 CN-AML patients with uniform treatment was used to validate findings in the first cohort. All patients received intensive double-induction and consolidation-chemotherapy in multicenter AMLCG-1999 trial from 1999 to 2003. The cohort age ranged from 17 to 83 years old and the median age was 57.5 years old. The gene expression data can be downloaded publicly (accession number: GSE12417) (26).

Microarray analysis

Gene expression was obtained from published microarray data using Affymetrix Human Genome 133 plus 2.0 as well as U133A Gene Chips (accession number: GSE9476, GSE1159, GSE6891, GSE12417) (24,25,27,28). Seventy-three CN-AML patients with mRNA, microRNA and methylation data were derived from The Cancer Genome Atlas (TCGA) (29). Design, data quality control

and normalization of microarray experiments were in accordance with the standard Affymetrix protocols. Expression of mRNA and microRNA were obtained by high throughout transcriptome sequencing (RNA-seq), while methylation data was obtained by Illumina Infinium 450K BeadChips. The expression level of *ANP32A* was standardized in normally distributed. Then the appropriate cut-off subdivision value was compared by the 4 quartiles of 185 CN-AML patients, which the median value showed evident distinction (*Figure S1A*,*B*,*C*). Therefore, median value of *ANP32A* expression was used to classify patients into $ANP32A^{high}$ and $ANP32A^{low}$ groups. The expression levels of *ERG*, *DNMT3A*, *BAALC*, *WT1* and other genes were obtained using the same strategy.

Statistical analysis

Overall survival (OS) was defined as the time from diagnosis date to death by any causes. Event free survival (EFS) was defined as the time from diagnosis date to the removal from the study because of the end of complete remission (CR), relapse or death (the censorship equals to 1 when death event was observed). The Kaplan-Meier method and the log-rank test were used to evaluate and validate the association between *ANP32A* expression and OS, EFS. The fisher exact and the Wilcoxon rank-sum test, analyzing categorical and continuous variables respectively, were used to estimate the association between *ANP32A* expression levels and the patients' clinical, molecular characteristics. Multivariate Cox proportional hazard models were used to assess the effect of *ANP32A* expression on OS and EFS with other known risk factors in presence. Except ANP32A and other 3 clinical & demographical variables were chosen, two gene mutations were further brought into our investigation which were shown to have a strong association with AML (30,31). Student's *t*-test as well as multiple hypothesis correction (False Discovery Rate, FDR) were used to identify discrepancy in gene expression, microRNA expression, and DNA methylation profiles between $ANP32A^{high}$ and $ANP32A^{low}$ groups. All analyses were performed using R3.2.3 and its related software packages.

Results

Overexpression of ANP32A in AML patients

ANP32A expression were analyzed in two microarray assays, including BM and peripheral blood samples (PB) in AML patients and healthy donors. The expression of *ANP32A* was remarkably high in AML BM than in Normal BM (*Figure 1A*, P=0.049, 7 AML BM samples *vs.* 10 normal BM samples, GEO accession number *GSE9476*). Overexpression of *ANP32A* was further validated by PB samples in the same microarray assay (*Figure 1B*, P=0.013, 19 AML PB samples *vs.* 10 normal PB samples). Furthermore, overexpression of *ANP32A* was found in different subgroups in AML patients than normal BM, including 116 CN-AML, 22 AML1-ETO, 17 MLL-translocation, and 19 CBFβ-MYH11 (*Figure 1C*, GEO accession number *GSE1159*). These results indicated that *ANP32A* was evidently overexpressed in AML patients.

Clinical and molecular characteristics between $ANP32A^{low}$ and $ANP32A^{high}$ group in AML patients

In a cohort of 185 CN-AML patients derived from GSE6891 assay (435 AML patients, no M3), $ANP32A^{high}$ group tends to have less FAB-M2 subtype patients (P=0.0007) and more FAB-M5 patients (P=0.0004). *ANP32A* was significantly overexpressed in patients with *FLT3-ITD* (P=0.029), while no significant differences between $ANP32A^{low}$ and $ANP32A^{high}$ group were observed in *CEBPA*, *NPM1* and *N-ras/K-ras* mutation groups (P=0.206, P=0.211, and P=0.817, respectively). Besides, $ANP32A^{high}$ group was more likely to have a higher expression of *MAPKBP1*, *RUNX1*, and *TCF4*, which were known adverse prognostic biomarkers in AML. Furthermore, patients in $ANP32A^{high}$ group was more likely to relapse than those in $ANP32A^{low}$ group (*Table 1*).

Overexpression of ANP32A was associated with unfavorable outcome in AML patients

As patients in $ANP32A^{high}$ group was more likely to relapse than those in $ANP32A^{low}$ group in the cohort of 185 CN-AML patients (*Table 1*, P=0.021), the survival analysis was further carried out in a whole cohort of CN-AML patients (n=185) and ELN Intermediate-I patients (n=115). $ANP32A^{high}$ group showed significantly shorter OS and EFS in CN-AML patients' cohort (*Figure 2A*,*B*, $ANP32A^{high}$ *vs.* $ANP32A^{low}$: median OS: 11.9 *vs.* 37.4 months, P=0.012; median EFS: 8.4 *vs.* 18.3 months, P=0.005) as well as in ELN Intermediate-I patients' cohort (*Figure 2C*,*D*, $ANP32A^{high}$ *vs.* $ANP32A^{low}$: median OS: 8.5 *vs.* 30.4 months, P=0.018; median EFS: 6.9 *vs.* 13.0 months, P=0.045). To further validate the prognostic significance of *ANP32A* expression, multivariable analysis was carried out in these

