Integrated analysis and validation reveal CYTH4 as a potential prognostic biomarker in acute myeloid leukemia

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Abstract. Acute myeloid leukemia (AML) is a clonal hematological malignancy with high mortality rates. The identification of novel markers is urgent for AML. Cytohesins are a subfamily of guanine nucleotide exchange factors activating the ADP-ribosylation factor family GTPases. While the important roles of cytohesins have been reported in various cancers, their function in AML remains unclear. The present study aimed to explore the prognostic impact of cytohesin-4 (CYTH4) and the underlying molecular functions. RNA sequencing and AML clinical data were obtained from The Cancer Genome Atlas and Gene Expression Omnibus databases to investigate gene expression and survival. Using the R software, differentially expressed genes were identified between the high- and the low-CYTH4 group. Functional enrichment analysis was conducted by Gene Ontology, Kyoto Encyclopedia of Genes and Genomes, and Gene Set Enrichment Analyses. The CIBERSORTx tool was used to explore the proportions of different immune cell types. The molecular function of CYTH4 was also validated *in vitro* by examining cell growth, cell cycle, apoptosis and colony-forming ability. CYTH4 was significantly upregulated in AML compared with other cancers and normal tissues. High CYTH4 expression was associated with high white blood count (P=0.004) and higher risk status (P<0.001). Patients with high CYTH4 expression had poor overall survival (OS; HR=2.19; 95% CI, 1.40-3.44; P=0.0006; high vs. low) and event-free survival (EFS; HR=2.32; 95% CI, 1.43-3.75; P=0.0006; high vs. low), and these patients could benefit from transplantation (HR=0.29; 95% CI, 0.18-0.47; P<0.0001; transplantation vs. chemotherapy). Multivariate analysis showed that high CYTH4 expression was independently associated with inferior OS (HR=2.49; 95% CI, 1.28-4.83; P=0.007) and EFS (HR=2.56; 95% CI, 1.48-4.42; P=0.001). Functional analysis showed that CYTH4 was involved in immunoregulation. *In vitro* validation showed knockdown of CYTH4 adversely affected cell growth and induced cell apoptosis, while overexpression of CYTH4 enhanced cell growth. Taken together, CYTH4 is expressed at high levels in AML and can potentially function as a prognostic biomarker.

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Abbreviations: AML, acute myeloid leukemia; CYTH4, cytohesin-4; HPA, Human Protein Atlas; CCLE, Cancer Cell Line Encyclopedia; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; FAB, French-American-British; ROC, Receiver Operating Characteristic curve; OS, overall survival; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; FC, fold-change; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; BM, bone marrow; WBC, white blood cell; PB, peripheral blood

Key words: CYTH4, acute myeloid leukemia, biomarker, clinical implications, functional analysis

Introduction

Acute myeloid leukemia (AML) is a heterogenous clonal hematopoietic malignancy characterized by arrest of myeloid cell maturation and disorder of differentiation, resulting in abnormal accumulation of immature malignant cells in the bone marrow (BM) and disruption of the normal hematopoietic process (1). Consequently, patients with AML exhibit a range of clinical symptoms such as fatigue, weight loss and frequent infections. The incidence of AML is about 3.4 to 5.0 cases per 100,000 individuals and the 5-year survival is poor (32.0-33.1%) (2). The clinical and genomic diversity make therapy challenging, and the development of novel biomarkers and treatment strategies is in urgent need. Over the past decades, the risk assessment and treatment selection for AML have relied on morphology, immunophenotype, cytogenetics and molecular features (1). Molecular testing is crucial because molecular changes often precede morphological abnormalities. However, clinical molecular testing mainly focuses on gene mutations and fusion genes (3). Recent studies have

highlighted the critical roles of transcriptional dysregulation in AML leukemogenesis (4-6), and genome sequencing might serve as an alternative (7) or complement (8) to traditional testing in the diagnosis and prognosis of the disease.

Cytohesins, including cytohesins 1-4 (CYTH1-4), are a subfamily of guanine nucleotide exchange factors. Cytohesins activate ADP-ribosylation factor family GTPases which are involved in several essential biological functions, such as cytoskeletal organization (9), cell migration (10,11) and cell signaling (12). CYTH1-4 share a similar structural organization with an N-terminal coiled-coil motif, a central Sec7 domain and a C-terminal pleckstrin homology domain (13). Data from several sources have demonstrated the effects of cytohesins in carcinogenesis and cancer progression. A study by Lee *et al (*14) showed that CYTH2 was upregulated in malignant melanoma and contributed to tumor growth. CYTH2 was also reported to be upregulated in colorectal cancer (15) and hepatocellular carcinoma (16), and it was associated with poor prognosis (15,16). This could be because CYTH2 enhanced the epidermal growth factor pathway (17). A study by Fu *et al* (18) demonstrated that CYTH3 was upregulated in hepatocellular carcinoma, and it was associated with tumor progression. Inhibiting cytohesins could inhibit the proliferation of gefitinib-resistant lung cancer cells, as reported by Bill *et al* (19). Moreover, Zhang *et al* (20) comprehensively analyzed public datasets and revealed that high CYTH4 expression was associated with worse survival in ovarian cancer.

Although numerous studies have reported the clinical and pathological implications of cytohesins in cancer, their roles in leukemia remain largely unexplored. A recent study reported that CYTH1 promotes leukemogenesis, and targeting CYTH1 overcomes resistance to venetoclax (21). Therefore, the present study aimed to investigate the expression of CYTH4 in AML and explore its potential clinical implications. Another aim was to identify genes associated with CYTH4 in AML to provide a promising prognostic biomarker for AML.

Material and methods

Gene expression analysis of CYTH4. In the current study, gene expression analysis of CYTH4 was carried out using various public datasets and online platforms. The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/ENSG00000100055-CYTH4/tissue, accessed on 14 October 2022) (22) was used to analyze the expression of CYTH4 in different healthy human tissues and cancer cell lines. The Cancer Cell Line Encyclopedia (CCLE) database (depmap.org/portal/interactive/, accessed on 15 October 2022) (23) was used to compare the expression of CYTH4 among various cancer types.

The Cancer Genome Atlas (TCGA) database (TCGA-LAML, https://portal.gdc.cancer.gov/, accessed on 26 October 2022) is a large public database containing both genome and clinical information spanning 33 cancer types (24). The Tumor Immune Estimation Resource version 2.0 (TIMER2.0) web resource (http://timer.cistrome.org/, accessed on 20 October 2022) (25) was used in the present study to compare the expression of CYTH4 between tumor and adjacent normal tissues from TCGA. The University of Alabama at Birmingham CANcer (UALCAN) data analysis portal (http://ualcan.path.uab.edu, accessed on 24 October 2022) (26) was used to visualize the expression data among different AML French-American-British (FAB) subtypes in TCGA database. The Gene Expression Omnibus (GEO) dataset GSE30029 (27) was adopted to compare the expression of CYTH4 between AML and healthy BM $CD34^+$ cells.

Survival analysis. TCGA database was used to investigate the survival significance of CYTH4 (24). A total of 151 AML samples from TCGA-LAML dataset with intact RNA sequencing data and survival status were included in the current study (24). Receiver Operating Characteristic (ROC) analysis was conducted, and the Youden index was calculated as the sum of sensitivity and specificity minus one. The expression level that achieves the maximum of the Youden index is referred to as the cut-off value to divide patients into the low- and the high-CYTH4 groups. Overall survival (OS) and event-free survival (EFS) were calculated using the Kaplan-Meier method and comparisons were carried out using the log-rank test. Univariate and multivariate survival analyses were performed using the Cox regression model and described with the hazard ratio (HR) and 95% confidence interval (CI). A stepwise forward procedure was used in the multivariate analysis. The datasets GSE10358 (28) and GSE14468 (29) from the GEO database were also used in the survival analysis.

Differential gene expression analysis and gene association analysis. DESeq2 package (Version 1.42.0, github.com/thelovelab/DESeq2) (30) in R software was used to screen differentially expressed genes between the low- and the high-CYTH4 groups in AML. Significantly differentially expressed genes were defined using an adjusted $P<0.05$ and $|\text{fold change (FC)}|>2$. Gene association analysis was carried out using LinkedOmics (http://www.linkedomics.org/login.php, accessed on 1 November 2022) (31), a publicly available portal for analyzing multi-omics data based on TCGA dataset. Pearson's correlation coefficient was calculated to search for CYTH4-associated genes. Significantly-associated genes were determined based on the criteria of False Discovery Rate (FDR)<0.05 and $|r|>0.5$.

