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ORIGINAL ARTICLE

Investigation of the expression profile of enhancer RNAs in acute myeloid leukemia based on RNA sequencing analysis

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ABSTRACT

Enhancer RNAs (eRNAs) are noncoding RNA. While dysregulation of eRNAs in the development of several cancers has been reported, the specific information regarding the landscape of eRNAs in acute myeloid leukemia (AML) is minimal. RNA-sequencing data were retrieved from the SRA database and aligned to the human reference genome (hg38) from Ensembl. Enhancer site annotations from the FANTOM5 project and gene annotations from Ensembl were used to obtain raw expression counts for eRNAs and protein-coding genes. Differential expression and co-expression analyses were performed to identify eRNAs associated with AML and their potential co-expressed genes. Functional enrichment analysis was used to determine biological pathways associated with the co-expressed genes of eRNAs. Two eRNAs associated with AML-related pathways were selected for experimental validation using real-time PCR. Two eRNAs, designated eRNA-7A (chr7:157299821–157300521, (hg38)) and eRNA-22A (chr22:29199767–29199953, (hg38)), were significantly upregulated in AML samples compared with normal controls. Real-time PCR confirmed their elevated expression levels in AML patients. Co-expression and functional enrichment analyses identified genes and pathways associated with these eRNAs, showing that genes positively correlated with eRNA-7A and eRNA-22A were significantly enriched in the Myc targets v1 and v2 pathways. Notably, several enriched genes in these pathways have been previously linked to AML pathogenesis. The eRNAs identified in this study, along with their associated genes and pathways, provide a preliminary understanding of the molecular mechanisms underlying AML development and progression. Moreover, they suggest the existence of promising candidates for future biomarker development in AML.

Keywords: Noncoding RNA, Enhancer RNA, AML, Differential expression analysis

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Introduction

Acute myeloid leukemia (AML) is a group of malignant diseases that arise from bone marrow hematopoietic stem cells. These diseases are defined by the aberrant development of these stem cells and the excessive growth of myeloid progenitor cells (1). Leukemia cells accumulate excessively, which inhibits the normal growth of hematopoietic cells, leukemia cell infiltration in the bone marrow, and the emergence of symptoms, including anemia, bleeding, and infection (2). AML typically begins in the bone marrow but rapidly spreads to the bloodstream. It can sometimes spread to other regions of the body, such as the lymph nodes, spleen, liver, central nervous system (brain and spinal cord), and testicles (3). AML patients have a low survival rate (4).

Treatment options for AML include general treatment, chemotherapy, targeted therapy, allogeneic and autologous hematopoietic stem cell transplantation, etc. (5, 6). Nevertheless, despite the comprehension of its pathophysiology, the mortality rates tend to be high. The undesirable outcomes can be attributed to delayed diagnosis and failure to achieve complete remission (7). This challenge underscores the immediate necessity to identify diagnostic and prognostic markers from RNA sequencing (RNA-Seq) data. The rapid advancements in high-throughput technology and omics approaches have led to the discovery of novel genetic abnormalities and molecular biomarkers associated with AML pathogenesis and clinical outcome (3). Recent studies have emphasized the significance of integrating molecular and genetic biomarkers to improve AML diagnosis, prognosis, and therapeutic stratification (8).

In the past decade, the rapid application and advancement of high-throughput sequencing technology have provided a new opportunity to study noncoding RNAs. A rising body of evidence suggests that the dysregulation of noncoding RNAs is associated with the emergence and progression of several major disorders. Of these, enhancer-derived RNAs, or eRNAs, have attracted a lot of attention because of their basic function in mediating gene transcription and enhancer activities, as well as their frequent overlap with noncoding risk loci related to various diseases (9).Enhancers are crucial cis-regulatory elements that facilitate the expression of genes in eukaryotes (9). eRNAs are primarily transcribed from active enhancers characterized by low DNA methylation, enrichment of histone modifications such as H3K4me1 and H3K27ac, open chromatin regions, and occupancy of transcription factors including TBP, TFII, and P300/CBP, as well as RNA polymerase II. These epigenetic and transcriptional features collectively define enhancer activity and drive eRNA transcription. (10).

eRNA transcription begins when specific transcription factors and coactivators bind to enhancers. Subsequently, they employ more transcription factors, intricate molecular complexes, and histone modifiers, such as P300/CBP. H3K27 undergoes acetylation by P300/CBP, causing the expansion of enhancer regions and enabling the binding of crucial proteins such as RNA pol II. BRD4 and similar cofactors facilitate RNA polymerase II elongation and eRNA processing (11). Based on their structure and transcription patterns, we can categorize eRNAs, which range in size from around 0.1 to 9 kb, into two groups. The first group consists of short, bidirectional RNAs that are not spliced or polyadenylated. The second group consists of long, unidirectional RNAs that are spliced, stable, and polyadenylated. Due to their lack of polyadenylation and instability, eRNAs are primarily found in the nucleus and in fractions enriched with chromatin (12).

Dysregulation of eRNAs can impact biological processes, including the cell cycle and the proliferation of cancer cells, or alter the expression of target genes. This suggests that eRNAs may serve as potential therapeutic targets for cancer treatment (9). A recent study has discovered that mutations in the eRNA element of ACTRT1 in basal cell carcinoma can diminish the enhancer's functionality and the expression of ACTRT1. This leads to the abnormal activation of Hedgehog signaling, which in turn promotes the development of carcinogenesis and tumor formation (13).

In T-cell acute lymphoblastic leukemia (T-ALL), it has been observed that the eRNA ARIEL is capable of attracting mediator proteins to the ARID5B enhancer, resulting in increased interaction between

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the enhancer and promoter and leading to the activation of ARID5B production. Myc oncogenic signals and the TAL1-induced transcriptional pathway were aberrantly activated as a result of these activities (14). NET1 is a crucial oncogene in the context of breast cancer. Prior studies have shown that the NET1-associated eRNA (NET1e) can increase the expression emergence of symptoms, including anemia, bleeding, and infection (2). AML typically begins in the bone marrow but rapidly spreads to the bloodstream. It can sometimes spread to other regions of the body, such as the lymph nodes, spleen, liver, central nervous system (brain and spinal cord), and testicles (3). AML patients have a low survival rate (4).

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Methods

Data collection and preprocessing

We obtained the AML RNA-seq samples for this study from two distinct bioprojects with accession numbers PRJNA576867 and PRJNA386992 from the Sequence Read Archive (SRA). Using the SRA Toolkit (v2.11.3)(17), transcriptome data were downloaded. PRJNA576867 was generated using a total RNA library preparation protocol, whereas PRJNA386992 employed a poly(A)-selected library preparation protocol. With the help of the "fastqdump" function from sratoolkit, the "sra" files were converted to fastq format, and quality checks were conducted with FastQC (v0.11.9). The ENSEMBL hg38 assembly was utilized as the genome reference, and sequence alignment was carried out using the STAR aligner (v2.7.11b)(18) with --runThreadN 14. SAMtools (v1.19.2)(19) was used to sort and index the "bam" files.