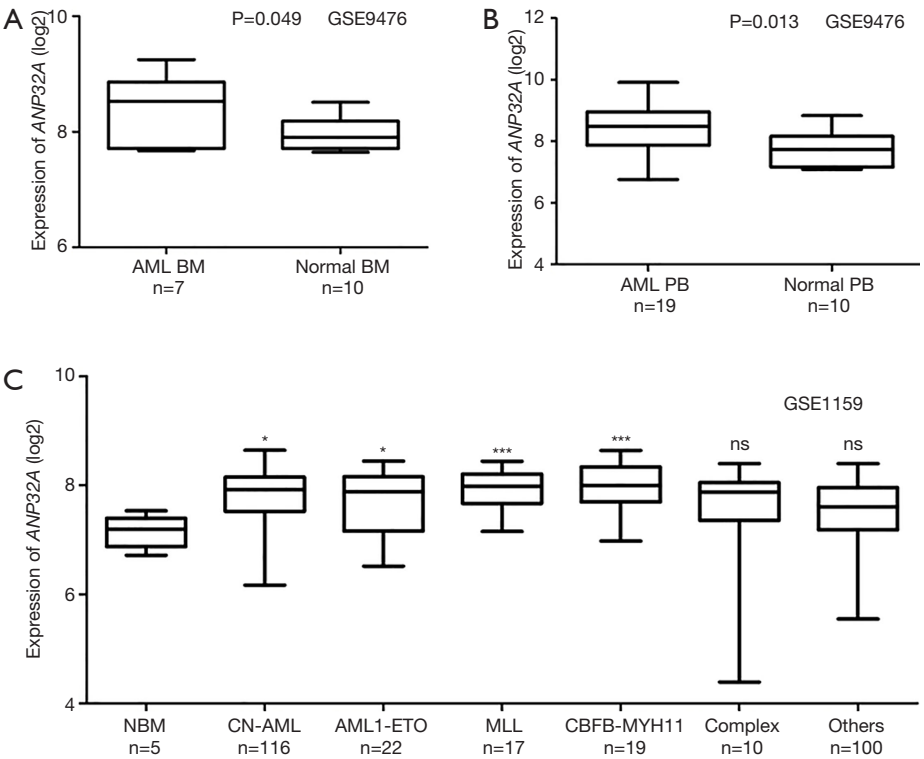

Figure 1 Different expression of *ANP32A*. (A) AML bone marrow samples (n=7) *vs.* normal bone marrow samples (n=10); (B) AML peripheral blood samples (n=19) *vs.* normal peripheral blood samples (n=10); (C) normal bone marrow samples (n=10), CN-AML bone marrow samples (n=116), AML1-ETO bone marrow samples (n=22), MLL-translocation bone marrow samples (n=17), CBFβ-MYH11 bone marrow samples (n=19), complex karyotype bone marrow samples (n=10), other karyotype bone marrow samples (n=100). *, 0.01<P<0.05; ***, P<0.001. AML, acute myeloid leukemia; CN-AML, cytogenetically normal AML.

two cohorts. It showed that *ANP32A*^{high} group had 0.47 times higher risks on EFS and 0.46 times higher risks on OS in CN-AML patients' cohort (*Table 2*, P=0.0088 and P=0.016, respectively). The presence of *FLT3-ITD* was another adverse factor (P=0.009 in EFS and P=0.019 in OS). As for the ELN Intermediate-I patients' cohort, *ANP32A*^{high} group had 0.45 times higher risks on EFS and 0.48 times higher risks on OS (*Table 2*, P=0.041 and P=0.051, respectively), indicating *ANP32A* expression had more prognostic significance in CN-AML cohort. In order to further investigate the prognostic value of *ANP32A* expression, survival analysis was also performed in 435 non-M3 AML patients and 255 NCCN Intermediate Risk AML patients. The results indicated a remarkable shorter OS and EFS of *ANP32A*^{high} group in 435 non-M3 AML patients' cohort (*Figure 2E,F*, *ANP32A*^{high} *vs.* *ANP32A*^{low}: median OS: 16.9 *vs.* 24.2 months, P=0.034; median EFS: 9.0 *vs.* 13.1 months, P=0.011) and 255 NCCN Intermediate Risk AML patients' cohort (*Figure 2G,H*, *ANP32A*^{high} *vs.* *ANP32A*^{low}: median OS: 16.5 *vs.* 24.7 months, P=0.048; median EFS: 9.0 *vs.* 14.1 months, P=0.039). However, the multivariate analysis in these two cohorts didn't confirm the independent prognostic value of ANP32A (see *Table S1*), further indicating the prognostic significance of ANP32A in CN-AML. To validate the unfavorable outcome associated with *ANP32A*^{high} in AML patients, another survival analysis was conducted in another independent cohort of 232 CN-AML patients derived from GSE12417 assay. *ANP32A*^{high} group showed significantly shorter OS in CN-AML patient cohort (*Figure S2*, *ANP32A*^{high} *vs.* *ANP32A*^{low}: median OS: 9.4 *vs.* 16.7 months, P=0.041).

Genome-wide gene-expression profiles associated with ANP32A expression

In order to further investigate the biological value of *ANP32A* in leukemogenesis, we derived *ANP32A*-associated gene expression profiles from genome-wide microarray

Table 1 Clinical and molecular characteristics of the patients in the 185 CN-AML patients' cohort according to ANP32A expression

Characteristics	ANP32A		U/χ^2	P
	Low expression group (n=92)	High expression group (n=93)		
Median age, years [range]	48 [16–60]	46 [17–60]	3889	0.2851
Gender, No. (%)			0.2653	0.6065
Male	48 (52.2)	45 (48.4)		
Female	44 (47.8)	48 (51.6)		
FAB subtype, No. (%)				
M0	1 (1.1)	2 (2.2)	0.5669	0.5669
M1	28 (30.4)	24 (25.8)	0.4903	0.4838
M2	27 (29.3)	9 (9.7)	11.42	0.0007
M4	10 (10.9)	18 (19.4)	2.592	0.107
M5	15 (16.3)	37 (39.8)	12.62	0.0004
M6	3 (3.3)	0 (0.0)	3.083	0.079
Other	8 (8.7)	3 (3.2)	2.474	0.116
CEBPA, No. (%)			3.164	0.206
Single mutation	4 (4.3)	2 (2.2)		
Double mutation	12 (13.0)	6 (6.5)		
Wild type	76 (82.6)	85 (91.4)		
NPM1, No. (%)			1.566	0.211
Mutation	48 (52.2)	57 (61.3)		
Wild type	44 (47.8)	36 (38.7)		
FLT3-ITD, No. (%)			4.732	0.029
Presence	31 (33.7)	46 (49.5)		
Absence	61 (66.3)	47 (50.5)		
N-ras/K-ras			0.053	0.817
Mutation	8 (8.7)	9 (9.7)		
Wild type	84 (91.3)	84 (90.3)		
ERG expression, No. (%)			0.655	0.418
High	49 (53.3)	44 (47.3)		
Low	43 (46.7)	49 (52.7)		
BAALC expression, No. (%)			0.049	0.825
High	47 (51.1)	46 (49.5)		
Low	45 (48.9)	47 (50.5)		
WT1 expression, No. (%)			0.913	0.339
High	43 (46.7)	50 (53.8)		
Low	49 (53.3)	43 (46.2)		

