Distinguishing acute leukemia subtypes: The role of hsa_circ_0012152 and hsa_circ_0020093 in peripheral blood

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Abstract. Acute leukemia (AL), a rapidly progressing hematological malignancy originating from the bone marrow, is primarily subclassified into acute myelocytic leukemia (AML) and acute lymphoblastic leukemia (ALL). Obtaining bone marrow samples can be challenging due to a number of reasons, including dilution or inaccessibility. Therefore, the present study focused on identifying novel diagnostic biomarkers in the peripheral blood for AL subgroups. Circular RNAs (circRNAs) are non-coding RNA molecules associated with various diseases. In the present study, to validate the distinct circRNA expression patterns distinguishing AML from ALL in peripheral blood, reverse transcription-quantitative polymerase chain reaction was employed. The diagnostic accuracy of hsa_circ_0020093 and hsa_circ_0012152 was then assessed using receiver operating characteristic curve analysis, and hsa_circ_0020093 was selected for further exploration using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses. The findings revealed that the expression patterns of hsa_circ_0020093 and hsa_circ_0012152 clearly differentiated ALL from AML in peripheral blood. The potential target genes of hsa_circ_0020093 identified were associated with critical biological processes such as protein serine kinase activity and cadherin binding. Furthermore, these genes are involved in signaling pathways including MAPK and mTOR. We propose that hsa_circ_0020093 plays a crucial role in initiating and promoting ALL by modulating downstream target genes through either hsa-microRNA (miR)-153-3p or hsa-miR-194-5p. The results of the present study demonstrate that hsa_circ_0020093 and hsa_circ_0012152 hold significant promise as diagnostic biomarkers for subclassifying AL into ALL or AML in peripheral blood. This discovery lays the foundation for future research endeavors aimed at elucidating the role of circRNAs in the pathogenesis and treatment of AL.

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Abbreviations: AL, acute leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; circRNA, circular RNA; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ROC, receiver operating characteristic; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ncRNAs, non-coding RNAs; miRNAs, microRNAs; AUC, area under the curve; FC, Fold Change; PPI, protein-protein interaction; ceRNA, competing endogenous RNA; 95% CI, 95% confidence interval

Key words: circRNA, peripheral blood, AL, diagnostic markers, bioinformatics

Introduction

Acute leukemia (AL), a highly heterogeneous malignant clonal disease affecting hematopoietic stem cells (1), is characterized by the infiltration of leukemia cells from the bone marrow into various organs and tissues, ultimately suppressing normal hematopoietic function. Based on the French-American-British classification, AL is divided into acute myelocytic leukemia (AML) and acute lymphoblastic leukemia (ALL) (2). While modern chemotherapy regimens have achieved high rates of complete remission, both AML and ALL are associated with decreased survival rates and poor prognosis due to recurrence within 5 years (3). The significance of the accurate identification of AML and ALL is paramount, as treatment strategies for the two subtypes notably differ (4). However, several challenges hinder the accurate diagnosis of leukemia worldwide, including limited medical resources, a scarcity of experienced technicians and the prohibitive cost of necessary equipment. These factors have led to instances of leukemia classification errors (5,6). Additionally, some patients with AL exhibit ambiguous expression of myeloid or lymphoid immune markers (7), while others have leukemia cells that simultaneously express both myeloid and lymphoid antigens (8). This complexity not only complicates the diagnostic process but also poses challenges in selecting appropriate chemotherapy regimens, ultimately impacting treatment outcomes. Given these challenges, there is an urgent need to identify a simple and reliable method for distinguishing AML from ALL.

Circular RNAs (circRNAs) constitute a unique subclass of non-coding RNAs (ncRNAs) that predominantly reside within the cytoplasm. These molecules are not affected by RNA exonuclease (9) and their expression shows marked stability and has been observed in a wide range of eukaryotic

organisms (10). A key mechanism by which circRNAs exert their biological influence is through the competitive adsorption of microRNAs (miRNAs) (11). circRNAs, with their higher abundance compared with mRNAs, have emerged as promising biomarkers for cancer diagnosis. A growing body of evidence suggests a strong association between circRNAs and the diagnosis as well as prognosis of AL (12-16). Certain studies have shown that circ-VIM (13) is significantly upregulated and hsa_circ_0004277 (14) is significantly downregulated in AML. In addition, the expression level of circPVT1 is increased in ALL (15). Meanwhile, Guo *et al* (16) utilized microarray analysis to compare circRNA expression profiles across different groups. Notably, hsa_circ_0012152 and hsa_circ_0001857 emerged as effective discriminators between AML and ALL in bone marrow samples, further underscoring their potential as diagnostic biomarkers. These findings underscore the increasing recognition of circRNAs as valuable players in the diagnosis and prognosis of AL.

However, current studies aiming to distinguish between AML and ALL predominantly rely on bone marrow samples, leaving peripheral blood as an underexplored alternative. The process of extracting bone marrow specimens is fraught with potential complications that can lead to unreliable test results, Lin *et al* (17) found that 11.8% of patients with bone marrow necrosis were still misdiagnosed after bone marrow aspiration. These factors include patient psychological issues, technical errors by the extraction personnel, the volume of extraction, age, puncture site and pathological conditions (18). Peripheral blood sampling offers a notably more convenient and less invasive approach compared with bone marrow sampling, potentially reducing patient discomfort (19). In suspected cases of leukemia, testing peripheral blood can provide earlier insights into AL subtypes, facilitating the timely determination of appropriate chemotherapy regimens. Chinese guideline for diagnosis and treatment of adult acute lymphoblastic leukemia (2024) (20) suggest using peripheral blood for essential tests when bone marrow aspiration yields no sample. This method is especially beneficial in scenarios where obtaining bone marrow samples poses challenges, such as in the presence of bone marrow fibrosis.

The present study aimed to identify peripheral blood-based circRNA biomarkers for minimally invasive distinction between AML and ALL. Using experimental validation and bioinformatic profiling, the diagnostic utility of hsa_circ_0012152 and hsa_circ_0020093 were assessed while exploring their potential regulatory roles in AL.

Materials and methods

Patients and specimen collection. The present study recruited 110 patients newly diagnosed with AL and 20 healthy individuals (10 males and 10 females; age range, 14-70 years) from January 2023 to September 2024 at the First Affiliated Hospital of Ningbo University (Ningbo, China). Specifically, the 110 patients with AL comprised 86 patients with AML (53 males and 33 females; age range, 11-87 years) and 24 patients with ALL (12 males and 12 females; age range, 2-74 years). The inclusion criteria required confirmation of diagnosis according to the 2022 World Health Organization Classification of Haematolymphoid Tumours (21,22) and the availability of peripheral blood samples. The exclusion criteria comprised patients with chronic myeloid leukemia, therapy-related AML, those who had undergone prior chemotherapy, individuals with concurrent malignancies and T cell ALL (T-ALL) cases. For the patients with ALL included in the study, a phased grouping strategy was applied. During the initial phase, all ALL cases were analyzed as a single unified group (n=24) and compared directly against the AML group. Subsequently, to investigate biomarker performance differences among intrinsic ALL subtypes, the ALL cohort was further stratified into two subgroups: ALL with myeloid antigen expression (n=12) and B-ALL (n=12) for independent comparative analysis. It should be noted that all specimens utilized in this investigation were prospectively collected residual peripheral blood samples obtained from the patients.

Screening target circRNAs. The circRNA microarray data analyzed in the present study were archived in the OMIX database (National Genomics Data Center; accession no: OMIX009143; https://ngdc.cncb.ac.cn/omix/release/OMIX009143) and were from our previous study (16). The sequencing data of hsa_circ_0012152 and hsa_circ_0020093 examined during the present study are also available in the GSA-human repository (https://bigd.big.ac.cn/gsa-human/browse/HRA007384). The R language (23) (version 4.1.2) limma package was utilized to standardize and analyze the raw circRNA microarray data. Using a fold change (FC) threshold of ≥10 and $P<0.05$ as criteria, those circRNAs with the most significant differential expression and higher expression abundance were selected when comparing AML to ALL as the focus of the present study.

