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Original Article

Prediction of gene signatures for diagnosing concurrent of acute myeloid leukemia and chronic lymphoblastic leukemia

Wen-Wei Xu[1], Cui-Zhen Yang[2], Jing Wang[3]

[1]Department of Hematology, Qianfoshan Hospital of Shandong Province, Jinan 250100, China; [2]Departments of Clinical Laboratory, [3]Nephrology, The Second Affiliated Hospital of Mudanjiang Medical College, Mudanjiang 157011, China

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Abstract: Acute myeloid leukemia (AML) was found concurrent with a diagnosis of chronic lymphoblastic leukemia (CLL). The aim of this study is to predict pathologic or diagnostic markers involved in concurrent AML and CLL. In order to improve the stability, two methods that identified differentially expressed genes (DEGs) were implemented in our study, one detected gene differential expressions in CLL and AML respectively, one screened integrated DEGs using RankProd method. The common DEGs were used to perform network analysis and functional analysis to identify key genes and pathways that might be related to concurrent AML and CLL. Last, reverse transcription polymerase chain reaction (RT-PCR) was implemented to verify several key genes in AML and CLL patients. In this study, a total of 101 common DEGs were identified in CLL and AML. Network analysis showed 5 hub genes and one transcription factor (TF) *CEBPB* in the final network, and these hub genes were all regulated directly by *CEBPB*. Functional analysis showed that the common DEGs were mainly involved in chemokine signaling pathway (P = 1.93E-04), leukocyte transendothelial migration (P = 3.60E-03) and hematopoietic cell lineage (P = 6.70E-03). Additionally, RT-PCR verified that the key genes, such as CEBPB, MAP3K3, LEF1, FRY, were all significantly differentially expressed in both AML and CLL patients. Our results predicted several underlying biomarkers for interpreting the pathogenesis of concurrent AML and CLL.

Keywords: Acute myeloid leukemia, chronic lymphoblastic leukemia, co-expression network, transcription factor, hub genes

Introduction

Leukemia is a malignant cancer of the blood-forming organs and is characterized by distorted proliferation and development of leukocytes and their precursors [1]. Chronic lymphoblastic leukemia (CLL) and acute myeloid leukemia (AML) are two of the most common types of leukemia in adults [2]. CLL is considered as a homogeneous disease of immature, immune-incompetent, minimally self-renewing B cells, which accumulates relentlessly through a faulty apoptotic mechanism [3]. AML is resulted from clonal transformation of hematopoietic precursors through the acquisition of chromosomal rearrangements and multiple gene mutations [4]. To date, previous studies have reported the importance role of transcriptome on diagnosis and treatment of AML and CLL respectively. It was pointed out that the use of gene expression profiling could improve the molecular classification of adult AML [5]. Also, it was reported that CLL can be characterized by a common gene expression "signature" using genomic-scale gene expression profiling [6]. Moreover, the phenotype of CLL can be recognized by analyzing their gene expression profiles [7].

Despite reported less at present, AML was found concurrent with CLL [8, 9]. Zhang et al. [9] identified the cytogenetic changes of a patient with concurrent AML and CLL by array-based comparative genomic hybridization (aCGH) combined with a cytogenetic analysis, and revealed the novel cytogenetic changes of t (2; 5; 11) and t (5; 11) as well as a deletion of TP53