Gene Ontology (GO), Gene Set Enrichment Analysis (GSEA), Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and CIBERSORT analyses. GO enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were carried out using the Database for Annotation, Visualization and Integrated Discovery online tool (Version v2023q3, https://david.ncifcrf.gov/, accessed on 20 October 2023) (32). GSEA was performed using the Molecular Signatures Database (Version 2023.1, https://www.gsea-msigdb.org/gsea/index.jsp, accessed on 21 October 2023) (33). Genes interacting with CYTH4 were also investigated using STRING (Version 11.5, https://string-db.org/, accessed on 19 November 2022). The CIBERSORTx tool (https://cibersortx.stanford.edu/, accessed on 22 October 2023) (34) was used to compare the difference in immune cell infiltration between the low- and high-CYTH4 groups.

Cell culture. MV4-11, HL-60, THP-1, U-937, Kasumi-1, K-562, RS4;11 and 293T cells were purchased from American

Type Culture Collection. HEL, Reh and MOLT-4 cells were purchased from the Chinese National Collection of Authenticated Cell Cultures. NOMO-1, MOLM-13, NB4, BALL-1, NALM-6, and SUP-B15 cells were purchased from Procell Life Science & Technology Co., Ltd. MV4-11, HL-60, THP-1, U-937, Kasumi-1, K-562, RS4;11, HEL, Reh, MOLT-4, NOMO-1, MOLM-13, NB4, BALL-1, NALM-6, and SUP-B15 leukemia cell lines were maintained in RPMI-1640 medium (VivaCell BIOSCIENCES), supplemented with 10% fetal bovine serum (FBS, TransGen Biotech Co., Ltd) and 1% penicillin-streptomycin (VivaCell BIOSCIENCES). 293T cells were maintained in DMEM medium (VivaCell BIOSCIENCES) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were cultured in a humidified incubator (Esco Lifesciences) with 5% CO_2 at a temperature of 37˚C. All the cell lines were tested and authenticated by using short tandem repeat matching analysis. No mycoplasma contamination was detected.

cDNA and short-hairpin (sh)RNA construction, lentivirus preparation and infection. Human CYTH4 was amplified from cDNA and cloned into the pLV3-EF1α-MCS-puro lentiviral construct (Wuhan MiaoLing Biotech Science Co., Ltd.). shRNA-targeting CYTH4 and non-targeting control were constructed using synthesized shRNA-encoded DNA oligos and cloned into the pLKO.1-puro vector (Addgene, Inc.). The designed target sequences were as follows: Scramble (TGAGGAAATTGCGGCTTATTT), shCYTH4 #1 (TRCN0000242587, CCGCCAAGGGTATCCAGTATT), shCYTH4 #2 (TRCN0000242586, TTGCACGGTTCCTGT ATAAAG). The lentivirus was produced in 293T cells by transfecting the designed plasmid together with the packing vectors pLP/VSVG and psPAX2 (Addgene, Inc.). Cells were subsequently infected with lentiviral particles via two rounds of 'spinoculation' with 8 μg/ml polybrene.

RNA isolation, complementary (c)DNA preparation and quantitative (q)PCR. The detection of mRNA expression level was carried out on day 2 after lentiviral infection to assess the overexpression and knockdown of CYTH4. Total RNA was isolated from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Isolated RNA was converted into cDNA using the TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR kit (One-Step gDNA Removal; cat. no. AT341; TransGen Biotech Co., Ltd.). The reaction was carried out by incubating the mixture at 42˚C for 15 min, followed by inactivation at 85˚C for 5 sec. The expression of CYTH4 was detected by qPCR using the KAPA SYBR® Fast Universal kit (cat. no. KK4601; Sigma-Aldrich; Merch KGaA) on the ABI Prism 7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The process included 3 parts: initial denaturation at 95˚C for 2 min, cycling stage (35 cycles) with denaturation at 95˚C for 15 sec and annealing plus extension at 60˚C for 1 min, and melt curve stage with 95˚C for 15 sec, 60˚C for 1 min, 95˚C for 30 sec and 60˚C for 15 sec. Expression of CYTH4 was determined by the comparative Cq method (35) using GAPDH for normalization. The following CYTH4 primer sequences were used: Forward, ATTGGGCGCAAGAAGTTCAAC; Reverse, TTTATA CAGGAACCGTGCAATGT. The following GAPDH primer sequences were used: Forward, CTCTGCTCCTCCTGTTCG AC; Reverse, GCCCAATACGACCAAATCC.

Western blotting. Western blotting was performed on day 2 after lentiviral infection. Cells were lysed using RIPA buffer supplemented with 1 mM phenylmethane sulfonyl fluoride (both Beijing Solarbio LIFE SCIENCES). Total protein concentration was measured using a BCA assay kit (Solarbio LIFE SCIENCES). Equal amounts of protein (~30 μg) were separated by 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 5% non-fat milk at room temperature for 1 h, then incubated with primary antibody CYTH4 (cat. no. H00027128-B01P; Novus Biologicals, LLC; Bio-Techne) at 4˚C overnight for about 12 h. The incubation of HRP-linked secondary antibody (cat. no. 7076; Cell Signaling Technology, Inc.) was carried out at room temperature for 1 h. After detecting CYTH4, the membrane was washed with stripping buffer (Solarbio LIFE SCIENCES) at room temperature for 30 min and blocked again. It was then incubated with primary β-Actin (HRP conjugate; cat. no. 5125; Cell Signaling Technology, Inc.) antibody for 2 h at room temperature. The primary antibody was diluted at 1:1,000 and the secondary antibody 1:3,000. TBST buffer with 0.05% Tween 20 (Solarbio LIFE SCIENCES) was used for washing. The immobilon western chemiluminescent HRP substrate (cat. no. WBKLS0100; MilliporeSigma) was added to the membrane, and blot signals were detected using a ChemiDoc XRS+ System (Bio-Rad Laboratories, Inc.).

Cell proliferation, cell cycle, apoptosis and in vitro colony formation assay. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK8; MedChemExpress) assay according to the manufacturer's instructions. At 72 h after infection, 5,000 viable cells counted by Trypan blue staining were seeded into 96-well plates. After incubating the media with CCK-8 reagent for 3 h, the absorbance was measured at 450 nm using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Inc.). The CCK-8 assay was performed at the same time for 5 consecutive days. At 72 h after infection, cells were harvested and fixed with 75% ice-cold ethanol at 4˚C for about 12 h. Cell cycle analysis was conducted using a Fluorescence-activated cell sorting (FACS) flow cytometer (Beckman Coulter, Inc.) after staining the samples with propidium iodide (PI) for 30 min. At 96 h after infection, apoptosis was detected using Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Briefly, cells were stained with Annexin V-FITC and PI at room temperature for 15 min and analyzed with a FACS flow cytometer (Beckman Coulter, Inc.). In FACS analysis, 10,000 cells were gated for cell cycle and apoptosis detection. Regarding the colony formation assay, cells were harvest at 72 h after infection with scramble or shCYTH4 lentivirus. Variable cells were seeded in methylcellulose medium (MethoCult™ H4434, Stemcell Technologies, Inc.) at a density of 1,000 cells/ml. Colonies (≥50 cells) were counted manually after 10 days.

Statistical analysis. SPSS (version 22.0; IBM Corp.) and GraphPad Prism (version 7; Dotmatics) were used for

statistical analyses. Clinical features between the two groups were compared using the χ^2 test for categorical variables and the Fisher's exact test for the expected frequency of an event (<5 in any cell of 2x2 tables). Continuous variables were compared using the non-parametric Mann-Whitney U test. One-way ANOVA was used to compare scramble cells and CYTH4-knockdown cells, with scramble cells serving as the control. Dunnett's multiple comparison test was used for testing. Two-way ANOVA followed by Sídák's multiple comparisons test were used to compare the cell viability between different groups on each day. Data are presented as mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

CYTH4 was upregulated in AML cell lines. The present study focused on CYTH4 because its expression was much higher than other cytohesins in AML (Fig. S1). The HPA dataset was first explored to examine the RNA tissue specificity of CYTH4 in healthy humans. Results showed that the expression of CYTH4 was enhanced in the BM and lymphoid tissues, while being expressed at low levels in other tissues (Fig. 1A).