Table 1 (*continued*)

Table 1 (*continued*)

Characteristics	ANP32A		U/χ^2	P
	Low expression group (n=92)	High expression group (n=93)		
DNMT3A expression, No. (%)			0.655	0.418
High	49 (53.3)	44 (47.3)		
Low	43 (46.7)	49 (52.7)		
DNMT3B expression, No. (%)			0.005	0.942
High	46 (50.0)	47 (50.5)		
Low	46 (50.0)	46 (49.5)		
MAPKBP1 expression, No. (%)			5.885	0.015
High	38 (41.3)	55 (59.1)		
Low	54 (58.7)	38 (40.9)		
ITPR2 expression, No. (%)			0.913	0.339
High	43 (46.7)	50 (53.8)		
Low	49 (53.3)	43 (46.2)		
ATP1B1 expression, No. (%)			0.135	0.714
High	45 (48.9)	48 (51.6)		
Low	47 (51.1)	45 (48.4)		
RUNX1 expression, No. (%)			12.98	0.0003
High	34 (37.0)	59 (63.4)		
Low	58 (63.0)	34 (36.6)		
TCF4 expression, No. (%)			4.545	0.033
High	39 (42.4)	54 (58.1)		
Low	53 (57.6)	39 (41.9)		
Relapse, No. (%)			5.342	0.021
Yes	58 (63.0)	73 (78.5)		
No	34 (37.0)	20 (21.5)		

High expression of ERG, BAALC, WT1, DNMT3A, DNMT3B, MAPKBP1, ITPR2, ATP1B1, RUNX1 and TCF4 was defined as above the median expression of all samples, respectively. CN-AML, cytogenetically normal acute myeloid leukemia; FAB, French-American-British classification; ITD, internal tandem duplication.

analysis in 73 CN-AML patients from TCGA dataset; 1,401 up-regulated and 543 down-regulated genes were found to be significantly associated with *ANP32A*^{high} group (FDR-adjusted P<0.001, *Figure 3A*,*B*). The up-regulated genes included: (I) genes encoded transcription factor proteins: *MYB*, *PHF10* (32); (II) independent unfavorable prognostic factor in CN-AML: *ERG* (33); (III) gene encoded cohesion complex: *SMC3* (34); (IV) gene involved in phosphatases: *PTPN11* (34); (V) genes regulated cell growth and signal transduction pathways: *ANXA* family genes (35); (VI) genes promoted tumorigenesis: *CDK4* (36); *ZPF91*, involved in leukemogenesis and could activate *HIF1a* via NF-kB to promote tumorigenesis (37); *SRSF* family genes (*SRSF1*, *SRSF3*, *SRSF10*), which were oncogene and overexpressed in multiple cancers to promote cell proliferation and transformation (38); (VII) gene related to ribosome biogenesis: *NPM1* (39); (VIII) gene involved in fine-tuning hematopoietic stem and progenitor cell homeostasis:

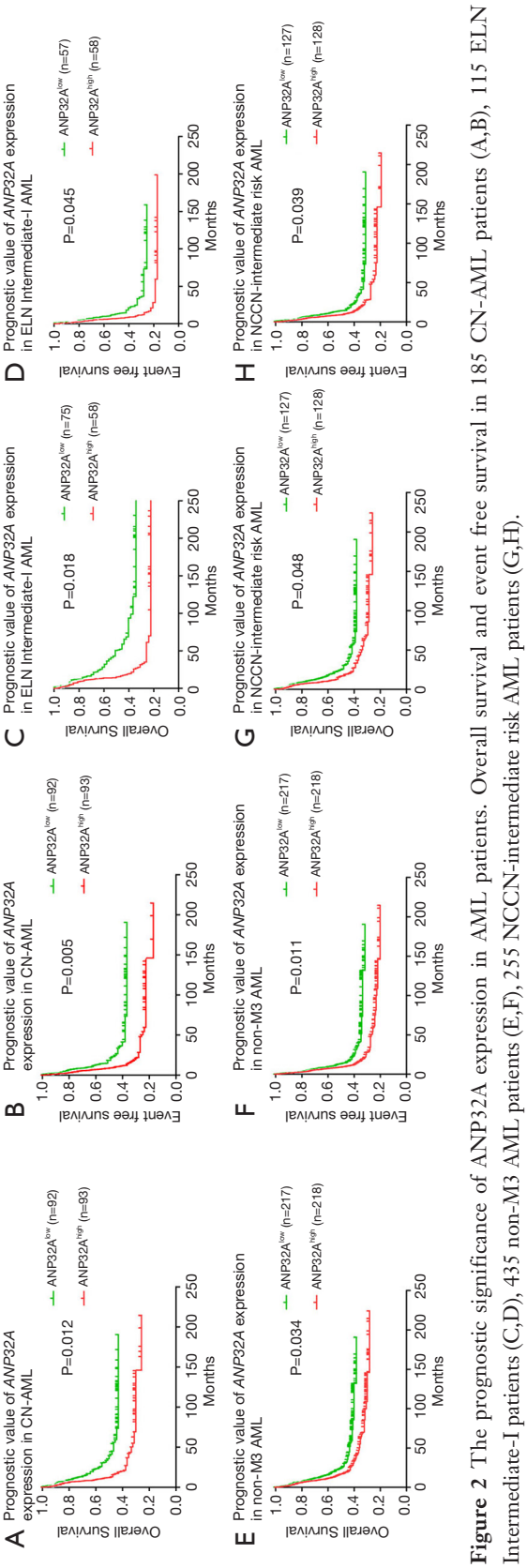

Figure 2 The prognostic significance of ANP32A expression in AML patients. Overall survival and event free survival in 185 CN-AML patients (A,B), 115 ELN Intermediate-I patients (C,D), 435 non-M3 AML patients (E,F), 255 NCCN-intermediate risk AML patients (G,H).

