Microarray profiling of bone marrow long non-coding RNA expression in Chinese pediatric acute myeloid leukemia patients

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Abstract. Long non-coding RNA (lncRNA) plays a role in gene transcription, protein expression and epigenetic regulation; and altered expression results in cancer development. Acute myeloid leukemia (AML) is rare in children; and thus, this study profiled lncRNA expression in bone marrow samples from pediatric AML patients. Arraystar Human LncRNA Array V3.0 was used to profile differentially expressed lncRNAs in three bone marrow samples obtained from each pediatric AML patient and normal controls. Quantitative polymerase chain reaction (qRT-PCR) was performed to confirm dysregulated lncRNA expressions in 22 AML bone marrow samples. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to construct the lncRNA-mRNA co-expression network. A total of 372 dysregulated lncRNAs (difference ≥10-fold) were found in pediatric AML patients compared to normal controls. Fifty-one mRNA levels were significantly upregulated, while 85 mRNA levels were significantly downregulated by >10-fold in pediatric AML, compared to normal controls. GO terms and KEGG pathway annotation data revealed that cell cycle pathway-related genes were significantly associated with pediatric AML. As confirmed by qRT-PCR, expression of 24 of 97 lncRNA was altered in pediatric AML compared to normal controls. In pediatric AML, ENST00000435695 was the most upregulated lncRNA, while ENST00000415964 was the most downregulated lncRNA. Data from this study revealed dysregulated lncRNAs and mRNAs in pediatric AML versus normal controls that could form gene pathways to regulate cell cycle progression and immunoresponse. Further studies are required to determine whether these lncRNAs could serve as novel therapeutic targets and bbdiagnostic biomarkers in pediatric AML.

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Introduction

Acute myeloid leukemia (AML) is the most common form of acute leukemia that occurs more frequently in adults, but relatively rare in children; and AML incidence increases with age (1). AML is characterized by the rapid growth of abnormal white blood cells that accumulates in the bone marrow, which inhibits production and differentiation of normal blood cells (1,2). Genetically, AML is defined as a clonal disorder caused by malignant transformation of bone marrow-derived cells, self-renewing stem cells, or progenitors that demonstrate a decreased rate of self-destruction and aberrant differentiation (3). Pediatric AML comprises of up to 20% of all childhood leukemia (4). Although overall AML prognosis has been improved in the last decade, further development and identification of prognostic markers or novel targets for AML treatment could improve the survival rate of AML patients, especially pediatric AML patients.

Long non-coding RNA (lncRNA) regulates gene transcription and protein expressions genetically and epigenetically, and altered expressions result in cancer development. Human genomic data has shown that ~75% of the human genome is transcribed into RNA, and only a few above 1% codes protein expressions; indicating that a large portion of the genome is dedicated as regulators (5,6). Many lncRNAs ranging from 0.2 to 100 kilobases (kb) in length are transcribed from the genome. Among these newly discovered RNA elements, lncRNAs have been identified to have functional roles in a diverse range of cellular functions such as development, differentiation, cell fate, as well as disease pathogenesis (7-11). Expression analyses of cancer versus normal cells have

revealed aberrant non-coding RNA (ncRNA) expression in various human cancers. For example, an altered PCGEM1 expression was associated with increased proliferation and colony formation in prostate cancer cells (12). MALAT1, also known as NEAT2, was originally identified as an abundantly expressed ncRNA during metastasis of early-stage non-small cell lung cancer; and its overexpression is a prognostic marker for poor patient survival rate (13,14). MALAT1 was also found to be highly expressed in hepatocellular carcinoma (15-17). The oncofetal H19 gene was the first imprinted ncRNA to be identified, and loss of imprinting (LOI) at chromosome 11p15.5H19/IGF2 locus leads to an imbalanced expression of H19 and IGF2. H19 dysregulation has been implicated in a variety of human cancers such as colorectal (18), HCC (19), breast (20), and bladder cancers (21,22).

Various lncRNAs were reported to be implicated in malignant hematopoiesis associated with blood cell neoplasms such as leukemia (23,24). H19 ncRNA was highly expressed in Bcr-Abl-transformed cell lines and primary cells derived from patients in a Bcr-Abl kinase-dependent manner (23). lncRNA MONC and MIR100HG were highly expressed in acute megakaryoblastic leukemia blasts (24). Thus, lncRNAs may be useful as diagnostic and prognostic markers in leukemia. In the present study, we profiled differential expression of lncRNAs in pediatric AML to better understand AML pathogenesis and identify biomarkers and novel therapeutic targets.

Materials and methods

Patients and samples. Bone marrow specimens were obtained from 22 pediatric AML patients at the Department of Hematology and Oncology, Children's Hospital of Soochow University, between 2011 and 2014. This study was approved by the Ethics Committee of the Children's Hospital of Soochow University (#SUEC2011-021 and #SUEC2014-037), and written informed consent was obtained from all parents or guardians. AML diagnosis was made in accordance with the revised French-American-British (FAB) classification (25). The main clinical and laboratory features of this cohort of patients are summarized in Table I. Additionally, bone marrow samples from 20 donors without leukemia were used as controls. Bone marrow mononuclear cells (BMNCs) were isolated using Ficoll solution within 2 h after bone marrow samples were harvested, and subjected for isolation of total cellular RNA.

Profiling of lncRNA expression using Arraystar Human LncRNA Array V3.0. Arraystar Human LncRNA Array V3.0 was used to profile expression of lncRNAs, which was performed by KangChen Bio-tech (Shanghai, China) according to a previous study (26). Briefly, RNA samples from BMNCs were further purified to remove rRNA, and transcribed into fluorescent cRNA as probes to hybridize to the Human LncRNA Array V3.0 (8660 K; Arraystar). The array contains 30,586 lncRNAs and 26,109 coding transcripts, which were collected from the most authoritative databases (such as RefSeq, UCSC, Knowngenes, and Ensembl) and related literature. Array data were then analyzed by MultiExperiment Viewer software for upregulation or downregulation of lncRNA expression in AML samples compared to control samples with a cut-off point of 2-fold for upregulation and a cut-off point of 0.5-fold for downregulation.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses. Gene Ontology (GO) functionally analyzes differentially expressed genes with GO categories (http://david.abcc.ncifcrf.gov/summary.jsp). Pathway analysis of differentially expressed genes was performed based on the latest Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg). Differentially expressed genes and gene product enrichment with particular attention to GO biological processes and molecular functions were grouped into gene pathways using a p-value ≤0.05, as shown below.