The level of CYTH4 expression was then analyzed in cell lines based on the latest next-generation sequencing data from the CCLE dataset (Table SI). Results showed that CYTH4 was expressed at high levels in lymphoma and leukemia cell lines, followed by thyroid cancer (Fig. 1B). The analysis of the HPA dataset also revealed that CYTH4 was expressed at high levels in myeloid cancer cells compared with other cancer cell lines such as brain, liver and kidney cancer cell lines (Fig. S2). In addition, the data from the CCLE leukemia cell lines were used to compare the expression level of CYTH4 in different types of leukemia, and it was found that CYTH4 was expressed at high levels in AML compared with acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML; Fig. 1C). The AML cell lines NOMO-1, MV4-11, HL-60, THP-1, MOLM-13, Kasumi-1, HEL, NB4 and U-937, the ALL cell lines RS4;11, BALL-1, Reh, NALM-6, MOLT-4 and SUP-B15, and the CML cell line K-562 were used in the present study to examine the expression of CYTH4. RNA was extracted from these cell lines and qPCR analysis was performed. Results showed that CYTH4 was expressed at high levels in NOMO-1, MV4-11, HL-60, THP-1 and MOLM-13 AML cell lines (Fig. 1D).

CYTH4 is upregulated in patients with AML. To investigate CYTH4 expression in human cancers, TCGA-LAML dataset was analyzed. Fig. 2A displays an overview of the different expression of CYTH4 between tumors and adjacent normal tissues across TCGA dataset, suggesting that CYTH4 expression was higher in patients with AML compared with that in patients with other tumors. These results for the AML samples matched those obtained from the cell lines (Fig. 1B). Analysis of the GSE30029 dataset showed CYTH4 expression was significantly upregulated in AML BM $CD34^+$ cells compared with that in normal BM $CD34^+$ cells (Fig. 2B). The French-American-British (FAB) classification system divides AML into 8 subtypes, designated Myeloid 0-7 (M0-M7), based on the morphology and the appearance of the leukemia cells. We compared the CYTH4 expression among different AML subtypes and patients with M3-AML had the lowest expression of CYTH4 (Fig. 2C).

High expression of CYTH4 is associated with poor survival in AML. To investigate the significance of CYTH4 expression in AML prognosis, survival was compared between the high- and low-CYTH4 expression groups in the different datasets. ROC analysis was performed to determine the cut-off value between the low- and high-CYTH4 expression groups (Fig. 3A). As shown in Fig. 3A and B, high CYTH4 expression was significantly associated with unfavorable OS (high vs. low; HR=2.19; 95% CI, 1.40-3.44; P=0.0006) and EFS (high vs. low; HR=2.32; 95% CI, 1.43-3.75; P=0.0006). This conclusion was validated in the GSE10358 dataset (Fig. 3C) and in the GSE14468 dataset (Fig. 3D). Next, survival was compared between the low- and high-CYTH4 expression groups by treatment using TCGA dataset. The results showed that high CYTH4 expression was associated with poor OS (Fig. 3E; high vs. low; HR=3.12; 95% CI, 1.82-5.34; P<0.0001) in patients treated with chemotherapy alone. However, in cases of patients who received both chemotherapy and transplantation, no significant difference was found (Fig. 3F; P=0.398). Survival was then compared between patients treated with chemotherapy alone and patients treated with chemotherapy plus transplantation grouped by CYTH4 expression level. Transplantation did not show a significant difference in the OS of the low-CYTH4 group (Fig. 3G; P=0.974), but significantly improved OS in the high-CYTH4 expression group compared with chemotherapy alone (Fig. 3H; transplantation vs. chemotherapy; HR=0.29; 95% CI, 0.18-0.47; P<0.0001). These findings suggest that transplantation may attenuate the adverse effect of high CYTH4 expression on patient survival in AML.

Clinical features of the low- and the high-CYTH4 groups. Based on the cut-off value in Fig. 3A, the characteristics of patients in the low- and the high-CYTH4 group were analyzed (Table SII). Both clinical features and gene mutations were listed in Table I. It was observed that the white blood cell count (WBC) varied significantly between the two groups, with patients in the high-CYTH4 group exhibiting higher WBC than those in the low-CYTH4 group (median WBC, 25.9 vs. 9.7; P=0.004). Moreover, in the M4-AML subtype, there were more patients with high CYTH4 expression than patients with low expression (P=0.001), while all patients with M3-AML were in the low-CYTH4 expression group (P<0.0001). This discovery is in line with the previous result that patients with M3-AML had the lowest CYTH4 expression (Fig. 2C). The low-CYTH4 group had higher percentages of cases with PML-RARA and RUNX1-RUNX1T1 karyotypes (P<0.0001). Regarding risk status, low expression of CYTH4 was significantly associated with a good-risk status (low vs. high, 58.8 vs. 9.4; P<0.0001). Besides, patients in the high-CYTH4 group tended to be older than those in the low-CYTH4 group (57 vs. 51 years; P=0.071). Patients in the high-CYTH4 group had a higher percentage of RUNX1 mutation (12% vs. 0; P=0.034). The percentage of patients with more than one mutation did not differ significantly between the low- and high-CYTH4 groups. No significant difference was found between the low- and high-CYTH4 group concerning sex, and BM and peripheral blood (PB) blasts.

To further explore the prognostic effect of CYTH4 in AML, univariate and multivariate survival analyses were

