Integrated analysis of microRNA and transcription factors in the bone marrow of patients with acute monocytic leukemia

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Abstract. Acutemonocytic leukemia (AMoL) is a distinct subtype of acute myeloid leukemia (AML) with poor prognosis. However, the molecular mechanisms and key regulators involved in the global regulation of gene expression levels in AMoL are poorly understood. In order to elucidate the role of microRNAs (miRNAs/miRs) and transcription factors (TFs) in AMoL pathogenesis at the network level, miRNA and TF expression level profiles were systematically analyzed by miRNA sequencing and TF array, respectively; this identified 285 differentially expressed miRNAs and 139 differentially expressed TFs in AMoL samples compared with controls. By combining expression level profile data and bioinformatics tools available for predicting TF and miRNA targets, a comprehensive AMoL-specific miRNA-TF-mediated regulatory network was constructed. A total of 26 miRNAs and 23 TFs were identified as hub nodes in the network. Among these hubs, miR-29b-3p, *MYC*, *TP53* and *NFKB1* were determined to be potential AMoL regulators, and were subsequently extracted to construct sub-networks. A hypothetical pathway model was also proposed for miR-29b-3p to reveal the potential co-regulatory mechanisms of miR-29b-3p, *MYC*, *TP53* and *NFKB1* in AMoL. The present study provided an effective approach to discover critical regulators via a comprehensive regulatory network in AMoL, in addition to enhancing understanding of the pathogenesis of this disease at the molecular level.

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Abbreviations: AMoL, acute monocytic leukemia; AML, acute myeloid leukemia; miRNA/miR, microRNA; TF, transcription factor; TFBS, transcription factor binding site; FBL, feedback loop; FFL, feed-forward loop; GO, Gene Oncology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, Database for Annotation, Visualization and Integrated Discovery; T-ALL, T-cell acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia

Key words: acute monocytic leukemia, microRNA, transcription factor, sequencing, array

Introduction

Acute monocytic leukemia (AMoL) is a distinct subtype of acute myeloid leukemia (AML) characterized by the uncontrolled proliferation of immature cells of myelo-monocytic origin (monoblasts, promonocytes or monocytes) within the bone marrow and peripheral blood; AMoL accounts for 5-10% of adult AML cases (1-3). Compared with other AML subtypes, such as M2, M3 and M4, AMoL is discriminated by its high leukocyte count, a tendency to infiltrate extramedullary sites and association with intravascular coagulation (1,3,4). It also differs from other AMoL subtypes in karyotype, genetics and immunophenotype (1). Patients with AMoL have been reported to have a worse prognosis compared with other AML subtypes, which is associated with hyperleukocytosis and extramedullary involvement (4,5). The 3-year overall survival rate and disease-free survival rate are reported to be only 31 and 26%, respectively (4). Consequently, there is an urgent need to discover novel therapeutic targets for AMoL. Further characterization of the critical factors and molecular mechanisms of AMoL may facilitate the development of new therapies. At present, different genetic mutations and chromosomal translocations have been demonstrated to serve prominent roles in the pathogenesis of AMoL, which include mutations in the genes encoding FMS-like tyrosine kinase 3, NRAS, nucleophosmin 1 and DNA methyltransferase 3A, and translocations in the gene encoding MLL on chromosome 11q23 (3-5). However, insight into the molecular characteristics of this disease remains limited (4). Specifically, the regulatory networks of AMoL gene expression level shave yet to be elucidated.

As two of the best-characterized regulators of gene expression levels, microRNAs (miRNAs/miRs) induce mRNA degradation or inhibit translation by binding the 3'-untranslated regions (3'-UTRs) of target mRNAs, whereas transcription factors (TFs) regulate gene transcription by

binding to functional TF binding sites (TFBSs) located within the promoter region of genes (6,7). It has been confirmed that TF-mediated transcriptional regulation and miRNA-mediated post-transcriptional regulation are tightly coupled, which implies that they share a common regulatory mechanism (7,8). miRNAs and TFs are capable of mutually regulating each other in the form of feedback loops (FBLs) or cooperatively regulating the same target gene in a combinatorial manner to form feed-forward loops (FFLs) (7,9). Previous studies have indicated that aberrations involving key TFs and miRNAs essential for the regulation of gene expression levels are major driving forces of leukemia pathogenesis (10-12). It has been reported that the TF STAT5 can upregulate miR-21 expression levels by directly binding the promoter region of the miR-21 locus, contributing to the subsequent downregulation of the miR-21 target programmed cell death protein 4in the AMoL cell line MOLM-13, which was found to participate in the antileukemic response induced by the tyrosine kinase inhibitor imatinib (10). miR-182 and the TF CCAAT enhancer binding protein α (CEBPA) regulate each other to form a negative FBL during the control of granulopoiesis progression, whereas disrupting this balance blocks granulocytic differentiation and is directly associated with AML initiation (11). miR-1246/1248 combined with the TFs WT1/SOX4/REL and their target gene Notch2 form regulatory modules that regulate T-cell acute lymphoblastic leukemia (T-ALL) cell proliferation (12). Nevertheless, the overall regulation of gene expression levels mediated by miRNAs and TFs in AMoL is still unclear. Therefore, integrated analysis of miRNAs and TFs specific for AMoL at the network level is critical.

The present study integrated miRNA sequencing and TF array technology, supported by bioinformatics analysis, to construct an AMoL-associated miRNA and TF regulatory network. By analyzing the network topology, hub miRNAs and TFs were identified from the network and their regulatory properties were investigated using a sub-network. These results may advance understanding of the complicated regulatory mechanisms underlying AMoL and may indicate potential biomarkers for AMoL in the future.

Materials and methods

Collection and storage of samples. Bone marrow samples were obtained from 10 patients with unexplained anemia or fever (six males, four females; age range, 10-73 years; mean age, 39.0±21.2 years) as controls, as well as from 10 patients with newly diagnosed and untreated AMoL (six males, four females; age range, 14-64 years; average age, 41.2±14.7 years) enrolled in the Department of Hematology of the Affiliated Hospital of Guangdong Medical University (Zhanjiang, China). Diagnosis of AMoL was confirmed according to the French-American-British criteria (1), whereas control patients had confirmed normal bone marrow morphology in bone marrow aspirates. The fresh bone marrow samples were frozen and stored in liquid nitrogen until use for RNA or nuclear protein extraction. All patients or their guardians provided written informed consent for voluntary participation in accordance with the Declaration of Helsinki, and ethical approval was granted by the ethics committee of the Affiliated Hospital of Guangdong Medical University (approval no. PJ2016081KT) (Zhanjiang, China).