Construction of the lncRNA-mRNA co-expression network. The construction of the lncRNA-mRNA co-expression network included three steps: i), lncRNA screening: lncRNAs that were upregulated or downregulated with a fold-change >3.0 and a p-value <0.05 were first selected to enhance data reliability. Sequences of lncRNAs that have not been recorded in ENCODE were removed. ii), lncRNA-miRNA interactions were predicted by miRcode (http://www.mircode.org/). iii), mRNAs targeted by miRNAs with experimental support were obtained from TarBase (http://www.microrna.gr/tarbase).

Network construction procedures included the following: i), preprocessing of data: if one coding gene has different transcripts, the median value was taken to represent the value of this gene expression without special treatment of lncRNA expression values; ii), data were screened and subset of data were removed according to the lists of differential lncRNA and mRNA expressions obtained from GO and KEGG pathway analyses; iii), Pearson's correlation coefficient was calculated, and the R value was used to calculate the correlation coefficient between lncRNA and coding genes; and iv), Pearson's correlation coefficient was used for screening, wherein, RNAs with a Pearson's correlation coefficient ≥0.98 were considered significant. The lncRNA-mRNA co-expression network was then constructed by Cytoscape software (The Cytoscape Consortium, San Diego, CA, USA).

RNA isolation and qRT-PCR. Total cellular RNA was isolated from bone marrow samples using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at -80˚C until use. RNA concentration was determined using a spectrophotometer (NanoDrop 2000) and purity was assessed by agarose gel electrophoresis. RNA samples were then reversely transcribed into cDNA using 4 μg of RNA samples in a 10-μl volume and SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's protocols. For qPCR, we first designed PCR primers according to the database of Real-time primers (Center for Medical Genetics, http://medgen.ugent.be/CMGG/) or using the online program, Primer 3 (www.fokker.wi.mit.edu/primer3/input.htm). Primer selection parameters were set to primer size: 20-26 nts; primer melting temperature: 60-64˚C; GC clamp: 1; and product size range: generally 120-240 bp, which went down to 100 bp if no appropriate primers could be identified. Primers were synthesized by Invitrogen. The qPCR amplification was set in a 20-μl

Table I. The main clinical and laboratory features of the pediatric AML samples.

	Gender	Age (years)	Diagnosis	AML typing	Chromosome analysis	Fusion gene	Mutation
1	F	11	AML	M5	46, XX,t(9;11)(P22;q23) [9]/46,XX[3]	MLL/AF9	
2	F	5	AML	M5	ns	ns	
3	M	7	AML	M2a	46, XY	AML/ETO	
4	M	5	AML	M2a	46, XY	(-)	CEBPA TAD1
5	F	6	AML	M4	46, XX	dupMLL, FLT-TKD	
6	M	6	AML	M2	45, X,-Y,t(8;21)(q22;q22) [7]/46,XY[5]	AML1/ETO	
7	F	12	AML	AML	46, XX	AML/ETO	
8	M	2	AML	M5	46, XY	46, XY	
9	F	10	AML	M2	46, XX,t(8;21)(q22;q22)	AML/ETO	C-Kit
10	M	5	AML	M4	46, XY	(-)	
11	F	2	AML	M5	ns	ns	
12	F	13	AML	M3	46, XX, t(15;17)(q22;q21)	PML/RARA	
13	M	12	AML	M2a	ns	ns	
14	F	2	AML	M4	46, XX	CBF/MYH11	
15	M	4	AML	M2	46, XY	AML/ETO	
16	F	12	AML	M2	45, X, -X,t(8:21)(q22;q22) [6]/46,XX[2]	AML/ETO	C-Kit
17	M	6	AML	M3	46,XY,t(15:7)(q22:q210 [9]/46,XY[3]	PML/RARA	
18	M	8	AML	M2a	46, XY	(-)	
19	F	1	AML	M5b	46, XX, t(6;11)(q27;q23)	MLL/AF6	
20	M	10	AML	M4	47, XY,+22, inv(16)(p13q22)	CBF/MYH11	
21	M	9	AML	M2a	46, XY	(-)	
22	M	7	AML	M3	46, XY	PML/RARA	

reaction volume containing 1 μl of cDNA, 0.2 mM of each primer, and 10 μl of SYBR Green Mix (Roche, Indianapolis, IN, USA); and was performed in a LightCycler 480 (Roche) using universal thermal cycling parameters (an initial 95˚C for 10 min and 45 cycles of 15 sec at 95˚C, 15 sec at 60˚C, and 60 sec at 72˚C. After that, the melting curve for 10 sec at 95˚C and 60 sec at 65˚C). For gene expression levels, we used the comparative Ct method. First, gene expression levels for each sample were normalized to the expression level of the housekeeping gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) within a given sample (-ΔCt); and the relative expression of each gene was calculated with 10^6 x log2 (-ΔCt). The difference between pediatric AML samples compared to control samples was used to determine 10^6 x log2 (-ΔCt).

Statistical analysis. SPSS v11.5 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Differentially expressed lncRNAs in AML samples were statistically compared with normal controls, and a cut-off point of 2-fold for upregulation and 0.5-fold for downregulation of lncRNA expressions were used in AML samples. For gene expression, we performed a Student's t-test; and a p-value ≤0.05 was considered statistically significant.

Results

Differentially expressed lncRNAs and mRNAs in pediatric AML. The Arraystar Human LncRNA 8x60k V3.01 microarray was used to profile differentially expressed lncRNAs and mRNAs in pediatric AML versus normal controls. A total of 2,335 differently expressed mRNAs were found in pediatric AML (Fig. 1A and B). Among them, 51 mRNAs were significantly upregulated and 85 mRNAs were significantly downregulated for >10-fold in pediatric AML compared to normal controls. Clustering analysis was used to visualize the relationships between the mRNA expression patterns present in the samples (fold-changes ≥10; Fig. 1C and D).

Moreover, a total of 2,413 differentially expressed lncRNA were identified in pediatric AML (Fig. 2A and B). Hierarchical clustering analysis of these differentially expressed lncRNAs with >10-fold difference is shown in Fig. 2C and D and Tables II and III.