Total RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation: 2 ml Ficoll solution (GE Healthcare) was vertically added to the bottom of a 15 ml centrifuge tube, followed by gentle layering of 4 ml EDTA-anticoagulated peripheral blood onto the Ficoll layer. After centrifugation at 500 x g for 30 min at 20˚C, the PBMC layer (opaque white interphase) was carefully aspirated. The harvested PBMCs were washed three times with 5 ml phosphate-buffered saline (PBS) (200 x g, 10 min each). Residual red blood cells, if present, were lysed using 5 ml Red Blood Cell Lysis Buffer (Beijing Solarbio Science & Technology Co., Inc.) at 37˚C for 10 min, followed by centrifugation (200 x g, 10 min). The pellet was resuspended in 1 ml PBS and transferred to RNase-free EP tubes for subsequent analysis. Total RNA was extracted from the PBMCs using RNAiso Plus reagent (Takara Bio, Inc.), following the manufacturer's instructions. The NanoDrop 2000 ultra microspectrophotometer (Thermo Fisher Scientific, Inc.) was then utilized to determine the concentration and purity of the RNA samples. RNA purity was deemed satisfactory when the optical density 260/280 ratio fell within the acceptable range of 1.8-2.1, with a concentration maintained at ~500 μg/ml. Following RNA extraction, cDNA synthesis was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) as per the manufacturer's protocol. Subsequently, qPCR was performed using the TB Green PCR reagent kit (Takara Bio, Inc.) on the StepOnePlus

Table I. Sequence of primers used in reverse transcription-quantitative PCR.

Gene	Primer sequences (5' to 3')
GAPDH	Forward: ATGGGGAAGGTGAAGGTCG
	Reverse: GGGTCATTGATGGCAACAATATC
hsa_circ_0012152	Forward: TCTCCCCACTTGCGCTTCTC
	Reverse: GCCAACCAGCACTTTGGGTC
hsa_circ_0020093	Forward: AATTGCGGCAGTCCAGATCA
	Reverse: TGGATAGCCTTCAATGAGCCA

circ, circular (RNA).

system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each 20 μl qPCR reaction mixture comprised 1 μl cDNA, 1.6 μl primer, 0.4 μl ROX, 10 μl TB Green and 7 μl diethylpyrocarbonate-treated (DEPC) water. The qPCR reaction program consisted of an initial pre-denaturation step at 95˚C for 60 sec, followed by 40 cycles of amplification. Each cycle included denaturation at 95˚C for 15 sec, primer annealing at 64˚C for 30 sec and extension at 72˚C for 32 sec, with fluorescence recording. Melting curve analysis was then performed, consisting of denaturation at 95˚C for 15 sec, annealing at 60˚C for 60 sec, re-denaturation at 95˚C for 15 sec and a final annealing step at 60˚C for 15 sec. For internal standardization, the MOLM-13 cell line (from The First Affiliated Hospital, Zhejiang University School of Medicine; RRID: CVCL_2119) was used as a reference for hsa_circ_0012152 detection, while the NALM-6 cell line (from The First Affiliated Hospital, Zhejiang University School of Medicine; RRID: CVCL_0092) served as a benchmark for hsa_circ_0020093 detection. GAPDH was employed as the reference gene and DEPC water was included as a negative control. Gene expression levels were analyzed using the comparative cycle threshold ($2^{-\Delta\Delta Cq}$) method (24). Primer sequences are provided in Table I. Post-PCR analysis involved confirming the absence of any jumps in the negative control, checking the corresponding Cq values of the reference gene (GAPDH), hsa_circ_0012152 and hsa_circ_0020093 as well as analyzing the melting curve. A single peak in the melting curve, with a melting temperature ranging between 80-90˚C, indicated good primer specificity. This was further verified through gel electrophoresis imaging, product sequencing and amplification efficiency validation. Adjustments were made to the baseline, amplification curve and threshold for each gene based on the observed amplification patterns, and all experimental data were recorded accordingly.

Construction of competing endogenous RNA (ceRNA) network. Using the CircInteractome database (https://circinteractome.irp.nia.nih.gov/) and starBase3.0 database (https://starbase.sysu.edu.cn/), the downstream target miRNA and miRNA-targeted genes of hsa_circ_0020093 were predicted. Given that the predictive analysis of hsa_circ_0012152 has been systematically established in a previous study (16), the current investigation specifically explored the prediction of hsa_circ_0020093 to advance novel findings. With the assistance of the STRING 11.5 database (https://string-db.org/), a protein-protein interaction (PPI) network was constructed, which was then visually represented using Cytoscape (25) (version 3.8.0). The essential hub genes were pinpointed in this PPI network. Furthermore, leveraging the capabilities of Cytoscape, the hsa_circ_0020093-miRNA-mRNA crosstalk network and the ceRNA network were crafted.

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway analyses for target genes. Using the interaction relationship within the ceRNA network as a foundation, GO and KEGG enrichment analyses on the miRNA target genes were performed employing R software. To visualize these enrichment results, the enrich plot package was utilized, generating graphical representations that provided insights into the functional and pathway associations of the target genes.

Statistical analysis. In the present study, SPSS 26.0 software (IBM Corp.) was harnessed to analyze the experimental data, while GraphPad Prism 9.0 (Dotmatics) was used to visualize the outcomes. To assess the diagnostic efficacy of hsa_circ_0012152 and hsa_circ_0020093 as auxiliary markers for AML and ALL in peripheral blood, as well as their utility in subtype classification for AL, the receiver operating characteristic (ROC) curve was employed to compute the area under the curve (AUC) value. The diagnostic cut-off values for both hsa_circ_0012152 and hsa_circ_0020093 were established using Youden's index, enabling the calculation of sensitivity, specificity, positive predictive value and negative predictive value. A comprehensive analysis of the diagnostic efficacy for AL subtypes was conducted using a range of experiments, including series and parallel tests, along with a multi-factor logistic regression model. AML cases were stratified into high- and low-expression groups based on the median expression level of hsa_circ_0012152, while ALL cases were similarly categorized according to hsa_circ_0020093 expression. Differences in laboratory indicators and clinical data between these groups were statistically analyzed. Qualitative data were subjected to the χ^2 test, while quantitative data with three or more groups were analyzed using the non-parametric Kruskal-Wallis H-test, followed by Dunn's multiple comparison test for post hoc pairwise comparisons. For datasets with 2 groups, the Wilcoxon rank sum test was directly employed. $P<0.05$ was considered to indicate a statistically significant difference.

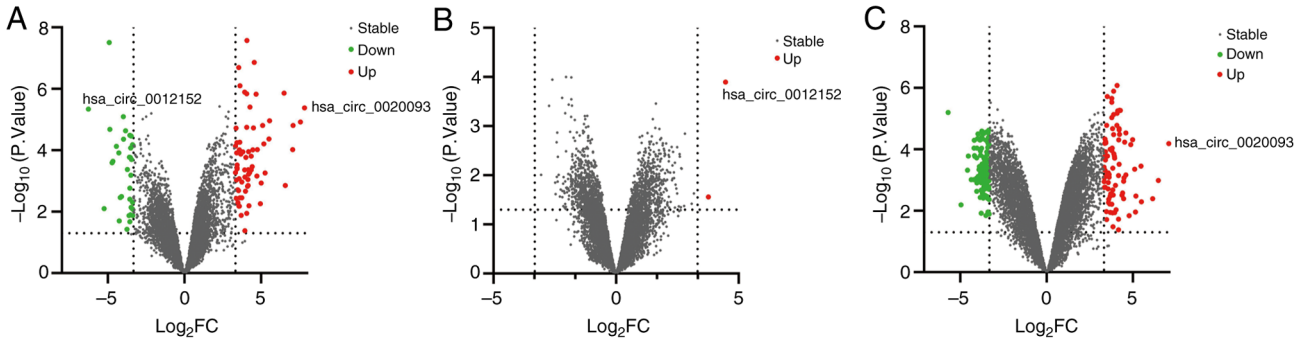

Figure 1. Volcano plot of differentially expressed circRNAs between ALL, AML and NC. 'log_2FC' is plotted on the horizontal axis and '-log_{10} (P-value)' is plotted on the vertical axis; that is, the larger the 'log_2FC' the greater the difference, and the larger the '-log_{10} (P-value)' the more significant the statistical difference. Red and green represent the upregulated and downregulated circRNAs, respectively, with $|log_2FC| \geq 10$ and $P<0.05$, while gray represents the circRNAs with $|log_2FC|<10$ or $P \geq 0.05$. (A) ALL vs. AML, (B) AML vs. NC and (C) ALL vs. NC. circRNA, circular RNA; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NC, normal control; FC, fold change.

Results

circRNA screening results. In our previous investigation utilizing circRNA microarray analysis (16), 10,171 circRNAs were identified. By applying stringent criteria of a FC ≥10 and a P<0.05, the selection was narrowed down to 101 differentially expressed circRNAs between ALL and AML. These included 67 circRNAs that were upregulated and 34 that were downregulated in ALL compared with AML. Notably, among this subset, hsa_circ_0012152 stood out as the most notably downregulated circRNA, exhibiting a 77-fold decrease and being the sole circRNA to exhibit a downregulation exceeding 50-fold. Conversely, hsa_circ_0020093 emerged as the most significantly upregulated circRNA, achieving a 228-fold increase and being the only one to surpass a 200-fold upregulation (Fig. 1A). When comparing the AML sample to the normal control, of the 2 differentially expressed circRNAs that were upregulated, hsa_circ_0012152 demonstrated the most significant upregulation, reaching a 22-fold increase (Fig. 1B). By contrast, when ALL was compared with the normal control, 201 circRNAs exhibited differential expression, comprising 88 upregulated and 113 downregulated circRNAs. Once again, hsa_circ_0020093 was the most significantly upregulated circRNA, achieving a 133-fold increase and being the only circRNA to exceed a 100-fold up-regulation (Fig. 1C). Notably, hsa_circ_0012152 and hsa_circ_0020093 exhibited the highest expression levels in AML and ALL, respectively, making them ideal candidates for further investigation. Consequently, these two circRNAs were selected as the focal targets of the present study.