RNA isolation. Total RNA was extracted from bone marrow samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and an RNeasy kit (Qiagen GmbH) with a DNase digestion step, according to the manufacturer's instructions. The purity and concentration of RNA was measured with a NanoDrop™ ND-1000 instrument (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

High-throughput miRNA sequencing and data analysis. Small RNA libraries were constructed using the NEBNext Multiplex Small RNA Prep Set for Illumina® Set 1 kit (cat. no. E7300S, New England BioLabs, Inc.), according to the manufacturer's instructions. Briefly, total RNA samples were ligated with 3' and 5' RNA adapters, reverse transcribed into cDNA (protocol was 50˚C for 60 min) and amplified by PCR (the thermocycling conditions were 94˚C for 30 sec, followed by 15 cycles at 94˚C for 15 sec, 62˚C for 30 sec and 70˚C for 15 sec). Subsequently, 130-155-bp PCR products (corresponding to 15-35-bp small RNAs) were separated and purified from 6% polyacrylamide gels. The quantity and length distribution of the sequencing library was determined by Agilent 2100 Bioanalyzer instrument (Agilent Technologies, Inc.) using an Agilent DNA 1000 chip kit (cat. no. 5067-1504; Agilent Technologies, Inc.) according to the manufacturer's instructions. Following denaturation with NaOH, the sequencing libraries were diluted to a final concentration of 8 pM and loaded on a cBot instrument to generate a clustered flowcell utilizing the TruSeq Rapid SR cluster kit (cat. no. GD-402-4001, both Illumina, Inc.). Single-end RNA sequencing (50 bp, 5' to 3' direction) of the flowcell was carried out on an IlluminaHiSeq 2000 sequencer using a TruSeq Rapid SBS kit (cat. no. FC-402-4002, Illumina, Inc.) for 36 cycles, following the manufacturer's recommendations. Analysis of sequencing images was performed with Off-Line Basecaller software (version V1.8.0; Illumina, Inc.). After passing through the Solexa CHASTITY quality control filter, the clean reads were trimmed to remove the adapter sequence. The remaining reads of ≥15 nucleotides were aligned to a known reference miRNA precursor in the miRBase database (release 19.0; www.mirbase.org/) using Novoalign software (version v2.07.11; www.novocraft.com/). Normalization of read counts, calculation of the relative expression levels of each miRNA and identification of differentially expressed miRNAs were performed as previously described (13).

TF array detection and data analysis. Nuclear proteins were prepared from bone marrow samples using a Nuclear Extract kit (cat. no. AY2002, Panomics, Inc.; Affymetrix; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. TranSignal Combo Protein/DNA Arrays (Panomics, Inc.; Affymetrix; Thermo Fisher Scientific, Inc.), which enable simultaneous quantitative analysis of 345 important TFs, were used according to the manufacturer's instructions. Briefly, nuclear proteins were incubated with a TF probe mixture containing 345 biotin-labeled DNA binding oligonucleotides (Panomics, Inc.; Affymetrix; Thermo Fisher Scientific, Inc.) for 30 min at 15˚C to form protein-DNA complexes. TF-bound probes were then separated from the non-bound probes using a spin column separation system (Panomics, Inc.; Affymetrix; Thermo Fisher Scientific, Inc.). The bound probes

Table I. miR-specific reverse transcription primers.

miR	Primer sequence (5'→3')
miR-155-5p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACCCCTA
miR-221-3p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGAAACCC
miR-378a-3p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCCTTC
miR-106b-5p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACATCTGCA
miR-142-3p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCCATAAA
miR-424-5p	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTTCAAA
U6 small nuclear RNA	CGCTTCACGAATTTGCGTGTCAT

miR, microRNA.

Table II. Primers for reverse transcription-quantitative PCR of miRs.

miR	Primer (5'→3')	Product length, bp
miR-155-5p	F: GGGGTAATGCTAATCGTGA	66
	R: CAGTGCGTGTCGTGGAG	
miR-221-3p	F: GGGAAGCTACATTGTCTGC	67
	R: CAGTGCGTGTCGTGGAGT	
miR-378a-3p	F: GGGGTCTGGACTTGGAGTCA	64
	R: GTGCGTGTCGTGGAGTCG	
miR-106b-5p	F: GGGGGTAAAGTGCTGACAGT	64
	R: GTGCGTGTCGTGGAGTCG	
miR-142-3p	F: GGGGGTGTAGTGTTTCCTA	68
	R: CAGTGCGTGTCGTGGA	
miR-424-5p	F: GGGCAGCAGCAATTCATGT	63
	R: GTGCGTGTCGTGGAGTCG	
U6 small nuclear RNA	F: GCTTCGGCAGCACATATACTAAAAT	89
	R: CGCTTCACGAATTTGCGTGTCAT	

F, forward; miR, microRNA; R, reverse.

were denatured and hybridized to a TranSignal Combo array membrane containing consensus binding oligonucleotides for TFs at 42˚C overnight. After three washes, membranes were incubated with HRP-conjugated streptavidin at room temperature for 5 min and exposed to ECL-Hyperfilm (Amersham Pharmacia Biotech; Cytiva). Signal intensities were quantified using a GBox Imaging System (Syngene Europe) with ScanAlyze software (version 1.0.3; www.graphics.stanford.edu/software/scanalyze/). The identification of differentially expressed TFs was performed as previously described (13,14).

Reverse transcription-quantitative (RT-q) PCR assay. Total RNA extracted from bone marrow samples was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (cat. no. 18080044, Invitrogen; Thermo Fisher Scientific, Inc.) with a temperature protocol of 65˚C for 5 min, followed by 50˚C for 60 min and 70˚C for 15 min according to the manufacturer's protocol. Stem-loop reverse transcription primers (Table I) and oligodT primers were used for cDNA synthesis of miRNAs and genes encoding TFs, respectively. qPCR assays were performed on cDNA samples using SYBR Green PCR Master Mix on an ABI Prism 7500 Real-time PCR System (both Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer sequences are listed in Tables II and III. Thermocycling parameters were 95˚C for 10 min, then 40 cycles of 95˚C for 10 sec and 60˚C for 1 min. The specificity of amplified products was evaluated via melt curve analysis following PCR amplification. U6 small nuclear RNA and 18S ribosomal RNA were selected as internal controls to normalize qPCR data for miRNA and TF-encoding gene expression levels, respectively. Fold-changes in expression levels were determined according to the $2^{-\Delta\Delta Cq}$ method (15).

Integrative analysis of miRNA and TF expression profiles

miRNA-mediated gene/TF regulation. In order to obtain miRNA-gene and miRNA-TF pairs, miRanda (version v5; www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), PicTar (release 2007; www.pictar.mdc-berlin.de/cgi-bin/PicTar_vertebrate.cgi) and TargetScan (version 6.2; www.targetscan.org/) algorithms were used to predict the target genes of all differentially expressed

Table III. Primers for reverse transcription-quantitative PCR of TFs.

TF	Primer (5'→3')	Product length, bp
MYC	F: ACACATCAGCACAACTACGC	159
	R: CCTCTTGACATTCTCCTCGGT	
NR2F1	F: ATCGAGAGCCTGCAGGAGAA	163
	R: CTACCAAACGGACGAAGAAGAG	
NFIC	F: TGCCACGTCAGACACTTCCT	154
	R: AGTCCTGCTGGTACTGCTTTG	
SRY	F: ATCCCAGAATGCGAAACTCA	180
	R: AATTCTTCGGCAGCATCTTC	
TP53	F: TTCTACAGTTGGGCAGCT	295
	R: GCAGTAAGCCAAGATCAC	
FOXO4	F: ATAGCACCACCTCCAGTCA	150
	R: CATGTCACACTCCAGGTTCTC	
18S rRNA	F: CCTGGATACCGCAGCTAGGA	112
	R: GCGGCGCAATACGAATGCCCC	

F, forward; NFIC, nuclear factor IC; NR2F1, nuclear receptor subfamily 2 group F member 1; R, reverse; rRNA, ribosomal RNA; SRY, sex-determining region Y; TF, transcription factor.