Altered lncRNA-related gene pathways in pediatric AML. Ontological pathway enrichment analysis was performed on differentially expressed lncRNA and mRNA, and a biological process enrichment analysis was performed using the DAVID tool to gain insights into their functions (GEO

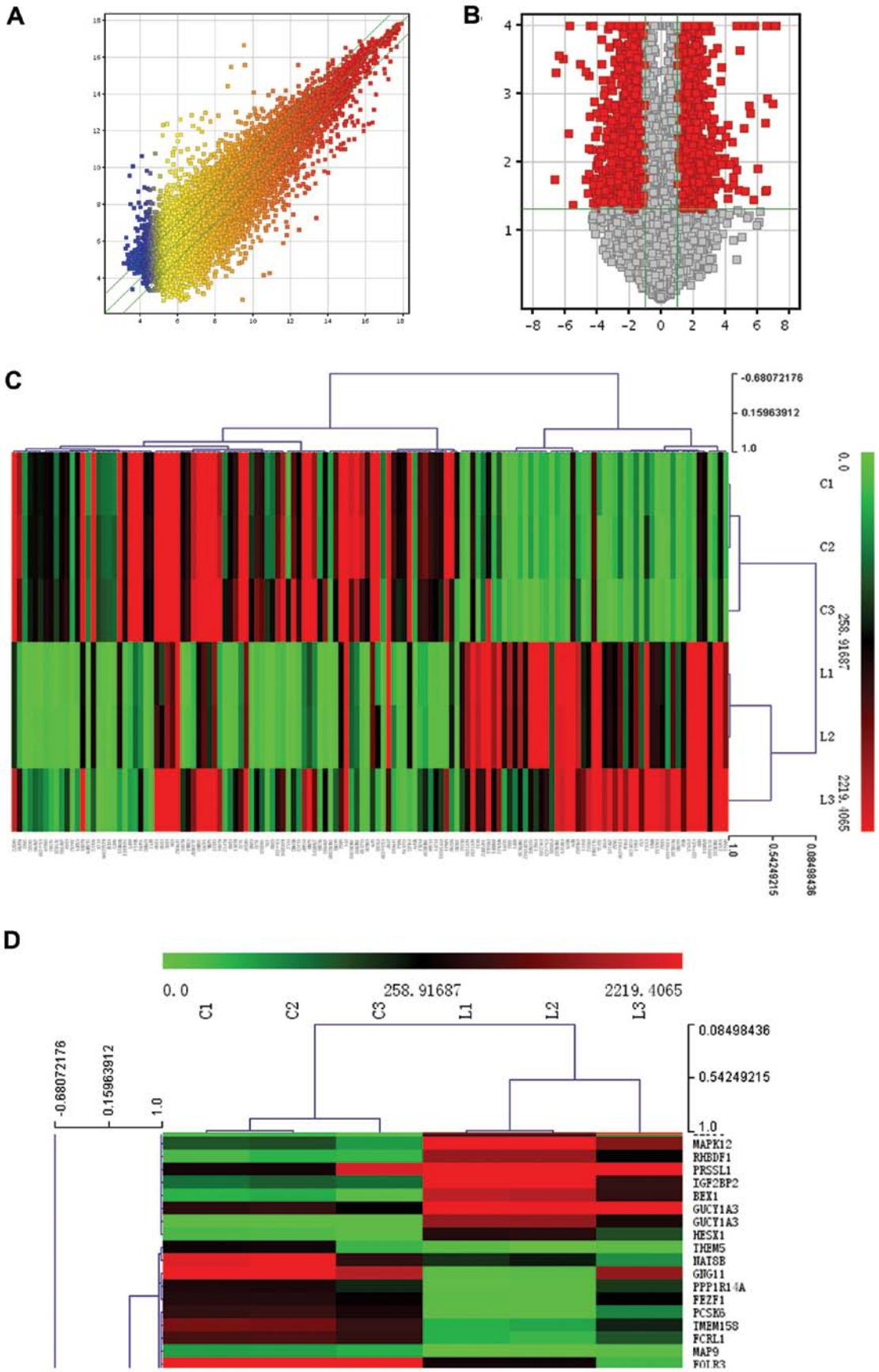

Figure 1. Cluster analysis of differentially expressed mRNAs in pediatric acute myeloid leukemia (AML). The Arraystar Human LncRNA 8x60k V3.01 microarray was utilized to profile and identify differentially expressed mRNAs in pediatric AML compared to normal controls. (A and B) Scatter-Plot assessment of mRNA expression is shown between pediatric AML and normal controls. Green lines are fold-change lines (the default fold-change value given is 2.0). The mRNAs above the top green line and below the bottom green line indicated a >2.0 fold-change of mRNAs between the two compared samples. (C) Hierarchical clustering analysis of the 136 significantly dysregulated mRNAs with ≥10 fold-changes in pediatric AML compared to normal controls. (D) Partial amplification of hierarchical clustering analysis of the 136 significantly dysregulated mRNAs with ≥10 fold-changes in pediatric AML compared to normal controls.