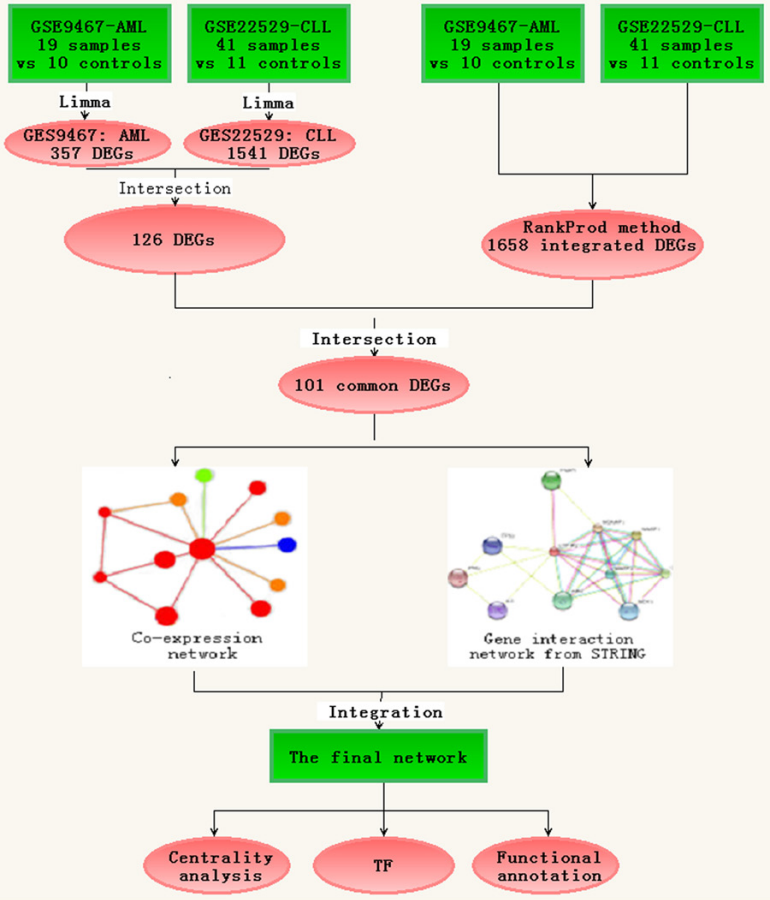

Figure 1. The flowchart showing a brief overview of the main experimental protocol.

and D13S319 simultaneously. DeFilipp et al. [8] also presented a case of AML with del (7q) occurring in a patient with a 4-year history of CLL. Recently, the number of cases with concurrent AML and CLL has increased as a result of the application of new technology, however, insights into treatment and prognosis in cases of this mixed leukemia is limited.

The current study attempted to identify the differences and correlation between AML and CLL, and improve the early diagnosis and treatment. In this study, first, gene differential expressions were investigated in patients with CLL and AML, respectively. The genes differentially expressed in both AML patients and CLL patients were recognize using RankProd package. Integrating differentially expressed genes (DEGs) obtained from individual study and RankProd method; the overlapped genes were selected as common genes, which were considered as robust biomarkers. Next, combining gene-gene interactions and co-expressions of these common genes, a robust network was constructed and analyzed. Last, key genes and functional associations involved in AML and CLL were investigated. The experimental protocol of this study was shown in **Figure 1**. This study provided a basis for researchers to reveal the underlying pathogenesis and novel treatments of leukemia.

Methods

Microarray data recruitment and preprocessing

In the current study, the microarray expression profiles of AML and CLL were downloaded from gene expression omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database under the access numbers of GSE9476 [10] and GSE22529 [11], respectively. In GSE22529, there were 104 samples, including 41 CLL samples and 11 age matched controls. In GSE9476, there were 64 samples, including 38 healthy donors and 26 AML patients. In our study, only peripheral blood samples were remained, including 19 AML patients and 10 healthy donors.

Identification of DEGs

Prior to analysis, the probe-level data in CEL files were converted into expression measures. Then, background correction and normalization were conducted using robust multichip average (RMA) method and quantiles algorithm, respectively. The platform annotation files provided by Affymetrix Company were used to map the relationship between the probes and gene symbols. A probe would be discarded if it could not match any gene symbol.

The primary comparison experiments between tumors (AML and CLL) and normal controls were conducted by linear models for microarray

data (LIMMA) package, respectively. DEGs were identified by assimilating a set of gene-specific t tests under the threshold of false discovery rate (FDR) < 0.01 and $|\log_2 FoldChange| > 2$.

Integrated analysis of DEGs

RankProd was used for integrated analysis of two datasets with different origins. RankProd can be used to determine the significance level for each gene stringently. It also allows for the flexible control of the FDR and family wise error rate in the multiple testing situation of a microarray experiment [12]. We can use this method to know how significant the changes are and how many of the selected genes are likely to be truly differentially expressed [12]. Considering a situation of the microarray experiment with two replicates (A and B), RankProd for a certain gene *g* will be $RP_g = \left(rank_g^{replicateA} / n\right) \times \left(rank_g^{replicateB} / n\right)$, where rank is the position of gene *g* in the list of genes in the replicate A. RP_g can be taken as a *p*-value when all ranks are equally likely, but cannot be used directly to assess the significance of an observed expression change. Therefore, a simple permutation-based estimation procedure is used to determine how likely it is to observe a given RP value or better in a random experiment, thus converting from the RP value to an *E* value [13]. Subsequently, for each gene *g*, a conservative estimate of the percentage of false-positive (pfp) is calculated if this gene is considered as significantly differentially expressed: $q_g = E(RP_g)/rank(g)$. Here, rank (*g*) denotes the position of gene g in a list of all genes sorted by increasing RP value. This method can decide how large a pfp will be accepted and extend the list of accepted genes up to the gene with this q_g value. In this study, a pfp cutoff value of < 0.01 was used.