Expression levels of hsa_circ_0012152 and hsa_circ_0020093 in the peripheral blood of patients with AL. To gain deeper insights into the expression profiles of hsa_circ_0012152 and hsa_circ_0020093 in AL, a sample validation study for these two circRNAs was undertaken. The results presented in Fig. 2A highlight that the expression levels of hsa_circ_0012152 were significantly elevated in the peripheral blood samples from patients with AML compared with ALL (median value, 8.40 vs. 0.51; P<0.0001). Furthermore, the expression of hsa_circ_0012152 was also significantly higher in patients with AML compared with the normal controls (median value, 8.40 vs. 0.29; P<0.0001). By contrast, the expression levels of hsa_circ_0020093were significantly increased in patients with ALL compared with AML (median value, 0.1601 vs. 0.0017; P<0.0001; Fig. 2B) and were also significantly higher in patients with ALL than in the normal controls (median value, 0.1601 vs. 0.0027; P=0.0003; Fig. 2B). These findings suggest that the expression levels of both hsa_circ_0012152 and hsa_circ_0020093 in peripheral blood could potentially serve as diagnostic and classification biomarkers for AML and ALL.

Upon further evaluation of their diagnostic efficacy using ROC curve analysis, it was found that hsa_circ_0012152 exhibited high sensitivity (0.9651), perfect specificity (1.0000) and an excellent AUC of 0.9878 [95% confidence interval (CI), 0.9660-1.0000; P<0.0001; Fig. 2C and Table II] as an adjunctive diagnostic marker for AML in peripheral blood. Similarly, hsa_circ_0020093 demonstrated good sensitivity (0.8333), perfect specificity (1.0000) and a promising AUC of 0.8708 (95% CI, 0.7489-0.9928; P<0.0001; Fig. 2D and Table II) as an adjunctive diagnostic marker for ALL in peripheral blood. Thus, both circRNAs showed promising potential as adjunctive diagnostic markers for AML and ALL in peripheral blood samples. Notably, the expression levels of hsa_circ_0012152 and hsa_circ_0020093 remained consistent across different patient demographics, including sex, age, laboratory parameters and survival outcomes (Table III). However, in patients with AML exhibiting high expression levels of hsa_circ_0012152, there was a higher proportion of primitive cells in the peripheral blood (P=0.008; Table III). This finding hints at a possible association between hsa_circ_0012152 expression and disease progression in patients with AML, which warrants further investigation.

hsa_circ_001252 and hsa_circ_0020093 can accurately discriminate AML from ALL in peripheral blood. Subsequently, ROC curves were constructed to evaluate the subtype-distinguishing capacity of hsa_circ_0012152 and hsa_circ_0020093 in differentiating AML from ALL (Fig. 3 and Table IV), extending their utility beyond initial diagnostic applications in leukemia detection. The findings revealed that in peripheral blood samples, hsa_circ_0012152 exhibited a sensitivity of 0.9651, a specificity of 0.6667 and an AUC of

Table II. Diagnostic efficacy of hsa_circ_0012152 and hsa_circ_0020093 in acute leukemia.

circRNA	Disease	Sensitivity	Specificity	PPV	NPV	Youden	AUC	95% CI	P-value
hsa_circ_0012152	AML	0.9651	1.0000	1.0000	0.8695	0.9651	0.9878	0.9660-1.0000	<0.0001
hsa_circ_0020093	ALL	0.8333	1.0000	1.0000	0.8333	0.8333	0.8708	0.7498-0.9928	<0.0001

The statistical analysis was performed using receiver operating characteristic analysis. AUC, area under curve; 95% CI, 95% confidence interval; PPV, positive predict value; NPV, negative predict value; circRNA, circular RNA; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia.

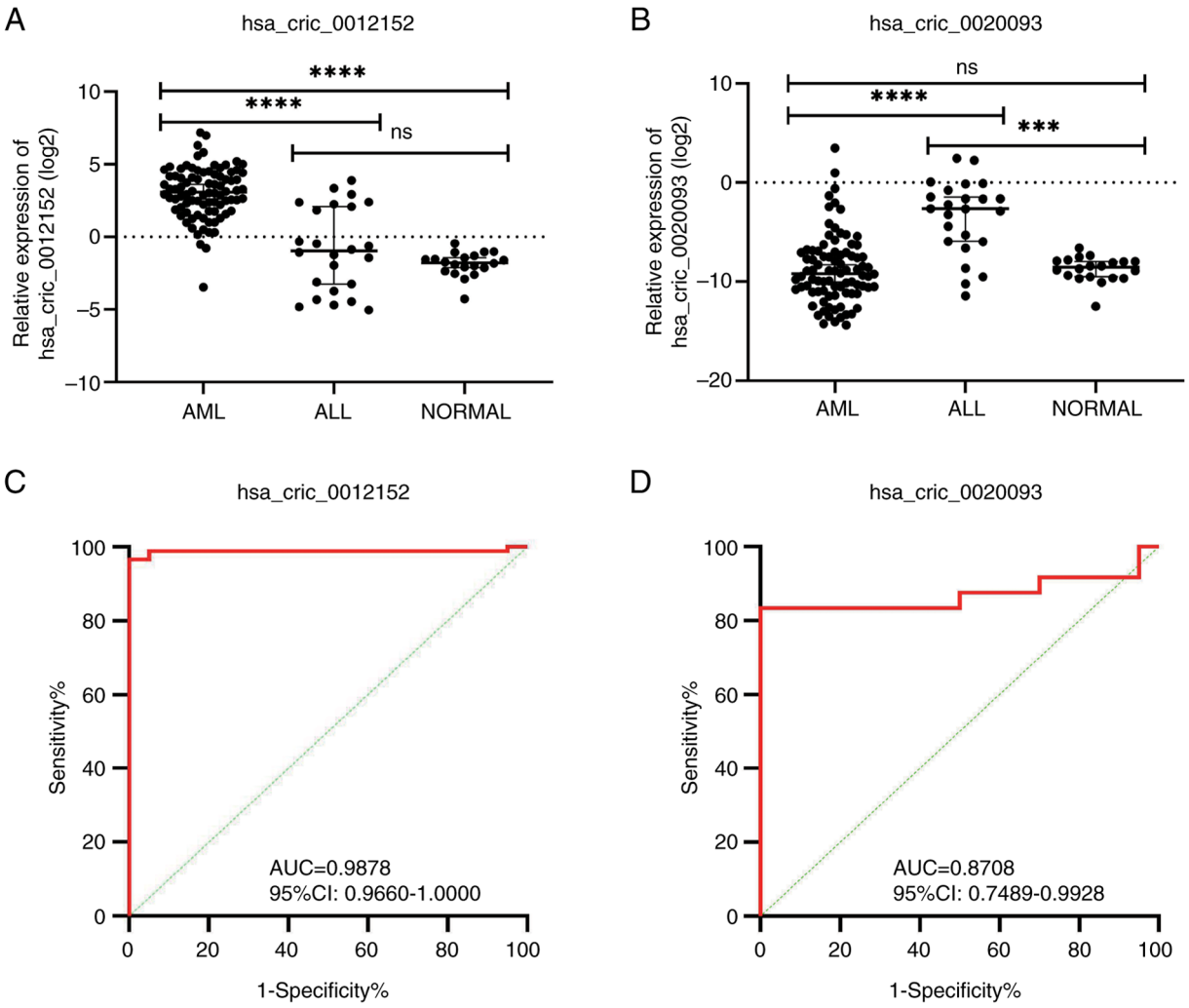

Figure 2. Relative expression levels of hsa_circ_0012152 and hsa_circ_0020093 in peripheral blood samples of patients with AL. (A) The relative expression levels of hsa_circ_0012152 in patients with AL, exhibiting significant differences. (B) The relative expression levels of hsa_circ_0020093 in patients with AL, highlighting distinct patterns. (C) ROC curve of hsa_circ_0012152, demonstrating its potential as a diagnostic biomarker for distinguishing AML from healthy individuals. (D) ROC curve of hsa_circ_0020093, emphasizing its diagnostic value in differentiating ALL from healthy controls. ****P<0.0001, ***P<0.001. ns, no significant difference; circ, circular (RNA); AL, acute leukemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AUC, area under the curve; 95% CI, 95% confidence interval; ROC, receiver operating characteristic.