Quantification of eRNAs and protein-coding genes

FeatureCounts in R (version 2.0.3) (20) was utilized to acquire the raw counts of eRNAs and protein-coding genes with paired-end mode enabled and multi-mapping reads excluded. The GTF annotation file (Homo sapiens, GRCh38, Ensembl release 111) was obtained from the ensemble database, and the annotation of the enhancer location was downloaded from the FANTOM5 project (F5.hg38.enhancers.bed.gz, released March 2016; https://fantom.gsc.riken.jp/5/datafiles/reprocessed/hg38_latest/extra/enhancer/) (21). Given that eRNA transcription regions could be wider than the enhancer ChIP-seq peaks, we defined the ± 3 kb regions around the middle point of the annotated enhancers as potential eRNA-transcribing regions (15, 22). We also filtered out those eRNA regions that overlapped with known coding regions and lncRNAs (with 1 kb extension from both transcriptions start site and transcription end site). This was done to exclude introns, exons, promoters, rRNA repeats, and other transcribed elements (15). RNA-seq data were mapped to these eRNA regions and expression levels were quantified using reads per million (RPM) metric.

Active eRNAs were defined as eRNAs with RPM ≥1 in at least one sample. This RPM-based criterion was applied exclusively for the identification of transcriptionally active eRNA regions, consistent with previously published eRNA-related studies (15, 22). RPM values were not used for downstream statistical analyses or differential expression testing. Raw count matrices were subsequently used as input for all downstream analysis.

Identification of differentially- expressed eRNAs and genes

Two distinct approaches implemented in the R packages edgeR (23) and limma (24) were used to identify those genes and eRNAs that are differentially expressed in the same direction (i.e., up- or down-regulated) between AML and normal samples. First, the filterByExpr function from the edgeR package was used to remove genes whose count per million (CPM) was less than 1 in 70% of the samples. Raw count data were then normalized using the trimmed mean of M-values (TMM) method implemented in edgeR. The normalized counts were transformed to log2 counts per million (logCPM) using the voom function in the limma package.

To account for potential batch effects arising from the integration of two independent SRA projects

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(PRJNA576867 and PRJNA386992), the ComBat function from the sva R package was applied to the merged logCPM expression matrix. The batch-corrected expression matrix was subsequently used for differential expression and downstream statistical analyses. For linear modeling, the lmFit function in the limma package was employed.

The threshold for the absolute log2 fold change (FC) was set at greater than 1 (|log2FC >1|), and the adjusted p value was less than 0.05 (adjusted $P<0.05$) to identify statistically significant differentially expressed genes and eRNAs. These thresholds were selected based on the commonly used criteria in transcriptomic differential expression studies to balance statistical significance and biological relevance while minimizing false-positive discoveries (25). In this study, genes and eRNAs with |log2FC| > 1 and adjusted P < 0.05 (FDR) were considered significantly differentially expressed.

Co-expression analysis

To investigate the associations between eRNAs and gene expression patterns, co-expression analysis was performed. In this manner, Pearson's correlation test between upregulated eRNAs and all genes was performed using the "cor" function in R. The genes that exhibited a correlation coefficient (r) > 0.6 and a FDR < 0.05 with each eRNA were selected. The co-expression network was visualized by Cytoscape (v.3.9.1)(26).

Functional enrichment analysis and protein-protein interaction (PPI) network

Functional enrichment analysis was performed for the differentially expressed genes (DEGs) and genes identified by co-expression analysis. Using the Enrichr web server (27), Molecular Signatures Database (MsigDB) pathway analysis and Gene Ontology (GO) functional analysis, including cellular component (CC), molecular function (MF), and biological process (BP), was conducted. The results were shown using the "clusterProfiler" package in the R (version 4.10.1)(28). Additionally, the STRING database (https://string-db.org/)(29) was utilized to identify known protein-protein interactions (PPIs) among genes co-expressed with eRNAs associated with AML-related signaling pathways. PPI networks were constructed with a minimum required interaction score of 0.4 (medium confidence). Only interactions supported by experimental evidence and curated databases were retained for network construction.

Selection of eRNAs for experimental validation

Following co-expression and enrichment analyses, two eRNAs with increased levels of expression and involvement in molecular pathways associated with AML were selected as candidates for the experimental investigation. The sequence of selected eRNAs was retrieved utilizing the Integrative genomics viewer (IGV) tool (30), and primers were designed using the Primer blast database in NCBI.

Epigenetic characterization of candidate eRNA loci

To further characterize the regulatory features of the candidate eRNA loci and their potential gene interactions, publicly available chromatin and regulatory data were examined using the UCSC Genome Browser (hg38). H3K27Ac signals (Layered H3K27Ac track, 7 cell lines), BRD4 ChIP-seq data from K562 cells (ENCSR583ACG), and GeneHancer regulatory element annotations (Double Elite) were visualized at both candidate eRNA loci. K562, a well-established leukemia cell line, was selected as a surrogate model given the limited availability of primary AML cell chromatin data in public databases.

Sample collection

For validation of the identified eRNAs, peripheral blood samples were collected from AML patients at Seyed al-Shohda Hospital (Isfahan, Iran). The cohort consisted of 15 patients diagnosed with non-acute promyelocytic leukemia (non-APL) AML, including 8 males and 7 females, with a mean age of 58.5 ± 6.4 years (Table 1). The patients did not get chemotherapy or biological treatment, and they had no history of autoimmune illnesses or underlying diseases. Also, 15 healthy control samples with no prior medical history of cancer were gathered. Patients and healthy participants provided written informed consent, and the Research Council and Ethical Committee of Isfahan University of Medical Sciences (IR.MUI.MED.REC.1402.121) confirmed this study.

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In ice tubes containing ethylenediaminetetraacetic acid (EDTA), 5 ml of peripheral blood from all patients and normal persons was collected. Ficoll's solution (Sigma-Aldrich, Germany) was used to isolate mononuclear cells (MNCs) according to the manufacturer's instructions.

Table 1. Demographic and clinical characteristics of AML patients included in the validation cohort.

Sample No.	Gender	Age (years)	AML Type
1	Male	67	Non-APL AML
2	Female	56	Non-APL AML
3	Male	50	Non-APL AML
4	Male	64	Non-APL AML
5	Female	64	Non-APL AML
6	Female	47	Non-APL AML
7	Male	64	Non-APL AML
8	Male	51	Non-APL AML
9	Female	53	Non-APL AML
10	Female	56	Non-APL AML
11	Female	65	Non-APL AML
12	Male	62	Non-APL AML
13	Male	57	Non-APL AML
14	Male	56	Non-APL AML
15	Female	65	Non-APL AML

RNA extraction, complementary DNA (cDNA) synthesis, and real-time PCR

Using a total RNA extraction kit (Parstos, Iran; Cat. No. A101231) and according to the manufacturer's directions, RNA was extracted from mononuclear cells. Briefly, cells were lysed using RL buffer, followed by chloroform extraction, column-based purification, and RNA elution in DEPC-treated water. A nanodrop spectrophotometer (Biochrom WPA Biowave II, England) was then used to determine the concentration and purity of the RNA samples. For cDNA synthesis, 1 ng–1 µg of total RNA was reverse-transcribed using the Easy cDNA Ultra-TM Synthesis Kit (Parstous, Iran; Cat. No. A101162)

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in a final reaction volume of 20 µL. Reverse transcription was performed at 25°C for 10 min, 55°C for 30 min, and 85°C for 5 min according to the manufacturer's protocol.

Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Real-Time PCR Master Mix (Parstous, Iran; Cat. No. C101081) on an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Each reaction was performed in a final volume of 20 µL and analyzed in triplicate using the primers listed in Table 2. Primer amplification efficiencies were evaluated using LinRegPCR software. Melt curve analysis was performed following amplification to verify the specificity of PCR products. No-template controls (NTCs) were included in all qRT-PCR runs to monitor potential contamination. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference gene for normalization because of its widespread use in AML and other hematological malignancy gene expression studies (31, 32).

Statistical Analysis

The results were normalized and statistically evaluated using Graph Pad Prism software version 10.2.2 (Graph Pad, USA) (33). Data normality was assessed using the Shapiro–Wilk and Kolmogorov–Smirnov tests. As the data followed a normal distribution, differences in expression levels between AML patients and healthy controls were evaluated using an unpaired Student's t-test. Relative gene expression was calculated using the 2^-ΔΔCt method. Data are presented as mean ± standard deviation (SD), and a p-value < 0.05 was considered statistically significant.

Results

Differential expression analysis

The study procedures are presented in Figure 1. A uniform pipeline, as described in the Materials and Methods section, was used to obtain RNAseq datasets from 29 subjects with AML and 10 healthy subjects from SRA. The characteristics of the RNA-seq datasets included in the present study are summarized in Supplementary Table 1. TMM normalization and voom transformation to logCPM were performed prior to ComBat-based batch correction of the merged expression matrix. Expression distributions before and after correction were visually inspected using boxplots, confirming successful normalization and removal of batch effects across samples (Figure 2). Following preprocessing, we have identified Differentially Expressed Genes (DEGs) in AML samples compared to normal samples. We have detected a total of 26,195 genes, out of which 2,697 genes were up-regulated and 1,827 genes were down-regulated (Figure 3).

These results were satisfied with a log2FC>1 and adjusted p-value < 0.05. Additionally, using logFC>1 and FDR<0.05 criterion, we detected 1538 loci associated with eRNA, comprising 6 up-regulated and 173 down-regulated eRNAs (Figure 3). There was no overlap between each of the detected eRNAs and the annotated genomic regions.

Co-expression analysis

Establishing a direct link between eRNA and its correlated genes remains a challenge. We constructed an eRNA-gene co-expression network between up-expressed eRNAs and all expression matrix genes (Pearson's correlation coefficient (r) > 0.6, FDR < 0.05). The set of upregulated eRNAs consists of specific locations.

The genomic coordinates are as follows: chr2:42968016-42968305 (hg38; eRNA-2A), chr2:241863497-241863832 (hg38; eRNA-2B), chr7:157299821-157300521 (hg38; eRNA-7A), chr16:88793718-88793872 (hg38; eRNA-16A), chr22:29199767-29199953 (hg38; eRNA-22A), and chrX:118717639-118718304 (hg38; eRNA-X1). Hereafter, these aliases are used throughout the manuscript. Co-expression results revealed that 206 genes had a strong positive correlation with eRNA-7A, 270 genes with eRNA-22A, 169 genes with eRNA-2A, 142 genes with eRNA-2B, 110 genes with eRNA-16A, and 132 genes with eRNA-X1. The co-expression network of eRNA-22A and eRNA-7A with their positively correlated genes are illustrated in Figure 4.

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Table 2. The sequences of primers used in qRT-PCR

Name	Forward primer 5′→3′	Reverse primer 5′→3′
eRNA-22A	AGAACGCTGTGAGTGGTGAAC	CACTCCACCCCTAGAAGGATGT
eRNA-7A	AACTCACTCAGGGTGGCATC	ATGGCAGCAGAGACTCGTAG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA

