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ORIGINAL RESEARCH

Screening of a Prognostic Gene Signature for Relapsed/Refractory Acute Myeloid Leukemia Based on Altered Circulating CircRNA Profiles

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Background: Relapsed/refractory acute myeloid leukemia (R/R-AML) has dismal prognosis due to chemotherapy resistance. Circular RNAs (circRNAs) have shown emerging roles in chemotherapy resistance in various cancers including hematologic malignancies. However, the potential roles of circRNAs in AML progression and drug resistance remain largely undetermined.

Methods: In this study, circulating circRNAs expression profiles were analyzed among R/R-AML, *de novo* AML and healthy controls (HC) using a human circRNA Array. Bioinformatic analysis was carried out to explore the differentially expressed circRNAs (DE-circRNAs). GO, KEGG pathway analysis, along with circRNA-miRNA-mRNA network analysis, were conducted to identify the potential biological pathways involved in R/R-AML. Finally, the UALCAN database was used to assess the prognosis of different target DE-circRNAs-related mRNAs.

Results: Forty-eight DE-circRNAs were upregulated, whereas twenty-seven DE-circRNAs were downregulated in R/R-AML samples. Up-regulated DE-circRNAs in R/R-AML samples were mainly enrichment in the biological processes and pathways of cell migration, microRNAs in cancers, Rap1 and Ras signaling pathways. Six DE-circRNAs were randomly selected to further explore their relationships with R/R-AML. GO and KEGG pathway analyses of the six candidate DE-circRNAs-related target mRNAs were mainly involved in the regulation of signal transduction and Ras signaling pathway. By overlapping our RNA-sequencing results of differentially expressed genes (DEGs) in R/R-AML samples with the candidate DE-circRNAs-predicted target mRNAs, we identified sixty-eight overlapping targeted mRNAs. Using UALCAN database analysis, we identified that AML patients with six upregulated DE-circRNA-related genes (ECE1, PI4K2A, SLC9A6, CCND3, PPP1R16B, and TRIM32) and one downregulated gene DE-circRNA-related genes (ARHGAP10) might have a poor prognosis.

Conclusion: This study revealed the overall alterations of circRNAs in R/R-AML. DE-circRNAs and their related genes might be used as potential early, sensitive and stable biomarkers for AML diagnosis, R/R-AML monitoring, and even as novel treatment targets for R/R-AML.

Keywords: Relapsed/refractory, acute myeloid leukemia, circular RNAs, biomarkers, prognosis

Introduction

Acute myeloid leukemia (AML) is characterized by a high incidence of relapse even after intensive consolidation chemotherapy. The current 5-year survival rate, as reported by the SEER database, stands at a mere 30%, underscoring the urgency for improved therapeutic strategies. Over half of AML patients relapse despite intensive consolidation therapy and the majority of relapsed patients exhibit resistance to chemotherapy.[1,2] Relapsed/refractory AML (R/R-AML) is a subset of malignant hematopoietic disease with dismal prognosis.[3,4] Early diagnosis, before the onset of

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relapse or refractory clinical indications, may be in selecting the most suitable treatment and delaying disease progression. However, the mechanisms leading to therapy resistance and relapse in AML are not fully understood.

Circular RNAs (circRNAs), a type of non-coding single-stranded RNA, have emerged as pivotal regulators in cancer biology, implicated in diverse cellular processes, including drug resistance.[5–7] CircRNAs are recognized as conserved and stable transcriptome elements, which can act as microRNA (miRNA) sponges and regulate transcriptional/post-transcriptional gene regulation, thereby emerging as potential biomarkers.[8] These circRNAs have been identified as dysregulated in AML and actively contributed to the pathogenesis and progression of this disease.[9–11] For instance, the up-regulated circRNA_0010984 in AML has been shown to enhance cell proliferation by targeting miR-375.[12] Competing endogenous RNAs (ceRNAs) regulate gene function by sequestering miRNAs, thereby controlling the accessibility of these miRNAs to their target mRNAs. CircSLC25A13 functions as a ceRNA to regulate AML progression via the miR-616-3p/ADCY2 axis, and the knockdown of circSLC25A13/miR-616-3p in AML cells has demonstrated inhibition of proliferation and an increase in cell apoptosis.[13] Up-regulated circ_0000370 and hsa_circ_0015278 have been identified as regulators of FLT3-ITD AML progression through interactions with miR-1299 or ferroptosis-related genes.[14] Additionally, the knockdown of Circ_0001187 has significantly promoted the proliferation and inhibited the apoptosis of AML cells in vitro and *in viv*o.[15] However, their role in AML, particularly in disease progression and resistance mechanisms, remains largely unexplored.

Limited investigations have been undertaken to elucidate the underlying functions of circRNAs in chemotherapy resistance in AML. Shang et al observed an elevation in circPAN3 levels in refractory and recurrent AML patient tissues, as well as in doxorubicin-resistant THP-1 AML cell lines.[16] Mechanistically, circPAN3 emerged as a crucial mediator of chemotherapy resistance in AML cells by regulating miR-153-5p/miR183-5p-XIAP (X-linked inhibitor of apoptosis) axis.[16] In a related study, Ding et al demonstrated that circNPM1 contributed to increased doxorubicin resistance in AML by modulating the miR-345-5p/FZD5 pathway.[17] The use of doxorubicin extends beyond AML to various solid tumors, including breast cancer, lung cancer, ovarian cancer, and sarcomas, where resistance to doxorubicin has also been observed. In triple-negative breast cancer, the upregulation of CircRNA-CREIT has been associated with mediating doxorubicin resistance through targeting the PKR/eIF2α signaling pathway.[18] Similarly, in breast cancer, Circ0006528 upregulation has been implicated in doxorubicin resistance by targeting the miR-1236-3p/CHD4 axis.[19] In osteosarcoma, CircANKIB1 has been found to bind miR-26b-5p and modulate EZH2, thereby promoting osteosarcoma progression and accelerating doxorubicin resistance.[20] In hepatocellular carcinoma (HCC), Circ_0000098, identified as an oncogenic circRNA, plays a pivotal role in HCC development through the miR-383/MCUR1 axis. Inhibiting Circ_0000098 in HCC cells has been shown to diminish doxorubicin resistance by reducing P-glycoprotein (P-gp, MDR1) expression and intracellular ATP levels.[21] Sorafenib, a kinase inhibitor crucial for tumor cell proliferation, is utilized in both HCC and AML.[22] Overexpression of the hsa_circRNA_102049 was found to enhance the sensitivity of HepG2 and Huh-7 cells to sorafenib, with hsa_circRNA_102049 upregulating the expression of RELN gene by sponging hsa-miR-214-3p.[23] These findings collectively suggest the potential application of circRNAs in reversing drug resistance. Nevertheless, the relationship between circRNAs and chemotherapy resistance in AML needs to be further investigated.