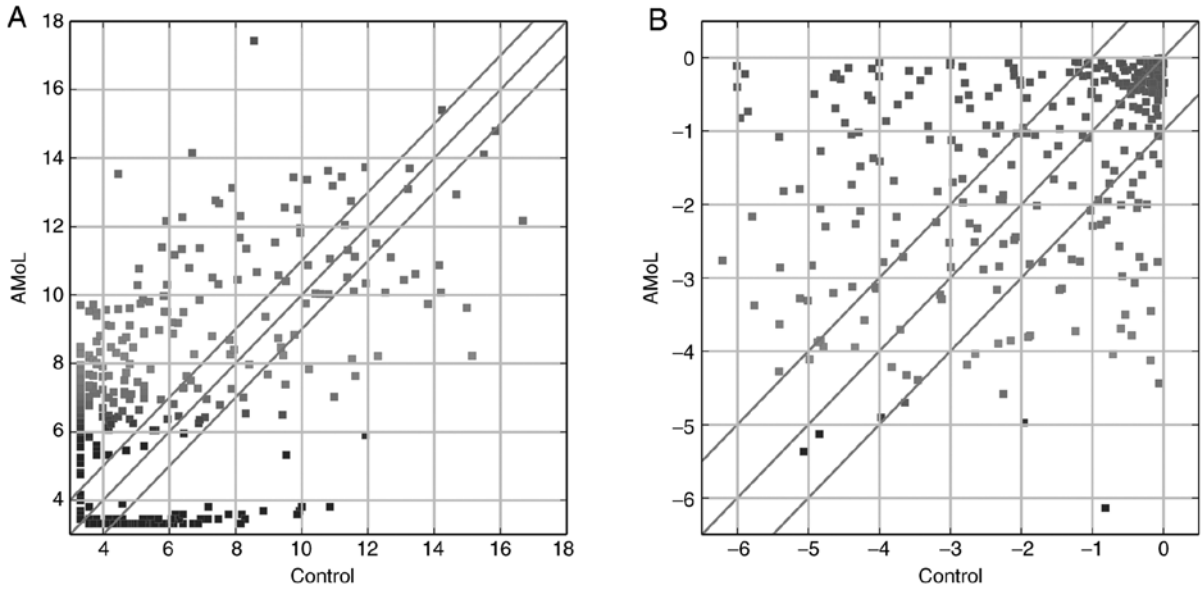

Figure 1. Scatter plot of miRNA and TF expression level profiles in bone marrow samples of patients with AMoL and controls. Expression levels of (A) miRNAs and (B) TFs were plotted on a $\log_2$ scale, normalized to controls. Oblique lines represent the fold-change lines (fold-change threshold value, 2.0). Spots above the top oblique line or below the bottom oblique line show a fold-change ≥2.0. miRNA, microRNA; TF, transcription factor; AMoL, Acute monocytic leukemia.

miRNAs identified by miRNA sequencing (14,16). In order to decrease the probability of false positives, the predicted miRNA-target pairs for further analysis were supported by ≥2 of the aforementioned databases (14,16). The predicted miRNA targets were then merged with the experimentally validated targets, acquired from miRTarBase (version 4.5; www.mirtarbase.mbc.nctu.edu.tw/) (17). After collecting AMoL candidate genes/TFs from the MalaCards database (version 1.08.564; www.malacards.org/) (18), these miRNA targets were overlapped with AMoL candidate genes/TFs to identify miRNA-mediated gene/TF regulatory associations. Finally, the extracted miRNAs and their target pairs were subjected to TFBS analysis.

TF-mediated gene/miRNA regulation. In order to identify the regulatory association between TFs and AMoL candidate genes or miRNAs, TFBS prediction was performed using the TFBS Conserved Track data tables (www.genome.ucsc.edu/cgi-bin/hgTables?hgsid=350051003&hgta_doSchemaDb=hg19&hgta_doSchemaTable=tfbsConsFactors) from the UCSC Genome

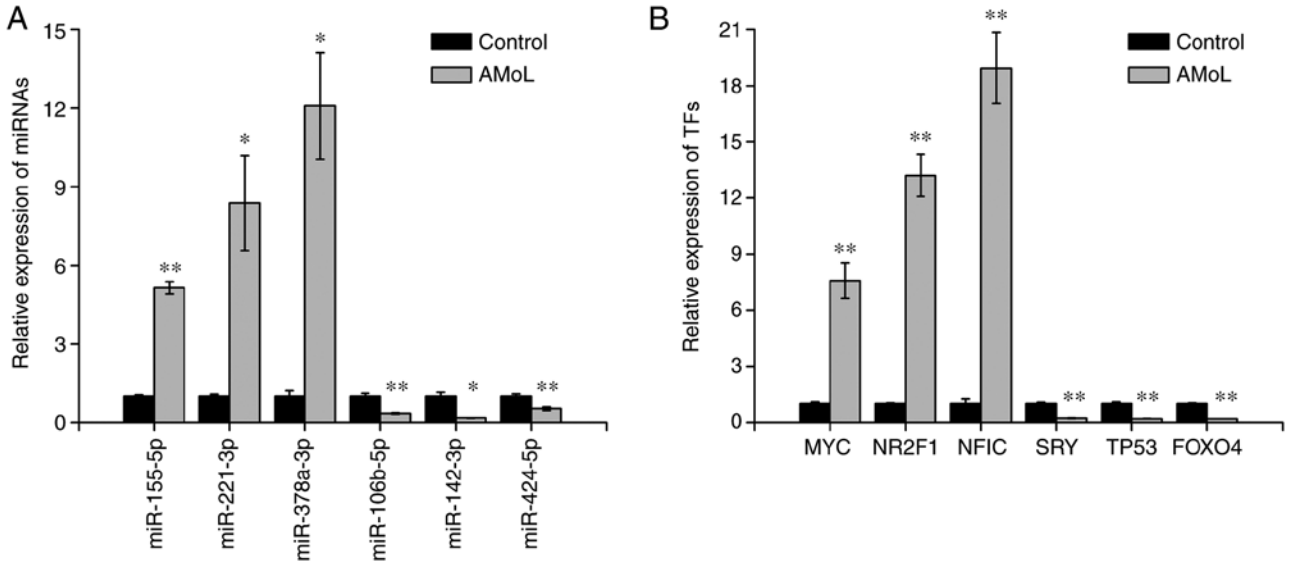

Figure 2. Confirmation of miRNA and TF expression level profiles via quantitative PCR. U6 small nuclear RNA and 18S ribosomal RNA were used to normalize expression levels of (A) miRNAs and (B) TFs, respectively. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 vs. control. Mir/miRNA, microRNA; TF, transcription factor; AMoL, acute monocytic leukemia; NR2F1, nuclear receptor subfamily 2 group F member 1; NFIC, nuclear factor IC; SRY, sex-determining region Y.

Browser, which contain the frequency and location of TFBSs conserved among the human/mouse/rat genome alignments (19). The putative promoter region (-5,000/+1,000 bp around the transcription start site) of AMoL candidate genes and precursor miRNAs were searched for TFBSs as described previously (13). Subsequently, the predicted TFs were overlapped with the differentially expressed TFs identified by TF array analysis to avoid redundancy and form TF-gene and TF-miRNA pairs.

Network construction, network node analysis and sub-network generation. After converging four types of regulatory pairs (TF-gene, TF-miRNA, miRNA-gene and miRNA-TF), an AMoL-associated miRNA and TF regulatory network was constructed. The resulting network was visualized with Gephi software (Release 0.8.1-beta; www.gephi.github.io/). Enriched Gene Ontology (GO; http://geneontology.org/docs/go-citation-policy/) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/kegg1.html) pathways for network nodes were examined using Database for Annotation, Visualization and Integrated Discovery (DAVID; version v6.7; www.david.abcc.ncifcrf.gov/) and were considered statistically significant at P<0.05. In order to assess the overall properties of the network, node degrees were computed based on the number of direct links of the node inside the network. Nodes with >15 total degrees (in-plus out-degrees) were defined as network hubs. The sub-networks were then established by extracting all directly linked nodes connected to the hubs.

Cell culture. The human AMoL cell line THP1, obtained from the American Type Culture Collection, was cultivated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (HyClone; Cytiva), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in 5% CO_2. Cells were routinely tested and had negative results for mycoplasma.