0.8634 (95% CI, 0.7778-0.9489; P<0.0001) in discriminating AML from ALL. Additionally, hsa_circ_0020093 demonstrated a sensitivity of 0.8333, a specificity of 0.8023 and an AUC of 0.8401 (95% CI, 0.7452-0.9350; P<0.0001) for the same purpose. To further enhance the diagnostic accuracy of these two circRNAs, series, parallel and logistic regression experiments were conducted for each. In the series experiments, the specificity and positive predictive value increased to 0.9444 and 0.9804, respectively, albeit with a slight decrease in sensitivity and negative predictive value (Table IV). Conversely, in the parallel experiments, the sensitivity and negative predictive value rose to 0.9931 and 0.9574, respectively, but there was a decrease in specificity and positive predictive value (Table IV). Using both hsa_circ_0012152

Table III. Relationship between high and low expression of hsa_circ_0012152 or hsa_circ_0020093 in peripheral blood with the clinical characteristics of patients with acute leukemia.

	hsa_circ_0012152 expression			hsa_circ_0020093 expression		
Characteristic	High	Low	P-value	High	Low	P-value
Sex, n (%)			0.121			0.414
Male	23 (43.40)	30 (56.60)		7 (58.33)	5 (41.67)	
Female	20 (60.61)	13 (39.39)		5 (41.67)	7 (58.33)	
Median age, years (IQR)	53 (11,78)	63 (14,87)	0.269	49.5 (3,66)	44.5 (2,74)	0.817
Median BMPC, % (IQR)	59.5 (25.5,93)	60.0 (23.0,89.5)	0.276	84 (59,92)	80.5 (46,96.5)	0.743
Median PBIC, % (IQR)	45 (4,90)	20 (1,90)	0.008[a]	36 (3,77)	50 (8,86)	0.744
Median WBC, $x10^9$/l (IQR)	15.3 (1.1,259.1)	8.2 (0.4,186.7)	0.141	11.9 (1.1,84.0)	16.5 (1.1,86.3)	0.954
Median RBC, $x10^9$/l (IQR)	2.2 (0.7,5.4)	2.32 (1.3,4.8)	0.638	3.91 (1.53,5.00)	2.67 (1.48,5.06)	0.386
Median Hb, g/l (IQR)	76 (26,131)	74 (46,142)	0.917	115.5 (54,136)	86.5 (56,153)	0.525
Median PLT, $x10^9$/l (IQR)	42 (9,507)	35 (4,316)	0.273	37 (4,173)	89 (8,294)	0.273
Median LDH, IU/l (IQR)	431 (102,1835)	324 (115,2540)	0.182	570 (199,7138)	525 (152,13207)	0.644
Median β2-MG, mg/l (IQR)	2.1 (1.2,8.5)	2.3 (1.0,8.6)	0.365	2.4 (1.2,3.4)	2.1 (1.1,5.2)	0.795
Median OS, days (IQR)	191 (1,632)	225 (5,747)	0.904	157 (105, 362)	136 (9,780)	0.670
Median EFS, days (IQR)	181 (1,632)	215.5 (5,747)	0.959	153 (105,362)	136 (9,780)	0.250

[a]Significant difference. χ^2 test was used for sex analysis and Wilcoxon rank sum test was used for the other analyses. IQR, interquartile range; circ, circular (RNA); BMPC, bone marrow primitive; PBIC, peripheral blood immature cells; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; PLT, platelet; LDH, lactate dehydrogenase; β2-MG, β2-microglobulin; OS, overall survival; EFS, event-free survival.

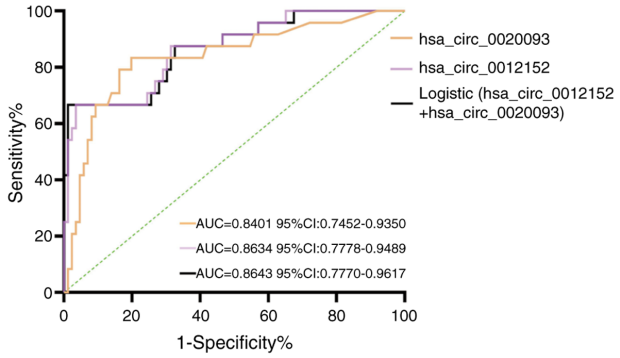

Figure 3. Receiver operating characteristic curve analysis for distinguishing acute myeloid leukemia from acute lymphoblastic leukemia utilizing hsa_circ_0012152 and hsa_circ_0020093 as diagnostic biomarkers. circ, circular (RNA); AUC, area under curve; 95% CI, 95% confidence interval.

and hsa_circ_0020093 as independent variables, a logistic regression model was established. The predictive probability derived from this model was then utilized as the diagnostic discriminator to construct an ROC curve. The results indicated that the sensitivity and AUC increased to 0.9884 and 0.8643 (95% CI, 0.7770-0.9617; P<0.0001; Table IV), respectively, with the logistic regression model offering a slight improvement in diagnostic discrimination compared with using these markers individually. Each type of testing (series, parallel and logistic regression) has distinct advantages and disadvantages. Series testing inherently reduces sensitivity as an intrinsic trade-off for specificity enhancement, while parallel testing inherently reduces specificity as an intrinsic characteristic to improve sensitivity (26). Logistic regression testing provides a notable enhancement in diagnostic accuracy (27). Therefore, it is recommended that these three tests be combined when distinguishing AML from ALL using a diagnostic marker, as this approach leverages the strengths of each method while mitigating their respective weaknesses.

Diagnostic efficacy of the hsa_circ_0012152 levels in peripheral blood in distinguishing the molecular subtypes of AML and the presence or absence of myeloid antigen expression in patients with ALL. Utilizing the 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms (21), which relies on cellular morphology, genetics and immunophenotype characteristics, enables more precise differentiation and consequently accurate guidance on the origin and prognostic assessment of AML. Against this backdrop, it was next determined whether the expression of hsa_circ_0012152 in peripheral blood could inform this WHO classification. Adhering to the WHO classification standards, 86 patients with AML were stratified into distinct groups. The expression profiles of hsa_circ_0012152 across these groups are presented in Fig. 4E. Notably, a statistically significant difference in hsa_circ_0012152 expression was observed across most groups, indicating the presence of a subset of patients with distinct expression patterns. Upon closer examination of the inter-group differences, it was found that the 'APL with PML-RARA or t (15;17)' group exhibited lower expression levels of hsa_circ_0012152 compared with the other groups (median value, 3.40 vs. 12.24; P<0.0001; Fig. 4A).

Table IV. Differential diagnostic efficacy of acute myeloid leukemia and acute lymphoblastic leukemia by hsa_circ_0012152 and hsa_circ_0020093.

Components and models	Sensitivity	Specificity	PPV	NPV	Youden	AUC	95% CI	P-value
hsa_circ_0012152	0.9651	0.6667	0.9121	0.8420	0.6318	0.8634	0.7778-0.9489	<0.0001
hsa_circ_0020093	0.8333	0.8023	0.5405	0.9452	0.6357	0.8401	0.7452-0.9350	<0.0001
Logistic (hsa_circ_0012152 + hsa_circ_0020093)	0.9884	0.6667	0.9140	0.9413	0.6550	0.8643	0.7770-0.9617	<0.0001
Parallel test	0.9931	0.5556	0.8890	0.9574	0.5487	-	-	-
Serial test	0.7742	0.9444	0.9804	0.5386	0.7186	-	-	-

The statistical analysis was performed using receiver operating characteristic analysis. circ, circular (RNA); AUC, area under curve; 95% CI, 95% confidence interval; PPV, positive predict value; NPV, negative predict value.