In summary, these investigations offer novel insights into the potential mechanisms underlying AML relapsed and drug-resistance based on the expression profiles of circRNAs. In the present study, circRNA expression profiles and associated biological processes were systematically analyzed in patients with R/R-AML, *de novo* AML patients and healthy controls (HC). The findings underscore the significant roles circRNAs may play in AML development, relapse, and drug resistance. Furthermore, circRNAs emerge as promising candidates for serving as early, sensitive, and stable biomarkers in AML diagnosis, monitoring R/R-AML, and may even offer new targets for therapeutic interventions in R/R-AML.

Materials and Methods

Patients and Samples

This study included 9 samples derived from 3 *de novo* AML patients, 3 R/R-AML patients and 3 HC. All samples were collected at Zhengzhou University People's Hospital. The Ethics Committee of Henan Provincial People's Hospital

approved this study (No.2018(51)). All *de novo* AML patients had not received chemotherapy or shown evidence of infection at the time of sample collection. The samples from R/R-AML patients were collected at the time of AML relapsed or refractory disease. Written consent was obtained from all individuals following the Declaration of Helsinki. The diagnosis of *de novo* AML and R/R-AML was established using FAB diagnostic criteria.

Cell Separation and RNA Extraction

Peripheral blood mononuclear cells (PBMCs) from all individuals were collected and isolated by density centrifugation (Ficoll-Hypaque). All specimens were derived from EDTA peripheral blood, and PBMCs were obtained within 4 hours, then preserved at −80°C. Total PBMC RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Briefly, 0.5 mL Trizol was added to homogenize the sample, followed by incubation at room temperature for 2~3 minutes. Subsequently, 1/5 volume of chloroform was added, and the mixture was vigorously shaken for 20~30 seconds, followed by incubation at room temperature for 2~3 minutes. The resulting solution was centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatant was carefully transferred to another tube. To this, 0.5 mL isopropanol was added, mixed and incubated at room temperature for 10 minutes. The mixture was then centrifuged at 12,000 rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol and air-dried. The pellet was dissolved in 50 μL DEPC-H2O, and the concentration was measured by OD260. The extracted RNA was stored at −80°C.

RNA-Sequencing and circRNA Microarray

Total RNA samples were quantified using NanoDrop. For RNA-sequencing (RNA-seq) library preparation, Illumina kits were employed, involving poly-A mRNA purification, random RNA fragmentation, random priming of mRNA to cDNA, second-strand cDNA synthesis, restriction enzyme digestion, sequencing adapter ligation and library PCR amplification. Following the quantification and quality assessment of the RNA-seq libraries, sequencing was conducted using Illumina HiSeq 4000 platform. CircRNAs were assessed using a circRNA array approach. Total RNA underwent treatment with RNase R to eliminate linear RNAs. Subsequently, the enriched circRNAs were amplified and transcribed into fluorescent cRNA. The labeled cRNA was hybridized onto a circRNA array (8×15 K, Arraystar V2). Array slides were washed, and the arrays were scanned using an Agilent Scanner G2505C.

CircRNAs and mRNA Data Analysis

The data of circRNAs array slides were extracted and analyzed by Agilent Feature Extraction and R software. Differentially expressed circRNAs (DE-circRNAs) between each pair of groups were visually represented using scatter plots, volcano plots and hierarchical clustering. CircRNAs with p-values<0.05 and fold changes≥1.5 were considered significantly differentially expressed. Similarly, the expression profiles of differentially expressed genes (DEGs) among different samples were presented using scatter plots, volcano plots, and hierarchical clustering. For mRNAs, those with p-values<0.05 and fold changes≥2 were considered differentially expressed.

GO and KEGG Pathway Analysis

To unravel the potential underlying biological procedures and pathways in R/R-AML, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis in the R environment. GO analysis was used to understand the biological functions associated with the DEGs. The KEGG database was utilized to analyze the DEGs, aiming to identify significant pathways relevant to AML and R/R-AML.

Establish circRNA/miRNA/mRNA Network

The interaction between circRNA and miRNA was predicted using TargetScan and miRanda. Detailed information about candidate circRNAs and their corresponding miRNA was presented. MiRanda was also utilized for assessing miRNA response elements (MREs). Additionally, mRNAs were predicted using DIANA-Tar-base, TargetScan, and microT. Only mRNAs identified in at least two of the three 3 databases (DIANA-TarBase, TargetScan, and microT) were considered potential target genes associated with the target miRNAs. Cytoscape was employed to construct circRNA/miRNA/mRNA networks comprising validated candidate circRNAs.

UALCAN Dataset Prognostic Analysis

The UALCAN platform was utilized to analyze TCGA data at the gene level, encompassing information from 163 AML patients. UALCAN aids in identifying potential biomarkers for diagnosis, prognosis evaluation, and treatment across various cancers.[24] To comprehensively investigate the role of candidate circRNA-related genes in AML relapse and drug resistance, we further overlapped the predicted mRNAs of candidate circRNAs with our RNA-sequencing profiles from R/R-AML samples. The overlapping genes were subsequently analyzed using UALCAN.

Statistical Analysis

Peripheral circRNA expression profiles among R/R-AML, *de novo* AML, and HC groups were compared using ANOVA to identify DE-circRNAs. Fisher's exact test or hypergeometric distribution test was employed to determine significant enrichment associated with specific biological processes or pathways. Kaplan-Meier curves and Log rank tests were used to evaluate the impact of DE-circRNAs-related mRNA on the prognosis of AML patients.

Results

Characteristics of DE-CircRNAs in R/R-AML

The expression levels of circRNAs were quantitated in 9 samples, comprising 3 from *de novo* AML patients, 3 from R/R-AML patients, and 3 from HC. Clinical parameters of these samples were provided in Table 1. The Arraystar Human circRNA Array V2. was employed for circRNA analysis, and expression variations among the samples were assessed using scatter plots, volcano plots and hierarchical clustering (Figure 1). CircRNAs with p-value$\leq$0.05 and fold changes$\geq$1.5 were thought to be significantly differentially expressed. In *de novo* AML patients compare to HC, 702 circRNAs were upregulated, and 1297 circRNAs were downregulated (Figure 1A–C). In R/R-AML patients compared to *de novo* AML patients, 48 circRNAs were upregulated and 27 circRNAs were downregulated (Figure 1D–F).

Further analysis of the chromosomal distribution of DE-circRNAs in *de novo* AML samples and R/R-AML samples showed that chromosome 1 harbored the largest number of DE-circRNAs (Figure 2A and B). Specifically, in *de novo* AML samples, the highest number of DE-circRNAs were observed on chromosome 1, 2, and 17 (Figure 2A), whereas in R/R-AML samples, they were found on chromosome 1, 3, and 12 (Figure 2B). The categorization of DE-circRNA is illustrated in Figure 2B and D, highlighting that the majority of DE-circRNAs in both *de novo* AML and R/R-AML samples originated from protein-coding exons, with a smaller proportion were from introns and sense overlapping regions (Figure 2C and D).