Luciferase reporter assay. For the miR-29b-3p promoter activity assay, miR-29b-3p promoter constructs containing either a wild-type (WT) or mutant (MUT) *MYC*-binding site (MBS) were cloned into pGL3-Basic reporter vectors from Guangzhou Land Unicomed Biotechnology Co., Ltd. In order to assess regulation of the miR-29b-3p promoter by *MYC*, THP1 cells ($4x10^4$ cells/well) were seeded into 24-well plates and co-transfected with 2 μg/ml promoter-luciferase reporter vectors (wild-type or mutant miR-29b-3p promoter vectors) and 2 μg/ml *MYC* overexpression vectors (pcDNA3.1-*MYC*), and the internal reference plasmid phRL-TK (100 ng/ml; Promega Corporation) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific Inc.). After 24 h transfection, a Dual-Luciferase Assay kit (Promega Corporation) was used to measure the luciferase activity according to the manufacturer's instructions. The values were normalized to those of *Renilla* luciferase. Experiments were performed in triplicate.

Statistical analysis. Statistical analysis was performed using SPSS statistical software version 16.0 (SPSS Inc.). Statistical data are presented as mean ± SD of three independent repeats. Differences between two groups were analyzed using Student's t-test. Data were analyzed using two-way ANOVA followed by Bonferroni's test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Differentially expressed miRNAs and TFs in AMoL samples compared with controls. In order to collect AMoL-associated miRNAs and TFs, miRNA deep sequencing and TF array analysis were performed to identify dysregulated miRNAs and TFs in AMoL samples compared with controls. A total of 285 miRNAs that exhibited aberrant expression levels between the groups were detected (Fig. 1A), with 199 up- and 86 downregulated in the AMoL group. The most abundantly

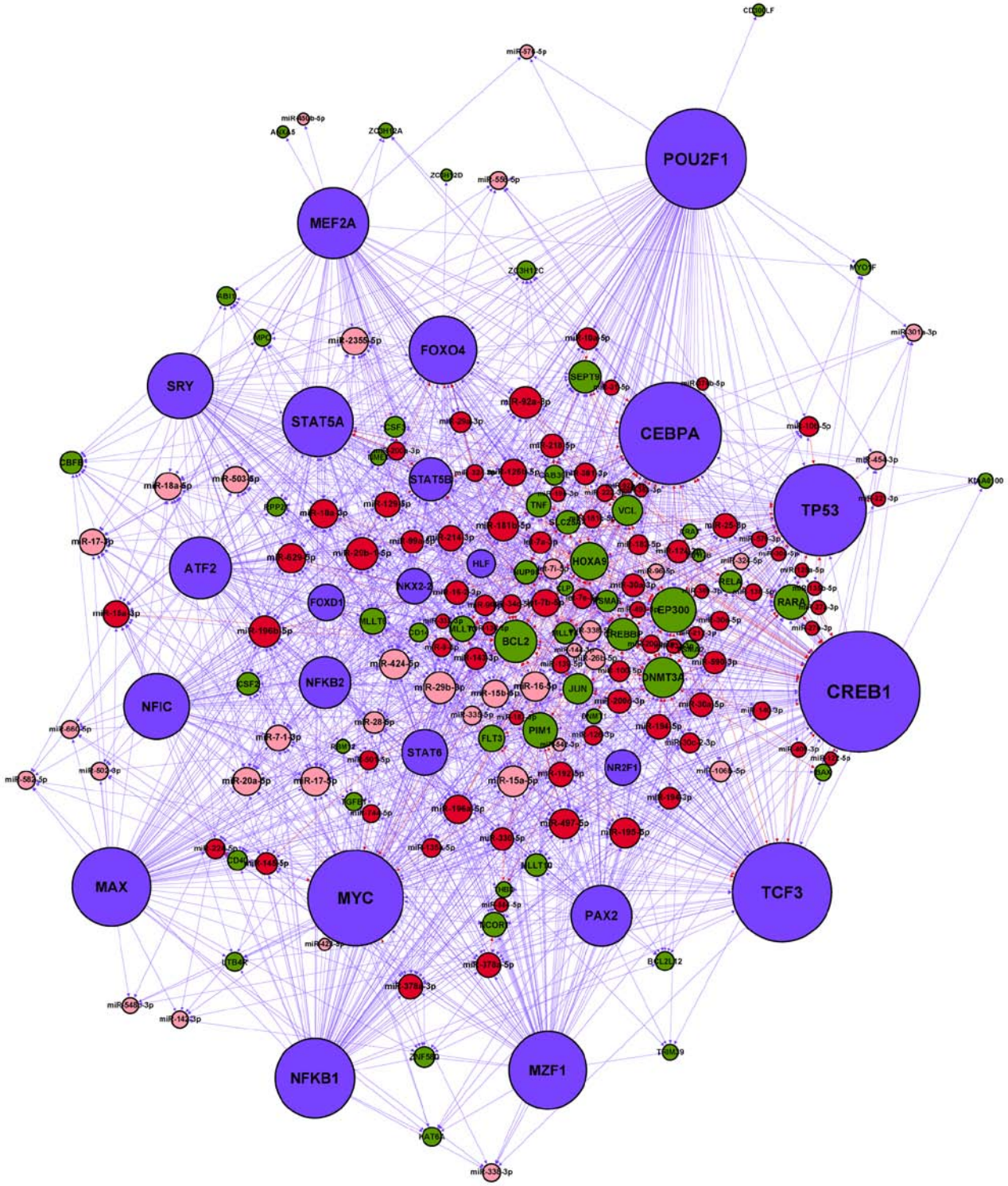

Figure 3. miRNA-TF regulatory network in AMoL. Red node, upregulated AMoL-associated miRNA; pink node, downregulated AMoL-associated miRNA; green node, AMoL candidate gene; blue node, TF. Red arrow, upregulated miRNA-gene; pink arrow, downregulated miRNA-gene; blue arrow, TF-miRNA or TF-gene. miRNA, microRNA; AMoL, acute monocytic leukemia; TF, transcription factor.

upregulated miRNA was miR-122-5p (fold-change, 542.2), whereas miR-941 (fold-change, 131.6) was the most downregulated miRNA. As shown in Fig. 1B, 139 TFs were differentially expressed (90 up- and 49 downregulated) between the control and AMoL samples. Of these TFs, nuclear factor IC *(NFIC;* fold-change, 94.1) was the most upregulated, whereas transcription factor AP4 (fold-change, 1,456.7) exhibited the highest degree of downregulation.

Validation of miRNA and TF expression level profiles via qPCR. In order to validate differentially expressed miRNAs and TFs identified by miRNA sequencing and TF arrays, respectively, six miRNAs and six TFs were selected to further determine their expression levels by qPCR. Similar to the miRNA sequencing results, miR-155-5p (P<0.01), miR-221-3p (P<0.05) and miR-378a-3p (P<0.05) were upregulated, and miR-106b-5p (P<0.01), miR-142-3p (P<0.05) and miR-424-5p

Table IV. Regulatory associations in the acute monocytic leukemia-associated miRNA and TF regulatory network.

Association	Number of pairs	Number of miRNAs	Number of genes	Number of TFs
miRNA-gene[a]	304	100	40	-
miRNA-TF[b]	81	57	-	8
TF-gene[c]	453	-	60	23
TF-miRNA[d]	807	102	-	23

miRNA repression of [a]gene and [b]TF expression levels. TF-mediated regulation of [c]gene and [d]miRNA expression levels. miRNA, microRNA; TF, transcription factor.

Table V. Summary of FFLs and FBLs based on acute monocytic leukemia-associated network data.

Module type	Number of modules	Number of nodes			Number of links			
		Genes	miRNAs	TFs	miRNA-gene	miRNA-TF	TF-gene	TF-miRNA
miRNA-FFL	99	22	38	7	73	54	46	-
TF-FFL	715	29	82	23	176	-	179	461
Composite-FFL	39	12	18	5	33	21	20	21
FBL	29	-	26	7	-	29	-	29

FFL, feed-forward loop; FBL, feedback loop; miRNA, microRNA; TF, transcription factor.