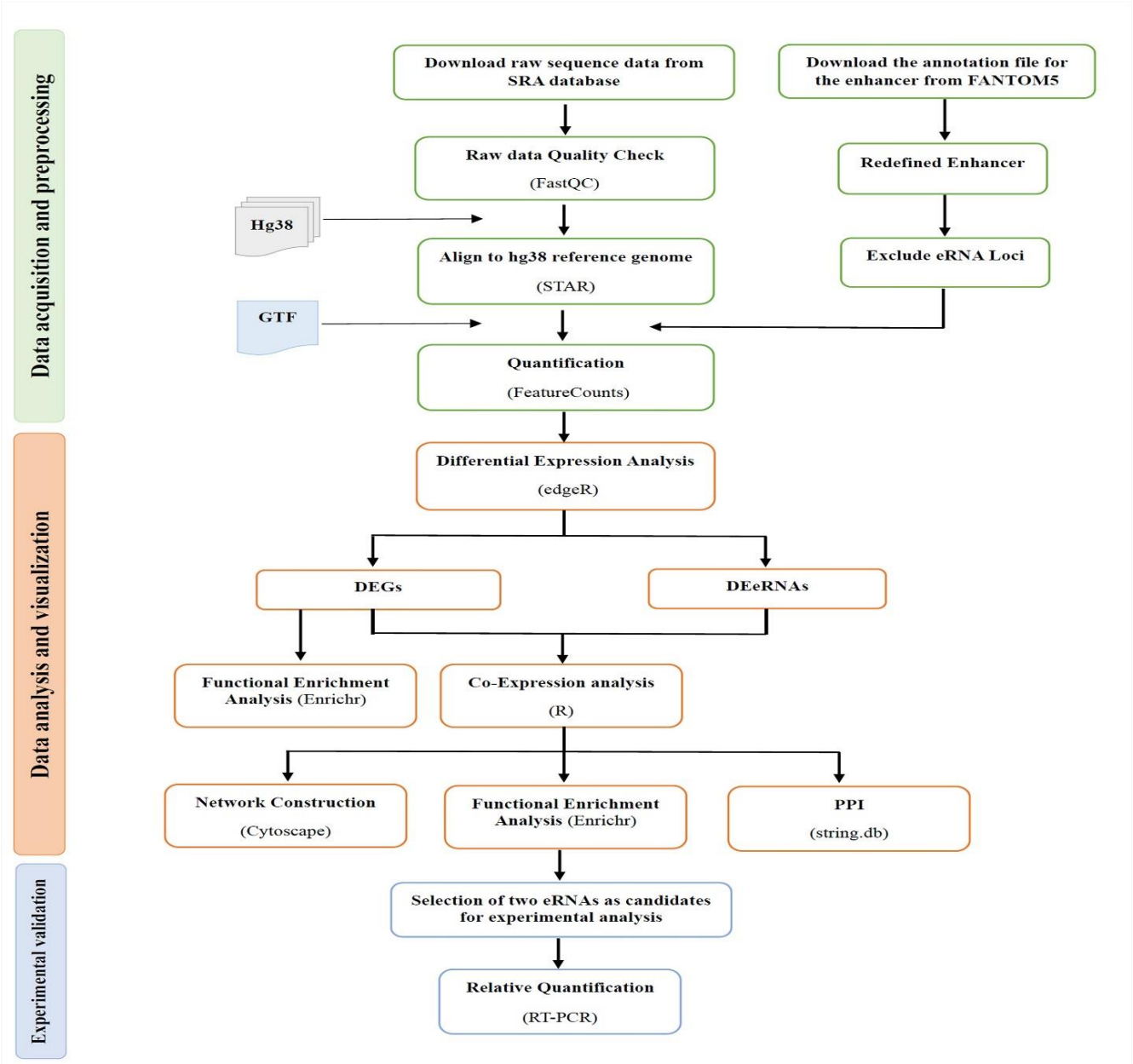

Figure 1. Schematic workflow of acquiring, processing, and analyzing RNAseq data

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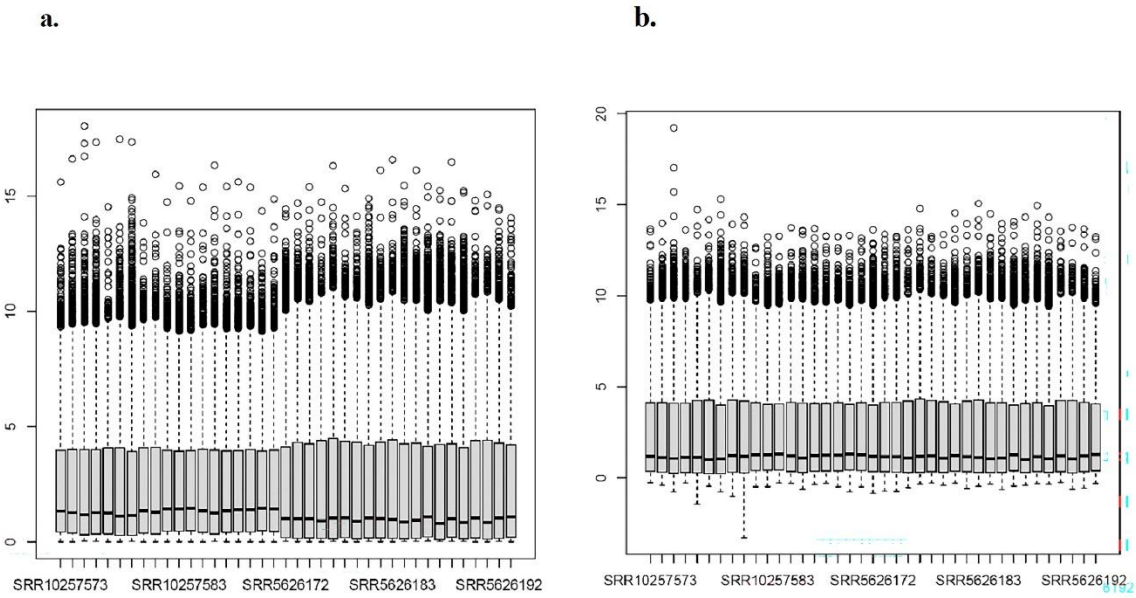

Figure 2. Expression distribution before and after batch effect correction. Boxplots showing the distribution of log2-transformed expression values across all samples a) before and b) after ComBat-based batch correction. Each box represents the interquartile range (IQR), with the horizontal line indicating the median expression value. Whiskers extend to 1.5× the IQR, and circles represent outliers. The increased uniformity of expression distributions after correction confirms successful removal of batch effects across samples from both bioprojects (PRJNA576867: SRR10257573–SRR10257591; PRJNA386992: SRR5626172–SRR5626193).

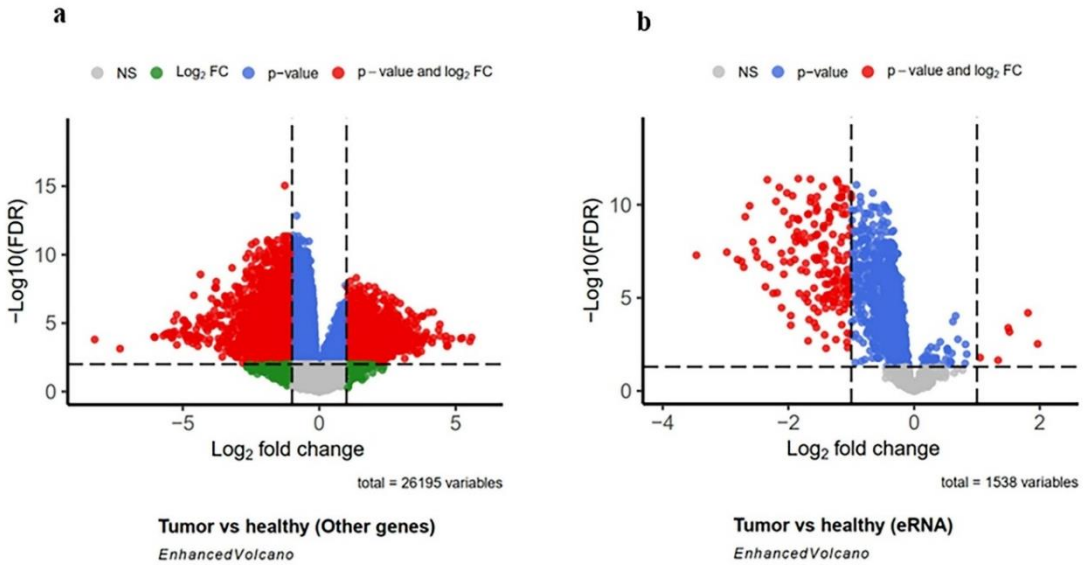

Figure 3. Volcano plots differentially expressed transcripts between AML and normal samples. a) Volcano plot of differentially expressed genes (DEGs), including 2,697 up-regulated (|logFC>1| and FDR<0.05) and 1,827 down-regulated (|logFC<-1| and FDR<0.05) genes. b) Volcano plot of differentially expressed eRNAs (DEeRNAs), including 6 up-regulated (|logFC>1| and FDR<0.05) and 173 down-regulated (|logFC<-1| and FDR<0.05) eRNAs.