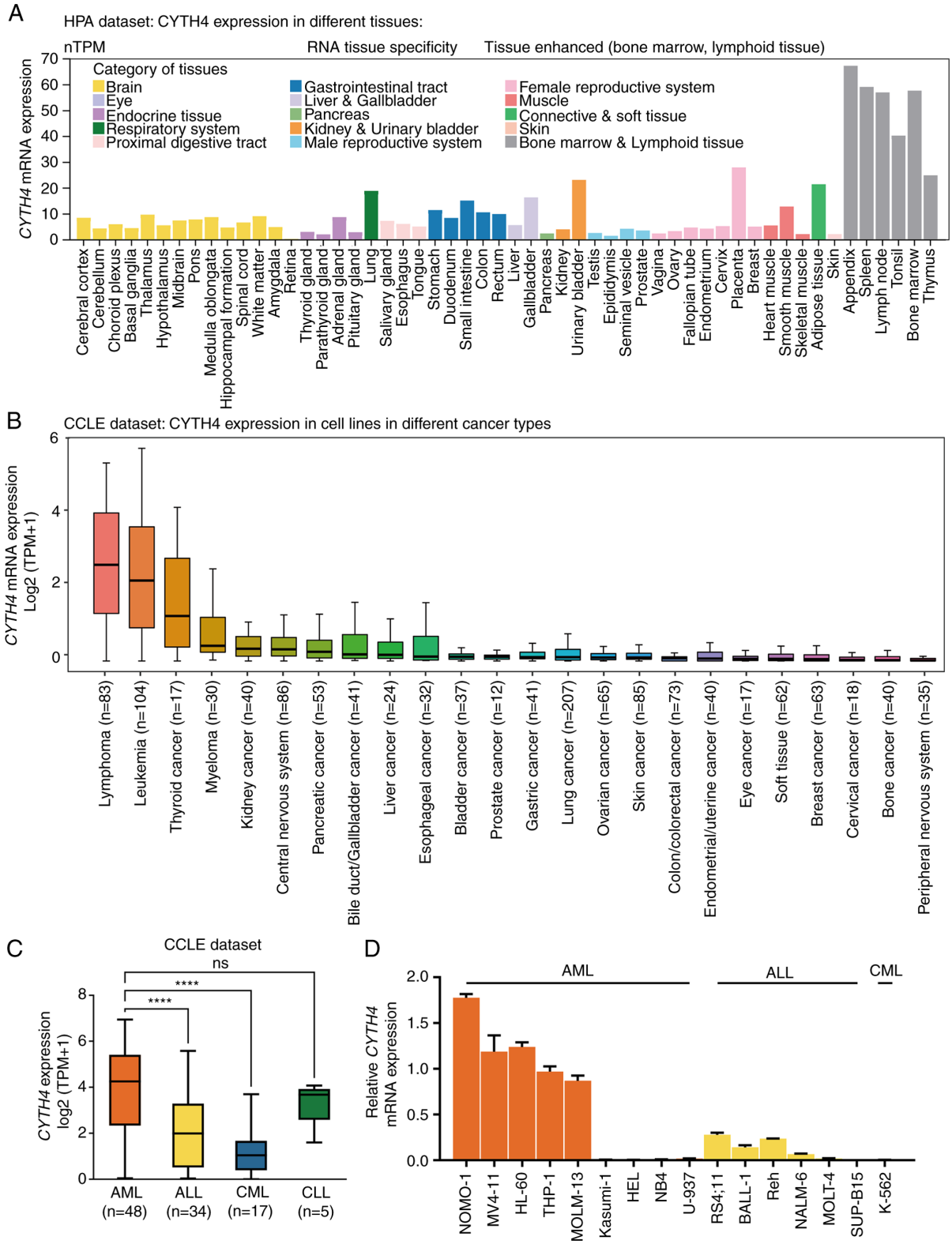

Figure 1. Expression of CYTH4 in human normal and cancer tissues. (A) Expression of CYTH4 in various human normal tissues in the HPA dataset. (B) Expression of CYTH4 in different categories of cancer cell lines, analyzed using the CCLE dataset. (C) Expression of CYTH4 in different types of leukemia cell lines, analyzed using the CCLE dataset. (D) Quantitative PCR showing the expression of CYTH4 in different leukemia cell lines. ****P<0.0001. HPA, Human Protein Atlas; CCLE, Cancer Cell Line Encyclopedia; CYTH4, cytohesin-4; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphoblastic leukemia.

performed using the Cox regression model (Table II). In the univariate analysis, high CYTH4, older age, high WBC, poor cytogenetics risk, FLT3, DNMT3A and TP53 mutations, and non-transplantation were associated with poor OS. High CYTH4, WBC, PB blasts, poor cytogenetics risk and DNMT3A mutation were identified as inferior prognostic

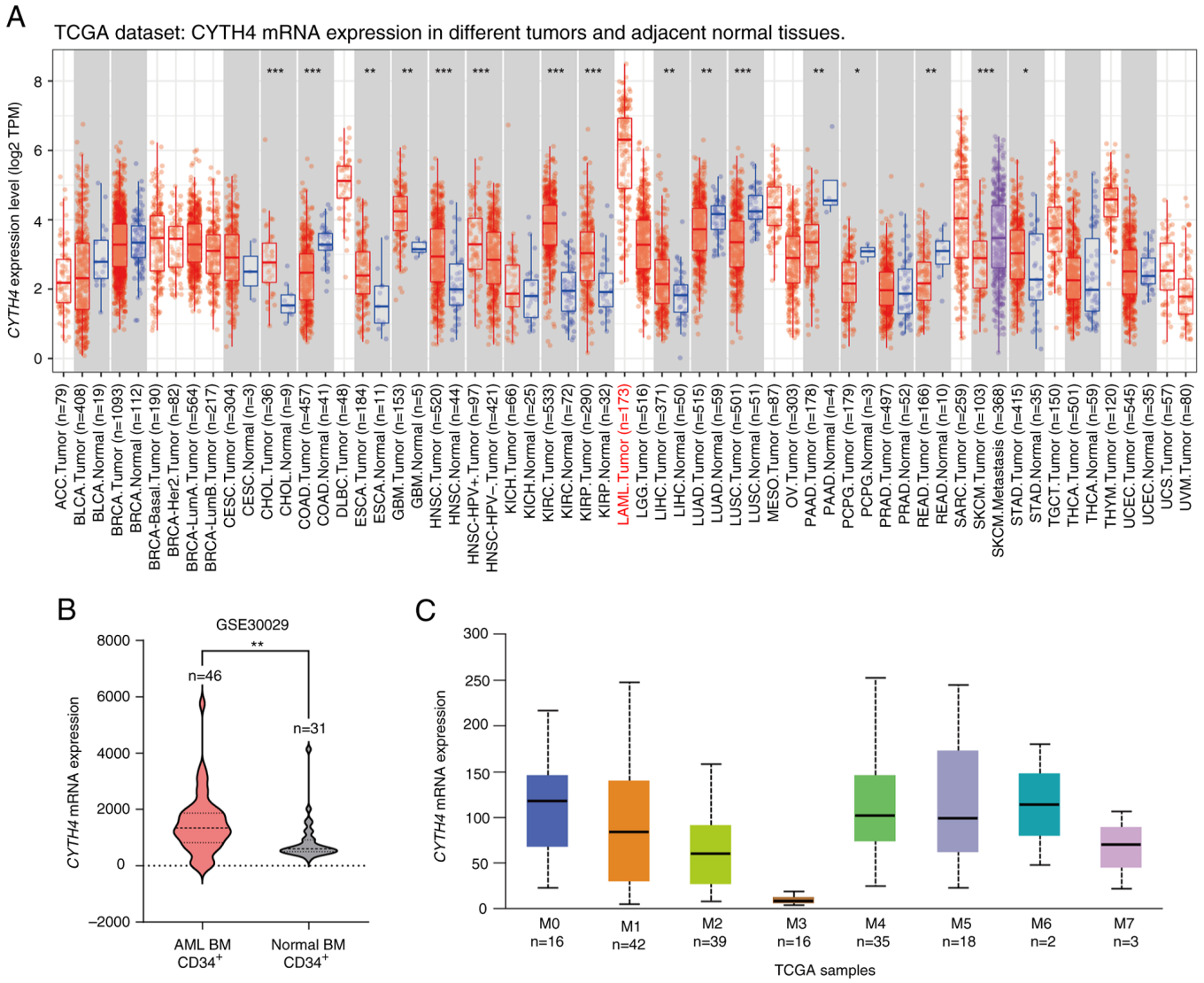

Figure 2. Expression of CYTH4 in patients with AML. (A) Expression of CYTH4 in tumor and adjacent normal tissues across all TCGA tumors, analyzed by TIMER 2.0. (B) Comparison of CYTH4 expression between AML BM CD34$^+$ cells and normal BM CD34$^+$ cells, analyzed using the GSE30029 dataset. (C) Expression of CYTH4 in different AML FAB subtypes, analyzed using UALCAN. *P<0.05, **P<0.01 and ***P<0.001. TCGA, The Cancer Genome Atlas; ACC, adrenocortical cancer; BLCA, bladder cancer; BRCA, breast cancer; CESC, cervical cancer; CHOL, bile duct cancer; COAD, colon cancer; DLBC, large B-cell lymphoma; ESCA, esophageal cancer; GBM, glioblastoma; HNSC, head and neck cancer; KICH, kidney chromophobe; KIRC, kidney clear cell carcinoma; KIRP, kidney papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, lower grade glioma; LIHC, liver cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian cancer; PAAD, pancreatic cancer; PCPG, pheochromocytoma & paraganglioma; PRAD, prostate cancer; READ, rectal cancer; SARC, sarcoma; SKCM, melanoma; STAD, stomach cancer; TGCT, testicular cancer; THCA, thyroid cancer; THYM, thymoma; UCEC, endometrioid cancer; UCS, uterine carcinosarcoma; UVM, ocular melanomas; BM, bone marrow; FAB, French-American-British classification system; CYTH4, cytohesin-4; TIMER 2.0, Tumor Immune Estimation Resource version 2.0; UALCAN, University of Alabama at Birmingham CANcer data analysis Portal.

factors for EFS. When age, WBC, cytogenetics risk, FLT3, DNMT3A and TP53 mutations, and transplantation were combined in the multivariate Cox regression analysis, results confirmed that high expression of CYTH4 was independently associated with inferior OS (HR=2.49; 95% CI, 1.28-4.83; P=0.007) and EFS (HR=2.56; 95% CI, 1.48-4.42; P=0.001).