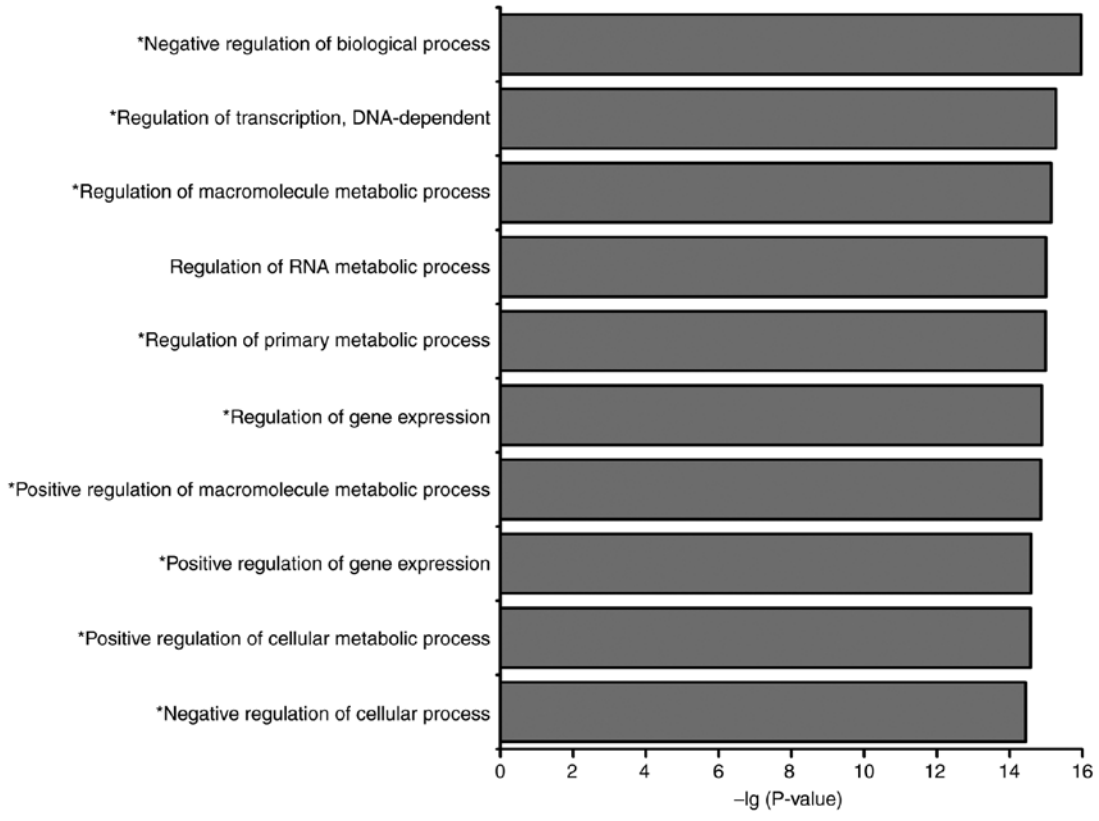

Figure 4. Top ten enriched biological process-associated GO terms for nodes in AMoL microRNA-transcription factor regulatory network. *P<0.05, associated with AMoL in GO. GO, Gene Ontology; AMoL, acute monocytic leukemia.

(P<0.01) were downregulated in AMoL samples compared with controls (Fig. 2A). qPCR analysis also demonstrated that *MYC*, nuclear receptor subfamily 2 group F member 1 gene and *NFIC* were overexpressed in AMoL samples, whereas

Table VI. Pathway analysis for nodes in acute monocytic leukemia-associated microRNA and transcription factor regulatory network.

Pathway ID	Term	Gene count	%	P-value
hsa05200	Pathways in cancer	18	23.68421	1.41×10^{-10}
hsa05221	Acute myeloid leukemia[a]	9	11.84211	1.24×10^{-8}
hsa05220	Chronic myeloid leukemia[a]	8	10.52632	1.72×10^{-6}
hsa04630	Jak-STAT signaling pathway[a]	9	11.84211	2.53×10^{-5}
hsa04010	MAPK signaling pathway[a]	11	14.47368	3.54×10^{-5}
hsa05215	Prostate cancer	7	9.210526	6.67×10^{-5}
hsa05222	Small cell lung cancer	6	7.894737	5.13×10^{-4}
hsa05210	Colorectal cancer	6	7.894737	5.13×10^{-4}
hsa04640	Hematopoietic cell lineage[a]	6	7.894737	5.71×10^{-4}
hsa04210	Apoptosis signaling pathway[a]	6	7.894737	6.03×10^{-4}
hsa04620	Toll-like receptor signaling pathway[a]	6	7.894737	1.19×10^{-3}
hsa05212	Pancreatic cancer	5	6.578947	2.52×10^{-3}
hsa04722	Neurotrophin signaling pathway	6	7.894737	2.95×10^{-3}
hsa04110	Cell cycle[a]	6	7.894737	3.05×10^{-3}
hsa04350	TGF-β signaling pathway[a]	5	6.578947	5.00×10^{-3}
hsa05014	Amyotrophic lateral sclerosis (ALS)	4	5.263158	8.62×10^{-3}
hsa04660	T cell receptor signaling pathway[a]	5	6.578947	1.06×10^{-2}
hsa05016	Huntington's disease	6	7.894737	1.40×10^{-2}
hsa04060	Cytokine-cytokine receptor interaction[a]	7	9.210526	1.73×10^{-2}
hsa05211	Renal cell carcinoma	4	5.263158	1.83×10^{-2}
hsa04520	Adherens junction	4	5.263158	2.35×10^{-2}
hsa04310	Wnt signaling pathway[a]	5	6.578947	3.23×10^{-2}
hsa04012	ErbB signaling pathway[a]	4	5.263158	3.23×10^{-2}

[a]Pathways associated with hematopoiesis or leukemia in Kyoto Encyclopedia of Genes and Genomes.

the expression levels of sex-determining region Y gene, *TP53* and *FOXO4* were decreased (all P<0.01; Fig. 2B), which was consistent with TF array results.

miRNA- and TF-mediated regulatory network in AMoL. Following amalgamation of the four types of regulatory association among TFs, miRNAs and their targets, an miRNA-TF regulatory network for AMoL was constructed (Fig. 3). Table IV shows the numbers of nodes and interaction pairs in the resultant network. Based on the master regulator, FFLs can be classified into three types: miRNA-FFLs, TF-FFLs and composite FFLs (7,9). MiRNA-FFL and TF-FFL can combine into a composite FFL, in which miRNA and TF regulate each other (7). The present study identified 853 FFLs and 29 FBLs in the network (Table V). Among these FFLs, 715 (83.8%) belonged to TF-FFLs, in which TFs serve as a master regulator to control the expression levels of miRNA and target genes, 99 (11.6%) were miRNA-FFLs and 39 (4.5%) belonged to composite-FFLs. Therefore, the TF-FFLs were the dominant motifs in the network.