These findings indicate significant differences in circRNA expression profiles in the peripheral blood between *de novo* AML patients and HC. Moreover, there are distinct differences in the expression of peripheral circRNAs between R/R-AML patients and *de novo* AML patients.

Table 1 Clinical Characters of de Novo AML, Relapsed/Refractory AML Patients and Healthy Controls

Group	Sex/Age	FAB	WBC	HGB	PLT	PB blasts (%)	Karyotype	Gene mutation	Phusion Gene
De novo AML-1	31/m	M2	90.79	112	10	5	46,XY[20]	–	–
De novo AML-2	24/m	M2	96.22	87	11	84	46,XY[20]	KIT, TET2	CBFβ-MYH11 /ABL=128.30%
De novo AML-3	35/m	M2	11.45	97	16	64	45,X,-Y,t(8;21)(q22;q22)[6]/46,XY[20]	FLT3, KIT, TET2	AML1-ETO /ABL=0.5862
R/R-AML-1	65/f	M2	52.8	75	50	81	45,XX,del(2)(q32),del(3)(q22),der(9;12)(q10;q10),add(11)(p15)[8]/46,XX[12]	–	–
R/R-AML-2	8/f	M5	3.47	83	15	95	46,XY[20]	NPM1, IDH2, TET2	–
R/R-AML-3	35/m	M2	90.99	107	30	40	46,XY,t(8;21)(q22;q22)[8]/46,XY[2]	TET2	WT1=35%, ETO=472.6%
Healthy control-1	28/m	–	7.60	142.6	225	–	N/A	N/A	N/A
Healthy control-2	50/f	–	4.50	132	196	–	N/A	N/A	N/A
Healthy control-3	39/f	–	6.10	121.2	295	–	N/A	N/A	N/A

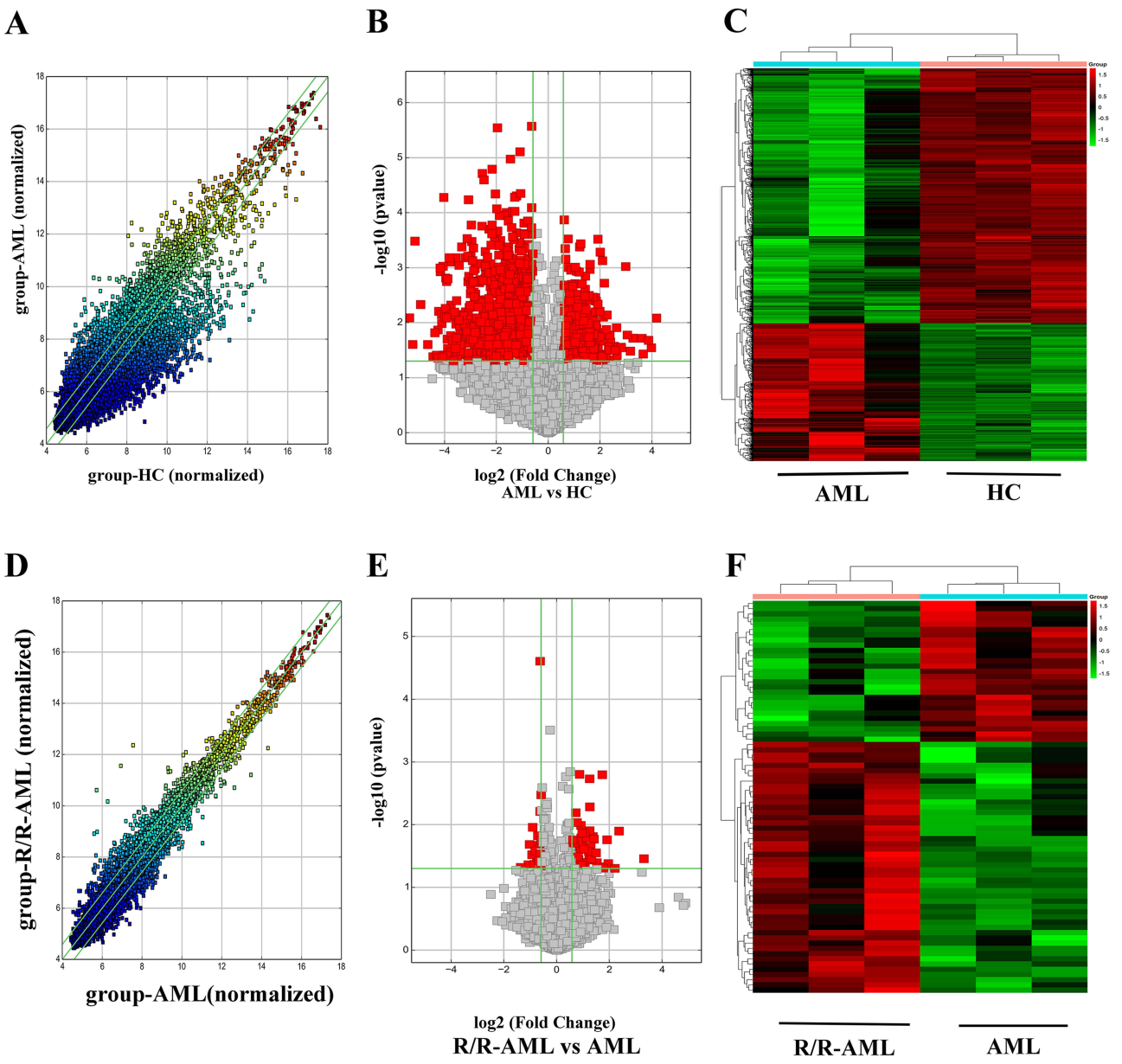

Figure 1 Expression profiles of circRNAs in de novo AML patients, R/R-AML and HC. (**A**) Scatter plots, (**B**) Volcano plots, and (**C**) Heat map showing differentially expressed circRNAs in de novo AML and HC. (**D**) Scatter plots, (**E**) Volcano plots, and (**F**). Heat map showing differentially expressed circRNAs in R/R-AML and de novo AML.

Functional Analysis of DE-CircRNAs and DEGs by GO and KEGG Pathway Analysis

The functional analysis of DE-circRNAs via GO and KEGG pathways reveals potential mechanisms in R/R-AML development and progression. Enrichment analysis of DE-circRNAs between R/R-AML and *de novo* AML showed involvement in GO biological processes such as positive regulation of cell migration (GO:0030335), GO cellular components including cytosol (GO:0005829), cell junction (GO:0030054), synapse (GO:0045202), nuclear body (GO:0016604), and nucleoplasm (GO:0005654) (Figure 3A and B). Regarding KEGG pathways, DE-circRNA were associated with proteoglycans in cancer (hsa05205), microRNAs in cancer (hsa05206), Rap1 signaling pathway (hsa04015), and Ras signaling pathway (hsa04014) (Figure 3A and B).