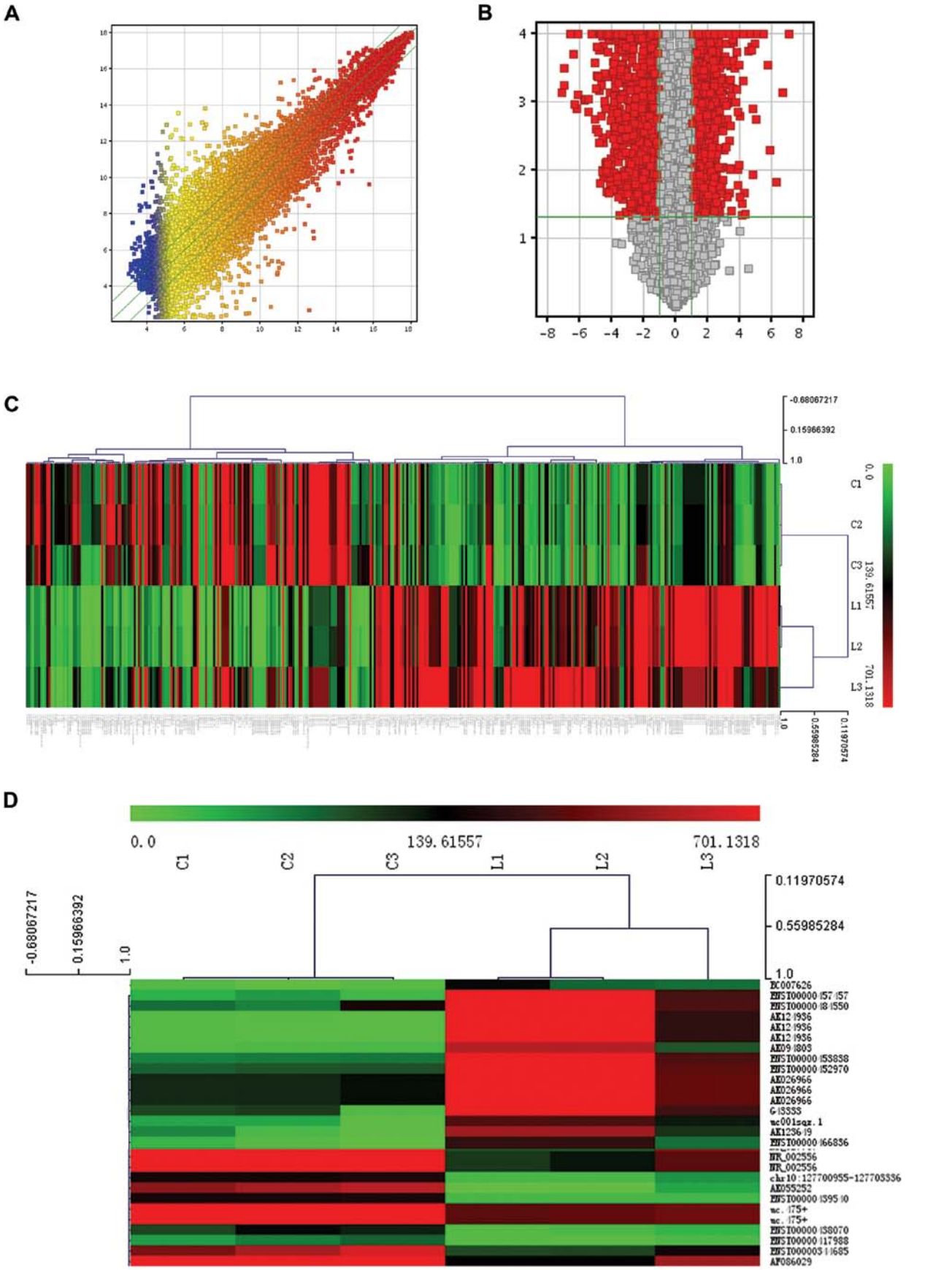

Figure 2. Cluster analysis of differentially expressed lncRNAs in pediatric acute myeloid leukemia (AML) is shown. (A and B) Scatter-Plot assessment of lncRNA expression is shown between pediatric AML and normal controls. Green lines are fold-change lines (the default fold change value given is 2.0). The lncRNAs above the top green line and below the bottom green line indicated a >2.0 fold-change of lncRNAs between AML and normal samples. (C) Hierarchical clustering analysis of the 372 significantly dysregulated lncRNAs for ≥10 fold-changes in pediatric AML compared to normal controls. (D) Partial amplification of hierarchical clustering analysis of the 372 significantly dysregulated lncRNAs for ≥10 fold-change in pediatric AML compared to normal controls.

Table II. lncRNA upregulated >10-fold in the pediatric acute myeloid leukemia compared with normal control.

	Seqname	Gene symbol	Associated gene name	Source	[C](raw)	[L](raw)	FC (abs)	P-value
1	AK124936	lincRNA-FAM47E	SCARB2	lincRNA	14.110494	1536.7562	59.8816	0.0072902
2	uc003ysq.2	CCDC26		UCSC_knowngene	34.0223	3260.5908	42.260292	0.0027339
3	uc002ehu.1	PNAS-108		UCSC_knowngene	20.46965	1096.482	39.467697	0.000351
4	BX952962	lincRNA-OBFC2A-2		lincRNA	17.117323	788.3796	35.642254	3.22E-06
5	ENST00000435695	AC002454.1	CDK6	Ensembl	127.27789	9728.407	34.028286	0.004122
6	NR_024259	LOC728606		RefSeq_NR	24.271034	1178.9098	33.306793	0.000145
7	ENST00000417463	KB-1592A4.14		Ensembl	32.294987	4614.054	30.944315	0.0318946
8	BC035649			misc_RNA	24.781305	1576.078	29.303568	0.0128913
9	AA635039	lincRNA-TARBP1-2		lincRNA	22.896029	899.75323	28.649506	0.0000286
10	BC022435		C9orf41	misc_RNA	35.91673	3247.7634	27.618906	0.0135179
11	ENST00000457457	AC016735.1		Ensembl	37.48748	1587.2334	26.119883	0.013067
12	AK127507			misc_RNA	16.645311	605.7293	21.048286	0.0024883
13	uc011mle.1	DM004476		UCSC_knowngene	26.679949	918.6805	21.00233	0.000982
14	uc002ufj.3	KLHL23	PHOSPHO2-KLHL23	UCSC_knowngene	133.91594	3545.8738	19.016815	0.0000236
15	ENST00000509500	U85056.1		Ensembl	24.437654	1016.5418	18.854773	0.0098472
16	ENST00000458624	AC007009.1	ICA1	Ensembl	35.76904	913.7956	18.405573	0.0051383
17	uc001guz.2	EF413001		UCSC_knowngene	100.33683	2223.7722	17.687159	0.000444
18	uc010wia.1	AK027091		UCSC_knowngene	443.38882	9681.8	15.688867	0.0075263
19	AY216265		ETS2	NRED	547.44006	11685.214	15.45025	0.0000294
20	AK000053		CLCC1	RNAdb	62.11948	1246.1871	14.599653	0.00033
21	uc002nrb.1	AK131472		UCSC_knowngene	33.197628	684.31696	13.896548	0.0102436
22	AK123638		COPG2	misc_RNA	41.249954	1012.7471	13.175612	0.0063138
23	uc001sqo.2	CR602022		UCSC_knowngene	204.72939	3436.1982	13.079311	0.0012676
24	AK094803			misc_RNA	20.941412	405.46933	12.166367	0.0237685
25	ENST00000453838	KRT18P28		Ensembl	69.50002	1220.4552	11.720998	0.0187785
26	BC035154			misc_RNA	143.23755	2030.2422	11.628016	0.000541
27	AK097063		ZNF850	misc_RNA	13.838933	242.19637	11.516952	0.0014993
28	AK123765			misc_RNA	61.605896	854.0999	11.402978	0.000336
29	uc002uwa.2	AOX2		UCSC_knowngene	33.613018	1187.9089	10.597581	0.0464274
30	AK123649			misc_RNA	31.297625	381.76712	10.589622	0.0417903
31	AK123319		NETO1	misc_RNA	38.455334	891.8227	10.473429	0.0190694
32	uc002ndq.1	CR605427	HSH2D	UCSC_knowngene	23.040321	295.48752	10.46813	0.000156
33	ENST00000452408	KRT18P1		Ensembl	19.09376	262.50006	10.44417	0.0088213
34	NR_001458	MIR155HG		RefSeq_NR	1211.0641	16439.678	10.089632	0.0018226

Table III. lncRNA downregulated >10-fold in the pediatric acute myeloid leukemia compared with normal control.