Functional evaluation of the synergistic regulatory network. Using the DAVID online tool, GO annotation and KEGG pathway analysis were performed for all nodes in the assembled miRNA-TF-based networks. GO analysis identified 10 highly

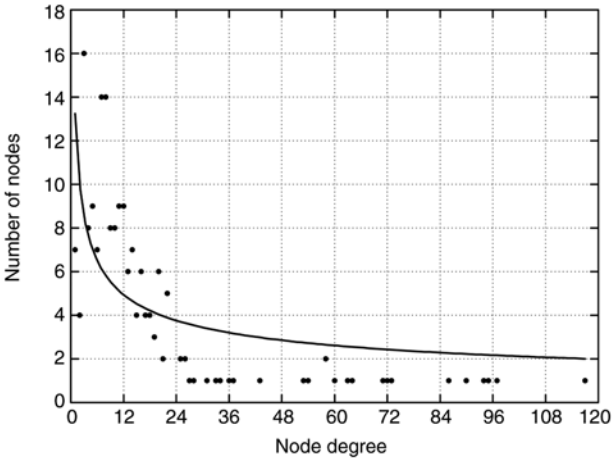

Figure 5. Node degree distribution of the network.

enriched biological process terms, of which nine were associated with the occurrence and development of AMoL (Fig. 4), namely 'negative regulation of biological process', 'regulation of transcription, DNA-dependent', 'regulation of macromolecule metabolic process', 'regulation of primary metabolic process', 'regulation of gene expression', 'positive regulation of macromolecule metabolic process', 'positive regulation of

Table VII. Hub miRs in acute monocytic leukemia-associated miRNA and transcription factor regulatory network.

miRNA	In-degree	Out-degree	Total degree
miR-29b-3p	19	3	22
miR-92a-3p	17	5	22
miR-29b-1-5p	21	1	22
miR-15a-5p	15	6	21
miR-196b-5p	19	2	21
miR-497-5p	16	4	20
miR-195-5p	16	4	20
miR-181b-5p	13	7	20
miR-424-5p	16	4	20
miR-17-5p	16	3	19
miR-16-5p	12	7	19
miR-629-5p	17	2	19
miR-20a-5p	16	2	18
miR-18a-3p	16	2	18
miR-196a-5p	16	2	18
let-7b-5p	8	9	17
miR-15b-5p	12	5	17
miR-18a-5p	16	1	17
miR-2355-5p	16	1	17
miR-17-3p	16	0	16
miR-503-5p	16	0	16
miR-15a-3p	15	1	16
miR-125b-5p	11	5	16
miR-129-5p	13	2	15
miR-214-3p	12	3	15
miR-7-1-3p	14	1	15

miR/miRNA, microRNA.

Table VIII. Hub TFs in acute monocytic leukemia-associated microRNA and TF regulatory network.

TF	In-degree	Out-degree	Total degree
CREB1	26	91	117
CEBPA	26	71	97
POU2F1	0	95	95
TCF3	18	76	94
MYC	18	72	90
TP53	26	60	86
NFKB1	0	73	73
MAX	0	72	72
MZF1	0	71	71
MEF2A	0	64	64
STAT5A	12	51	63
FOXO4	15	45	60
SRY	0	58	58
NFIC	0	58	58
ATF2	0	54	54
PAX2	0	53	53
NFKB2	0	43	43
STAT6	0	37	37
STAT5B	15	19	34
FOXD1	0	27	27
NR2F1	0	26	26
NKX2-2	0	26	26
HLF	0	18	18

TF, transcription factor.

gene expression', 'positive regulation of cellular metabolic process' and 'negative regulation of cellular process'. KEGG pathway analysis indicated that the network nodes were significantly enriched in 23 different signaling pathways (Table VI), of which 13 were associated with hematopoiesis or leukemia. Among these pathways, 'MAPK signaling pathway', 'apoptosis signaling pathway', 'Toll-like receptor signaling pathway' and 'TGF-β signaling pathway' have previously been implicated in AMoL (20-25).

Network hub identification and sub-network construction. The overall properties of the network were evaluated by analyzing the node degrees and their distributions. The average degree values of miRNAs, TFs and genes were 9.8 (range, 1-22), 61.6 (range, 18-117), and 11.6 (range, 1-36), respectively. Furthermore, only a few nodes exhibited a high node degree (hubs, nodes with >15 total degrees), whereas the majority of nodes interacted with a relatively low number of other nodes, implying that the network was scale-free (Fig. 5). These results also indicated that hub nodes may play critical roles in sustaining the global connectivity and stability of the network. Nodes were sorted in descending order and hub nodes were identified according to their total degrees. From the miRNA-TF regulatory network, 26 hub miRNAs (Table VII) and 23 hub TFs (Table VIII) were obtained. Among these hub miRNAs, eight (miR-15a-5p, miR-15a-3p, miR-15b-5p, miR-16-5p, miR-195-5p, miR-424-5p, miR-497-5p and miR-503-5p) belonged to the miR-15 family, indicating a potential role of the miR-15 family in the AMoL-associated miRNA and TF regulatory network. Notably, one hub miRNA (miR-29b-3p) and three hub TFs (*MYC*, *TP53* and *NFKB1*) have previously been found to be associated with AMoL (26-32). In order to further investigate their regulation inside the network, sub-networks were constructed by extracting miR-29b-3p, *MYC*, *TP53*, *NFKB1* and all their directly connected nodes from the network (Fig. 6). In the subnetworks, miR-29b-3p was potentially targeted by *MYC* (Fig. 6A and B), *TP53* (Fig. 6A and C) and *NFKB1* (Fig. 6A and D), which may establish cross-talk between these four sub-networks.

Direct targeting of the promoters of miR-29b-3p by MYC. In order to evaluate whether miR-29b-3p is a direct target of *MYC*, luciferase reporter assays were performed in THP1 cells with different promoter constructs of miR-29b-3p. Luciferase activity in WT-MBS constructs was significantly decreased when cells were transfected with the *MYC* overexpression vector (pcDNA3.1-*MYC*) compared with those transfected with