Identification of co-expression genes

Many genes holding together can accomplish biological functions, and high co-expressed genes take part in similar biological process and pathway. In this study, differentially co-expressed genes and links (DCGL) 2.0 package in R was applied to identify differentially co-expressed genes and links. DCGL 2.0 is a R Package for revealing differential regulation from differential co-expression. It contains four modules: gene filtration module, link filtration module, differential co-expression analysis (DCEA) module and differential regulation analysis (DRA) module. Differential co-expression profile (DCp) and differential co-expression enrichment (DCe) are involved in DCEA module for extracting differentially co-expressed genes (DCGs) and differentially co-expressed links (DCLs). DCp worked on the filtered set of gene co-expression value pairs, where each pair was composed of two co-expression values worked out under two different conditions separately. The subset of co-expression value pairs related with a particular gene, in two groups for the two conditions separately, was written as two vectors X and Y: $X = (x_{i1},x_{i2},...,x_{in})$, $Y = (y_{i1},y_{i2},...,y_{in})$, n is co-expression neighbors for a gene.

We used a length-normalized Euclidean distance to measure differential co-expression (dC) of this gene.

$$dC_n(i) = \sqrt{\frac{(x_{i1} - y_{i1})^2 + (x_{i2} - y_{i2})^2 + ... + (x_{in} - y_{in})^2}{n}}$$

A permutation test was performed to assess the significance of *dC*. In this test, the disease samples and normal controls were randomly permuted, new pearson correlation co-efficient (PCC) was calculated, gene pairs were filtered based on the new PCCs, and new *dC* statistics were calculated. The sample permutation was repeated N times, and a large number of permutation *dC* statistics formed an empirical null distribution. The *p*-value for each gene could then be estimated.

DCe was also used to identify DCGs and DCLs, which based on the 'Limit Fold Change' (LFC) model. First, we divided correlation pairs into 3 parts according to the pairing of signs of co-expression values and the multitude of co-expression values: pairs with same signs (N_1), pairs with different signs (N_2) and pairs with differently-signed high co-expression values (N_3). The first two parts were processed with the 'LFC' model separately to produce two subsets of DCLs (K_1, K_2), while the third part (N_3) added to the set of DCLs directly. Therefore, a total of $K = N_3+K_1+K_2$ DCLs were determined from a total of *N* gene links. For a gene (g_i), the total number of links (n_i) and DCLs in particular (k_i) associated with it were counted. Binomial probability model was used to estimate the significance of the gene being a DCG.

$$p(g_i) = \sum_{x=k_i}^{n_i} c_{n_i}^{x} \left(\frac{K}{N}\right)^x \left(1 - \frac{K}{N}\right)^{n_i - x}$$

We implemented differentially co-expression summarization (DCsum) to combine the results from DCp and DCe methods.

Identification of transcription factors (TFs)

Regulatory impact factor (RIF) metric was used to identify critical TFs from gene expression data. RIF can combine the change in co-expression between the TFs and the DEGs. It can simultaneously integrate three sources of information (the extent of differential expression; the abundance of DEGs; and differential co-expression between TF and its differentially expressed target genes) into a single measure to evaluate which TFs are consistently most differentially co-expressed with the highly ample and highly DEGs [14]. The expression for RIF was shown as:

$$RIF(TF_i) = \frac{1}{n_{de}} \sum_{j=1}^{j=n_{de}} \left[(e1_j \times r_{ij})^2 - (e2_j \times r2_{ij})^2 \right]$$

Where n_{de} is the number of DEGs; *e1 (e2)* is the expression value of DEGj in condition 1 (condition 2); $r1_{ij}$ ($r2_{ij}$) is the correlation of *TFi* and DEGj in condition 1 (condition 2).

Network construction and analysis

First, the co-expression network was constructed using the integrated DEGs from RankProd method. Then common genes were mapped on the general network. However, this network might be imprecise because of the small sample size in our study. The search tool for the retrieval of interacting genes (STRING) database is a public resource of known and predicted gene direct and indirect interactions, which is used widely. So, in order to improve the accuracy of the network, we combined our result with the known gene interaction data from STRING database. Then, the final network was obtained by combining co-expression network we constructed and gene interaction network from STRING database. In the network, genes with high degree (highly connected) were considered as hub genes, which were interacted with several other genes, suggesting a central regulatory role. Based on the distribution of degree values, all common DEGs were permuted in descending order. In our study, the top 5% genes were considered as hub genes.