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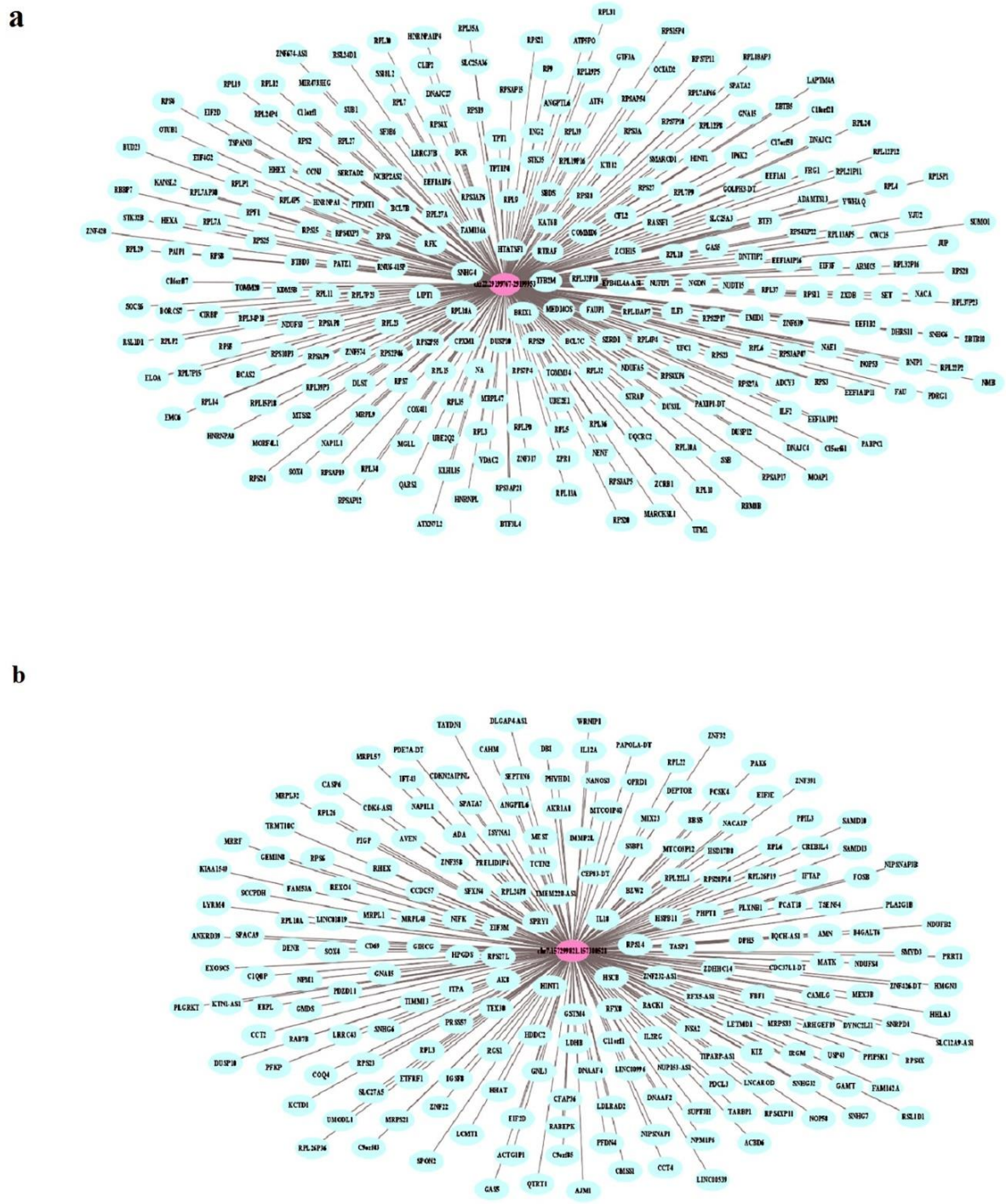

Figure 4. Co-expression networks illustrating the correlation between selected eRNAs and their related genes. a) eRNA-22A; b) eRNA-7A. Nodes represent genes, and edges represent significant correlations (r > 0.6, FDR < 0.05).

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Enrichment analysis and construction of PPI networks

The functional enrichment analyses for DEGs and genes acquired from co-expression analysis are presented in Figure 5, respectively. The enrichment analyses for DEGs indicate their involvement in cancer-related pathways, including allograft rejection, Apoptosis, unfold protein response, estrogen response early, TNF-alpha signaling via NF-Kb, inflammatory response, IL-2/STAT5 signaling, Myc targets v1, Myc targets v2, and the p53 pathway. Also, the enrichment results for genes obtained from co-expression analysis were statistically significant only for genes that had a positive correlation with the eRNAs eRNA-7A and eRNA-22A.

These eRNAs correlated genes were implicated in the Myc targets v1 and v2 signaling pathways. These two eRNAs were selected as candidates for experimental validation. PPI network analysis demonstrated significant interaction enrichment among genes co-expressed with both candidate eRNAs. The network associated with eRNA-22A consisted of 193 nodes and 2418 edges (expected number of edges = 724), with an average node degree of 25.1 and a highly significant PPI enrichment p-value (< 1.0e-16). Similarly, the network associated with eRNA-7A comprised 137 nodes and 199 edges (expected number of edges = 59), with an average node degree of 2.91 and a significant PPI enrichment p-value (< 1.0e-16) (Figure 6).

Epigenetic characterization of candidate eRNA loci

To further support the enhancer activity of the two candidate eRNA loci and their potential gene interactions, publicly available chromatin and regulatory data from ENCODE and GeneHancer were examined. Both eRNA-7A and eRNA-22A loci exhibited strong H3K27Ac signals across multiple cell lines and were annotated as candidate Cis-Regulatory Elements (cCREs) in the ENCODE4 registry. Furthermore, BRD4 ChIP-seq data from K562 cells revealed BRD4 occupancy at both loci, providing additional support for their putative enhancer activity. GeneHancer Double Elite annotations indicated that both loci are recognized as regulatory elements (GH07J157298 and GH22J029195, respectively) with predicted gene interactions. Notably, EMID1, one of the genes predicted to interact with the eRNA-22A locus by GeneHancer, was also independently identified in our co-expression analysis, providing convergent computational evidence for enhancer-gene interactions at this locus. Although ATAC-seq data from untreated hematopoietic or AML cells were not available in public databases for these loci, the combined evidence from H3K27Ac enrichment, ENCODE4 cCRE annotations, BRD4 occupancy, and GeneHancer regulatory annotations are consistent with the classification of both regions as putative active enhancer elements (Supplementary Figure 1).

Extracting the sequence of candidate eRNAs for primer design

Using IGV tools (v2.17.4), we searched for the genomic position of candidate eRNAs in BAM files. We determined the target sequence for primer design based on two criteria: it had the maximum number of aligned reads and existed in all BAM files. Table 2 shows the sequence of the designed primers.

Relative quantification eRNA-22A and eRNA-7A expression in AML samples

To perform experimental validation, we measured the expression levels of eRNA-7A and eRNA-22A in peripheral blood samples collected from healthy individuals (n = 15) and AML patients (n = 15). As shown in Figure 7, eRNA-22A expression was significantly increased in AML samples compared with controls. The mean relative expression level in AML samples was 10.63 (95% CI: 9.81–11.44), corresponding to a 10.63-fold increase relative to healthy controls ($p < 0.0001$). Similarly, eRNA-7A expression was significantly elevated in AML patients. The mean relative expression level in AML samples was 4.79 (95% CI: 4.17–5.41), corresponding to a 4.79-fold increase relative to healthy controls ($p < 0.0001$). These findings are consistent with the results of our bioinformatic analyses.