	Seqname	Gene symbol	Associated gene name	Source	[C](raw)	[L](raw)	FC (abs)	P-value
1	NR_002712	CXCR2P1		RefSeq_NR	6307.8667	94.28683	95.082214	0.0001815
2	ENST00000340794	MAGEA13P	Ensembl	655.6455	33.92397	54.03028	0.0099015	
3	AK055252		C15orf38-AP3S2	misc_RNA	522.1257	18.751987	53.95123	0.0019818
4	CR592555		DHFR	RNAdb	52353.11	2405.9045	28.411339	0.0017334
5	NR_024420	LOC389634	RefSeq_NR	2986.3733	443.83182	27.338875	0.0243679	
6	ENST00000511213	RP11-362F19.1	Ensembl	390.45132	20.542099	26.359552	0.0014037	
7	NR_003191	GGTA1		RefSeq_NR	5235.906	306.32895	23.206347	6.774E-05
8	BC035072			misc_RNA	264.2821	13.62112	22.975542	0.0019559
9	uc003iir.1	AK124399		UCSC_knowngene	584.21124	38.04964	20.08043	0.000707
10	uc003yse.1	LOC727677	POU5F1B	UCSC_knowngene	210.80397	12.041702	19.793795	0.0070152
11	AK128410			misc_RNA	9238.451	1925.9315	15.957043	0.0250141
12	M18204	TEA ncRNAs	lncRNAdb	3398.365	756.7373	14.61877	0.0316519	
13	AL832164		RORA	RNAdb	10414.999	2412.2832	13.646528	0.0276052
14	AK125371			misc_RNA	121.759	16.427212	13.455201	0.0147761
15	ENST00000417930	AC092580.4	Ensembl	1074.5836	187.77055	13.370509	0.0223116	
16	ENST00000406090	AC009494.3	Ensembl	111.52717	17.403128	13.112155	0.0121482	
17	AK095282			misc_RNA	581.82513	140.42796	12.77245	0.0465396
18	NR_027767	TNIK	TNIK	RefSeq_NR	1542.7079	210.12633	12.649625	0.0047896
19	uc001wby.2	TCRA		UCSC_knowngene	229.08073	36.00486	12.01228	0.011543
20	ENST00000425002	AC079325.6	Ensembl	150.33525	17.702103	11.682941	0.0063381	
21	chr1:22577463-22595288+	lincRNA-ZBTB40	lincRNA	591.5555	134.24306	11.504897	0.0407799	
22	AK092053			misc_RNA	590.2487	103.68743	11.401361	0.0197635
23	chr8:128772715-128786539-	lincRNA-MYC-6	lincRNA	298.0089	32.098663	11.27972	0.000242	
24	ENST00000447519	AP001189.4	LRRC32	Ensembl	575.1246	64.24173	10.889111	0.0001103
25	uc001rpo.1	AX747844		UCSC_knowngene	3992.0068	857.19037	10.817992	0.017896
26	ENST00000419624	AC005392.13	Ensembl	207.65968	24.788124	10.610993	0.0003014	
27	ENST00000464405	TRBC1		Ensembl	469.0503	109.8695	10.379145	0.0380851
28	AK023096		PCSK5	NRED	1533.9199	419.10873	10.367031	0.0380955
29	AK097103			misc_RNA	2026.3512	261.61304	10.004671	0.0020644

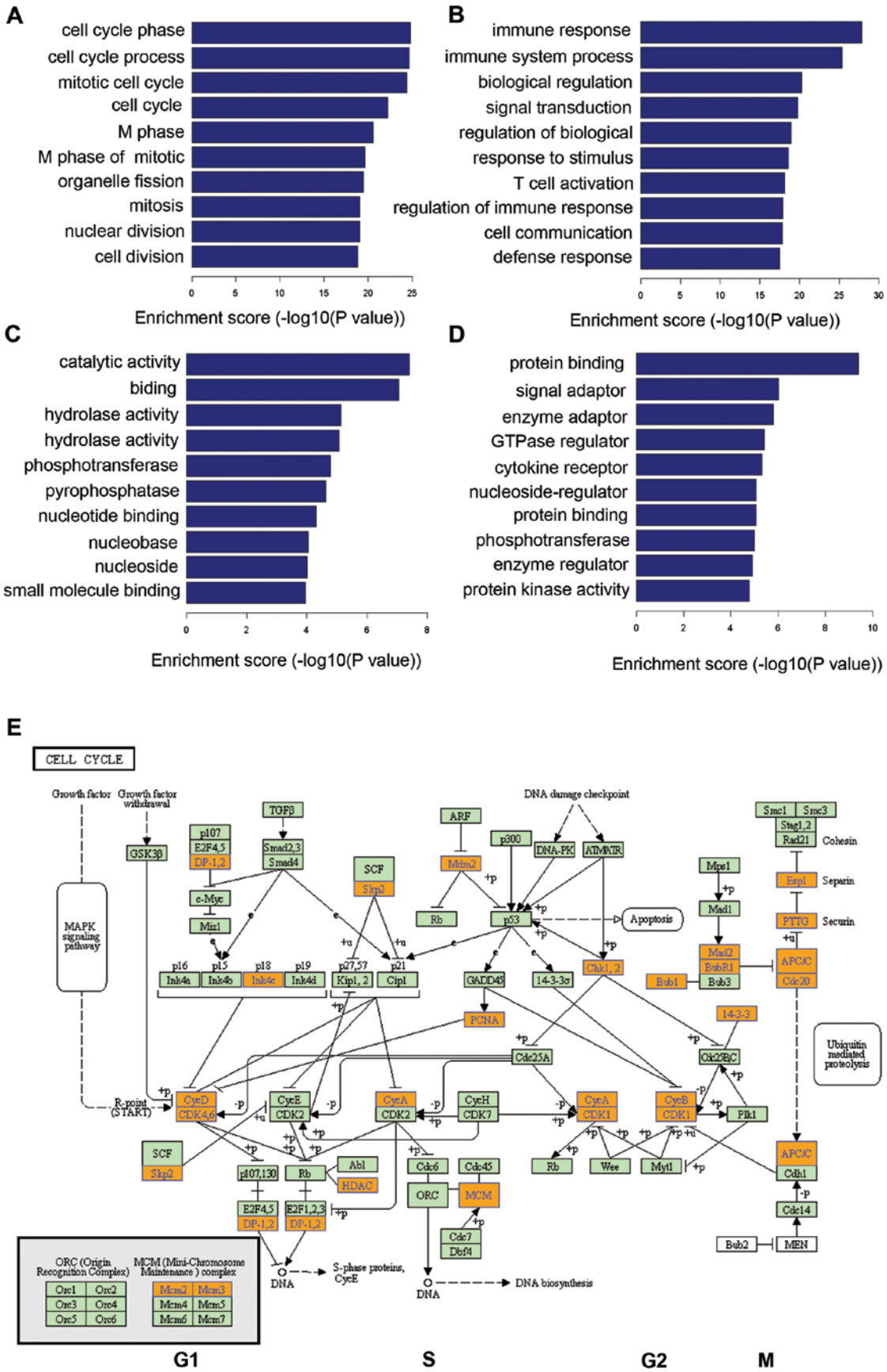

Figure 3. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed mRNAs in pediatric acute myeloid leukemia (AML). (A) The most enriched GO terms targeted by upregulated transcripts were involved in a variety of functions. (B) The most enriched GO terms targeted by downregulated transcripts were involved in a variety of functions. (C) The most significant molecular function (MF) enrichment scores for upregulated transcripts. (D) The most significant MF enrichment scores for downregulated transcripts. (E) KEGG pathway annotations of the most enriched cell cycle pathways with a p-value of $7.22764E^{-10}$. Cell cycle pathway proteins included *ANAPC11*, *ANAPC13*, *ANAPC7*, *BUB1*, *BUB1B*, *CCNA2*, *CCNB1* and *CCNB2*.