Functional and pathway enrichment analysis

The gene ontology (GO) functional enrichment and kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis were performed using the online tool DAVID [15]. DAVID bioinformatics resources could systematically extract biological meaning from a large number of genes or proteins. GO terms and KEGG pathways with *P* value less than 0.05 were considered as significant enrichment based on expression analysis systematic explorer (EASE) test applied in the DAVID [16].

Reverse transcription polymerase chain reaction (RT-PCR) validation

To verify the key genes, we obtained 5 peripheral blood samples from AML and CLL patients, respectively, in Qianfoshan Hospital of Shandong Province between October 2015 and March 2016. Normal samples were obtained from health people undergoing physical examination. The ethical clearance was approved by the Institutional Ethical Committee. Written informed consents were obtained from patients who agreed to participate in this study.

We firstly isolated total RNA from peripheral blood samples using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Then, cDNA was synthesized using AMV reverse transcriptase and oligo (dT18) primers (Invitrogen). Amplification of the reverse transcribed RNA was performed in a reaction system of 4 mM dNTPs, 2.5 U/μg Taq DNA Polymerase High Fidelity (Invitrogen), 0.5 μM of each forward and reverse primer. The cDNA from each sample was used as template, with β-actin as a reference. Each reaction was performed in three replicate samples. RT-PCR products were analyzed by 1.5% agarose gels electrophoresis and imaged by Quantity One software (Bio-Rad, Hercules, CA). The statistical analysis was carried out by SPSS 19.0 (SPSS, Chicago, IL). The Student's t-test was performed to determine the statistical significance of differences between groups. A difference with $P < 0.05$ was considered statistically significant.

Results

Identification of DEGs

Under the criterion of FDR < 0.01 and $|\log_2 \text{FoldChange}|$, a total of 357 DEGs in AML and 1541

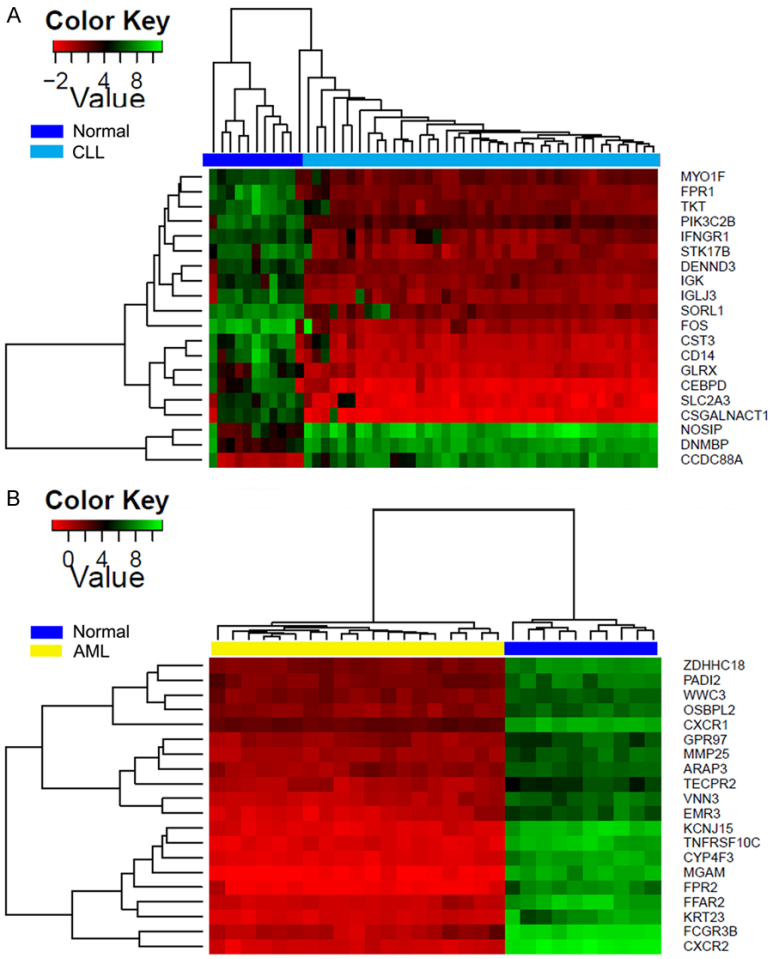

Figure 2. Hierarchical clustering of the top 20 differentially expressed genes. A: Chronic lymphoblastic leukemia; B: Acute myeloid leukemia.