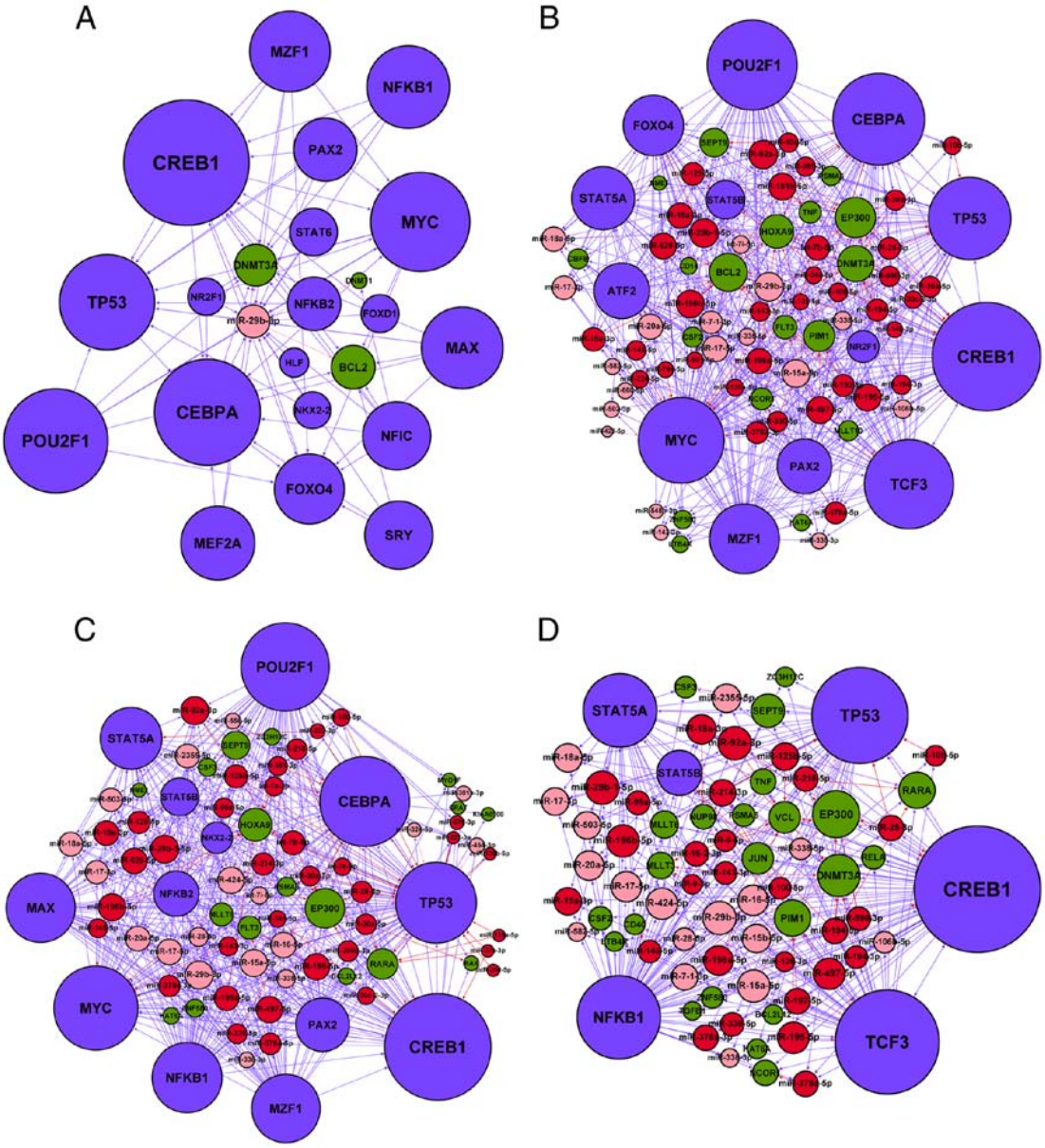

Figure 6. Subnetworks of the four acute monocytic leukemia-associated hubs in the miR-transcription factor regulatory network. All directly linked nodes were extracted to establish the sub-networks of the (A) miR-29b-3p, (B) *MYC*, (C) *TP53* and (D) *NFKB1* hubs. Red node, upregulated AMoL-associated miRNA; pink node, downregulated AMoL-associated miRNA; green node, AMoL candidate gene; blue node, TF. Red arrow, upregulated miRNA-gene; pink arrow, downregulated miRNA-gene; blue arrow, TF-miRNA or TF-gene. miR, microRNA.

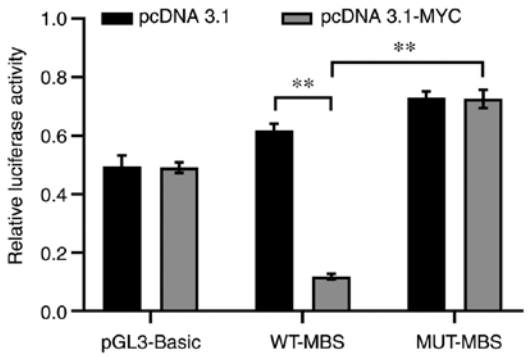

Figure 7. Verification of miR-29b-3p as a *MYC* target via luciferase reporter assay. *MYC* overexpression resulted in decreased luciferase activity in WT-MBS constructs. By contrast, MUT-MBS resulted in enhanced luciferase activity in *MYC*-overexpressing THP1 cells. **P<0.01. WT, wild-type; MBS, MYC-binding site; MUT, mutant.

the pcDNA3.1 plasmid (P<0.01; Fig. 7), indicating inhibition of miR-29b-3p transcriptional activity by *MYC*. In addition, luciferase reporter assays also revealed that MUT-MBS exhibited increased luciferase activity when *MYC* was overexpressed (P<0.01), demonstrating that the MBS within the miR-29b-3p promoter serves a key role in the regulatory effects of *MYC* on miR-29b-3p gene transcriptional activity.

Discussion

The mechanisms underlying AMoL are poorly understood, which obstructs the design of therapeutic strategies to treat this disease. Instead of focusing on *a priori* candidate genes, systems biology combines experimental data and computational analysis to investigate molecular interactions and explain biological behavior at the network level, which may

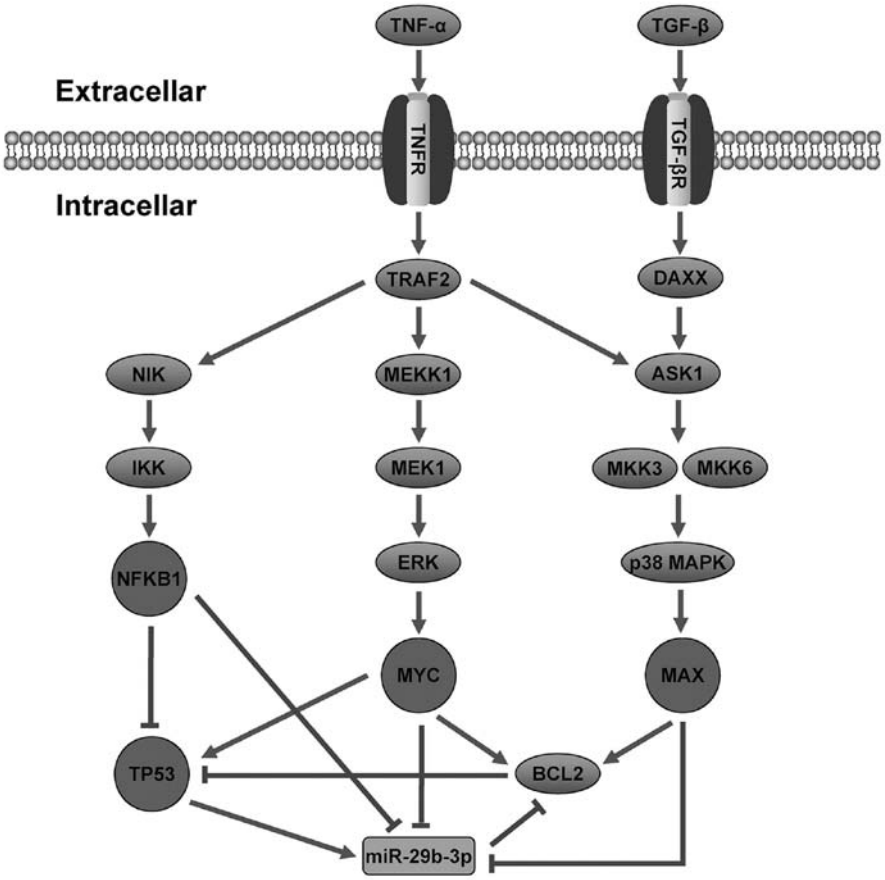

Figure 8. Hypothetical model of miR-29b-3p involving signaling pathways and regulatory networks in AMoL. Following AMoL, expression levels of TNF-α and TGF-β increase, and MAPK and apoptosis signaling pathways are activated. These signal transductions trigger TFs such as *MAX*, *MYC*, *TP53* and *NFKB1* to regulate the transcription of miR-29b-3p. Rectangle, miRNA; ellipse, gene; circle, TF. Sharp arrow, activation; T-shaped arrow, repression. miR, microRNA; AMoL, acute monocytic leukemia; MAX, MYC-associated X protein; TF, transcription factor.

provide deeper insight into disease pathogenesis and accelerate the discovery of key elements and regulatory motifs associated with a disease (33-35). The present study used miRNA sequencing, TF array and bioinformatics technology to perform a systems biology-based integrative analysis and construct a comprehensive miRNA-TF regulatory network for AMoL. Subsequently, miRNA-TF-mediated regulatory motifs, including 853 FFLs and 29 FBLs, were identified from the network, which have previously been demonstrated to be significantly overrepresented in the mammalian gene regulatory network (9,36). FFLs and FBLs are key motifs in gene regulatory networks that serve important roles in multiple types of leukemia, including AML, T-ALL and B-cell acute lymphoblastic leukemia (B-ALL) (7,13,37). FFLs may serve as the core for the entire gene regulatory network, which has been used to identify cancer-associated miRNAs or genes in multiple types of tumor, including T-ALL and B-ALL (9,13,37). The majority of FFLs in the present network were found to be TF-FFLs, where in a TF serves as the primary regulator to control the expression levels of miRNAs and their target genes. This is consistent with previous studies showing that TF-FFLs are the prevalent motif in the miRNA-TF regulatory network of glioblastoma and B-ALL (13,38); this has been demonstrated to be a powerful tool to determine the etiology of these diseases, thus unveiling the important role of TF-FFLs in AMoL pathogenesis.