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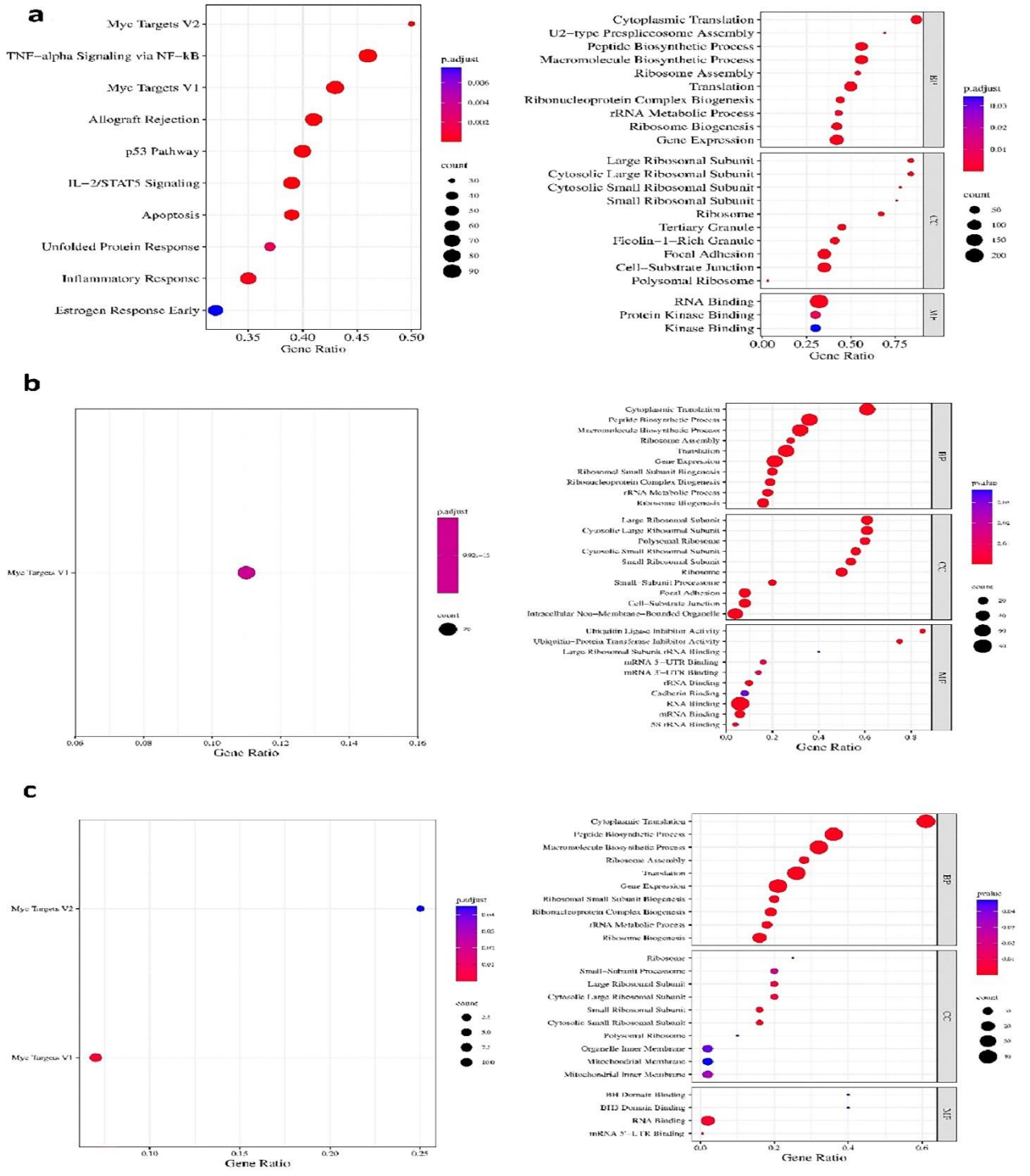

Figure 5. GO/MsigDB enrichment analysis of DEGs and eRNA-related genes. MsigDB pathway analysis and GO terms enriched in the categories "biological process," "molecular function," and "cellular component" for a) DEGs, b) eRNA-22A, and c) eRNA-7A. The size and color of dots are determined by the number of genes (Count) and the adjusted P value (p. adjust), respectively. (FDR is less than 0.05, and the figure reports the pathways and GO terms with the highest significance.)

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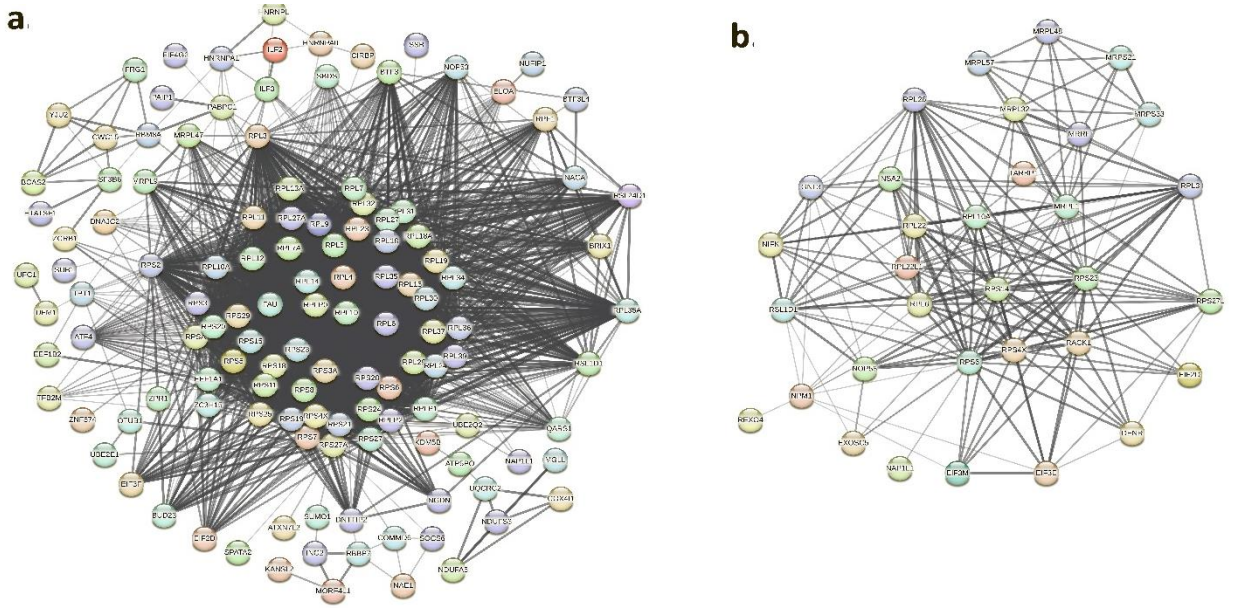

Figure 6. Protein–protein interaction networks of genes co-expressed with the candidate eRNAs. a) Genes correlated with eRNA-22A (193 nodes, 2418 edges; average node degree = 25.1). b) Genes correlated with eRNA-7A (137 nodes, 199 edges; average node degree = 2.91). Networks were generated with a minimum interaction score of 0.4, considering only experimentally validated and database-supported interactions. Nodes represent genes co-expressed with the candidate eRNAs, whereas edges represent known protein–protein interactions obtained from the STRING database.