PHF6 (40). The down-regulated genes included: (I) gene inactivated in solid tumor and acute lymphoblastic leukemia (ALL): *CREBBP* (41); (II) gene improved tumor immunogenicity and enhanced Fas-induced apoptosis: *TNFRSF14* (42); (III) genes interacted with NAD metabolism and p53 signaling: *SIRT* family genes (*SIRT2*, *SIRT7*) (43); (IV) genes interacted with WNT-pathway and B-cell receptor signaling mediators: *IRF9*, *BCL9L*, *SIT1* (44).

There is evidence that the uncovering of unique gene expression profiles with the identification of dysregulated signaling pathways is now providing new insights into the leukemogenesis of CN-AML (45). Therefore, data from *MSigDB* was used to investigate cell signaling pathways associated with *ANP32A* (46). A total of 6 down-regulated and 22 up-regulated pathways were found to be significantly associated with *ANP32A*^{high} group (P<0.05, *Figure 3C*). The up-regulated pathways included: (I) important molecular and biological pathways, such as RNA transport, ribosome biogenesis in eukaryotes, spliceosome, ribosome, DNA replication, protein export, proteasome, cell cycle, and RNA polymerase; (II) metabolic pathways of proteins, amino acid and nucleic acid, such as basal transcription factors, carbon metabolism, pyrimidine metabolism, purine metabolism, and valine, leucine, isoleucine degradation; (III) lipid metabolic pathways, such as fatty acid degradation and fatty acid metabolism. On the contrary, the down-regulated pathways were mostly immune-related ones, such as T and B cell receptor signaling pathway, TNF signaling pathway, Toll-like receptor signaling pathway. These dysregulated signaling pathways might explain the involvement between *ANP32A* and the leukemogenesis of CN-AML.

Genome-wide microRNA profiles associated with ANP32A expression

MicroRNAs have been increasingly used to diagnose and assess prognosis in hematopoietic malignancy. Dysregulated microRNAs could promote aggressive tumor phenotype, identify risk classification, and reflect therapeutic effects (47). The genome-wide microRNA profiles were analyzed and 61 microRNAs were identified to be significantly correlated to *ANP32A* expression, including 51 positive and 10 negative microRNAs (P<0.05, *Figure 4A,B*). Positively correlated microRNAs included miR-17, miR-19b-1, miR-20a, miR-25, miR-106a, miR-106b, miR-125b-1, miR-99a, miR-301b, miR-199b, miR-191, miR-629, etc. MiR-17, miR-19b-1 and miR-20a, the members of miR-17-92 cluster, were found to be

Table 2 Multivariable analysis of OS and EFS in 185 CN-AML and 115 ELN Intermediate-I patients

Variables in final model by end point	EFS			OS		
	HR	95% CI	P value	HR	95% CI	P value
All CN-AML, n=185						
ANP32A expression, high *vs.* low	1.60	1.1–2.3	0.0088	1.58	1.1–2.3	0.016
Risk, poor *vs.* non-poor	2.12	0.49–9.2	0.32	3.50	0.81–15.2	0.09
Age, per 10-y increase	1.04	0.90–1.2	0.58	1.08	0.93–1.3	0.32
Gender, male *vs.* female	1.18	0.83–1.7	0.34	1.37	0.95–1.9	0.1
FLT3-ITD, presented *vs.* others	1.64	1.1–2.4	0.009	1.59	1.1–2.3	0.019
N-ras, mutated *vs.* wild type	1.66	0.88–3.1	0.12	1.24	0.63–2.4	0.54
ELN Intermediate-I, n=115						
ANP32A expression, high *vs.* low	1.57	1.02–2.4	0.041	1.59	0.99–2.5	0.051
Risk, poor *vs.* non-poor	1.43	0.31–6.6	0.64	1.92	0.42–8.7	0.4
Age, per 10-y increase	1.15	0.96–1.4	0.12	1.19	0.99–1.4	0.07
Gender, male *vs.* female	1.17	0.77–1.8	0.46	1.45	0.92–2.3	0.11
FLT3-ITD, presented *vs.* others	1.17	0.74–1.9	0.5	1.10	0.68–1.8	0.69
N-ras, mutated *vs.* wild type	1.59	0.62–4.0	0.33	1.43	0.56–3.6	0.45

EFS, event free survival; OS, overall survival; CN-AML, cytogenetically normal acute myeloid leukemia.

upregulated in AML and helped apoptotic resistance in K562 cells (48). MiR-25 had statistically higher expression in c-kit subgroup and might be involved in leukemogenesis in pediatric AML (49). MiR-106a and miR-106b, paralogs of miR-17-92 cluster, were found to enhance proliferation of T-ALL cells and significantly upregulated in relapsed pediatric MLL-AML, respectively (50). By targeting genes associated with tumorigenesis, miR-125b-1 might act as an onco-microRNA and its overexpression alone in mice could induce tumors in multiple hematopoietic lineages (51). MiR-99a upregulation was proven to be associated with poor prognosis of AML, leading to cell expansion and progression of AML (52). MiR-301b and miR-199b were found upregulated in AML patients at diagnosis and trended to normalization after chemotherapy (53). Overexpression of miR-191 had significantly worse OS and EFS than those with low expression (54). MiR-629 was found upregulated in pediatric T-ALL, and might be used for distinguishing pediatric ALL subtypes (55). Negatively correlated microRNAs included miR-24-2, miR-26b, miR-197. MiR-24-2 was proved to be down-regulated in acute leukemia cell lines compared to hematopoietic stem progenitor cells (56). Decreased expression of miR-26b was found in T-ALL cells and it functioned as a tumor suppressor in T-ALL. Furthermore, it could inhibit the PI3K/AKT pathway, reduce proliferation and promote apoptosis of T-ALL cells (57). Overexpression of miR-197 inhibited tumor growth and prolonged OS in subcutaneous mouse xenograft model in multiple myeloma, revealing its novel role as tumor suppressor (58). These results indicated that microRNA expression might link to unfavorable outcome in $ANP32A^{bigh}$ group.