Additionally, enrichment analysis of differentially expressed mRNAs (DE-mRNAs) between R/R-AML and *de novo* AML (Figure 3C) revealed their involvement in GO biological processes, particularly immune response (GO:0006955)

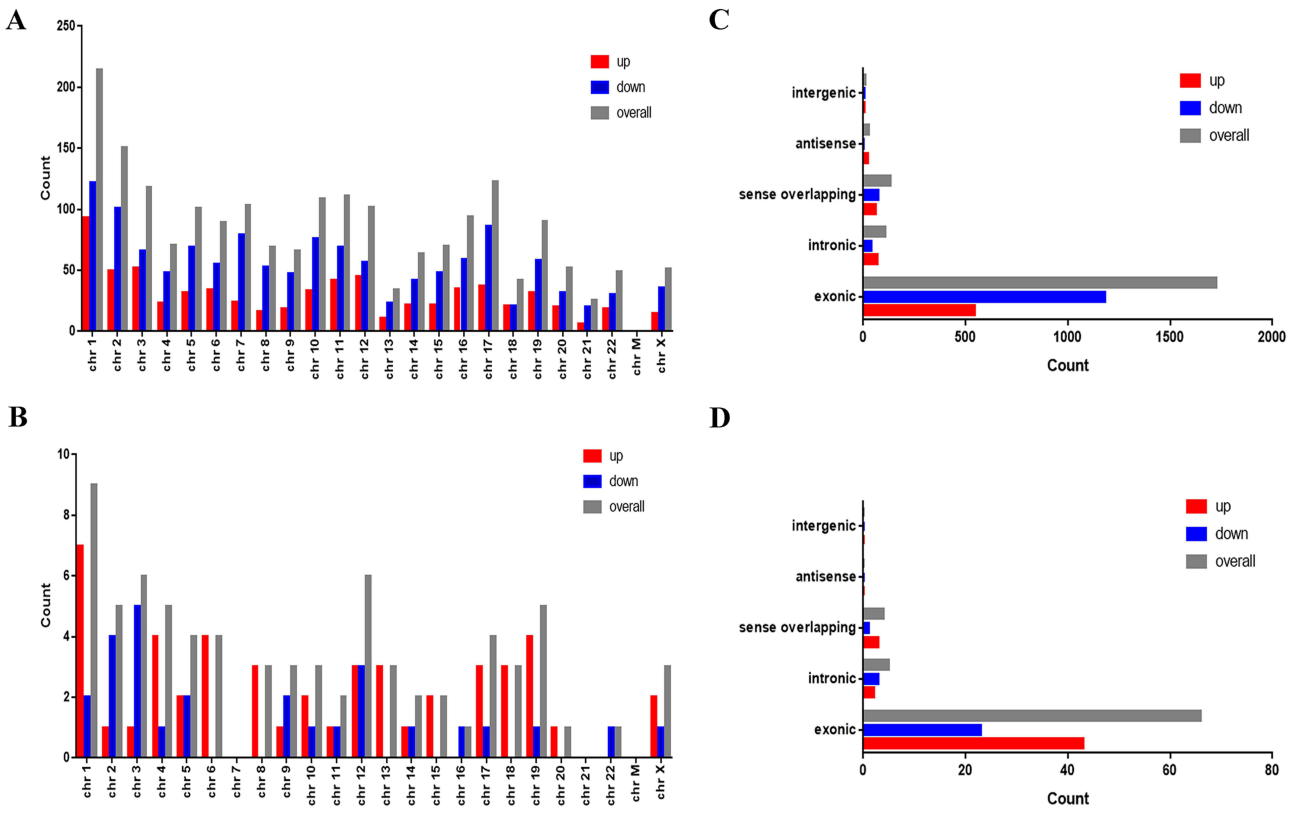

Figure 2 Distribution and category of differentially expressed circRNAs (DE-circRNAs) in AML. (**A**) Distribution of DE-circRNAs in human chromosomes in de novo AML patients. (**B**) The percentage of DE-circRNAs derived from different genomic loci (exonic, intronic, antisense, intergenic, and sense overlapping) in de novo AML patients. (**C**) Distribution of DE-circRNAs in human chromosomes in R/R-AML patients. (**D**) The percentage of DE-circRNAs derived from different genomic loci (exonic, intronic, antisense, intergenic, and sense overlapping) in R/R-AML patients.

and innate immune response (GO:0045087). DE-mRNAs were associated with KEGG pathways such as osteoclast differentiation (hsa04380), B cell receptor signaling pathway (hsa04662), Hematopoietic cell lineage (hsa04640), Epstein-Barr virus infection (hsa05169), and Human immunodeficiency virus 1 infection (hsa05170) (Figure 3C).

These findings highlight the roles of DE-circRNA and DE-mRNAs in R/R-AML, implicating processes like cell migration, microRNAs in cancers, Rap1 and Ras signaling. Moreover, DE-mRNAs in R/R-AML are pivotal in immune response and B cell receptor signaling pathways.

Candidate DE-circRNAs Associated with R/R-AML Development

To identify potential circRNAs critical in R/R-AML, we overlapped DE-circRNAs between R/R-AML vs *de novo* AML group and *de novo* AML vs HC group. This revealed 30 overlapping DE-circRNAs. These 30 overlapping DE-circRNAs were identified between R/R-AML vs *de novo* AML and *de novo* AML vs HC samples (Figure 4A). Of these, 21 circRNAs were upregulated in the R/R-AML vs *de novo* AML and downregulated in *de novo* AML versus HC. Additionally, 5 circRNAs showed downregulation in R/R-AML vs *de novo* AML and upregulation in *de novo* AML vs HC. Two circRNAs showed the highest expression, while two circRNAs exhibited the lowest expression in the R/R-AML group (Figure 4A). A heatmap depicting the expression patterns of these 30 circRNAs was generated (Figure 4B). We then selected 3 upregulated circRNAs (has-circRNA-051239, has-circRNA-102491, and has-circRNA -101539) and 3 downregulated circRNAs (has-circRNA-101330, has-circRNA-406642, and has-circRNA-101819) to explore their potential roles in R/R-AML (Table 2).