DEGs in CLL were identified, respectively. Among the two groups of DEGs, 126 DEGs were the same. By RankProd package, a total of 1658 integrated DEGs, including 837 up-regulated genes and 821 down-regulated genes, were identified. Combining the 126 DEGs from two individual studies and 1658 integrated DEGs from RankProd method, a total of 101 DEGs were common DEGs, which might be more credible indicator.

In the present study, we also performed a hierarchical clustering analysis to assess the classification performance for samples using top 20 DEGs in CLL and AML, respectively. The clustering effects were shown in **Figure 2**. It could be easily found that the samples could be classified into two major clusters in both CLL and AML. In CLL study, the top 20 DEGs could separate CLL patients from normal subjects with an accuracy of 98% (**Figure 2A**). While the top 20 DEGs of AML could completely separate AML patients from normal cases with the accuracy of 100% (**Figure 2B**). Generally, the top 20 DEGs showed a good classification efficiency, indicating that an applicable method was implemented in this study.

Network construction of common DEGs

First, based on the integrated DEGs from RankProd method, a total of 417 DCGs and 65,535 DCLs were summarized by DCsum based on DCp and DCe results. Also we obtained 28 critical TFs using RIF. The 101 common DEGs were mapped on the general network to form a co-expression network of common DEGs, which was shown in **Figure 3A**. In the co-expression network of common DEGs, there was only one TF, i.e. *CEBPB*. Next, we uploaded the common genes to the STRING database to generate a gene-gene interaction network (**Figure 3B**). Then, the final network of common DEGs was obtained by combining co-expression network we constructed and gene interaction network from STRING database (**Figure 4A**).

In the final network, the top 5% genes were considered as hub genes. In this work, a total of 5 genes in the network were selected as hub genes, which might play important roles in the biological processes of AML and CLL. The gene *MAP3K3* showed the highest degree (degree = 69) in the network, followed by *LEF1* (degree = 68), *FRY* (degree = 67), *CHST15* (degree = 66), and *PTPN12* (degree = 66). Interestingly, all these 5 hub genes directly interacted with the only TF *CEBPB* in the network (**Figure 4B**).

Functional enrichment analysis

To annotate the 101 common DEGs, GO functional analysis and KEGG pathway analysis