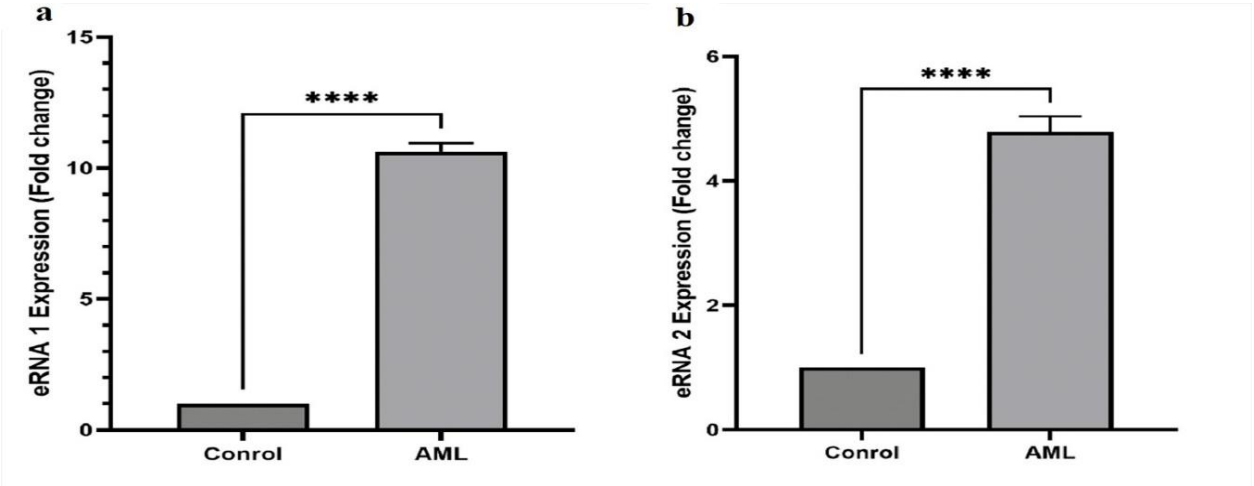

Figure 7. qRT-PCR validation of the candidate eRNAs in AML patients (n = 15) and healthy controls (n = 15). a) eRNA-22A (eRNA1); b) eRNA-7A (eRNA2). Relative expression levels were calculated using the 2^-ΔΔCt method and are presented as mean ± SD. Statistical significance between groups was assessed using an unpaired Student's t-test. Both eRNAs showed significantly increased expression in AML samples compared with healthy controls (p < 0.0001).

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Discussion

The complex genetic and molecular pathways underlying AML make it an extremely heterogeneous disease. The rates of complete remission (CR) and long-term survival of AML patients remain poor despite remarkable advancements in targeted therapy (34). Therefore, it is imperative to identify useful prognostic biomarkers. The present study investigated the link between eRNAs, a new group of long non-coding RNAs, and AML disease.

In this study, gene expression data relating to AML was analyzed to extract DEGs and DEeRNAs. Subsequently, co-expression analysis was conducted to identify genes associated with upregulated eRNAs. Molecular pathways associated with the genes co-expressed with eRNAs were identified by functional enrichment analyses. The results indicated that genes positively correlated with two upregulated eRNAs, eRNA-22A and eRNA-7A, were significantly enriched in the Myc-targets v1 and Myc-targets v2 pathways. Notably, both eRNAs were consistently upregulated in AML samples and were further validated by qRT-PCR in an independent cohort. Although the precise biological functions of these eRNAs remain to be experimentally determined, their reproducible overexpression and association with AML-relevant transcriptional programs suggest a potential role in AML-associated molecular alterations. *Myc*, one of the most studied cancer genes, is involved in the development, maintenance, and progression of many cancer types. *C-Myc*, *L-Myc*, and *N-Myc* are the three members of the *Myc* gene family. They are all members of the basic helix-loop-helix leucine zipper (bHLHLZ) DNA-binding proteins superfamily.

Majority of Myc proteins' roles in the cell are transcriptional modulators; they regulate genes that are involved in a variety of cellular processes, such as immune response, angiogenesis, stromal remodeling, cell growth, differentiation, apoptosis, and metabolism (35, 36). In normal cells, *Myc* expression is strictly regulated. However, among solid and hematopoietic neoplasias, *Myc* aberrant activity is one of the most frequent occurrences (37). For leukemias induced by several fusion genes e.g., BCR-ABL1, RUNX1-RUNX1T1, and AML1-ETO and FLT3-ITD, *Myc* is necessary. Also, overexpression of *Myc* has the opposite effect of *Myc* suppression, which promotes myeloid differentiation and decreases the proliferation of AML cells. Accordingly, several human AML patient cohorts have demonstrated a positive correlation between elevated *Myc* expression at the mRNA and protein levels, immature myeloid differentiation markers, greater blast numbers, and a poor prognosis. Taken together, these pre-clinical and clinical findings imply that *Myc* is critical to the maintenance of AML and may influence the course of treatment and clinical results (38, 39).

Some of the genes positively correlated with these eRNAs in the Myc target v1 and Myc targeted v2 pathway include ribosome biogenesis genes (such as *RPL22* and *RPL34*), *NPM1*, *UBE2E1*, *GNL3*, and *RACK1*. The previous research has established the involvement of each of these genes in the etiology of AML.The capacity of *Myc* to both initiate and maintain malignancies may be causally linked to its capacity to regulate ribosomal genes (40). Ribosomal proteins (RPs) have significant functions in the proliferation and development of eukaryotic cells (41). Ribosomopathies are a group of disorders that occur due to mutations and deletions in ribosomal proteins. Together, these diseases are marked by inefficient hematopoiesis, failure of the bone marrow, and an increased risk of developing myelodysplastic syndrome (MDS) and later AML. This observation emphasizes the significance of the malfunctioning of this group of proteins in the formation and advancement of myeloid neoplasms (42). Wang et al. (41) confirmed the prognostic significance of *RPL34* in AML and showed its upregulation in the disease. Patients who achieved a complete response following induction therapy and experienced long-term survival exhibited significantly lower levels of *RPL34* compared to those who did not respond to induction therapy and experienced recurring disease relapses.

NPM1 is a phosphoprotein located in the nucleolus, which has been characterized as a histone chaperone protein (43). *NPM1* is known to control various cellular processes, such as biogenesis and transport, maintenance of genomic stability, duplication of centrosomes, modification of chromatin structure, DNA repair, assistance in folding histones, and regulation of the ARF-p53 tumor suppressor pathway. Studies show that *NPM1* gene mutations, which affect between 25–35% of patients with AML, are among the most prevalent genetic mutations (44).

Cytoplasmic dislocation is a prevalent feature observed in all *NPM1* mutants. This condition serves

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as the primary stimulant for leukemogenesis by activating the c-myc oncogene, affecting the positive regulation of *HOXA* and *HOXB* genes, inhibiting cell proliferation, and reducing apoptosis. Furthermore, apart from mutations in the NPM1 gene, several chromosomal translocations of the *NPM1* gene have been identified in AML cases.

These translocations include t(5;10)(q35;q23), t(5;18)(q34;q12), and t(5;6)(q35;q23), resulting in the formation of the NPM1/RPP30, NPM1/SETBP1, and NPM1/CCDC28A fusion transcripts (45). *UBE2E1* is an E2 class ubiquitin-conjugating enzyme that controls the expression of the *HOX* gene via histone ubiquitination. The actual function of *UBE2E1* in cellular signaling remains mostly unknown. Nevertheless, the regulation exerted by *UBE2E1* on the *HOX* gene could potentially provide crucial insights into the prognostic significance of *UBE2E1* in AML, given that disruptions in the expression of *HOX* genes have been detected in AML patients (40, 46). Research finding suggests that AML leukemogenesis increased by *HOX* gene overexpression, specifically *HOXA10* and *HOXA9*. Additionally, the level of the transcription factor *HOXA11* may be associated with resistance to AML therapies (40).