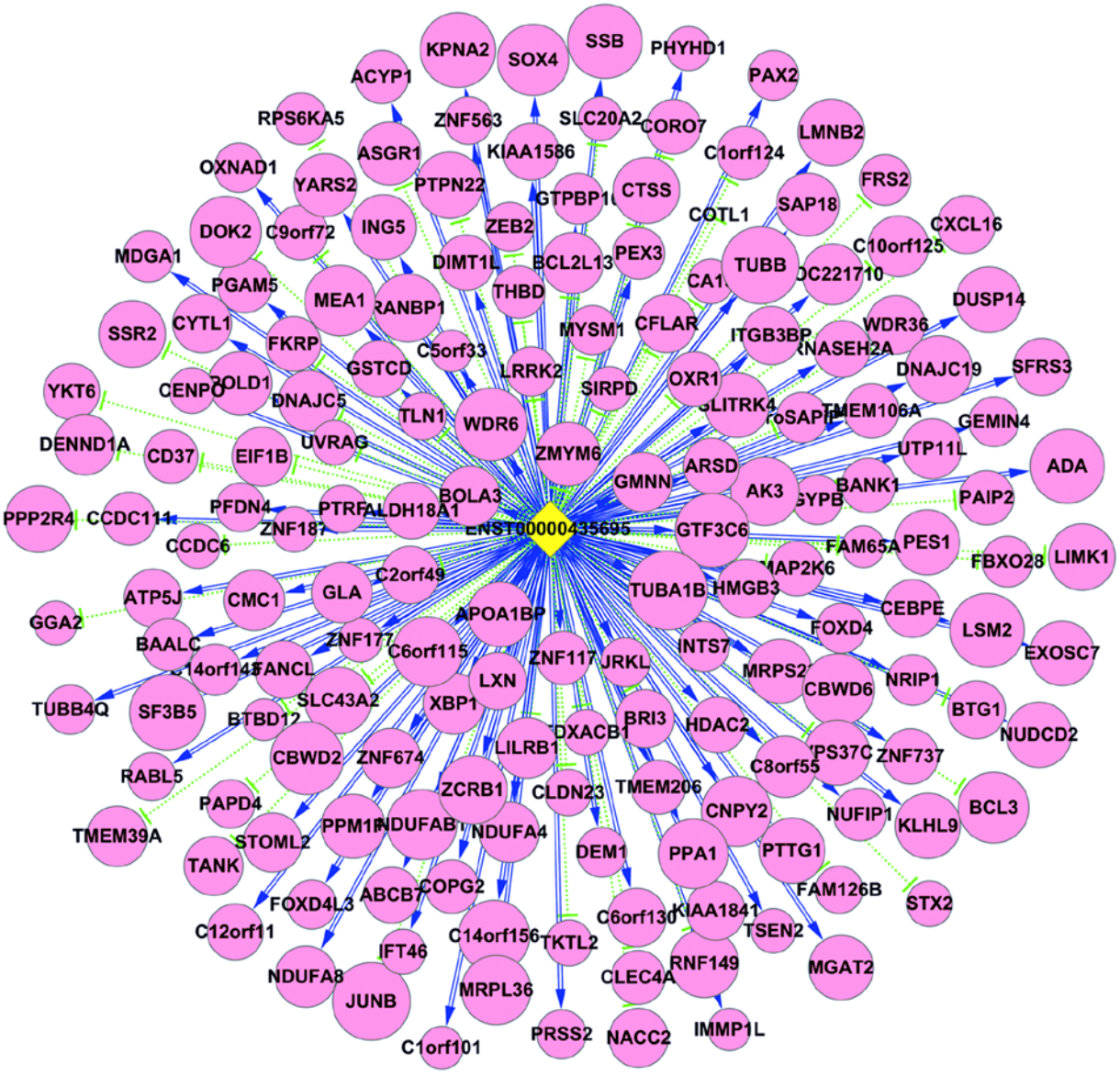

Figure 4. The lncRNA-mRNA co-expression network. Pearson's correlation coefficient was calculated using the R value to estimate the correlation coefficient between lncRNA ENST00000435695 and coding genes. A Pearson's correlation coefficient value ≥0.98 was considered a significant correlation of the lncRNA-mRNA co-expression network. Data were analyzed and constructed by Cytoscape software (The Cytoscape Consortium, San Diego, CA, USA). This co-expression network suggests an inter-regulation of lncRNAs and mRNAs in acute myeloid leukemia development.

accession no. GSE64975). The most enriched GO targeted by upregulated and downregulated transcripts were involved in cell cycle regulation, immune response, and immune system process (Fig. 3A and B). The most significant molecular function (MF) enrichment scores are shown in Fig. 3C and D. KEGG annotations of the most enriched pathways are shown in Fig. 3E, and KEGG pathway annotations of the most enriched pathways were cell cycle regulation with a p-value <7.22764E^{-10}. Cell cycle pathway proteins included *ANAPC11*, *ANAPC13*, *ANAPC7*, *BUB1*, *BUB1B*, *CCNA2*, *CCNB1* and *CCNB2*.

lncRNA/mRNA co-expression network in pediatric AML. Next, we constructed a coding and non-coding gene expression network based on the correlation analysis between differentially expressed lncRNAs and mRNAs. Pearson's correlation coefficient analysis was performed using a coefficient no less than 0.98 to construct the network (Fig. 4). The expression network indicated that one mRNA or lncRNA may correlate with one to tens of lncRNAs. The co-expression network may suggest the inter-regulation of lncRNAs and mRNAs during AML development.