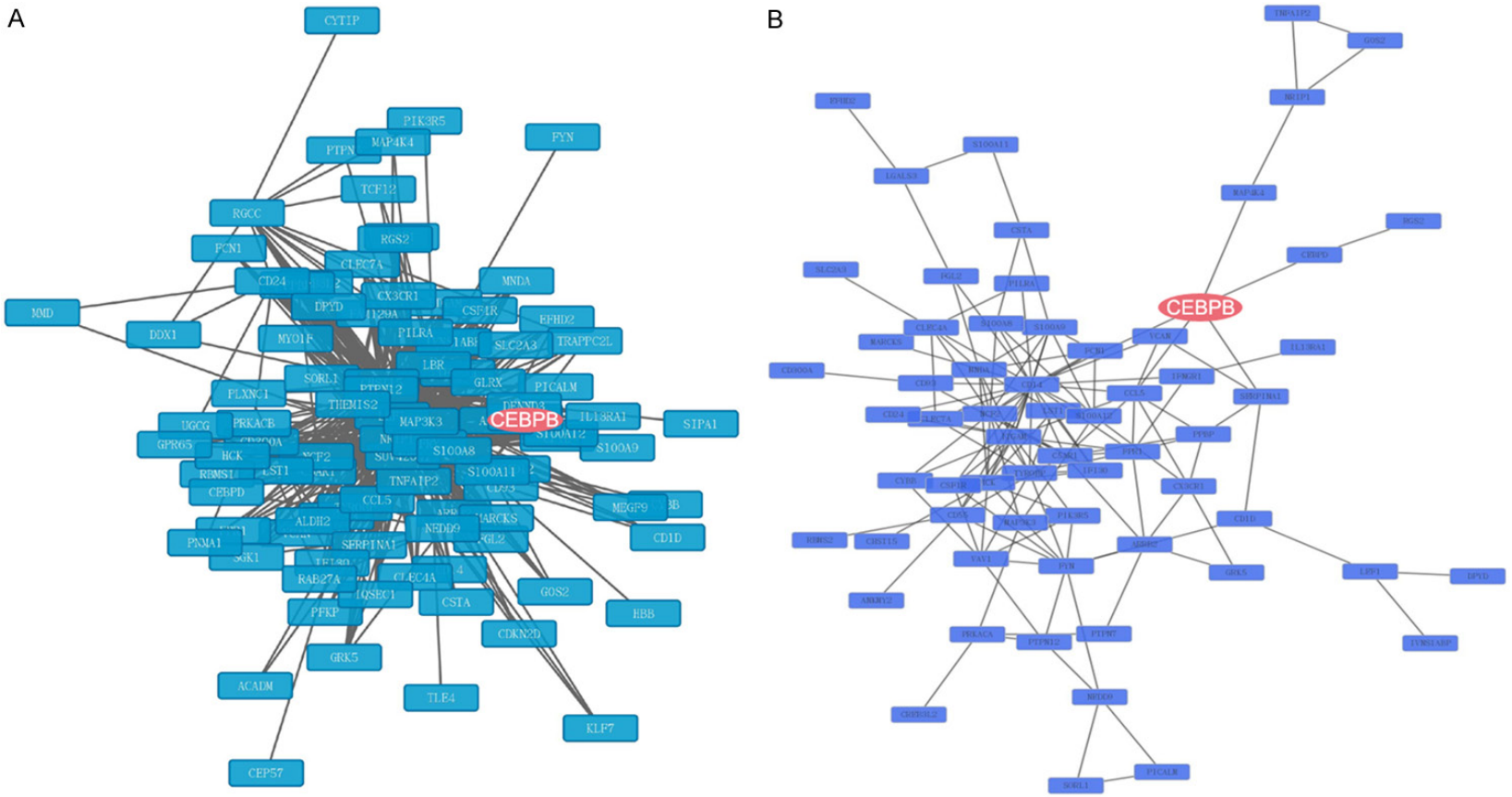

Figure 3. Co-expression network and gene-gene interaction network of common differentially expressed genes. A: Co-expression network constructed by differentially co-expressed genes and links package; B: Gene-gene interaction network based on the search tool for the retrieval of interacting genes database. The red node represents the transcription factor.