To further validate the results of the microarray platform and microRNAs, interaction network analysis was used to explore the interaction and correlation between microRNAs and their target genes (*Figure 4C*). The results showed the tumor suppressors repressed by the up-regulated microRNAs, led to worse outcome. For example, *SIRT7*, targeted by miR-629, possessed tumor-suppressing properties in breast cancer, pancreatic cancer, head and neck squamous cell carcinoma, etc. (59). *KLF13*, targeted by miR-106b, inhibited the transcription of *MYB* and *BCL2* in ALL (60). Additionally, many oncogenes were the targets of the down-regulated microRNAs. *SUGT1*, targeted by miR-24-2, contributed to cancer development by stabilizing oncoproteins (61). *FBXO45*, targeted by miR-26b, exerted anti-apoptotic effect by interfering the proteasome-

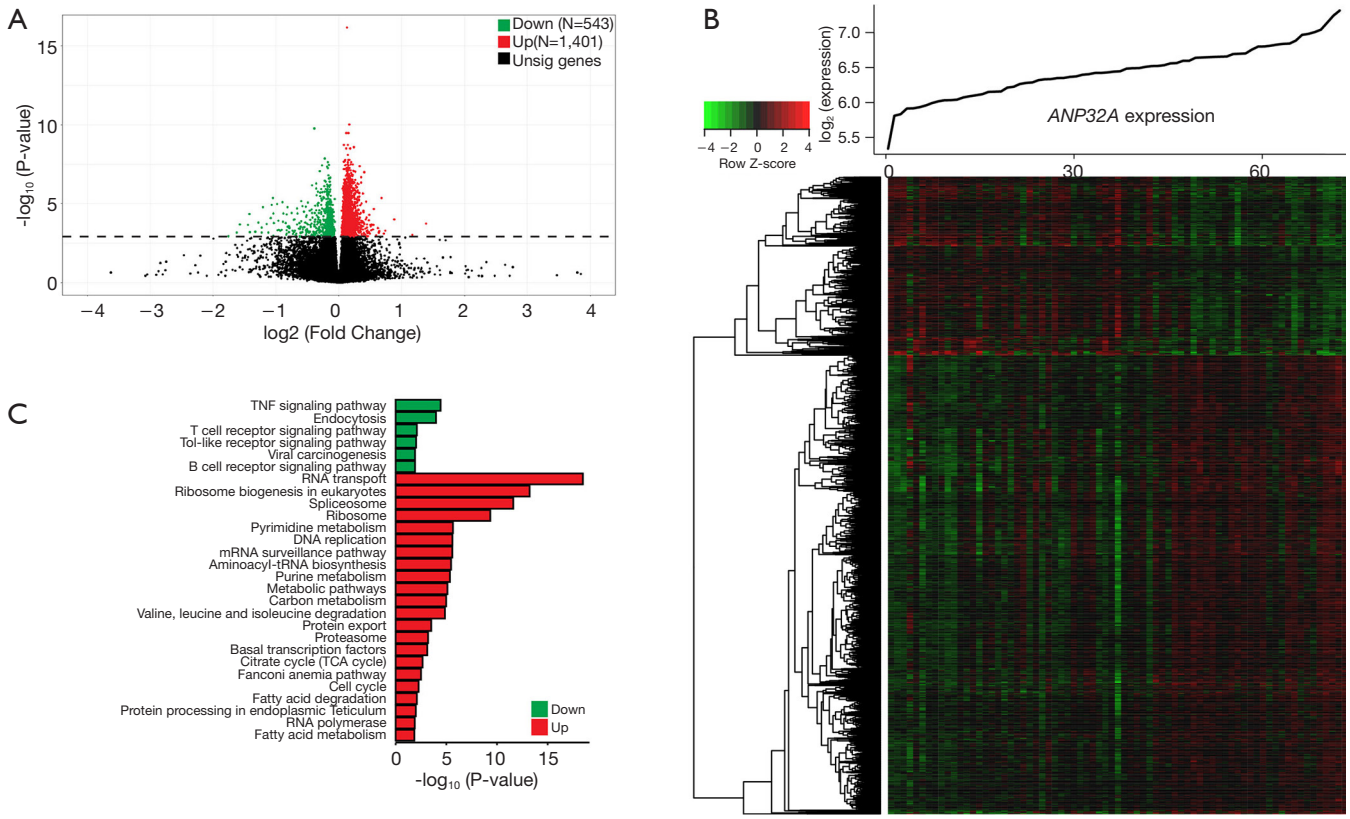

Figure 3 Genome-wide genes/cell signaling pathways associated with ANP32A expression. (A) Volcano plot of genome-wide gene profiles between *ANP32A*^{high} group and *ANP32A*^{low} group; (B) gene expression heatmap. The top curve indicates expression distribution of *ANP32A* in 73 CN-AML samples; (C) cell signaling pathways associated with *ANP32A* expression.

dependent degradation of *p73*, a known pro-apoptotic factor (62). These results suggested why *ANP32A* acted as an unfavorable prognosticator and could help provide a comprehensive view of the molecular mechanisms of *ANP32A*.

Genome-wide methylation profiles associated with ANP32A expression

Differential methylated regions (DMR) were further analyzed to uncover the different methylation patterns between *ANP32A*^{high} group and *ANP32A*^{low} group of CN-AML. A total of 733 hypomethylation and 31 hypermethylation DMRs were discovered from the comparison between *ANP32A*^{high} group and *ANP32A*^{low} group (P<0.05, |Log2(FC)| >1, *Figure 5A*). In addition, position distributions around CpG islands were compared in these aberrant DMRs. It showed that significantly more hypomethylation DMRs were found in the CpG islands (P=0.03, *Figure 5B*). Furthermore, position distributions of different structural fragments of genes were analyzed. The results indicated relatively more hypomethylation DMRs lied in 1st Exon regions (P=0.08, *Figure 5B*). Finally, the heatmaps showed *ANP32A*-associated DMRs enriched on islands and gene promoter regions (*Figure 5C,D*).