Confirmation of dysregulated lncRNAs in pediatric AML versus normal control samples. To confirm the microarray data, we selected 97 dysregulated lncRNAs from the microarray analysis of 22 pediatric AML samples and 20 control samples using qRT-PCR. Our data revealed that the lncRNA expression profile in pediatric AML was significantly different from normal controls (Fig. 5A). A total of 20 genes were successfully clustered using lncRNA samples (Fig. 5B). A total of 24 lncRNAs were confirmed to be dysregulated in pediatric AML (Table IV), and detailed expressions of each upregulated and downregulated lncRNA in pediatric AML are shown in

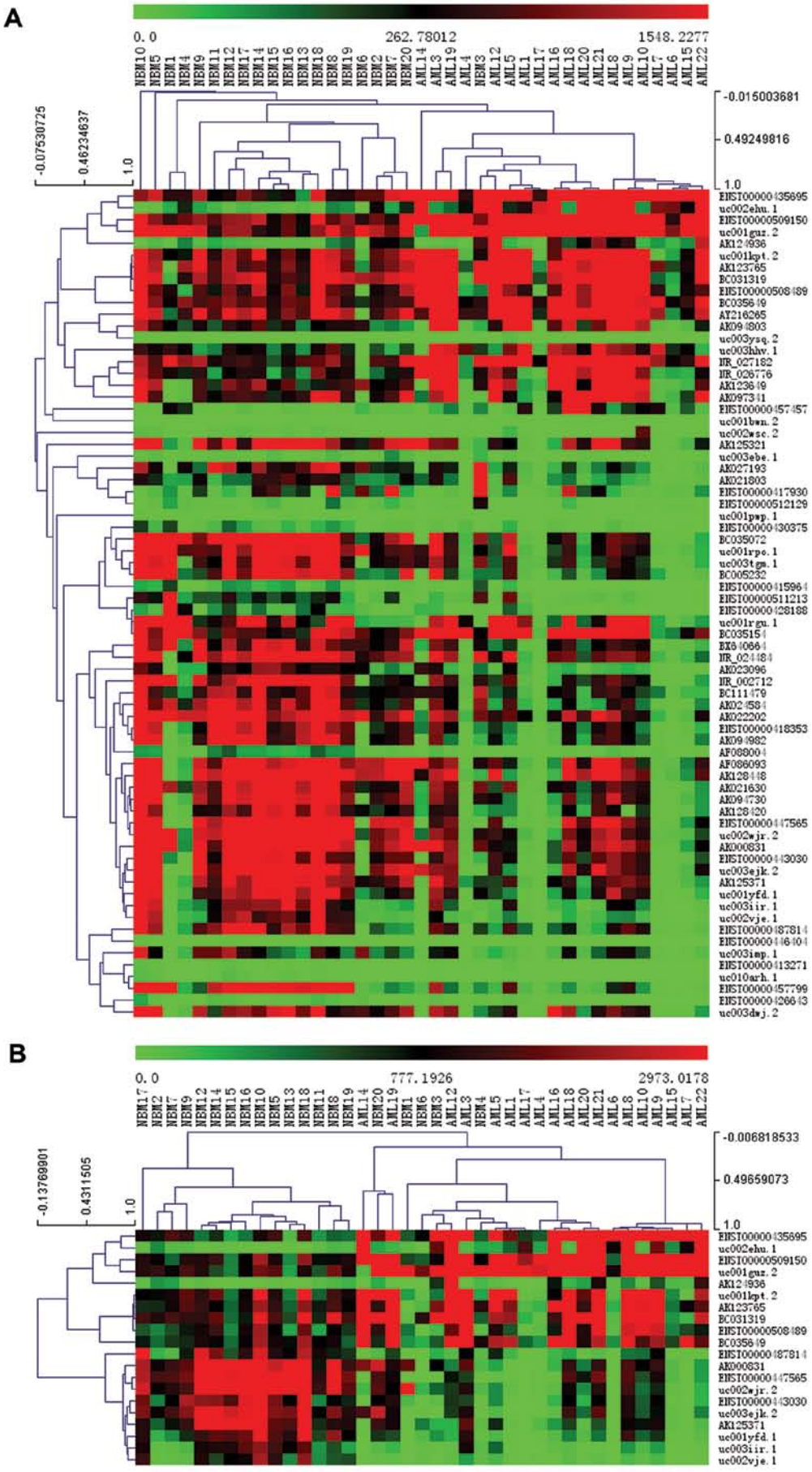

Figure 5. Verification of differentially expressed lncRNAs in pediatric acute myeloid leukemia (AML) vs. normal control samples. (A) Cluster analysis. lncRNA expression between AML samples and normal control samples were analyzed using qRT-PCR and MultiExperiment View (MEV) cluster software. (B) Twenty lncRNAs were successfully clustered between AMLs and normal controls.

Table IV. lncRNAs most dysregulated in the pediatric acute myeloid leukemia compared with normal control.

	Seqname	N	AML	FC	P-value
1	ENST00000435695	991.3334	65836.24647	66.41181	0.001962
2	uc002ehu.1	157.1266	9872.330406	62.83042	0.040465
3	ENST00000509150	1056.795	41764.2482	39.51973	0.026976
4	uc001guz.2	2078.731	76903.56747	36.99544	0.00817
5	ENST00000457457	41.08492	1197.166609	29.13883	0.021802
6	uc001kpt.2	1255.126	25614.56252	20.40796	0.027621
7	NR_027182	878.2717	9092.321414	10.35252	0.008923
8	AK123765	1042.844	9066.866062	8.694369	0.014631
9	BC031319	1911.99	15009.11256	7.849995	0.021456
10	AK124936	104.2268	612.3396304	5.87507	0.017449
11	uc003hhv.1	283.4595	1530.759093	5.400274	0.043802
12	NR_026776	394.2164	2050.126185	5.20051	0.008692
13	AK027193	816.4939	105.3354471	0.129009	0.000302
14	AK024584	1589.484	203.7112857	0.128162	0.000796
15	BC005232	1683.387	213.9596056	0.127101	0.000347
16	AK094982	1550.97	196.5648444	0.126737	0.009100
17	uc003ebe.1	18.21419	2.279349604	0.125141	0.013112
18	ENST00000512129	41.8703	4.359070151	0.104109	0.008417
19	AF088004	63.27258	6.537745743	0.103327	0.000117
20	uc002vje.1	610.3662	48.49298993	0.079449	0.001854
21	uc010arh.1	6.662799	0.504782	0.075761	0.006096
22	ENST00000428188	247.9221	18.21257952	0.073461	0.001850
23	ENST00000457799	2389.248	169.9657438	0.071138	0.000823
24	ENST00000415964	56.81532	3.721491039	0.065502	0.000550

Figs. 6 and 7. The most upregulated lncRNA in pediatric AML is ENST00000435695 and the most downregulated lncRNA in pediatric AML is ENST00000415964.