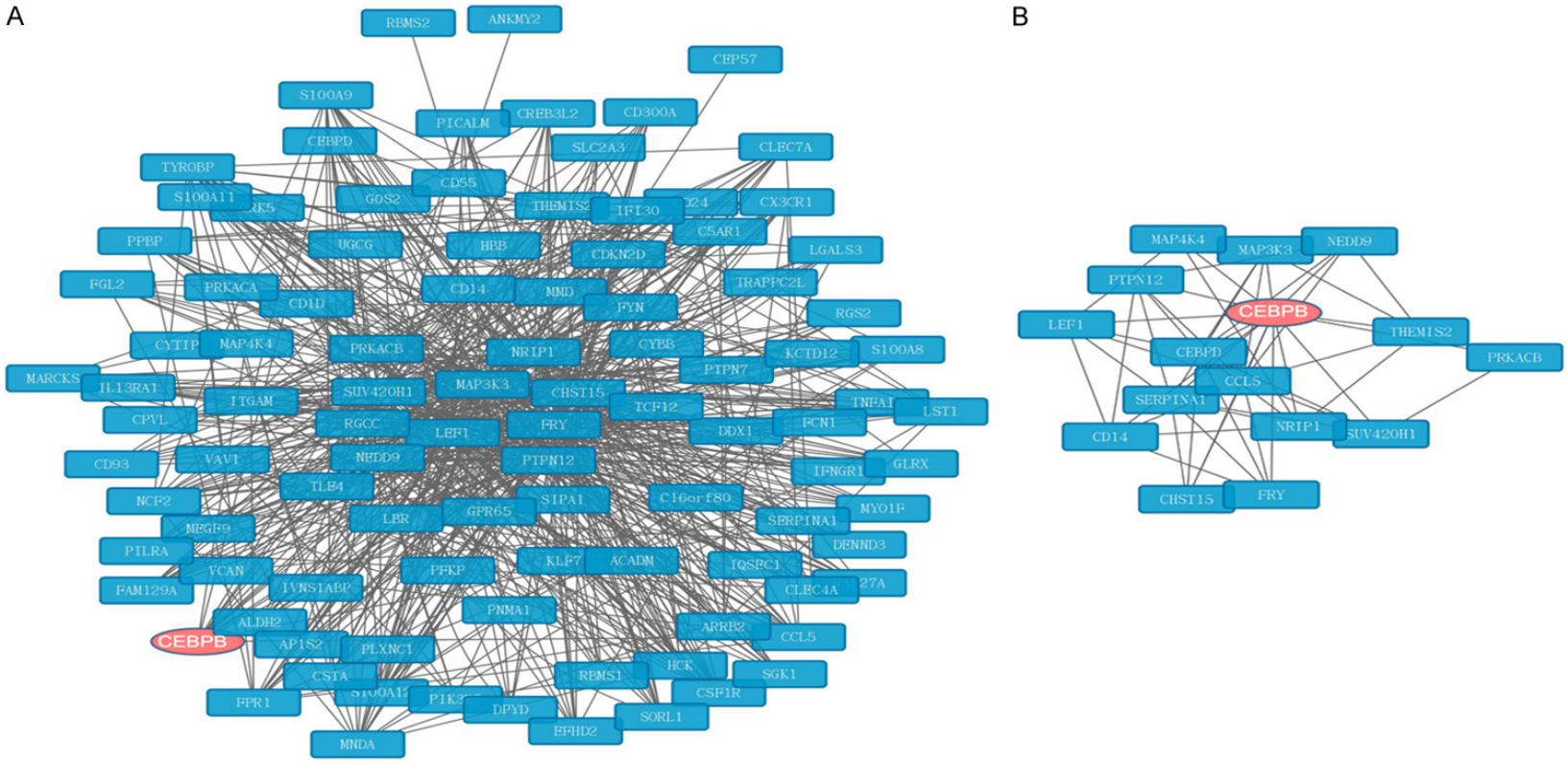

Figure 4. The final network obtained by combining the co-expression network and gene-gene interaction network. A: The final network; B: The regulatory network of transcription factor CEBPB.

Table 1. The significantly enriched pathways of common differentially expressed genes

KEGG ID	Term	*P* value	Genes
hsa04062	Chemokine signaling pathway	1.93E-04	ARRB2, PPBP, HCK, CX3CR1, PIK3R5, GRK5, PRKACB, CCL5, VAV1
hsa04670	Leukocyte transendothelial migration	3.60E-03	CYBB, NCF2, SIPA1, PIK3R5, VAV1, ITGAM
hsa04640	Hematopoietic cell lineage	6.70E-03	CD55, ITGAM, CD14, CSF1R, CD1D
hsa00410	beta-Alanine metabolism	1.58E-02	ACADM, ALDH2, DPYD
hsa04650	Natural killer cell mediated cytotoxicity	2.91E-02	FYN, PIK3R5, VAV1, IFNGR1, TYROBP
hsa05020	Prion diseases	3.79E-02	FYN, PRKACB, CCL5

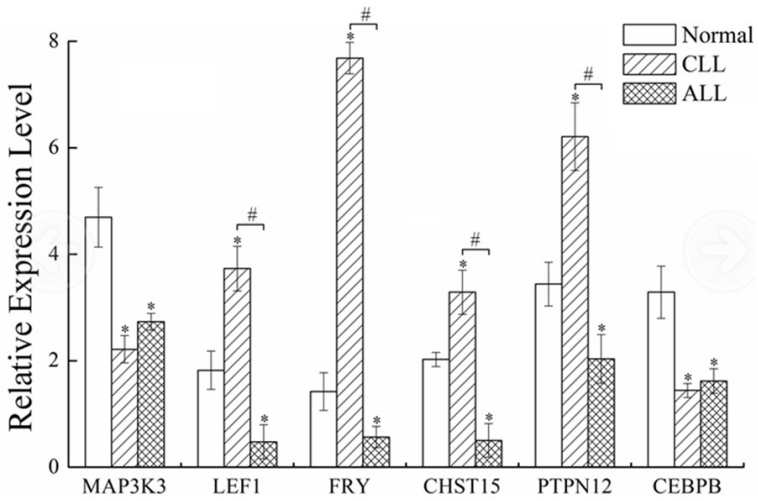

Figure 5. Relative expressions of key genes in acute myeloid leukemia and chronic lymphoblastic leukemia. *P < 0.05, relative to normal; #P < 0.05, acute myeloid leukemia *vs* chronic lymphoblastic leukemia.

were performed using DAVID. By GO enrichment analysis, we can concluded that these genes significantly participated in defense response (P = 1.77E-9), immune response (P = 7.02E-8) and membrane invagination (P = 1.57E-6). Pathway analysis based on KEGG database showed that these genes significantly enriched in 6 terms (**Table 1**). The most significant terms were chemokine signaling pathway (P = 1.93E-04), leukocyte transendothelial migration (P = 3.60E-03) and hematopoietic cell lineage (P = 6.70E-03).