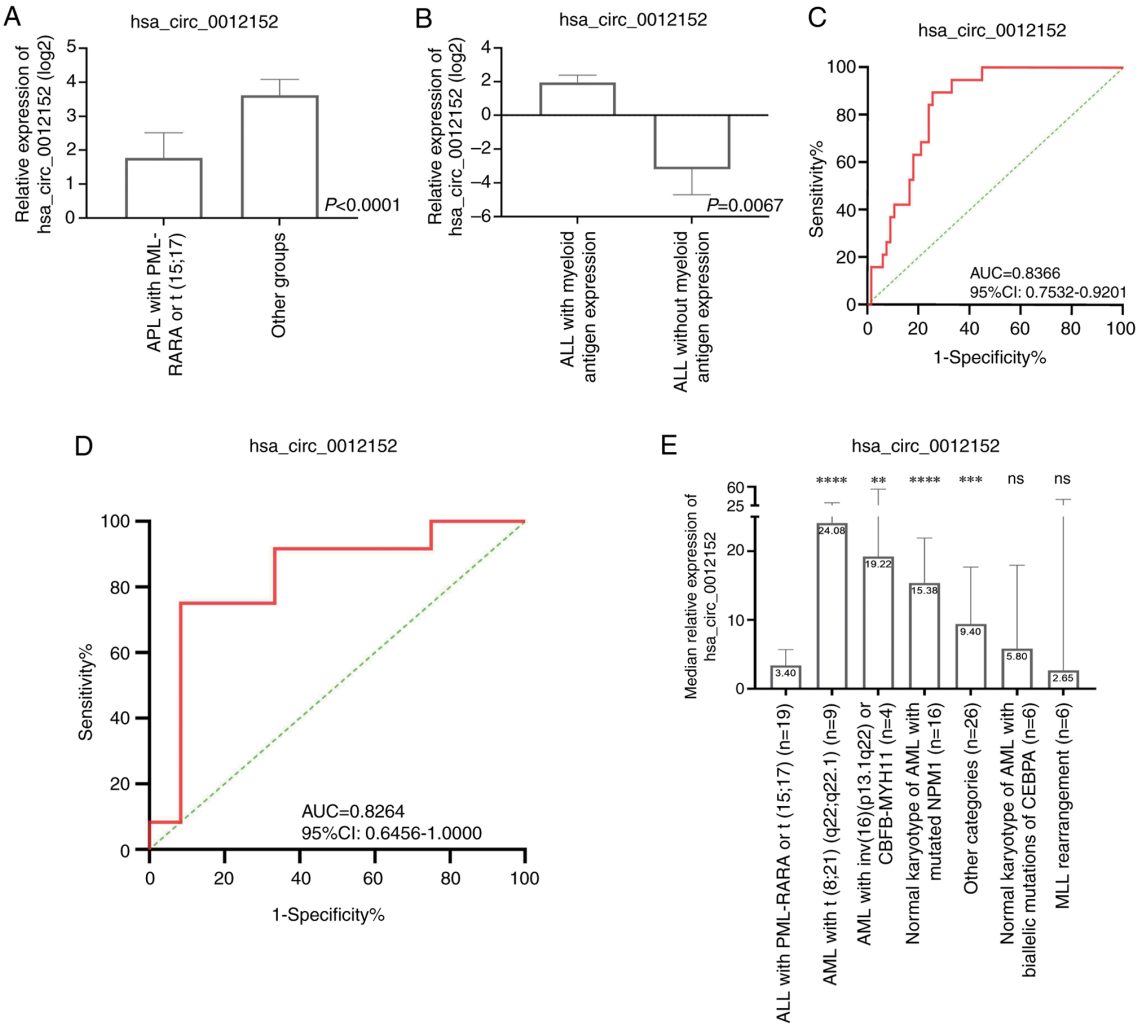

Figure 4. Diagnostic Efficacy of hsa_circ_0012152 levels in peripheral Blood for distinguishing molecular subtypes of AML and assessing myeloid antigen expression in patients with ALL. (A) The expression levels of hsa_circ_0012152 specifically in 'APL with PML-RARA or t (15;17)' compared with the other AML subtypes, highlighting significant differences. (B) The expression patterns of hsa_circ_0012152 in patients with ALL with and without myeloid antigen expression, indicating potential diagnostic utility. (C) ROC curve analysis for identifying 'APL with PML-RARA or t (15;17)' within the AML population based on the hsa_circ_0012152 expression levels. (D) ROC curve analysis for differentiating myeloid antigen expression in patients with ALL using hsa_circ_0012152 as a diagnostic biomarker. (E) Classification of hsa_circ_0012152 expression in patients with AML according to the World Health Organization criteria. ****P<0.0001, ***P<0.001, **P<0.01, all groups were compared with APL with PML-RARA or t (15;17). ns, no significant difference circ, circular (RNA); AUC, area under curve; 95% CI, 95% confidence interval; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ROC, receiver operating characteristic; PML, promyelocytic leukemia; RARA, retinoic acid receptor α; APL, acute promyelocytic leukemia; CBFB, core-binding factor subunit β; MYH11, myosin heavy chain 11; NPM1, nucleophosmin 1; MLL, mixed lineage leukemia gene; CEBPA, CCAAT/enhancer binding protein α.

Table V. Diagnostic efficacy of the levels of hsa_circ_0012152 in peripheral blood in distinguishing the molecular subtypes of AML and the presence or absence of myeloid antigen expression in patients with ALL.

Identity	Sensitivity	Specificity	PPV	NPV	Youden	AUC	95% CI	P-value
APL with PML-RARA and other groups in AML	0.8947	0.7463	0.5000	0.9615	0.6410	0.8366	0.7532-0.9201	<0.0001
Presence or absence of myeloid antigen expression in ALL	0.7500	0.9167	0.9000	0.7857	0.6667	0.8264	0.6456-1.0000	0.0067

The statistical analysis was performed using receiver operating characteristic analysis. circ, circular (RNA); AUC, area under curve; 95% CI, 95% confidence interval; PPV, positive predict value; NPV, negative predict value; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; PML, promyelocytic leukemia; RARA, retinoic acid receptor α.

Table VI. Comparison of the expression levels of hsa_circ_0012152 in APL with PML-RARA and other groups of AML.

Molecular subtype of AML	Expression level of hsa_circ_0012152	P-value
APL with PML-RARA vs.		
AML with t (8;21) (q22; q22.1)	3.40 vs. 24.08	<0.0001[a]
AML with inv (16) (p13.1q22) or CBFB-MYH11	3.40 vs. 19.22	0.0021[a]
Normal karyotype of AML with mutated NPM1	3.40 vs. 15.38	<0.0001[a]
Other categories	3.40 vs. 9.40	0.0006[a]
MLL rearrangement	3.40 vs. 2.65	0.3302
Normal karyotype of AML with biallelic mutations of CEBPA	3.40 vs. 5.8	0.3094

[a]Significant difference. The statistical analysis was performed using the non-parametric Kruskal-Wallis H-test, followed by Dunn's multiple comparison test for post hoc pairwise comparisons. circ, circular (RNA); ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; CBFB, core-binding factor subunit β; MYH11, myosin heavy chain 11; NPM1, nucleophosmin 1; MLL, mixed lineage leukemia gene; CEBPA, CCAAT/enhancer binding protein α.

This observation suggests that hsa_circ_0012152 may serve as a diagnostic marker, differentiating this specific subgroup from others when examining peripheral blood samples. With a sensitivity of 0.8947, a specificity of 0.7463 and an AUC of 0.8366 (95% CI, 0.7532-0.9201; P<0.0001; Fig. 4C and Table V), its diagnostic potential is further highlighted.

In addition, when comparing the 'APL with PML-RARA or t (15;17)' group to other specific AML subgroups, such as 'AML with t (8;21) (q22; q22.1)' and 'AML with inv (16) (p13.1q22) or CBFB-MYH11', the expression levels of hsa_circ_0012152 were significantly reduced (P<0.0001 and P=0.0021, respectively; Table VI). Similarly, the 'APL with PML-RARA or t (15;17)' group showed significantly lower expression compared with the 'Normal karyotype of AML with mutated NPM1' and 'Other categories' groups (P<0.0001 and P=0.0006, respectively; Table VI). However, no significant differences were observed when compared with the 'MLL rearrangement' and 'Normal karyotype of AML with biallelic mutations of CEBPA' groups, potentially due to the limited sample size within these subgroups (Table VI).

Furthermore, ALL encompasses various subtypes, including B-ALL, T-ALL and mixed-phenotype ALL expressing myeloid antigens. The present study aimed to investigate whether the expression of myeloid antigens in ALL influences the expression of hsa_circ_0012152. To this end, patients with ALL were categorized based on the presence or absence of myeloid antigen expression. Notably, it was found that hsa_circ_0012152 expression was significantly elevated in ALL samples expressing myeloid antigens compared with those without (median value, 3.88 vs. 0.11; P=0.0067; Fig. 4B). With a sensitivity of 0.75, a specificity of 0.9167 and an AUC of 0.8264 (95% CI, 0.6456-1.0000; P=0.0067; Fig. 4D and Table V), this finding suggests that hsa_circ_0012152 may aid in distinguishing between ALL subtypes based on myeloid antigen expression. However, it should be noted that no significant difference in hsa_circ_0020093 expression was observed between these two ALL groups (0.03 vs. 0.27, P=0.149).