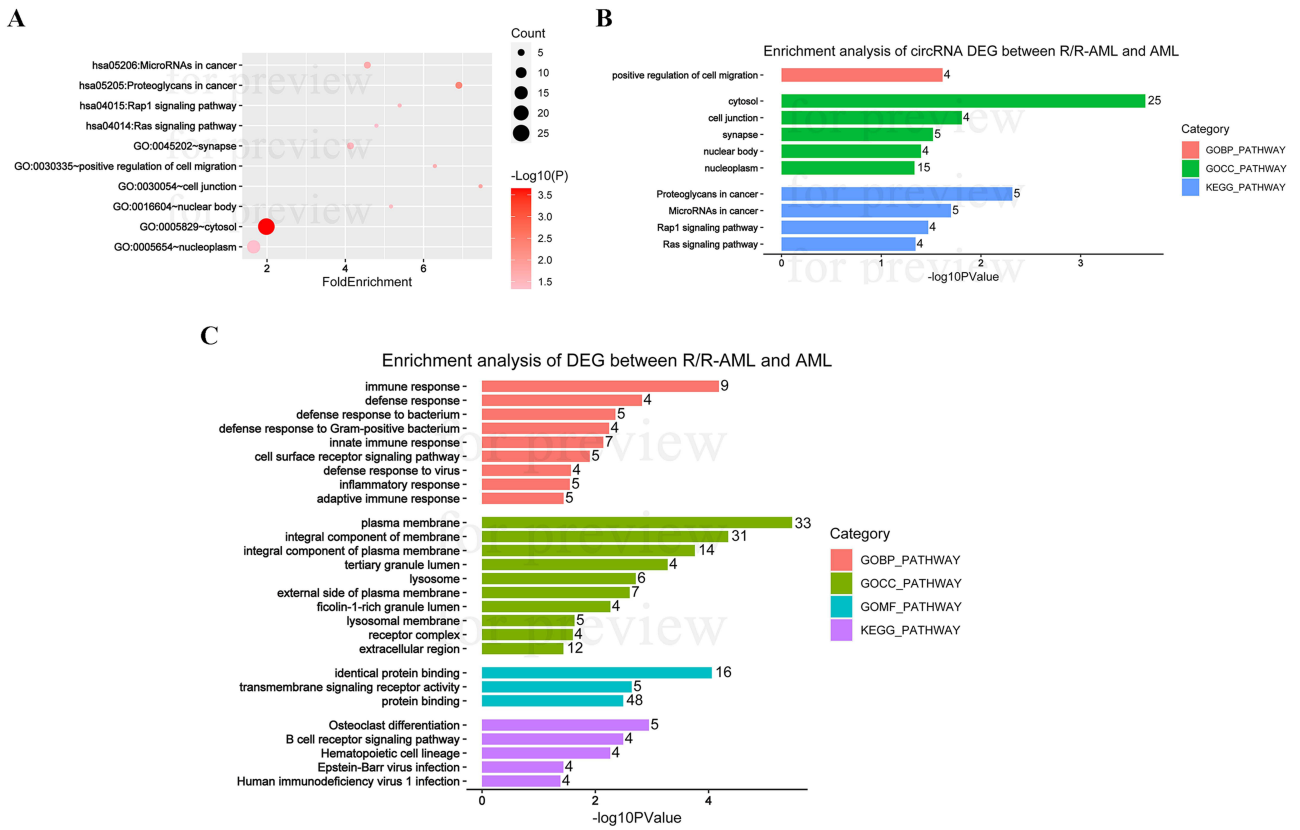

Figure 3 The GO and KEGG enrichment analysis of DE-circRNAs and DEGs between R/R-AML and de novo AML. (**A** and **B**). Enriched GO and KEGG terms of DE-circRNAs in R/R-AML patients. (**C**) Enriched GO and KEGG terms of DEGs in R/R-AML patients.

CircRNA/miRNA/mRNA Regulatory Network Construction

To further associate circRNAs with biological processes, we integrated the 6 candidate DE-circRNAs using miRTarBase and TargetScan databases to identify corresponding miRNAs and target mRNAs. Subsequently, regulatory networks involving circRNAs, miRNAs, and mRNAs were constructed using Cytoscape software (version 3.6.0). The predicted circRNA-miRNA-mRNA interaction network for the 6 candidate DE-circRNAs was visualized and is shown in Figure 5. In the network, circRNAs were represented as yellow nodes, miRNAs as blue nodes, and their target genes as red nodes.

Based on the outlined analysis strategy, we predicted the target mRNAs and miRNAs of the candidate DE-circRNAs. GO and KEGG pathway analysis of the mRNAs associated with the 6 candidate DE-circRNAs are depicted in Supplementary Figure 1. The targeted mRNAs were primarily involved in the regulation of plasma membrane bounded cell projection assembly (GO: 0120032), regulation of signal transduction (GO: 0009966) and positive regulation of development process (GO:0051094) in biological process (Supplementary Figure 1A). In the KEGG pathway analysis, the targeted mRNAs were primarily enriched in the Ras signaling pathway (Supplementary Figure 1B and c).

Bioinformatics analysis revealed that the candidate circRNA has-circRNA-101819 harbored miRNA response elements (MREs) for has-miR-15a-5p, has-miR-15b-5p, has-miR-1301-5p, has-miR-615-5p, and has-miR-433-3p (Supplementary Figure 2 A-E). This suggested that has-circRNA-101819 might act as a sponge for the miR-15 family, potentially influencing R/R-AML progression.

The Prognostic Value of the DE-circRNAs-Related Genes in R/R-AML

To comprehensively explore key genes associated with AML relapse and drug resistance, we overlapped RNA-sequencing results from R/R-AML samples with the predicted target mRNAs of candidate DE-circRNAs (Figure 6A). This analysis revealed 68 overlapping targeted mRNAs, comprising 40 upregulated mRNAs and 28 downregulated