CYTH4-associated gene analysis. To better understand the role of CYTH4 in AML, the transcriptomes were compared between the high- and the low-CYTH4 groups in TCGA dataset. A total of 552 genes showed significantly different expression (adjusted P<0.05; |FC|>2) including 394 and 158 genes significantly upregulated and downregulated in the high-CYTH4 group, respectively (Fig. 4A; Table SIII). LinkedOmics tools were then used to perform correlation analysis, and a total of 451 significantly co-expressed genes with a cut-off value of FDR<0.05 and |r|>0.5 were identified (Fig. 4B; Table SIV). Of these co-expressed genes, 326 and 125 genes were positively and negatively correlated with CYTH4 expression, respectively (Table SIV). By integrating the results of these two analyses, 159 genes were identified that were upregulated in the high-CYTH4 group and positively correlated with CYTH4 expression (Fig. 4C). By contrast, only 17 genes were found to be both downregulated in the high-CYTH4 group and negatively correlated with CYTH4 expression (Fig. 4D). The overlapping genes were further analyzed for their biological functions.

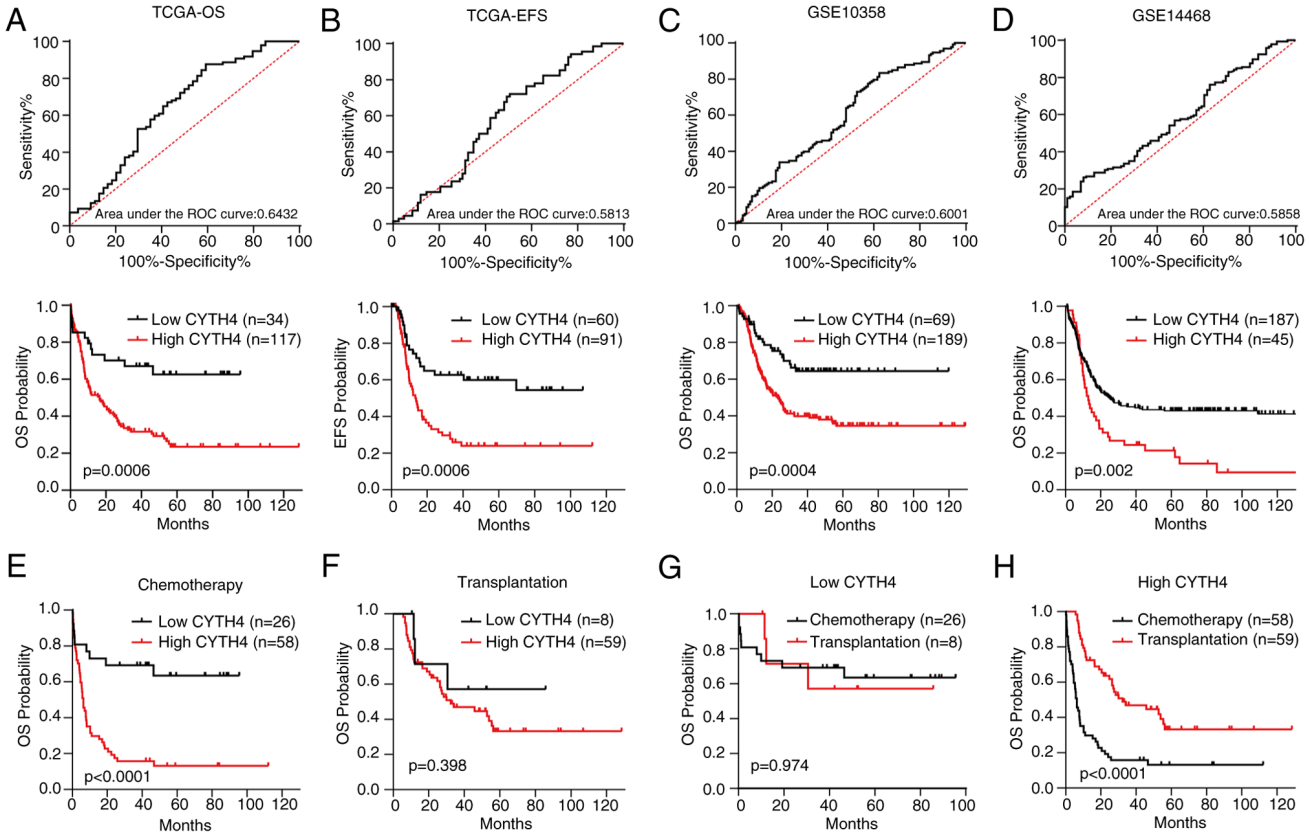

Figure 3. Survival analysis based on the expression level of CYTH4. Comparison of (A) OS and (B) EFS between the high- and the low-CYTH4 group in TCGA dataset. Comparison of OS between the high- and the low-CYTH4 group in (C) GSE10358 and (D) GSE14468 datasets. The upper plots in A-D show the ROC curves of the effect of CYTH4 expression on survival. The bottom plots in A-D show the Kaplan-Meier survival curves. Comparison of OS between the high- and the low-CYTH4 group in (E) patients treated with chemotherapy alone or (F) treated with chemotherapy plus transplantation. Comparison of OS between chemotherapy and transplantation in patients (G) with low CYTH4 expression or (H) with high CYTH4 expression. Two-sided P<0.05 was considered to indicate a statistically significant difference. OS, overall survival; EFS, event-free survival; TCGA, The Cancer Genome Atlas; ROC, Receiver Operating Characteristic curve; CYTH4, cytohesin-4.

Functional enrichment analysis of overlapping genes. Next, the possible biological function of CYTH4 was explored. GO and KEGG pathway enrichment analyses were performed (Fig. 5A and B). These overlapping genes were significantly associated with 'innate immune response', 'inflammatory response', 'signal transduction', 'apoptotic process', 'phagosome' and 'allograft rejection'. GSEA and the enrichment plot showed that these overlapping CYTH4-associated genes were significantly enriched in the gene set related to the immune response (Fig. 5C). Additionally, STRING analysis was used to investigate the genes that interacted with CYTH4 (Fig. 5D). Then, the fractions of 22 distinct immune cell types were estimated using the CIBERSORTx algorithm. Results showed that high CYTH4 expression was significantly correlated with $CD4^+$ memory T cells resting (P<0.01), monocytes (P<0.0001) and mast cells resting (P<0.0001; Fig. 5E).

In vitro validation of the function of CYTH4. To further investigate the function of CYTH4 in AML, *in vitro* validation was carried out. CYTH4 knockdown was conducted in the AML cell lines MOLM-13, NOMO-1 and THP-1. These cell lines were chosen because they exhibit relatively high expression of CYHT4 (Fig. 1D). Lentivirus-expressing shRNA significantly reduced the mRNA and protein expression levels of CYTH4 (Fig. 6A; Fig. S3). Cell proliferation analysis showed that CYTH4 knockdown significantly suppressed the cell growth of AML cells (Fig. 6B). Cell cycle assays revealed a significant G0/G1 phase arrest in all three AML cell lines (Fig. 6C). The results of the colony-forming assays demonstrated that the silencing of CYTH4 significantly impaired the clonogenic potential of the three leukemia cell lines (Fig. 6D). Increased apoptosis was also recorded in the leukemia cell lines following transfection with CYTH4 shRNA (Fig. 6E). Furthermore, CYTH4 was overexpressed by lentivirus in the U-937 (acute monocytic leukemia) and Kasumi-1 (acute myeloblastic leukemia with maturation) AML cell lines (Fig. S4A and B), in which the CYTH4 expression was low (Fig. 1D). Results showed that the overexpression of CYTH4 enhanced the cell growth of the U-937 and Kasumi-1 cell lines (Fig. S4C). Taken together, these results indicated that CYTH4 plays an oncogenic role in AML cells.

Discussion

AML is a clonal hematological malignancy characterized by abnormally rapid proliferation, maturation arrest and differentiation block of myeloid precursors (36). Patients diagnosed with AML usually have poor outcomes and high mortality rates (2). At present, the diagnosis of AML mainly relies on the analysis of BM morphology, immunophenotype, cytogenetics

Table I. Characteristics of patients with AML (n=151) in TCGA dataset grouped by CYTH4 expression; low-CYTH4 (n=34) and high-CYTH4 (n=117).