Construction of the ceRNA network for hsa_circ_0020093. hsa_circ_0020093 and hsa_circ_0012152 have been identified as potential markers for distinguishing patients with ALL

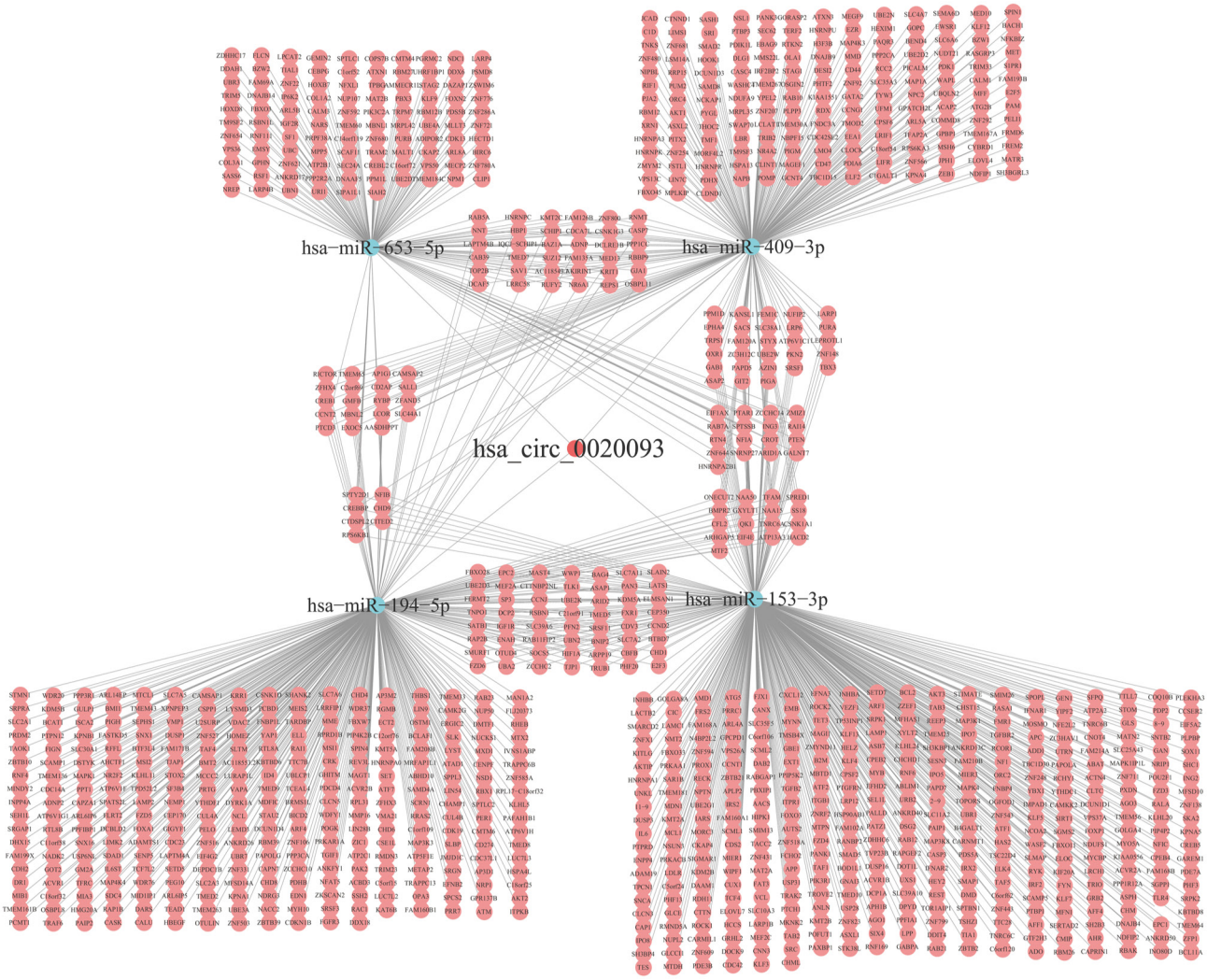

Figure 5. Elucidating the interplay between hsa_circ_0020093, downstream miRNAs and their target genes. A complex network of interactions that underlie the functional mechanisms of hsa_circ_0020093, shedding light on its potential role in gene regulation and cellular processes. circ, circular (RNA); miR, microRNA.

from patients with AML and healthy individuals, respectively. Based on this observation, we hypothesize that these circRNAs may be involved in the underlying mechanisms of ALL and AML pathogenesis. While the preliminary research team has conducted comprehensive bioinformatics analyses on hsa_circ_0012152 (16), hsa_circ_0020093 was chosen as the focus of further investigations on. Using the CircInteractome and StarBase 3.0 databases, it was predicted that hsa_circ_0020093 can bind to four miRNAs: hsa-miR-153-3p, hsa-miR-194-5p, hsa-miR-409-3p and hsa-miR-653-5p. Subsequently, the starBase3.0 database was utilized to identify a total of 1,146 downstream target genes for these miRNAs. The intricate interactions between hsa_circ_0020093, the miRNAs and their target genes are visualized in Fig. 5. To gain a deeper understanding of these target genes, a PPI network was constructed using the STRING 11.5 database (Fig. 6). Within this network, the top 10 hub genes were identified using the Degree algorithm in Cytoscape (v3.8.0): *SRC*, *RAC1*, *MAPK1*, *UBC*, *PIK3R1*, *AKT1*, *CDC42*, *GRB2*, *CREB1* and *ITGB1* (Fig. 7A). Furthermore, a ceRNA network was constructed based on the interactions between hsa_circ_0020093, the miRNAs and their target genes (Fig. 7B). Within this network, it was found that hsa-miR-153-3p targets 5 genes, while hsa-miR-194-5p targets 3 genes. This leads us to consider that hsa_circ_0020093 may play a critical role in ALL by regulating downstream target genes through its interaction with hsa-miR-153-3p or hsa-miR-194-5p.

GO and KEGG analysis for hsa_circ_0020093. Using the enrich plot package in R, GO and KEGG enrichment analyses were conducted for the 843 target genes of hsa-miR-153-3p and hsa-miR-194-5p (Fig. 8). Through GO analysis, it was observed that these target genes play crucial roles in several biological processes, including 'positive regulation of cellular catabolic processes' and 'amoeboid-type cell migration'. In terms of cellular components, they are involved in 'cell-substrate junction' and 'cytoplasmic ribonucleoprotein granule'. Within the molecular functions category, they exhibit activities such as 'DNA-binding transcription factor binding', 'protein serine kinase activity' and 'cadherin binding', among others. KEGG analysis further suggested that these target genes might be implicated in key pathways such as the 'Neurotrophin signaling

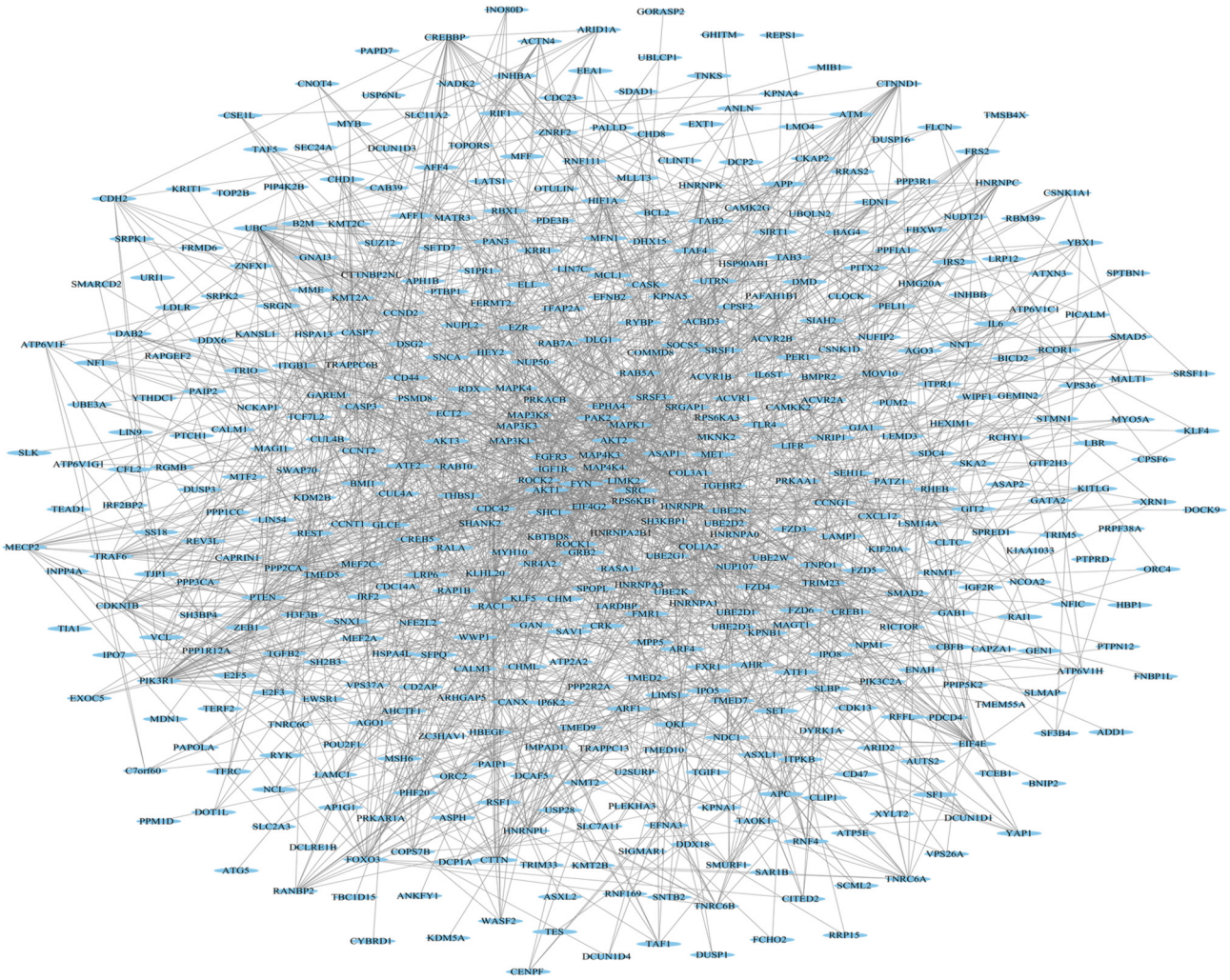

Figure 6. PPI network of target genes. An intricate web of PPIs among the target genes, revealing their functional interconnectedness and potential roles in cellular processes. PPI, Protein-protein interaction.