Consequently, *UBE2E1* expression in AML inversely correlates with survival. According to studies conducted by A. Bauer et al., patients with high UBE2E1 expression have a poorer prognosis and do not respond to chemotherapy, whereas those with lower *UBE2E1* expression are more responsive and more likely to achieve complete remission (47). *Nucleostemin* (*NS*), often referred to as *GNL3*, is a GTPase protein that shuttles between the nucleolus and nucleoplasm. It plays a crucial role in various processes related to embryonic development, pluripotency reprogramming, telomere maintenance, and tissue regeneration (48). Additionally, interacting with nucleolar proteins involved in pre-rRNA processing contributes to ribosome biogenesis. *NS* levels are elevated in several solid and hematologic tumors, such as AML. Ali et al. (49) performed a study that revealed a significant link between a higher level of *NS* in patients with AML and the percentage of immature blast cells and hematopoietic stem/progenitor cell surface markers. Furthermore, AML patients with high levels of *NS* transcripts tend to have a poor prognosis.

These findings suggest that *NS* expression could serve as an indicator of the immature characteristics of AML cells. Receptor for activated C kinase 1 (*RACK1*) is a widely conserved protein in eukaryotes. Several studies have indicated that *RACK1* contributes to cell proliferation in a context-dependent manner. For instance, *RACK1* exhibits a pro-tumorigenic function in hepatocellular carcinoma while inhibiting gastric carcinogenesis. Recent findings indicate that *RACK1* increases the activity of GSK3b, which could help the survival of leukemic progenitor cells in AML. As a result, focusing on *RACK1* could have significant therapeutic implications for AML management (50). Likewise, a study conducted by Call et al. (51) revealed that a specific group of extremely active super-enhancers (SEs), known as enhancer clusters, are distributed across extensive genomic areas and work together to preserve strong gene transcription. In AML, epigenetic reprogramming causes the aberrant activation of super-enhancer (SE) landscapes that drive the expression of important oncogenes, such as the oncogenic *Myc* pathway, and contribute to the progression of AML and resistance to chemotherapy. The SEs were recognized as potential therapeutic targets and were specifically targeted using the NR4A-inducing drug dihydroergotamine (DHE). In AML, DHE modulates overlapping gene expression patterns and suppresses the transcription of a specific group of leukemic oncogenes associated with super-enhancers, such as *Myc*.

The AML-selective SE cluster that controls *Myc* transcription interacts with NR4As to decommission its activation status by excluding crucial SE-bound coactivators such as BRD4, Mediator, and p300. As a result, Pol 2-dependent eRNA transcription and p300-dependent H3K27 acetylation are lost.

Based on our findings, we hypothesize that the two identified eRNAs may contribute to AML pathogenesis by modulating the transcriptional activity of MYC target genes. Given that active enhancers are known to recruit transcriptional coactivators such as BRD4 and Mediator complex, and that eRNAs have been shown to facilitate enhancer-promoter looping in other cancer contexts (52). It is plausible that these eRNAs may similarly act to sustain MYC-driven transcriptional programs in AML cells. However, the present study does not provide direct mechanistic evidence for such a role, and functional experiments, including CRISPRi-mediated perturbation and

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chromatin interaction analyses, will be required to evaluate this hypothesis.

In addition to the six upregulated eRNAs identified in this study, we detected a substantially larger group of downregulated eRNAs (n = 173) in AML samples. Although these eRNAs were not investigated further due to the study's focus on experimentally validating the most prominent upregulated candidates, their widespread reduction may reflect global alterations in enhancer activity and transcriptional regulation in AML. Previous studies have suggested that disruption of enhancer landscapes is a common feature of malignant transformation (53). Therefore, the observed predominance of downregulated eRNAs may indicate the loss of normal enhancer functions associated with hematopoietic differentiation and gene regulation. Future studies are needed to characterize the biological significance of these downregulated eRNAs and their potential contribution to AML pathogenesis.

Despite these promising findings, several limitations should be acknowledged. First, AML is a genetically and clinically heterogeneous disease, and eRNA expression patterns can differ among molecular and cytogenetic subtypes. In addition, the validation cohort was relatively small and primarily intended as an exploratory validation cohort. Although statistically significant differences were observed, larger independent cohorts with broader clinical and molecular characterization are required to further validate the reproducibility and generalizability of these findings across AML subtypes. Additionally, detailed molecular and cytogenetic information was not available for the validation cohort, which limited subtype-specific analyses and clinicopathological correlation assessments.

Second, differences in sample sources may have affected the comparability of the discovery and validation cohorts. the discovery cohort included samples from bone marrow (PRJNA576867) and peripheral blood or bone marrow (PRJNA386992), whereas the validation cohort consisted exclusively of peripheral blood mononuclear cells (PBMCs).

Third, post-treatment samples were not available in this cohort, precluded the assessment of eRNA expression changes following treatment. Future longitudinal studies with paired pre- and post-treatment samples are necessary to evaluate their potential utility as biomarkers of treatment response.

Fourth, the present study was primarily based on transcriptomic and co-expression analyses, and no functional experiments were performed to validate the biological roles of the identified eRNAs. Moreover, although GeneHancer analysis provided computational support for enhancer-gene interactions at both loci, direct experimental confirmation using chromatin conformation capture techniques such as promoter capture Hi-C was not performed in this study. Therefore, future mechanistic studies including CRISPRi-based knockdown or overexpression in AML cell lines together with chromatin interaction analyses, will be needed to clarify the potential contribution of these candidate eRNAs to AML pathogenesis and MYC-associated transcriptional programs.

Finally, the analyzed RNA-seq datasets were generated using different library preparation protocols, including total RNA sequencing and poly(A)-selected RNA sequencing. Because eRNAs include both polyadenylated and non-polyadenylated transcripts, these protocols may differ in their ability to capture particular eRNA subsets. Consequently, some enhancer-associated transcripts may have been underrepresented in the poly(A)-selected dataset. Nevertheless, the candidate eRNAs highlighted in this study were detected across both datasets and were subsequently validated by qRT-PCR in an independent cohort, supporting the robustness of the observed expression patterns.

Overall, the findings of our investigation are consistent with the studies mentioned above. Therefore, it can be said that our results, along with other studies, can contribute to an improved comprehension of the molecular underlying mechanisms of AML, and the knowledge of all of this may contribute to future studies aimed at improving AML diagnosis, risk stratification, and therapeutic development. Our findings indicated that the expression levels of eRNA-7A and eRNA-22A are elevated in AML samples as compared to control samples. These eRNAs and their co-expressed genes represent promising candidates for future biomarker evaluation in AML. Further validation in larger independent cohorts, alongside comprehensive clinical and experimental investigations, will be required to determine their diagnostic and prognostic value and shed light on the underlying molecular mechanisms of AML.

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All raw data and annotation resources used in this study are publicly available. RNA-seq datasets are deposited in the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra/) under accession numbers PRJNA576867 and PRJNA386992. The reference genome (hg38) and GTF annotation were obtained from Ensemble database, and enhancer annotations were retrieved from the FANTOM5 project. These publicly accessible resources ensure full reproducibility of the analyses. The processed data and analysis code are available from the corresponding author upon reasonable request

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