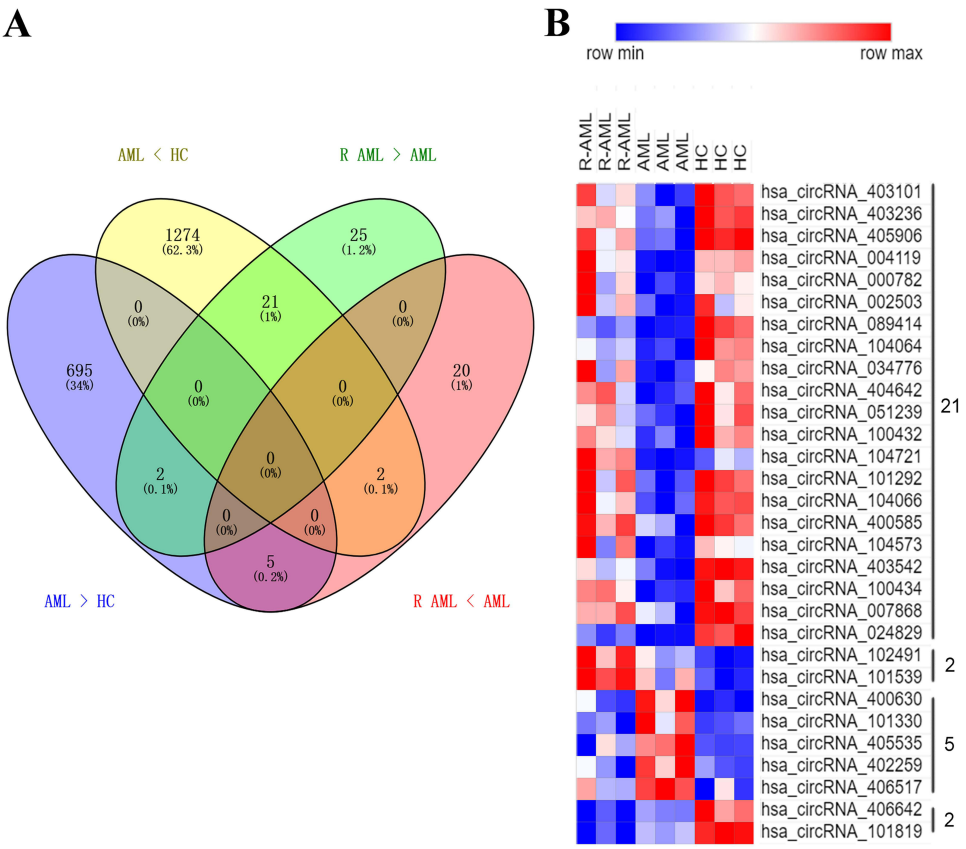

Figure 4 Candidate DE-circRNAs associated with R/R-AML. (**A**) Venn diagram of candidate DE-circRNAs. (**B**) Heatmap of the 30 overlapping DE-circRNAs.

mRNAs in R/R-AML (Figure 6B). To investigate the prognostic value of DE-circRNAs-related genes in AML, UALCAN was used to analyze the expression levels and prognostic values of the overlapped target genes.

The analysis identified 7 upregulated DE-circRNA-related genes (ECE1, PI4K2A, SLC9A6, CCND3, PPP1R16B, and TRIM32) (Figure 7A–F) and 1 downregulated DE-circRNA-related gene (ARHGAP10) (Figure 7G) associated with a poor prognosis in AML (Table 3). The data accessed from UALCAN indicate the potential prognostic values of DE-circRNAs-related genes in R/R-AML. However, further in vitro and in vivo investigations are needed to elucidate the roles and mechanisms of these DE-circRNA-related genes in the development of R/R-AML.

Discussion

Circular RNAs (circRNAs) exhibit structural conservation, remarkable stability, and are abundantly present in the human body, suggesting their potential as biomarkers for various cancers. In AML, circRNAs have been identified as deregulated players implicated in disease pathogenesis and progression. Despite these insights, their specific roles in

Table 2 Characters of the 6 Selected Candidate circRNAs That Might Responsible for R/R AML

circRNA	Alias	Chrom	Strand	Txstart	TxEnd	Circrna_type	best_transcript	GeneSymbol
hsa_circRNA_051239	hsa_circ_0051239	chr19	–	41,938,372	41,945,481	exonic	uc010xwb.2	ATP5SL
hsa_circRNA_101330	hsa_circ_0031419	chr14	+	31,097,414	31,122,794	exonic	NM_016106	SCFD1
hsa_circRNA_406642		chr5	+	96,434,640	96,438,798	sense overlapping	ENST00000509481	CTD-2215E18.1
hsa_circRNA_101819	hsa_circ_0039522	chr16	+	57,050,985	57,054,919	exonic	NM_032206	NLRC5
hsa_circRNA_102491	hsa_circ_0008135	chr19	-	19,049,161	19,049,858	exonic	NM_004838	HOMER3
hsa_circRNA_101539	hsa_circ_0005402	chr15	-	60,648,117	60,674,640	exonic	NM_004039	ANXA2