pathway', 'Axon guidance', 'Human T-cell leukemia virus 1 infection', 'HIF-1 signaling pathway', 'MAPK signaling pathway', 'mTOR signaling pathway' and 'Focal adhesion'. These findings provided valuable insights into the potential functions and regulatory mechanisms of these target genes in various biological processes and pathways.

Discussion

AL represents a highly diverse group of blood malignancies, necessitating precise classification. While the conventional Morphology, Immunology, Cytogenetics and Molecular Biology examination can differentiate most AL cases (28-30), it requires considerable expertise from diagnosticians and costly equipment, posing significant challenges to leukemia diagnoses globally (4,5). Consequently, researchers have been actively pursuing innovative and more efficient methods to improve AL diagnosis and subtype identification. Studies have demonstrated the potential of long ncRNAs (31,32), miRNAs (33-35) and circRNAs (16) in the diagnosis and prognosis of AL. However, the exploration in AL diagnostic typing is primarily limited to bone marrow samples. Challenges arise when bone marrow fibrosis results in dry pumping, rendering samples unavailable. Therefore, there is an unmet need to investigate the expression levels of circRNAs specifically in peripheral blood samples and their role in AL. Peripheral blood samples provide convenient access to the circRNAs, which can greatly reduce the patient's suffering. Addressing this gap could notably contribute to advancing the field and improving patient outcomes.

The present study aimed to develop a streamlined approach for diagnosing AL by focusing exclusively on peripheral blood samples. The present study delves into the role of circRNAs in AL classification and preliminarily explores their underlying mechanisms. Extensive sample analysis revealed that the expression levels of hsa_circ_0012152 in peripheral blood were higher in patients with AML compared with patients with ALL and healthy individuals. This finding allows for the differentiation of patients with AML from healthy subjects with high accuracy (AUC, 0.9878; P<0.0001) and serves as a potential marker for distinguishing AML from ALL (AUC, 0.8634; P<0.0001). Similarly, hsa_circ_0020093 expression is elevated in patients with ALL, enabling differentiation from patients with AML and healthy individuals (AUC, 0.8708; P<0.0001) and serving as another potential marker for AML-ALL classification (AUC, 0.8401; P<0.0001). Previous research has identified circ-PVT1 (15) as a

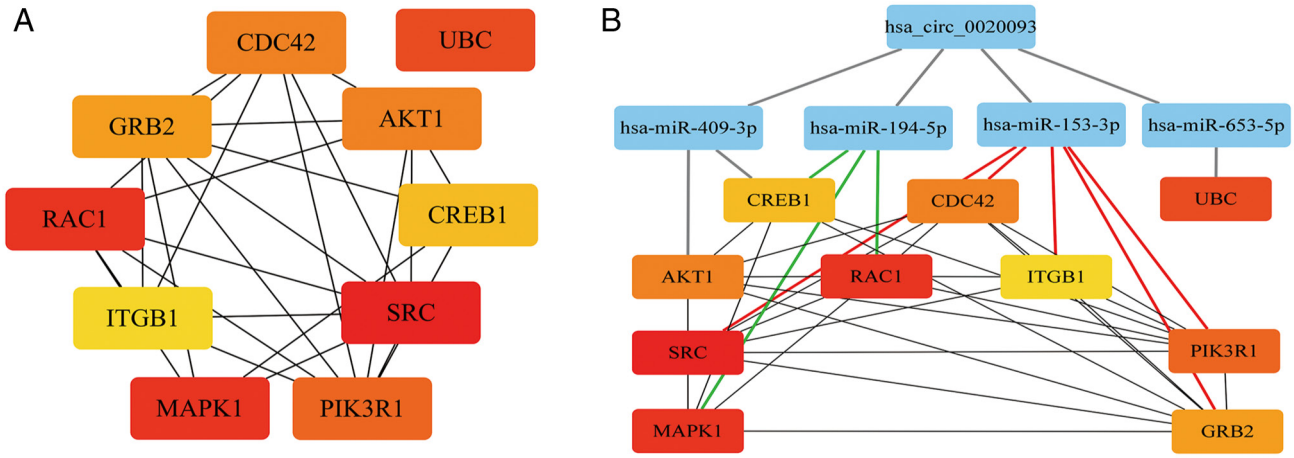

Figure 7. Construction of the ceRNA network for hsa_circ_0020093. (A) The top 10 hub genes. (B) Elucidating the potential functional mechanism of hsa_circ_0020093 through a comprehensive ceRNA network. In this schematic representation, hsa_circ_0020093 occupies the topmost tier, serving as the central player. The middle tier shows the 4 miRNAs specifically targeted by hsa_circ_0020093, highlighting their crucial roles as mediators. The bottom tier encompasses the genes regulated by these miRNAs, further emphasizing the intricate web of interactions within the ceRNA network. Connecting lines between the various tiers illustrate the intricate relationship and functional interplay among these RNA molecules, shedding light on the potential mechanisms underlying the biological functions of hsa_circ_0020093. circ, circular (RNA); miRNA/miR, microRNA; ceRNA, competing endogenous RNA.

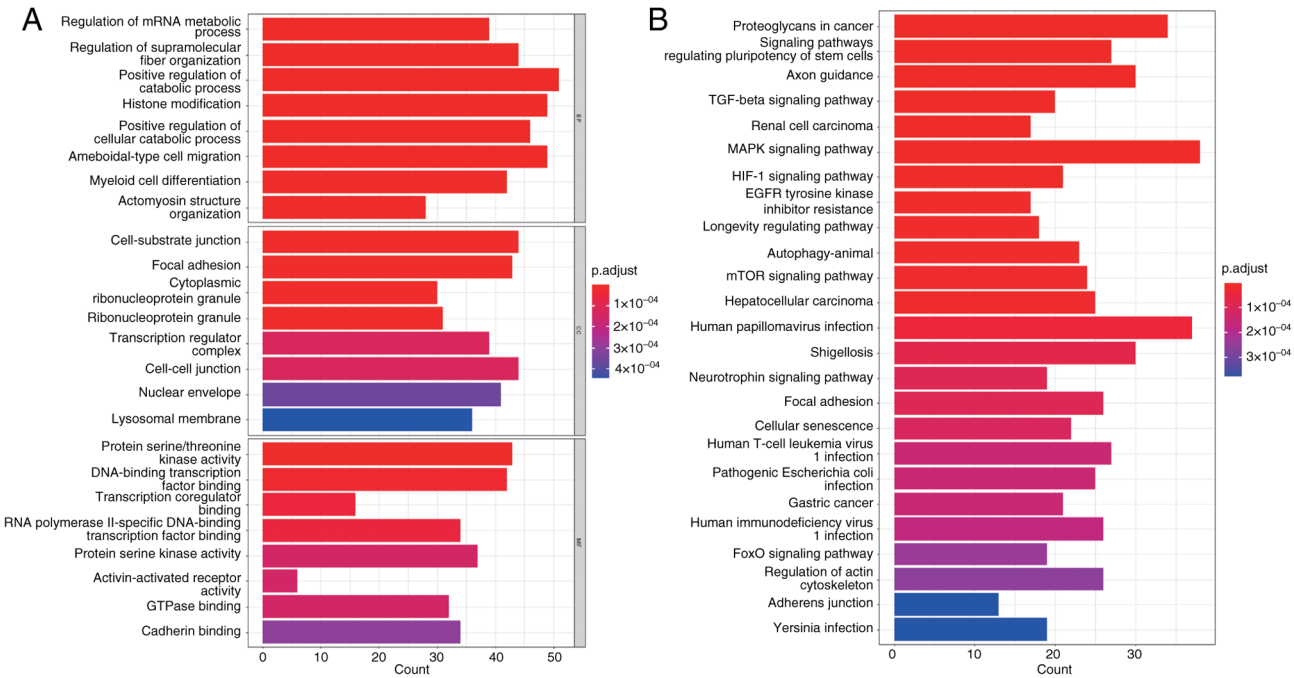

Figure 8. Functional annotation of target genes from the hsa_circ_0020093 competing endogenous RNA network through GO and KEGG pathway analysis. (A) GO functional annotation of the target genes, encompassing their roles in biological processes, molecular functions and cellular components. This comprehensive annotation provides a holistic understanding of the functional landscapes of the target genes. (B) KEGG functional annotation of the target genes, highlighting the signaling pathways in which they may be involved. Each row represents a distinct signaling pathway, revealing the potential impact of hsa_circ_0020093 on these critical cellular networks. circ, circular (RNA); GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