Discussion

Recent study provided strong evidence that *ANP32A* was a key factor to deregulate histone H3 acetylation, and played a role as an oncogene in leukemogenesis (23). It could interact with DNA-binding transcription factors, thus influencing gene expression (63). *ANP32A* acted as an oncogene in certain solid tumors, such as colorectal cancer, liver cancer, ovarian cancer, etc. (19,64). Besides, study showed *ANP32A* might repress *ERK* and subsequently inhibit *RUNX1* and *FLI1* to promote megakaryocyte differentiation in acute megakaryoblastic leukemia (65). In

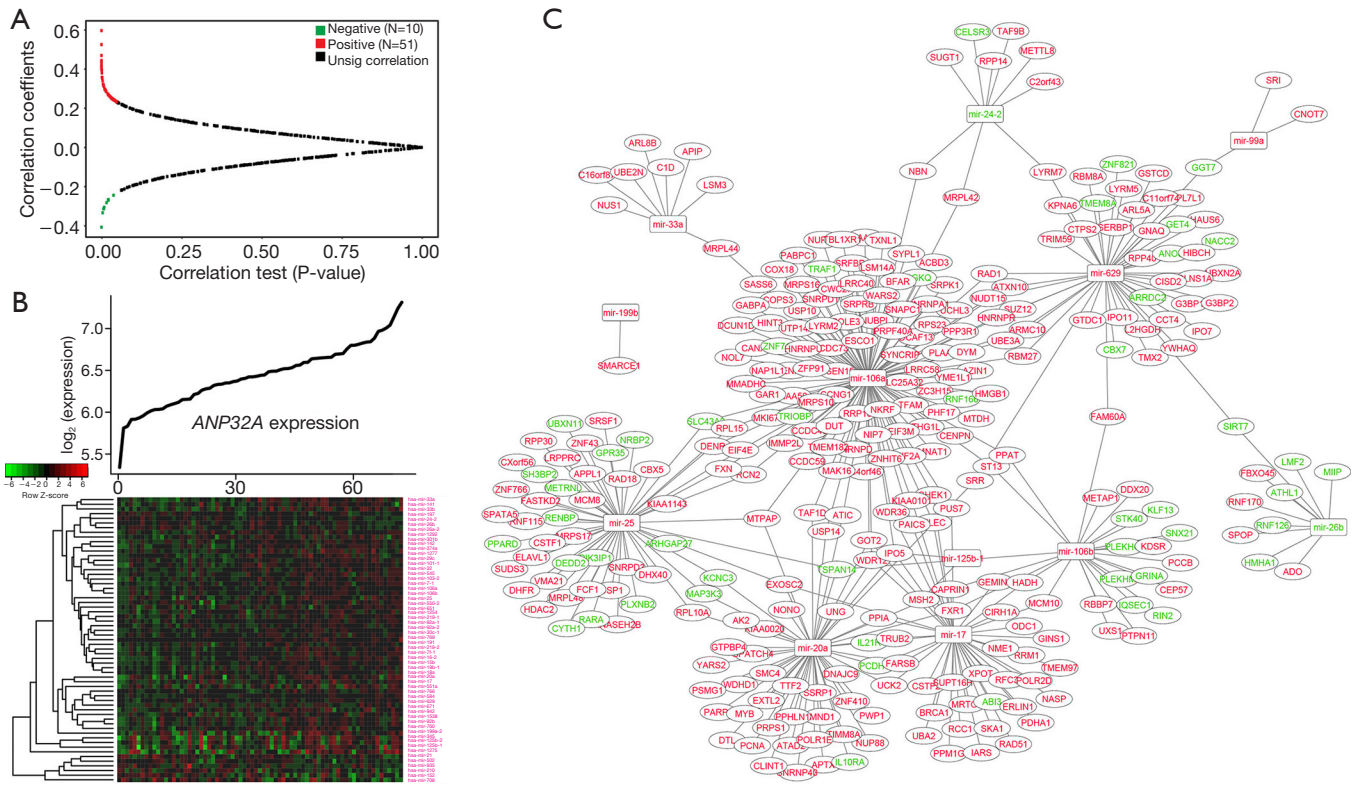

Figure 4 Interaction network analysis of genome-wide microRNAs and their target genes associated with ANP32A expression. (A) Volcano plot of genome-wide microRNAs profiles between *ANP32A*^{high} group and *ANP32A*^{low} group; (B) microRNAs expression heatmap. The top curve indicates expression distribution of *ANP32A* in 73 CN-AML samples; (C) microRNA-mRNA interaction network analysis. (red: up-regulation; green: down-regulation; rectangle: microRNAs; ellipse: mRNAs).

our study, we provided direct evidence that high expression of *ANP32A* predicted unfavorable outcomes for CN-AML. Firstly, high expression of *ANP32A* was shown in AML BM and PB than in normal BM and PB samples, as well as in CN-AML BM than in normal BM samples (*Figure 1A,B,C*). Secondly, survival prognosis with gene expression were well studied previously (66,67) as well as the various functionality of omics data (68-73). After validating in relatively large independent CN-AML cohort, high expression of *ANP32A* was shown to associate with unfavorable outcomes. Similar results were also shown in ELN Intermediate-I cohort, NCCN-Intermediate Risk cohort, and non-M3 AML cohort, all containing a variety of karyotypes of AML. These results were consistent with earlier findings of *ANP32A*, suggesting its potential role of a prognosticator and therapeutic target of AML. Furthermore, our results could provide evidence for further fine stratification of AML, especially in CN-AML, ELN Intermediate-I, NCCN-Intermediate Risk patients.