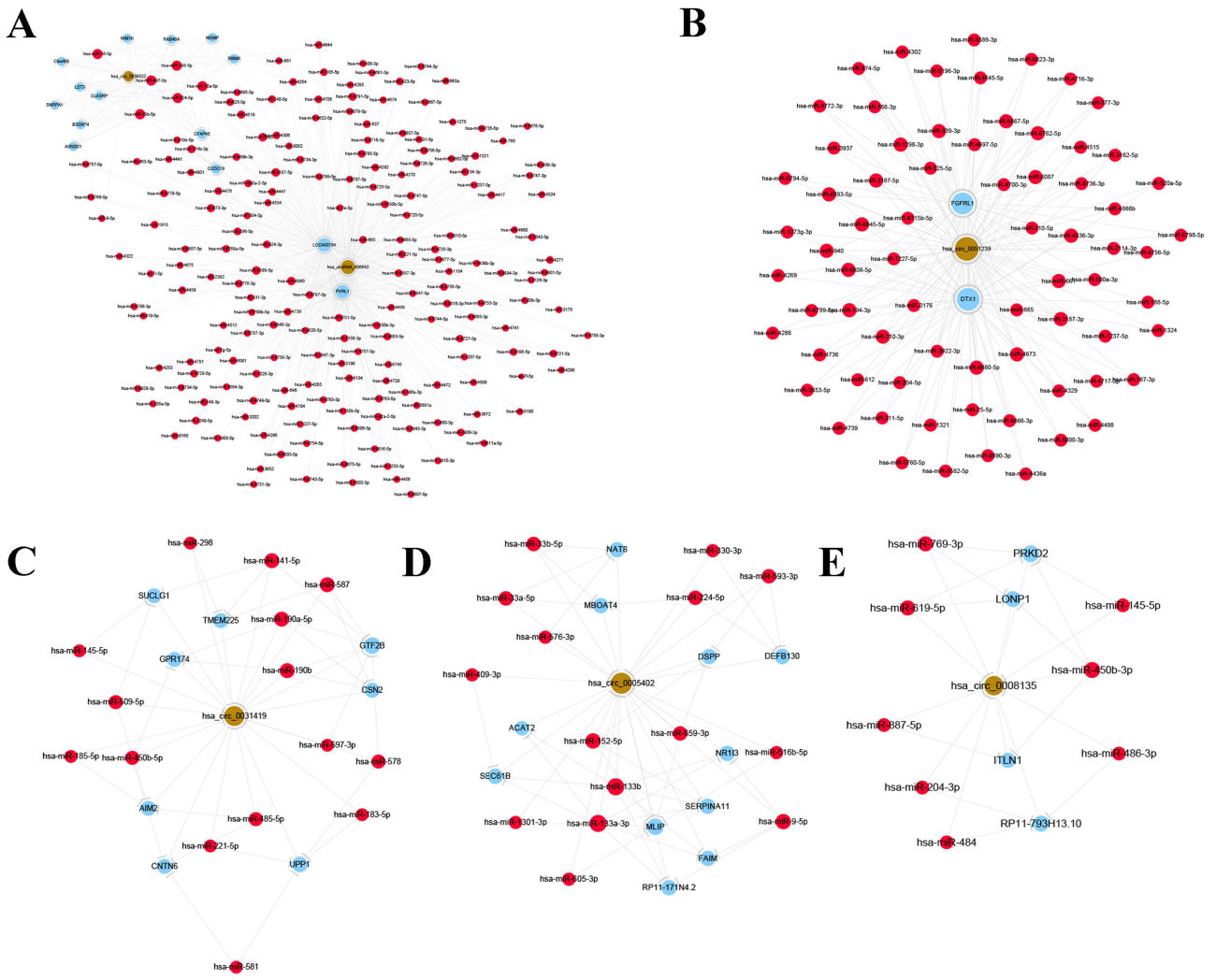

Figure 5 CircRNAs/miRNAs/mRNAs network analysis of candidate DE-circRNAs. Predictions of circRNA-miRNA-mRNA interaction network, showing 6 DE-circRNAs: (**A**) has-circRNA-101819 and has-circRNA-406642, (**B**) has-circRNA-051239, (**C**) has-circRNA-101330, (**D**) has-circRNA-101539 and (**E**). has-circRNA-10249. (circRNAs were shown as yellow nodes, miRNAs were shown as blue nodes, and their target genes were shown as red nodes).

AML, particularly in disease progression and resistance mechanisms, remain largely unexplored. This study aimed to address this gap by confirming alterations in circRNA expression in relapsed and drug-resistant AML. Previous studies[15–17] have made initial attempts to elucidate the underlying functions of circRNAs in chemoresistance in AML. Our findings contribute to a growing body of evidence supporting circRNAs' involvement in the complex landscape of AML, particularly in the context of disease relapse and resistance to therapies.

In this study, we identified 48 upregulated and 27 downregulated DE-circRNAs in R/R-AML patients. Most DE-circRNAs originated from protein-coding exons, with a smaller fraction from introns and sense overlapping regions. Bioinformatics analysis revealed that the candidate circRNA has-circRNA-101819 harbored MREs for has-miR-15a-5p, has-miR-15b-5p, has-miR-1301-5p, has-miR-615-5p, and has-miR-433-3p. Previous studies have validated the involvement of the miR-15 family of miRNAs in the development of various cancers.[25] Previous researches have highlighted the regulator roles of circRNAs as ceRNAs in AML progression, particularly through interactions with miRNAs or mRNAs. Key circRNA-miRNA interactions, such as circ0010984/miR-375, circSLC25A13/miR-616-3p, circ0000370/miR-1299, and CircSLC25A13/miR-616-3p/ADCY2, have been identified as critical in AML development.[12–14] However, our understanding of circRNAs' specific functions in AML chemoresistance remains limited.

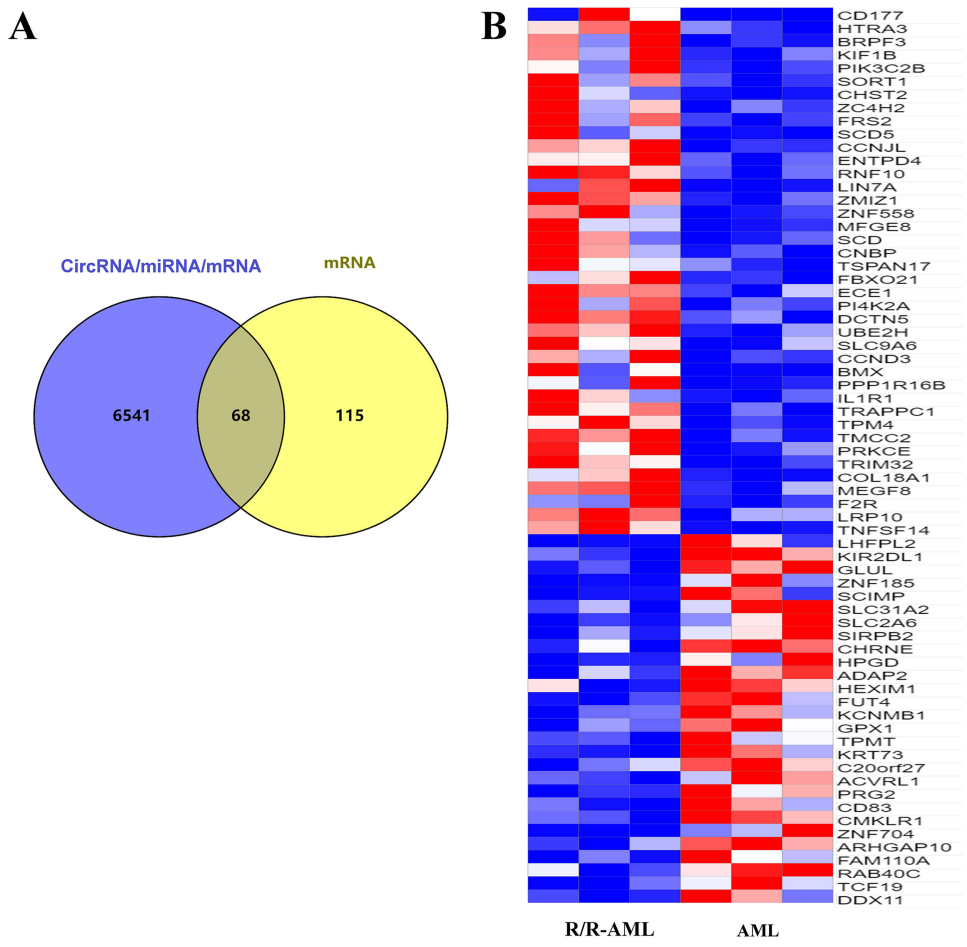

Figure 6 Overlapping of the predicted target mRNAs and our ceRNA. (**A**) Venn diagram of predicted target mRNAs and our RNA-sequencing profiles of R/R AML samples. (**B**) Heatmap of the 68 overlapping mRNAs.

The enrichment analysis of DE-circRNA between R/R-AML and *de novo* AML in our study revealed significant associations with biological processes such as cell migration, cell junction, and Ras signaling pathway. DE-mRNAs in R/R-AML were predominantly associated with biological processes related to immune response and B cell receptor signaling pathway. In the context of chemoresistance, circNPM1 has been associated with increased resistance to adriamycin in AML through its regulatory effects on the miR-345-5p/FZD5 pathway.[17] Additionally, upregulated circRNAs, including CircRNA-CREIT in the PKR/eIF2α signaling pathway, and CircANKIB1 in the miR-26b-5p pathway, have been implicated in mediating doxorubicin resistance.[18–20] In contrast, inhibition of the oncogenic Circ_0000098 has been shown to mitigate doxorubicin resistance by decreasing P-glycoprotein expression and intracellular ATP levels.[21] Despite these findings, the mechanism of circRNAs in chemoresistance of AML require to be further investigated. Our results suggest potential avenues for future research aimed at unraveling the mechanisms underlying chemoresistance in AML.