Clinicopathological characteristics	Low-CYTH4	High-CYTH4	P-value
Sex			0.567
Male	17	65	
Female	17	52	
Median age, years (range)	51 (25-76)	57 (21-88)	0.071
Median BM blasts, % (range)	79 (33-100)	71 (30-99)	0.212
Median WBC, x10^9 cells/l (range)	9.7 (0.4-90.4)	25.9 (0.7-223.8)	0.004
Median PB blasts, % (range)	36 (0-97)	39 (0-96)	0.471
FAB classifications, n (%)			
M0	1 (2.9)	14 (12.0)	0.192
M1	7 (20.6)	29 (24.8)	0.613
M2	10 (29.4)	27 (23.1)	0.450
M3	14 (41.2)	1 (0.9)	<0.0001
M4	0 (0.0)	29 (24.8)	0.001
M5	0 (0.0)	15 (12.8)	0.028
M6	0 (0.0)	2 (1.7)	1.000
M7	1 (2.9)	0 (0.0)	0.225
NA	1 (2.9)	0 (0.0)	0.225
Fusion gene, n (%)			
Normal karyotype	7 (20.6)	55 (47.0)	0.006
BCR-ABL1	2 (5.9)	1 (0.9)	0.064
MYH11-CBFB	0 (0.0)	10 (8.5)	0.170
PML-RARA	14 (41.2)	1 (0.9)	<0.0001
MLL translocation	0 (0.0)	7 (6.0)	0.319
RUNX1-RUNX1T1	7 (20.6)	0 (0.0)	<0.0001
Complex karyotype	1 (2.9)	17 (14.5)	0.125
Others	3 (8.8)	26 (22.2)	0.141
Molecular risk level, n (%)			
Good	20 (58.8)	11 (9.4)	<0.0001
Intermediate	9 (26.5)	72 (61.5)	<0.001
Poor	5 (14.7)	31 (26.5)	0.156
NA	0 (0.0)	3 (2.6)	1.000
Gene mutation, n (%)			
TET2	1 (2.9)	11 (9.4)	0.220
DNMT3A	4 (11.8)	32 (27.4)	0.060
IDH1/IDH2	6 (17.6)	23 (19.7)	0.793
CEBPA	2 (5.9)	11 (9.4)	0.520
RUNX1	0 (0.0)	14 (12.0)	0.034
NPM1	6 (17.6)	32 (27.4)	0.251
TP53	3 (8.8)	8 (6.8)	0.695
WT1	3 (8.8)	7 (6.0)	0.558
FLT3	9 (26.5)	34 (29.1)	0.768
>1 mutation	31 (91.2)	102 (87.2)	0.765

TCGA, The Cancer Genome Atlas; BM, bone marrow; WBC, white blood cell; PB, peripheral blood; FAB, French-American-British; NA, not applicable/not available. Two-sided P<0.05 was considered to indicate a statistically significant difference.

and molecular features, which also form the basis for risk stratification (1,8). The complexity and heterogeneity of AML shed light on the importance of precision medicine and the detection of robust biomarkers. Recent studies highlight the feasibility of gene expression assay in AML management, with an improvement in risk stratification efficiency and prognostic

Table II. Univariate and multivariate analyses of OS and EFS in patients with AML in TCGA dataset (n=151).

	OS				EFS			
Characteristics	Univariate, HR (95% CI)	P-value	Multivariate, HR (95% CI)	P-value	Univariate, HR (95% CI)	P-value	Multivariate, HR (95% CI)	P-value
CYTH4 high vs. low	2.79 (1.52-5.12)	0.001	2.49 (1.28-4.83)	0.007	2.47 (1.45-4.21)	0.001	2.56 (1.48-4.42)	0.001
Sex	0.98 (0.66-1.46)	0.924			1.07 (0.66-1.72)	0.796		
Age	2.07 (1.33-3.20)	0.001	1.02 (1.00-1.04)	0.028	1.39 (0.85-2.26)	0.187		
WBC	1.00 (1.00-1.01)	0.020			1.01 (1.00-1.01)	0.003		
BM blast	1.00 (0.99-1.01)	0.977			1.00 (0.98-1.01)	0.529		
PB blast	1.00 (0.99-1.00)	0.485			1.01 (1.00-1.02)	0.007	1.01 (1.00-1.02)	0.006
Cytogenetics risk					1.11 (1.01-1.23)	0.033		
Inter vs. good	3.12 (1.59-6.14)	0.001	2.71 (1.29-5.68)	0.008	2.94 (1.48-5.84)	0.002		
Poor vs. good	4.42 (2.13-9.16)	<0.001	4.43 (1.81-10.83)	0.001	1.76 (0.71-4.33)	0.222		
Gene mutations								
FLT3	1.54 (1.01-2.38)	0.045	2.30 (1.45-3.65)	<0.001	1.59 (0.95-2.67)	0.078		
DNMT3A	1.74 (1.11-2.71)	0.015			1.76 (1.03-3.01)	0.039	1.71 (1.00-2.92)	0.050
NPM1	0.87 (0.56-1.37)	0.554			0.71 (0.43-1.20)	0.200		
TP53	5.09 (2.64-9.85)	<0.001	3.80 (1.69-8.57)	0.001	3.18 (0.98-10.36)	0.054	7.55 (2.16-26.41)	0.002
Transplantation	0.53 (0.36-0.81)	0.003	0.38 (0.23-0.63)	<0.001	1.55 (0.95-2.53)	0.082		

OS, overall survival; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; WBC, white blood count; BM, bone marrow; PB, peripheral blood; TCGA, The Cancer Genome Atlas. Two-sided P<0.05 was considered to indicate a statistically significant difference.

capacity (7,8). In the present study, OS and EFS were used to evaluate the prognosis of patient survival. It was shown that high expression of CYTH4 was associated with poor survival in AML, and it might be used as a prognostic biomarker. Other clinical outcomes such as the chemotherapy response, graft-versus-host disease (GVHD), and relapse rate were not discussed in the present study. Whether these outcomes are influenced by CYTH4 requires further investigation.

Cytohesins have been reported to play a pivotal role in various cancers, including but not limited to hepatocellular carcinoma, colorectal, lung and ovarian cancer (10,14,16-19,37). The present study focused on CYTH4 due to its higher expression in AML compared with that of other cytohesins. The tissue distribution of CYTH4 showed that CYTH4 expression was predominantly enhanced in the BM and lymphoid tissues. The distribution partly contributes to the high expression of CYTH4 in leukemia and lymphoma. Furthermore, CYTH4 expression in AML BM is higher than that in healthy people. The high expression and BM specificity provided prerequisites for CYTH4 to be a possible biomarker in AML. Furthermore, the limited tissue specificity made it reasonable to hypothesize that it might be used as a therapeutic target. The study by Bill *et al* (19) found that inhibition of cytohesins improved the treatment of gefitinib-resistant lung cancer. A recent study reported that the cytohesin inhibitor SecinH3 showed anti-leukemic effects both *in vitro* and *in vivo* (21). Furthermore, reduced expression of CYTH4 was observed in patients with PML-RARA and RUNX1-RUNX1T1. By contrast, cell lines with MLL-rearrangement such as NOMO-1, MOLM13 and MV4-11 exhibited high levels of CYTH4 expression. This suggests that the regulation of CYTH4 may be influenced by fusion proteins and their associated signaling pathways. This hypothesis is in line with the study by Stengel *et al*, which identified CYTH4 as a target regulated by the fusion protein RUNX1-RUNX1T1 (38).