It is at present unclear about the pathogenesis of AML. However, previous studies showed the molecular biology of leukemia and identified favorable or unfavorable prognosticators, facilitating the understanding of leukemogenesis. In our study, associations between *ANP32A* expression and previously known prognosticators were explored in a cohort of 185 CN-AML patients. We found that *ANP32A*^{high} group contained significantly more M5 (P=0.004) and less M2 (P=0.007) FAB subtype, suggesting that *ANP32A*^{high} expressers carried more mature cells. Furthermore, *ANP32A*^{high} group had significantly more *FLT3-ITD* mutation (P=0.029), and remarkably high expression of *MAPKBP1* (P=0.015), *RUNX1* (P=0.0003) and *TCF4* (P=0.033). all of which were known independently adverse prognosticators in AML (25). Besides, *ANP32A*^{high} group had more relapse patients than *ANP32A*^{low} group (P=0.021). These results indicated that *ANP32A* might be another independent biomarker of unfavorable outcome for CN-AML.

There is a growing number of evidences demonstrated that

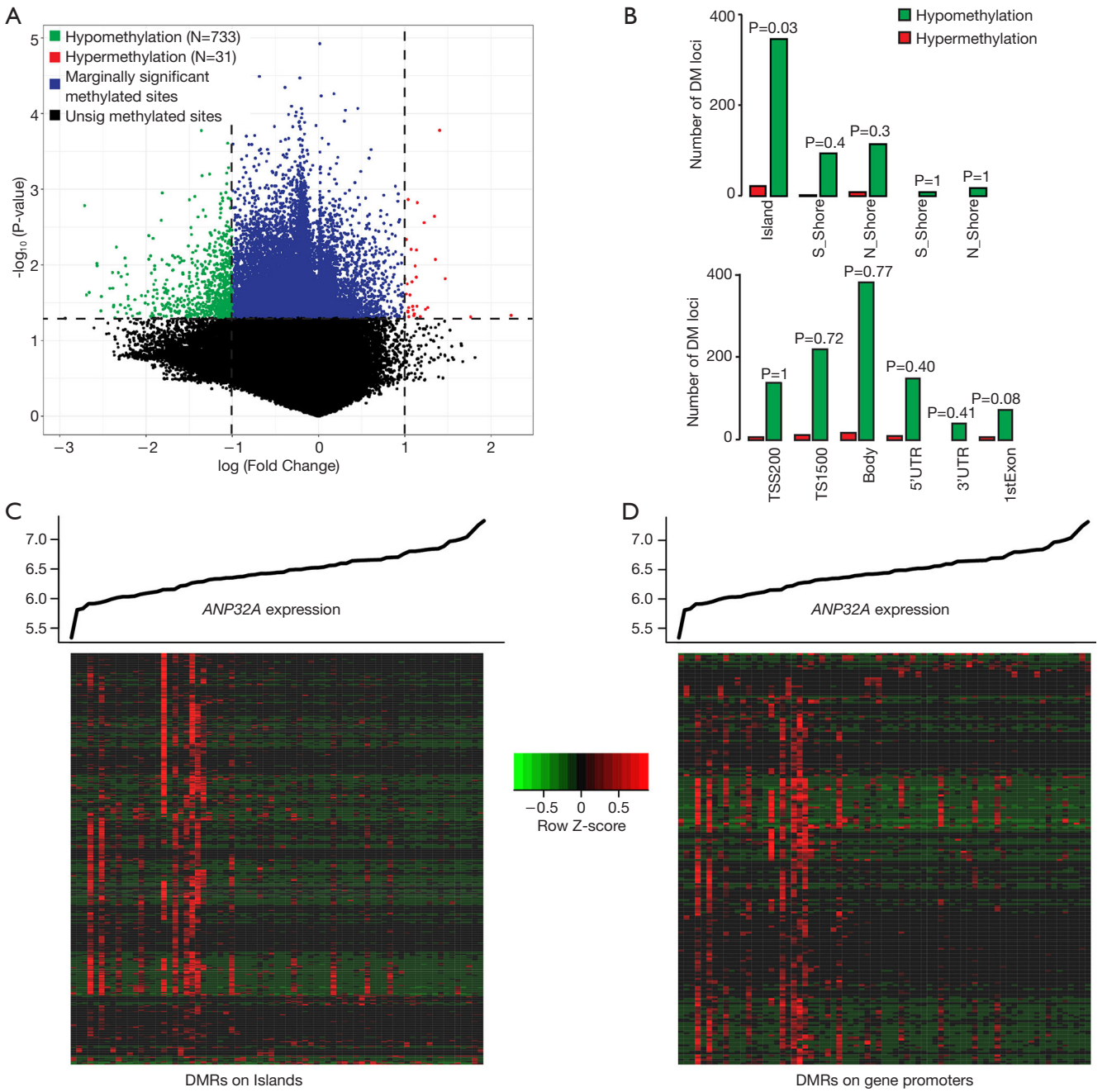

Figure 5 Genome-wide methylation profiles associated with ANP32A expression. (A) A total of 733 hypomethylation and 31 hypermethylation differential methylated regions were discovered from the comparison between *ANP32A*^{high} group and *ANP32A*^{low} group; (B) more hypomethylation differential methylated regions were found in the CpG islands and more hypomethylation differential methylated regions lied in 1st Exon regions; (C,D) the heatmaps showed *ANP32A*-associated differential methylated regions enriched on islands and gene promoter regions.

aberrant gene expression, microRNA post-transcriptional regulation and DNA methylation played significant role in leukemogenesis (74-77). Our study further discussed how overexpression of *ANP32A* affected prognosis from these three aspects. Firstly, we investigated genome-wide genes and cell signaling pathways that associated with *ANP32A*. Genes promoted tumorigenesis were up-regulated, such as *CDK4*, *ZPF91*, *SRSF* family genes, as well as *ERG*, an independent unfavorable prognosticator in CN-AML. On the contrary, genes inactivated in tumor and immunogenicity were down-regulated, such as *CREBBP* and *TNFRSF14*, respectively. Secondly, cell signaling pathway analysis results were in accordance with *ANP32A*'s unfavorable prognosticator role. Results showed up-regulation of cell cycle pathway, lipid metabolic pathway, metabolic pathways of proteins, amino acid and nucleic acid as well as down-regulation of immune-related pathways of T cell, B cell and Toll-like receptor signaling pathway and TNF signaling pathway. All these results further validated the previous study that *ANP32A* was a member in nuclear protein family implicated in multiple cellular pathways (78).