In order to further evaluate the functional properties of the present network, GO functional classification and KEGG pathway analysis were performed to analyze the enrichment of network nodes. Of the 10 highly enriched biological processes terms, nine (90%) were associated with the initiation and progression of AMoL. Furthermore, >50% of the significantly enriched signaling pathways were associated with hematopoiesis or leukemia. Among them, four have previously been reported to be involved in AMoL pathogenesis, namely 'MAPK signaling pathway', 'apoptosis', 'Toll-like receptor signaling pathway' and 'TGF-β signaling pathway'. These results demonstrated the effectiveness and reliability of the network construction.

Hubs are highly connected nodes in molecular interaction networks that serve pivotal roles in sustaining network structure and function (39). From the miRNA-TF regulatory network, 26 hub miRNAs and 23 hub TFs that exert the largest impact on network overall behavior were obtained; these may serve important regulatory roles in AMoL. In order to investigate this massive and highly complex network and mine the critical regulators, subnetworks of the four hubs (miR-29b-3p, *MYC*, *TP53* and *NFKB1*) known to be involved in AMoL were subsequently constructed.

As a positive regulator of granulocytic and monocytic differentiation, miR-29b-3p has been found to be downregulated in chronic lymphocytic leukemia and AML, and is a known

tumor suppressor miRNA in leukemogenesis (26,40,41). The enforced expression of miR-29b-3p markedly inhibits cell proliferation and induces apoptosis in the THP1 AMoL cell line (26).

MYC is an essential TF of the basic-helix-loop-helix-zipper family that regulates numerous biological processes, including cell proliferation, differentiation, metabolism, cell cycle progression and apoptosis (42,43). High levels of *MYC* expression are frequently observed in multiple types of malignant hematopoietic cell, including AMoL (42,44). Treatment with 10058-F4, a small molecular *MYC* inhibitor that disrupts the association between *MYC* and *MYC*-associated X protein, induces cell-cycle arrest and monocytic differentiation of AMoL U937 cells (27). Similarly, the inhibition of *MYC* expression levels by small interfering RNA also induces cell apoptosis, enhances the sensitivity of cells to mitoxantrone and abolishes stroma-mediated drug resistance in U937 cells (28).

TP53 is a nuclear TF that plays a key role in tumor suppression via the regulation of DNA repair, cell cycle progression, differentiation, apoptosis, chemosensitivity and senescence (45). The synergistic induction of *TP53*-mediated apoptosis and autophagy by valproic acid and nutlin-3 in the AMoL cell line MOLM-13 also inhibits leukemia progression in an *in vivo* xenograft model of MOLM-13 (29). Furthermore, the mutual interaction of p53, Runt-related TF1 and core-binding factor subunit β contributes to the acquisition of resistance to cytarabine in AMoL MV4-11 cells (30).

The TF *NFKB1* plays an important role in the regulation of cell proliferation, apoptosis, angiogenesis and immune responses, and is upregulated in numerous types of hematological malignancy, including AMoL (46). The blockade of *NFKB1* activity in combination with nutrient depletion yields synergistic cytotoxicity in MOLM-13 and U937 cells (31). Furthermore, the inhibition of *NFKB1* and heme oxygenase-1 in combination also results in significant cytotoxicity in THP1 cells (32). Among these hubs, miR-29b-3p was notable as a pivotal regulator owing to its direct link with three other hubs (*MYC*, *TP53* and *NFKB1*) inside the subnetworks, which also revealed crosstalk between these subnetworks.

In the subnetworks, miR-29b-3p was potentially targeted by *MYC*, *NFKB1*, *TP53* and *CEBPA*. Furthermore, *MYC* and miR-29b-3p were predicted to coordinately regulate their common target gene *BCL2*, which established a TF-FFL composed of *MYC*, miR-29b-3p and *BCL2*. It has been reported that *MYC* and *NFKB1* inhibit miR-29b-3p expression levels at the transcriptional level via direct binding to the promoter region of the miR-29b-3p locus (47). The present study also verified that miR-29b-3p was a direct target of *MYC* in AMoL THP1 cells. The downregulation of miR-29b-3p expression levels mediated by *MYC* may enhance expression of their target gene *BCL2*. Thus, it is hypothesized that *MYC* may cooperate with miR-29b-3p to promote *BCL2* expression levels in a *MYC*/miR-29b-3p/*BCL2* TF-FFL. *BCL2* is a member of the *BCL2* gene family, which was the first apoptosis regulator found to be associated with cancer (48). The overexpression of *BCL2* has been detected in numerous types of hematological malignancy, including AMoL (48,49). Selective *BCL2* inhibition by ABT-199 rapidly inhibits cell growth and induces apoptosis in the MOLM-13 AMoL cell line and markedly inhibits leukemia progression in an aggressive mouse xenograft model of MOLM-13 cells (50). A *MYC*/miR-29b-3p/*BCL2* TF-FFL was identified as a potential key motif in the present network, which may serve an important role in the occurrence and progression of AMoL.

Notably, *TP53* has previously been demonstrated to be a direct transcriptional activator of *CEBPA* (51), whereas *CEBPA* activates transcription of the miR-29b-3p gene (52), which is in accordance with the regulatory association in the present network, and establishes a regulatory circuit in which *TP53* may regulate miR-29b-3p expression levels via *CEBPA*. *CEBPA* was one of the hub TFs in the network and is known to serve a crucial role in myeloid transformation, hematopoietic stem cell self-renewal and cell cycle control throughout the process of hematopoiesis (53,54). *CEBPA* is downregulated in numerous types of human cancer, including AML, chronic myeloid leukemia, pancreatic cancer and hepatocellular carcinoma (55). *CEBPA* loss also results in the development of AML with complete penetrance in a *CEBPA*-deficient mouse model (55,56). Consistent with these reports, decreased expression levels of *CEBPA* were also observed in the TF array analysis in the present study, implying a potential association between *CEBPA* and AMoL. It is hypothesized that miR-29b-3p is a key miRNA involved in the AMoL miRNA-TF regulatory network, which may play an important tumor suppressive role in AMoL.

In order to investigate the regulatory mechanism of AMoL, a hypothetical model of miR-29b-3p was proposed, involving signaling pathways and regulatory networks in AMoL (Fig. 8). However, the majority of AMoL-related miRNAs and genes used in the present study have not been confirmed to be causal, and the regulatory associations among miRNAs, TFs and genes were neither complete nor unbiased. Further experimental validation is warranted to confirm these hypotheses in future studies.

To the best of our knowledge, the present study is the first to demonstrate an miRNA and TF synergistic regulatory network specifically for human AMoL, which may provide valuable information to identify critical elements and regulatory motifs in AMoL and improve understanding of gene regulatory mechanisms in AMoL.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HTZ and XCL conceived and designed the study. XCL and QY wrote the manuscript. YMZ and NL made substantial contributions to the collection and storage of bone marrow samples. XCL, QY, YMZ, NL and HD conducted the experiments. XCL, WYF and LBL performed the bioinformatics analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Affiliated Hospital of Guangdong Medical University (approval no. PJ2016081KT; Zhanjiang, China), and all patients or their guardians provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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