As for clinical characteristics, high CYTH4 expression was significantly correlated with high WBC, higher risk status and RUNX1 mutation. These unfavorable factors are known to adversely affect the prognosis of patients with AML (39,40), indicating that CYTH4 might act as a negative prognostic factor. The survival analysis provided ultimate evidence that high expression of CYTH4 was associated with poor survival, validated in three different datasets. The multivariate analysis also confirmed the adverse prognostic effect of CYTH4. Additionally, it was observed that in patients with high CYTH4 expression, those who received chemotherapy plus transplantation had better survival outcomes than those who received chemotherapy alone. It suggested that CYTH4 might serve as an indicator to guide therapy, and transplantation could potentially overcome the adverse effect of high CYTH4 expression. BM evaluation is used through the diagnosis, management and follow-up of AML (41). However, in the present univariate and multivariate analysis, results showed that BM blast was not a risk factor for AML. The reason might be that ~80% (n=119/151) of the patients had a high percentage (>50%) of BM blasts at diagnosis. Therefore, the prognostic value of BM blasts was not significant in that particular cohort. *In vitro* functional analysis further confirmed that CYTH4 exerted oncogenic effects on AML cell lines, thereby underscoring its potential value as a prognostic biomarker in

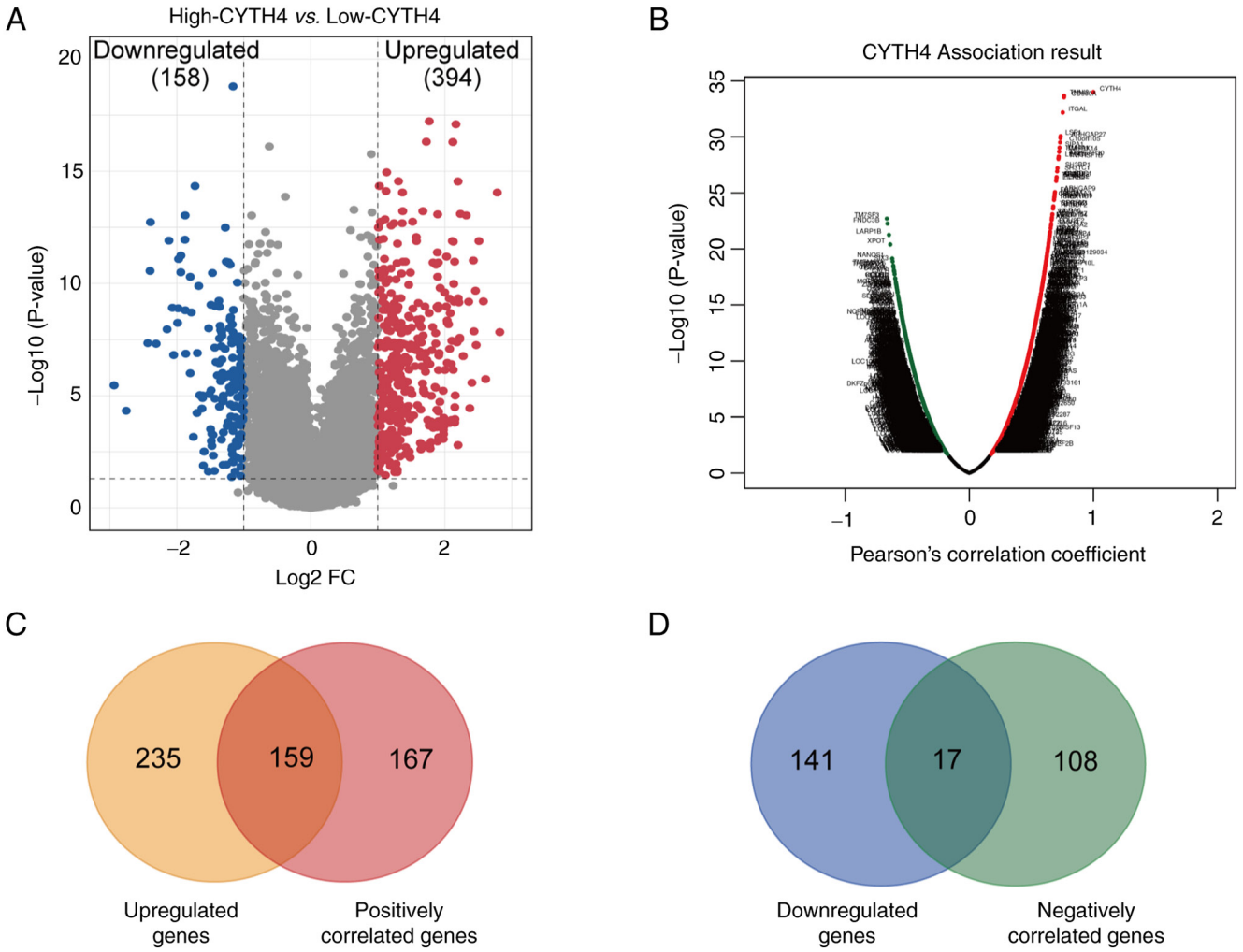

Figure 4. Identification of CYTH4-associated genes. (A) Volcano plot of differentially expressed genes between the low- and the high-CYTH4 group. The red and blue dots represent genes significantly upregulated and downregulated genes in the high-CYTH4 group, respectively. Adjusted P<0.05 and |FC|>2 indicated statistically significantly expression. (B) CYTH4 association analysis result by Linkedomics. Red and green dots represent genes positively and negatively correlated with CYTH4, respectively. (C) Venn diagram showing the overlap of upregulated genes and positively correlated genes. (D) Venn diagram showing the overlap of downregulated genes and negatively correlated genes. FC, fold change; CYTH4, cytohesin-4.

AML. These findings were consistent with previous studies that reported cytohesins have a variety of biological activities and are involved in cell proliferation (16), migration (18) and invasion (15) during carcinogenesis. Ren *et al* (21) reported that inhibiting CYTH1 could reduce the expression of the anti-apoptotic protein MCL1. Due to their identical structural organization (21), CYTH4 may also play a role in leukemogenesis by regulating essential molecules and pathways associated with cell proliferation (16,18) and apoptosis (21). Moreover, GO analysis and GSEA revealed that genes associated with CYTH4 were involved in cell defense response, signal transduction and apoptosis. Therefore, the present study suggests that CYTH4 is upregulated in AML and may play a crucial role in AML leukemogenesis. However, the specific mechanism by which CYTH4 contributes to leukemogenesis requires further investigation.

In AML, the data of the current study showed that CYTH4-associated genes were largely involved in the immune response such as antigen processing and presentation, and positive regulation of T cell proliferation and differentiation. This is consistent with the study by Wang *et al* (10) which demonstrated CYTH2 participated in immunoregulation. The KEGG analysis and GSEA also proved that CYTH4 was involved in immune response, but the exact mechanism needs to be further explored. Immunotherapy, including checkpoint inhibitors and chimeric antigen receptor-T therapy, is playing an increasingly important role in the treatment of leukemia (42). Further investigation of the role of CYTH4 in immunoregulation might provide novel insight into improving therapeutic efficacy and overcoming obstacles encountered in immunotherapy. Apart from the immunoregulation effect, the KEGG result showed that CYTH4 was related to allograft rejection and involved in GVHD in patients with AML undergoing transplantation. Further investigation of CYTH4 might help us improve the success rate of transplantation. However, this hypothesis requires further study.

The current study has some limitations that need to be addressed. Firstly, the results were mainly based on bioinformatics analysis of public datasets; additional experimental validations, especially *in vivo*, are required to confirm the findings. Secondly, it was suggested that CYTH4 expression might be used as a prognostic biomarker in AML. However,