Furthermore, in our study, *ANP32A* was found to correlate positively or negatively to microRNAs, such as members of miR-17-92 cluster and miR-24-2, which played novel role in leukemogenesis or tumor suppression, respectively. Also, synchronized changes of microRNA-mRNA pairs along with *ANP32A* expression might contribute to the aggravation of CN-AML malignancy. For instance, the pair of positive correlated miR-629 and down regulated *SIRT7*, the pair of negative correlated miR-26b and up regulated *FBXO45*, etc. These genes, microRNAs, pathways, microRNA-mRNA pairs might be attributable to the adverse outcome of CN-AML.

Finally, genome-wide methylation profiles associated with *ANP32A* expression were analyzed. Results showed that more hypomethylation DMRs lied around CpG islands and in 1st Exon regions. Hypomethylation in different genic regions had been proven to exhibit a significant adverse effect on gene expression, resulting in malignant transformation and tumor progression (79). For example, in chronic lymphocytic leukemia (CLL), epigenomic analysis detected widespread DNA hypomethylation in the gene body, implicating functional and clinical roles of DNA hypomethylation in leukemogenesis (80). The hypomethylation DMRs detected in our study might possibly contribute to the unfavorable prognosis and provide deeper understanding in leukemogenesis.

In summary, we had performed a large-scale analysis of *ANP32A* in CN-AML. Combined with recent study that *ANP32A* could promote leukemogenesis, we further validated that overexpression of *ANP32A* was an unfavorable biomarker for CN-AML. We provided evidence that *ANP32A*^{high} patients had different kinds of adverse molecular characteristics. Furthermore, we highlighted the power of multi-omics analysis to provide new insights into the leukemogenic mechanisms of CN-AML and define their clinical implications.

Conclusions

We proved ANP32A is a novel, potential unfavorable prognosticator and therapeutic target for AML.

Acknowledgments

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the local institutional review boards. In accordance with the Declaration of Helsinki, all patients provided written informed consent.

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Supplementary

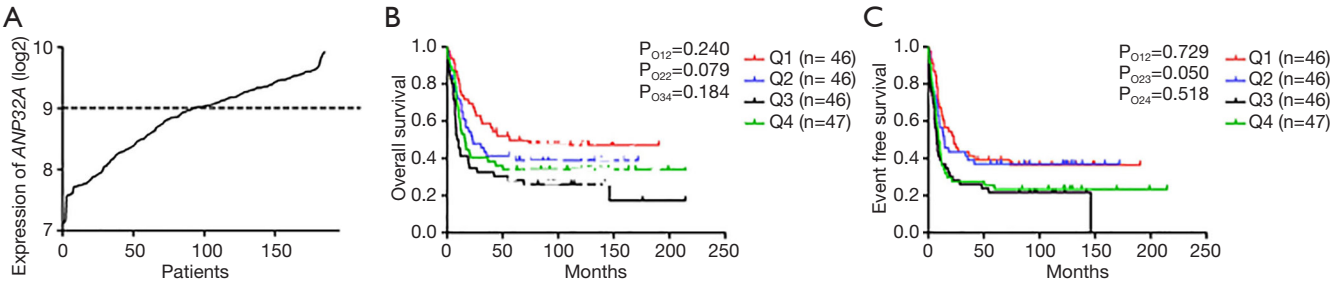

Figure S1 Median value of ANP32A expression as the cut-off. (A) Normally distribution of ANP32A expression. (B) OS and (C) EFS of CN-AML patients, the patients were subdivided into four quartiles based on the quartile of ANP32A expression.

Table S1 Multivariable analysis of OS and EFS in 435 AML and 225 NCCN intermediate patients

Variables in final model by end point	EFS			OS		
	HR	95% CI	P value	HR	95% CI	P value
All AML, n=435						
ANP32A expression, high *vs.* low	1.09	0.90–1.32	0.358	1.14	0.93–1.39	0.201
Risk, poor *vs.* non-poor	2.24	1.71–2.93	5.54E-09	2.16	1.64–2.86	5.77E-08
Age, per 10-y increase	1.07	0.98–1.18	0.140	1.14	1.04–1.26	0.008
FLT3-ITD, presented *vs.* others	1.66	1.27–2.15	0.0002	1.73	1.32–2.27	8.32E-05
Gender, male *vs.* female	0.94	0.75–1.18	0.601	1.07	0.84–1.35	0.581
N-ras, mutated *vs.* wild type	1.20	0.83–1.74	0.340	1.06	0.70–1.58	0.796
NCCN intermediate, n=255						
ANP32A expression, high *vs.* low	1.16	0.92–1.46	0.208	1.25	0.98–1.60	0.069
Risk, poor *vs.* non-poor	1.07	0.86–1.33	0.535	1.13	0.90–1.43	0.289
Age, per 10-y increase	1.19	0.71–2.01	0.507	1.17	0.68–2.02	0.572
FLT3-ITD, presented *vs.* others	1.42	0.83–2.41	0.201	1.46	0.83–2.56	0.188
Gender, male *vs.* female	0.87	0.50–1.51	0.613	0.93	0.52–1.66	0.803
N-ras, mutated *vs.* wild type	NA	NA	NA	NA	NA	NA

EFS, event free survival; OS, overall survival; AML, acute myeloid leukemia.

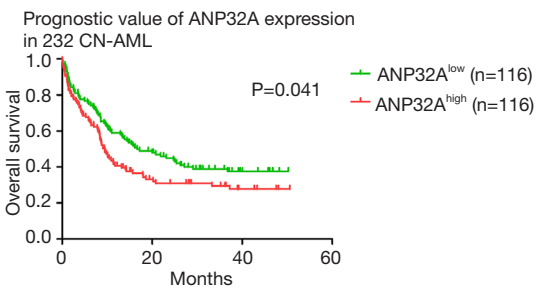

Figure S2 The validation of the unfavorable outcome associated with ANP32Ahigh in a cohort of 232 CN-AML patients.