diagnostic marker for ALL, while circ-VIM (13), circAF4 (36) and circ-FOXO3 (37) can diagnose AML; however, these studies were primarily based on bone marrow samples. The results of the present study suggest that hsa_circ_0012152 and hsa_circ_0020093 can serve as auxiliary and differential diagnostic markers for AML and ALL, respectively, enriching the circRNA expression profile for AL diagnosis and classification. Furthermore, the present study demonstrated that the circRNA expression profiles in the peripheral blood of newly diagnosed patients with AL can be used for auxiliary diagnosis, offering a more convenient alternative to bone marrow extraction. To improve the diagnostic efficacy of hsa_circ_0012152 and hsa_circ_0020093 in identifying AL, serial and parallel testing approaches were evaluated. Both methods respectively increased specificity and sensitivity, but with trade-offs. To enhance diagnostic efficiency comprehensively, logistic regression models were explored (27), which integrate multiple indicators into a single evaluation index. Combining hsa_circ_0012152 and

hsa_circ_0020093 using logistic regression models improved the sensitivity in discriminating AL to 0.9884, resulting in a slightly improved AUC of 0.8643. While the increase is modest, it may be due to the limited number of ALL cases in the present study, necessitating further sample collection for validation. Additionally, it was found in the present study that hsa_circ_0012152 can distinguish 'APL with PML-RARA or t (15;17)' from other AML groups using peripheral blood (AUC, 0.8366; P<0.0001), and differentiate whether ALL expresses myeloid antigens in peripheral blood (AUC, 0.8264; P=0.0067), further enriching the circRNA expression profiles for AML subtype differentiation, and providing valuable diagnostic assistance for identifying patients with AL harboring chaotic immunological marker expression. Overall, the findings of the present study suggest that circRNAs hold promise as diagnostic and classification markers for AL, offering a more convenient and less invasive alternative to bone marrow-based methods.

The involvement of circRNAs in the onset and progression of diseases is significantly influenced by their function as miRNA sponges. Through the ceRNA network, circRNAs bind to miRNAs, effectively modulating the expression of downstream target genes that are crucial for disease development. For instance, Han *et al* (38) demonstrated that hsa_circ_0001947 inhibits AML cell proliferation via the hsa_miR-329-5p/CREBRF axis. Similarly, Jamal *et al* (39) found that the circRNA-100290/miRNA-293/Rab10 axis can upregulate the RAS signaling pathway, accelerating AML progression. In pediatric AML, circRNA-0004136 adsorbs miRNA-142, promoting tumor cell proliferation (40). These findings underscore the ability of circRNAs to regulate target genes through miRNA sponging, thereby influencing disease progression and prognosis. In the present study, the expression levels of hsa_circ_0012152 and hsa_circ_0020093 were significantly elevated in AML and ALL, respectively, suggesting their involvement in the pathogenesis of these diseases and potential diagnostic utility. Guo *et al* (16) conducted bioinformatics analyses on hsa_circ_0012152, speculating that it may contribute to AML development through the activation of the MAPK pathway via the hsa_circ_0012152/miR-491-5p/miR-512-3p/EGFR/mitogen-activated protein kinase 1 (MAPK1) axis. Meanwhile, bioinformatics analysis of hsa_circ_0020093 in the present study, revealed its potential involvement in ALL pathogenesis through miRNA sponging effects involving hsa-miR-153-3p or hsa-miR-194-5p and subsequent regulation of downstream target genes. A previous study has established a negative correlation between growth factor receptor-bound protein 2 (GRB2) expression and overall survival in patients with ALL, highlighting its prognostic significance (41). *GRB2* plays a crucial role in tyrosine kinase signaling, leading to the activation of *MAPK1* and *MAPK3* (42). Inhibition of *GRB2* has been shown to suppress ALL progression, making it a promising therapeutic target (42). Another study implicated reduced miR335 expression in elevated *MAPK1* levels and poor prognosis in pediatric patients with ALL, emphasizing the prognostic significance of *MAPK1* expression levels (43). Signaling pathway analysis has revealed an association between the ATP binding cassette subfamily B member 1 gene and poor diagnosis and prognosis in ALL, mediated through MAPK pathway activation (44). Furthermore, a study has shown that deferoxamine may inactivate hypoxia-inducible factor 1 (HIF1) and inhibit ALL progression via MAPK signaling pathways (45), further highlighting the contribution of MAPK and HIF1 signaling in ALL development and progression. Regarding the mammalian target of rapamycin (mTOR) signaling pathway, numerous studies have linked mTOR dysregulation to malignant cell proliferation (46-48). Activation of the mTOR signaling pathway is often associated with poor prognosis and chemoresistance in ALL. Taken together, these findings underscore the complexity and interconnectedness of molecular mechanisms underlying ALL pathogenesis and highlight the potential diagnostic and therapeutic implications of circRNA-mediated miRNA sponging in these diseases. In the expansion of KEGG pathway prediction analysis for hsa_circ_0020093 downstream target mRNA genes in the present study, it was determined that *PIK3R1*, *MAPK1* and *GRB2* are intricately linked to the 'mTOR signaling pathway', whereas *MAPK1*, *GRB2* and *CDC42* play a part in the 'MAPK signaling pathway'. Based on these findings, we hypothesize that hsa_circ_0020093 might regulate the expression of downstream genes, namely *CDC42*, *GRB2* and *MAPK1*, by competitively binding to either hsa-miR-153-3p or hsa-miR-194-5p. This, in turn, could activate crucial signaling cascades such as MAPK and mTOR, ultimately driving the onset and progression of ALL. Notably, this implicates not just *CDC42*, *GRB2* and *MAPK1* as potential therapeutic targets for ALL, but also highlights hsa_circ_0020093, hsa-miR-153-3p and hsa-miR-194-5p as precise therapeutic candidates for the disease.

The present study prospectively investigated the expression patterns of hsa_circ_0012152 and hsa_circ_0020093 in diagnosing AL and distinguishing its subtypes, thereby offering valuable tools to enhance clinical management. These findings hold notable significance in mitigating patient suffering and refining the diagnostic categorization of AL. However, owing to the limited availability of ALL samples, the study did not include T-ALL, necessitating larger sample sizes and extended follow-up durations in future research to validate the findings. Additionally, further exploration is warranted to elucidate the association of hsa_circ_0012152 or hsa_circ_0020093 with gene mutations, chemotherapy resistance and their mechanistic role in AL.

In conclusion, the present study demonstrated that hsa_circ_0020093 may regulate downstream target genes via hsa-miR-153-3p or hsa-miR-194-5p, thereby contributing to the initiation and progression of ALL. The combination of hsa_circ_0012152 with hsa_circ_0020093 in peripheral blood can facilitate the differentiation between AML and ALL, highlighting their potential utility as diagnostic biomarkers for the disease and providing a less invasive alternative to bone marrow aspiration.

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Availability of data and materials

The sequencing data of hsa_circ_0012152 and hsa_circ_0020093 examined in the present study may be found in the GSA-human repository under the accession number HRA007384 or at the following URL: https://bigd.big.ac.cn/gsa-human/browse/HRA007384. The circRNA microarray data analyzed in the present study may be found in the OMIX database (National Genomics Data Center, China) under the accession number OMIX009143 or at the following URL: https://ngdc.cncb.ac.cn/omix/release/OMIX009143. All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

QM was responsible for the study design and framework development; QY, DL, YC and QM secured funding; DL conducted the data collection and developed the statistical analysis methods; YC performed chart and diagram creation. QM supervised the overall project coordination and provided critical oversight. QY led the writing of the original manuscript and QY and YC contributed to data analysis and interpretation. QM reviewed and edited the manuscript. QY, DL, YC and QM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First Affiliated Hospital of Ningbo University (Ningbo, China; approval no. 159A for Research in 2023). All patients and volunteers involved in the study provided written informed consent, which includes permission for the use of remaining peripheral blood specimens in this research. For those patients who were minors, written consent was obtained from their parents or legal guardians, and these minors also provided assent where applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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