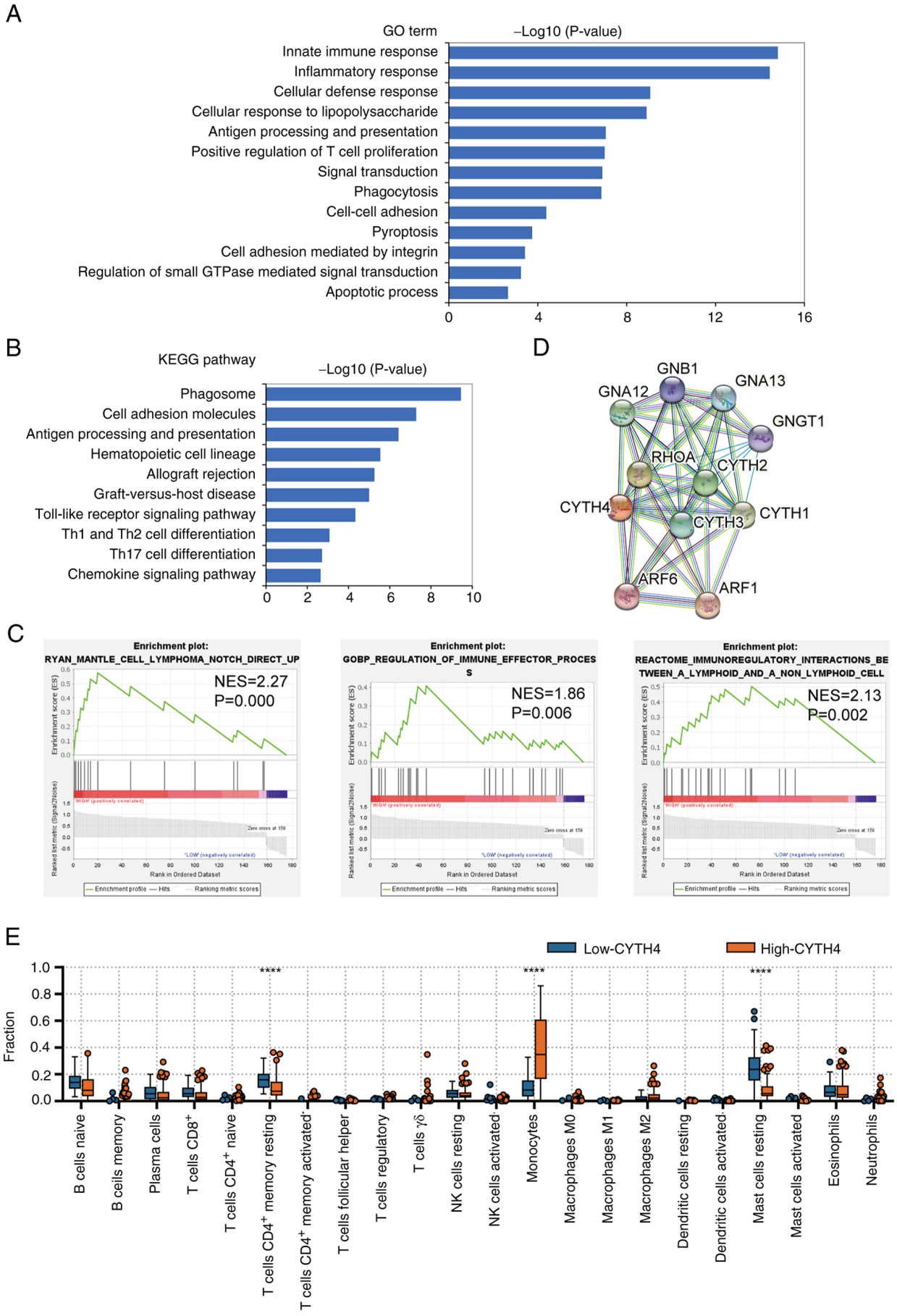

Figure 5. Functional enrichment analysis of CYTH4-associated genes. (A) GO analysis result. (B) KEGG pathway enrichment analysis. (C) GSEA enrichment plots. (D) Genes interacting with CYTH4 in STRING. (E) CIBERSORTx result showing the different fraction of immune cells between the low- and the high-CYTH4 groups. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; CYTH4, cytohesin-4.

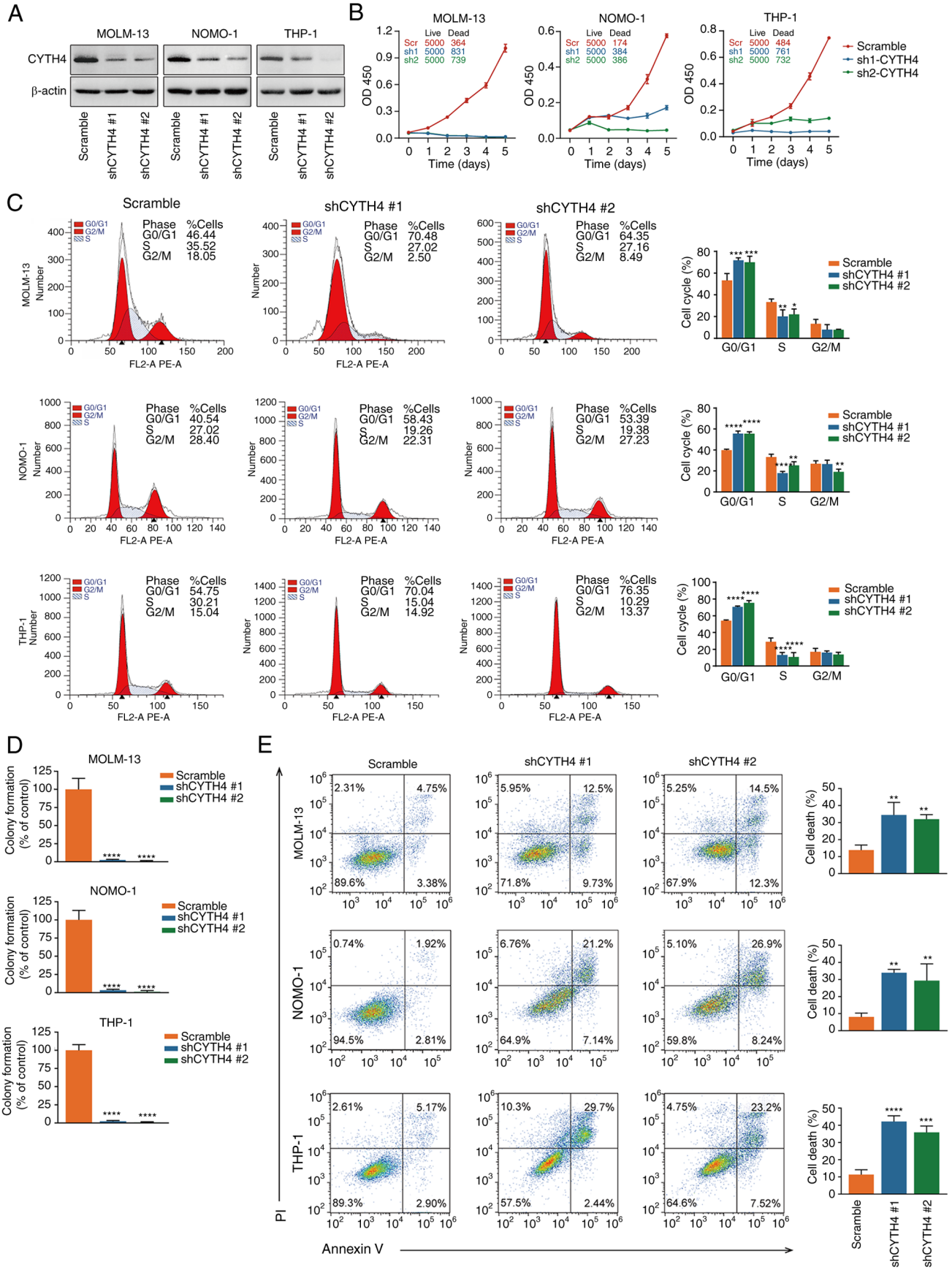

Figure 6. *In vitro* validation of the function of CYTH4. (A) Western blot showing reduced CYTH4 protein expression level after shRNA-mediated CYTH4 knockdown. (B) CCK-8 assay displaying the inhibition of cell growth in CYTH4-silenced cells compared with control cells. The number of living cells and dead cells on day 0 is displayed. (C) Flow cytometry analysis of cell cycle distribution in CYTH4-silenced and control cells. (D) Colony formation assays showing growth inhibition in CYTH4-silenced cells compared with control cells. (E) Flow cytometry showing increased apoptosis in CYTH4-silenced cells compared with control cells. Bars in (C), (D) and (E) show the mean ± SD from three independent biological replicates. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. CCK8, Cell Counting Kit-8; CYTH4, cytohesin-4; shRNA, short hairpin RNA.

compared with gene mutation and cytogenetic abnormities, there is more to consider before the gene expression profile could be used as a biomarker. For instance, the optimal cut-off value between high and low expression of CYTH4 is difficult to determine, because expression is a relative concept. The accuracy, feasibility and clinical utility of the CYTH4 expression need to be well demonstrated in larger patient cohorts. Nonetheless, the present study provides valuable insights into the potential role of CYTH4 as a prognostic biomarker in AML and prompts further investigations into its clinical relevance and therapeutic potential.

CYTH4 is upregulated in AML, and the high expression of CYTH4 is associated with poor survival. CYTH4 can potentially be used as a prognostic marker in AML.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HW and YL designed the research. HW, YX and WZ acquired and analyzed the data, and performed statistical analysis. HW and YX performed *in vitro* function validation. HW drafted the manuscript. YX, WZ and YL revised the manuscript. HW and YL confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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