RT-PCR validation

In the present study, the key genes (*MAP3K3*, *LEF1*, *FRY*, *CHST15*, *PTPN12*, and *CEBPB*) were screened to perform the validation experiment by RT-PCR. We found that all of them were significantly differentially expressed in both AML and CLL patients relative to normal subjects (P < 0.05) (**Figure 5**). However, it could be easily found that the relative expression values of several genes were various. Two genes (*MAP3K3* and *CEBPB*) showed coincident expression between AML and CLL. While, the other four key genes present inverse trend, all of them showed higher expression in CLL than that in AML (P < 0.05). It could be proposed that the differential expression of these key genes and the dys-regulation among them might the underlying pathogenesis of AML and CLL.

Discussion

Although there were many studies conducted to find molecular mechanism of AML and CLL [2, 5, 17], few reported the biological processes and cytogenetic changes that occurred with concurrent AML and CLL. In this study, RankProd method, TFs, functional analysis, and network analysis were implemented to provide clues for discovering the underlying mechanism of concurrent AML and CLL.

In the present study, RIF was used to identify critical TFs. RIF is stable and can integrate three sources of information into a single measure simultaneously. Reverter et al [14] showed that RIF had universal applicability, and was advocated as a promising hypotheses generating tool for the systematic identification of novel TF not yet documented as critical. In our analysis, 28 TFs were identified. Among them, only *CEBPB* (CCAAT/enhancer-binding protein beta) was mapped on the final network. *CEBPB* was a basic leucine zipper domain (bZIP) TF that could bind as a homodimer to certain DNA regulatory regions. Recently, a large amount of *CEBPB* target genes had been confirmed by previous studies, such as *TNF-α* [18], *IL-6* [19], and *CREB1* [20]. Many studies had showed its important roles in leukemia [21-23]. Watanabe-

Okochi et al. [24] reported that *CEBPB* could collaborate with ecotropic viral integration site 1 (Evi1), which was one of the master regulators in the development of AML, to promote leukemogenesis using a Evi1-related leukemia mouse model. Moreover, *CEBPB* directly interacted with all 5 hub genes in the network (**Figure 4B**), suggesting the crucial role in regulation of AML and CLL.

By network analysis, 5 genes were considered as hub genes. Thereinto, *MAP3K3* (Mitogen-activated protein kinase kinase kinase 3) showed the highest degree (degree = 69) in the network. It was reported that *MAP3K3* was dysregulated in some human tumors, such as esophageal squamous cell carcinoma [25] and breast cancer [26]. *MAP3K3* was a key mediator of T cell receptor signals, and played an important role in regulating helper T cell differentiation [27]. *LEF1* (Lymphoid enhancer binding factor 1) also showed high degree in the network (degree = 68). Previous studies had shown the clinical significance of *LEF1* expression in AML [28, 29] and CLL [11, 30]. In this work, the other 3 hub genes were few reported about the relationship with AML and CLL. While, all of them were interacted with *MAP3K3* and *CEBPB*, indicated the underlying correlation with AML and CLL. The differential expression of these hub genes might contribute the uncontrollable cellular process in the development of leukemia, and deeper research should focus on them.

Pathway analysis revealed that the common genes were significantly enriched in 6 biological pathways, including chemokine signaling pathway, leukocyte transendothelial migration and hematopoietic cell lineage. Most of them have been reported the correlations with the development and progression of leukemia. Several reports suggested that they might play essential roles in the pathogenesis of AML and CLL [31-33]. The results also suggested the applicability of our strategy.

Conclusion

Since AML and CLL were concurrent in some cases, and originated from the same clone with similar clinical characterization. Many researches were performed to find diagnostic biomarkers and hopeful candidates of drug targets for this mixed leukemia. We used systems biology approach to understand how signaling networks and co-expressed genes were involved in concurrent AML and CLL. This might help us to predict key aspects of the pathogenesis in concurrent AML and CLL. In conclusion, this study would help us to understand the pathogenesis of concurrent AML and CLL, which might be useful for the diagnosis and treatment of concurrent AML and CLL.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Cui-Zhen Yang, Department of Clinical Laboratory, The Second Affiliated Hospital of Mudanjiang Medical College, No. 15, East Xiaoyun Street, Aimin District, Mudanjiang 157011, China. Tel: +86-13945380666; E-mail: cuizheny157011@yeah.net

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