Traditional cytogenetic classification categorizes AML into favorable, intermediate, and unfavorable; however, even within the favorable or intermediate categories, a considerable number of patients eventually progress to R/R-AML.[26] Early diagnosis of R/R-AML before onset of relapse or refractory clinical manifestations is crucial for potentially delaying disease progressions. We identified 30 overlapping DE-circRNAs-related genes in the R/R-AML *versus de novo* AML samples. Prognostic analysis of DE-circRNAs-related genes in R/R-AML identified 7 DE-circRNAs-related genes (ECE1, PI4K2A, SLC9A6, CCND3, PPP1R16B, TRIM32 and ARHGAP10) associated with a poor prognosis in AML. Data accessed from UALCAN indicated the potential prognostic value of the 7 DE-circRNAs-related genes in R/R-AML. Previous studies[27–30] have also implicated ECE1, CCND3, PPP1R16B, and TRIM32 in AML progression

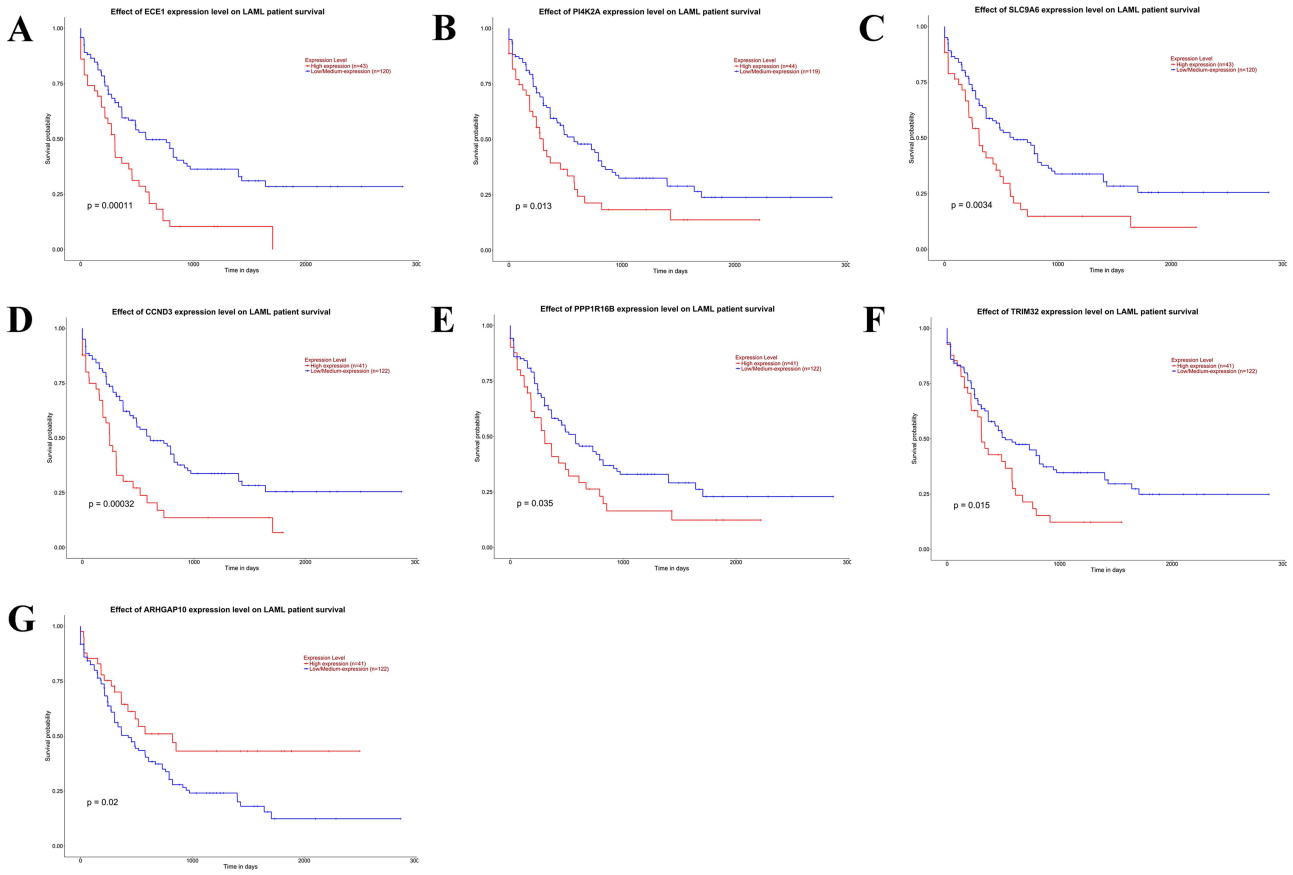

Figure 7 Identify the prognostic gene signature of R/R-AML by the UALCAN data portal based on DE-circRNA profiles. AML with overexpression of 6 genes indicated a poor prognosis: (**A**) ECE1, (**B**) PI4K2A, (**C**) SLC9A6, (**D**) CCND3, (**E**) PPP1R16B, (**F**) TRIM32 and (**G**). ARHGAP10. ($p < 0.05$).

and myelofibrosis pathogenesis. However, due to the limitations of our study, additional in vitro and in vivo investigations into the underlying mechanisms are warranted.

In summary, our investigations provide novel insights into the potential biological characteristics associated with relapse and refractory processes in AML, focusing on circRNA and mRNA expression profiles. Utilizing circRNA microarray analysis, our study revealed dysregulation of circRNAs in patients with R/R-AML. CircRNAs and their associated mRNAs may play pivotal roles in the early diagnosis of R/R-AML and could serve as potential targets for treatment interventions in this challenging disease.

Table 3 Prognostic Genes in Relapsed/Refractory Acute Myeloid Leukemia Based on Altered Circulating CircRNA Profiles

Gene_Name	Experssion	Locus	Fold_Change	p_value	R/R-AML_FPKM	AML_FPKM
SCD	Up	chr10:102,106,881–102,124,591	3.293140176	0.04856219	3.432308546	1.712844623
ECE1	Up	chr1:21,543,740–21,671,997	2.798308367	0.026461452	3.403848727	1.919293774
PI4K2A	Up	chr10:99,400,443–99,436,191	2.229820568	0.037668142	2.023627622	0.8667
SLC9A6	Up	chrX:135056000–135,129,428	1.80645387	0.044186541	1.59830978	0.745149366
CCND3	Up	chr6:41,902,671–42,018,095	1.523669903	0.027017109	3.629783823	3.02223344
PPP1R16B	Up	chr20:37,434,348–37,551,667	2.720360333	0.038299865	3.021231557	1.577433796
ARHGAP10	Down	chr4:148,653,214–148,993,931	0.570312099	0.011738765	0.334734947	1.144911403

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Disclosure

The authors report no conflicts of interest in this work.

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