Discussion

Our present study profiled differentially expressed lncRNAs and mRNAs in pediatric AMLs. We demonstrated for the first time the expression profiles of human lncRNAs in pediatric AML by microarray; and identified a collection of aberrantly expressed lncRNAs in pediatric AML compared to normal controls. It is likely that these dysregulated lncRNAs play a key or partial role in the development and/or progression of pediatric AML. Previous genome-wide profiling studies revealed that many transcribed non-coding ultra conserved regions exhibit distinct profiles in various human cancers. For example, a genome-wide RNA sequencing (RNAseq) analysis evaluated the differential expressions of lncRNAs in a cohort of 102 prostate cancer versus benign samples (27); and identified 121 unannotated transcripts that could accurately discriminate benign, localized and metastatic samples. Other lncRNA expression profile studies were conducted in >100 paired esophageal squamous cell carcinoma and normal samples (28), 5 pairs of liver cancer and normal tissues (29), and 6 pairs of renal clear cell carcinoma and corresponding normal tissues (26); which revealed large numbers of lncRNAs that were significantly dysregulated in cancer tissues. Recent studies have started to reveal the importance of lncRNAs in leukemia tumorigenesis. For example, lncRNA H19 was highly expressed in Bcr-Abl-transformed cell lines and primary cells derived from patients in a Bcr-Abl kinase-dependent manner (23). lncRNA MONC and MIR100HG were highly expressed in acute megakaryoblastic leukemia blasts (24). However, it remains to be determined how these lncRNAs participate and contribute to leukemia development or progression. In this study, we found that some of these differentially expressed lncRNAs regulated cell cycle progression and immunosystem functions. Future study is required to investigate whether manipulating lncRNA expressions could control AML progression and be used as a therapeutic target to control AML.

Our present study profiled and identified a group of dysregulated lncRNAs and mRNAs in bone marrow samples obtained from pediatric AML patients versus normal controls. We also verified 97 lncRNAs in >20 AML and normal control samples; and found 24 dysregulated lncRNAs in pediatric AML. These lncRNAs could regulate cell cycle progression and immunoresponses that could be associated with AML development or progression. Further study is required to determine whether these lncRNAs may serve as new therapeutic targets and diagnostic markers for pediatric AML.

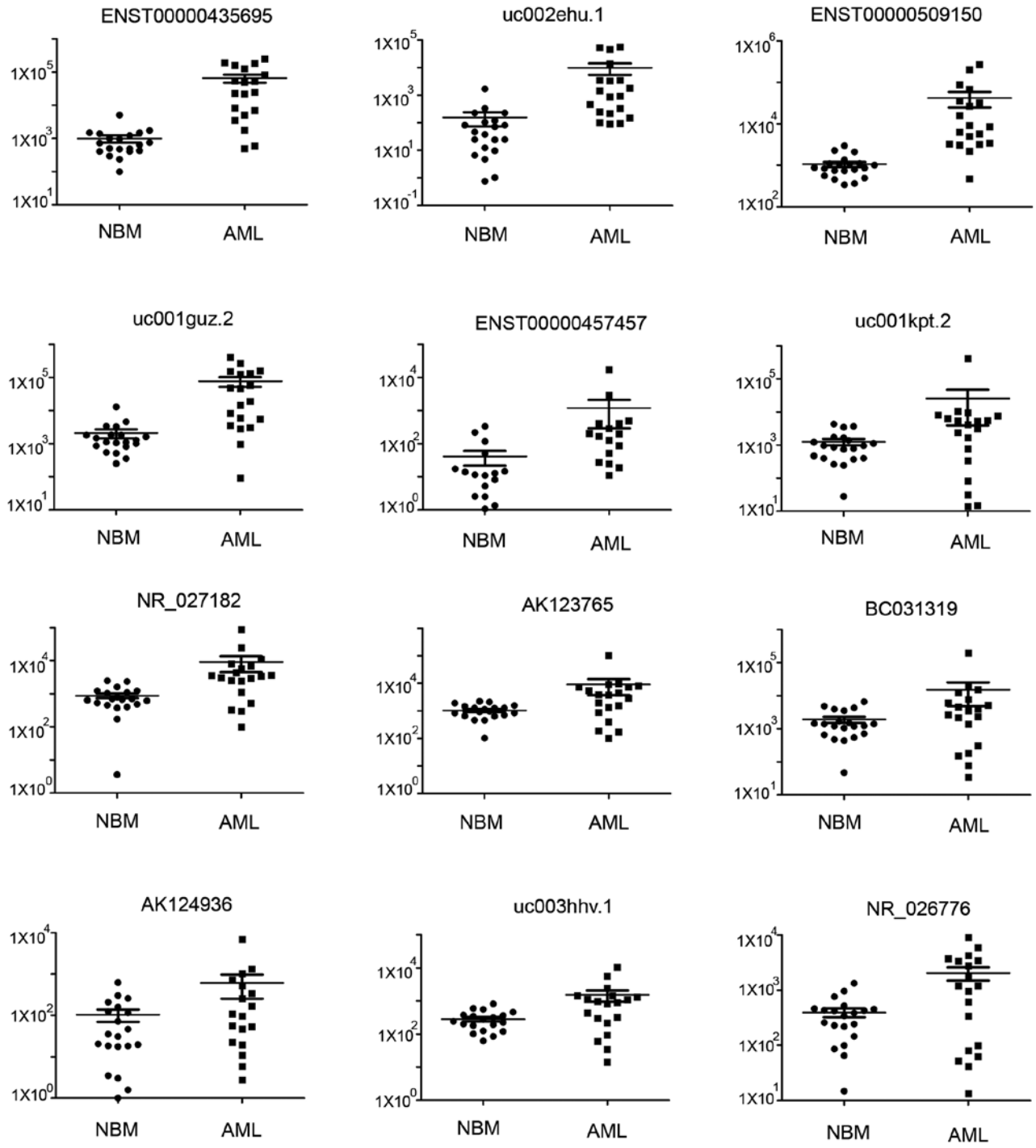

Figure 6. Verification of upregulated lncRNA expression in pediatric acute myeloid leukemia (AML). Expression of pediatric AML samples vs. control samples was analyzed using qRT-PCR, and summarized as mean average ± standard error (SE). P<0.05 was considered statistically significant.

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References

1. Appelbaum FR, Baer MR, Carabasi MH, Coutre SE, Erba HP, Estey E, Glenn MJ, Kraut EH, Maslak P, Millenson M, *et al*; National Comprehensive Cancer Network: NCCN practice guidelines for acute myelogenous leukemia. Oncology (Williston Park) 14: 53-61, 2000.

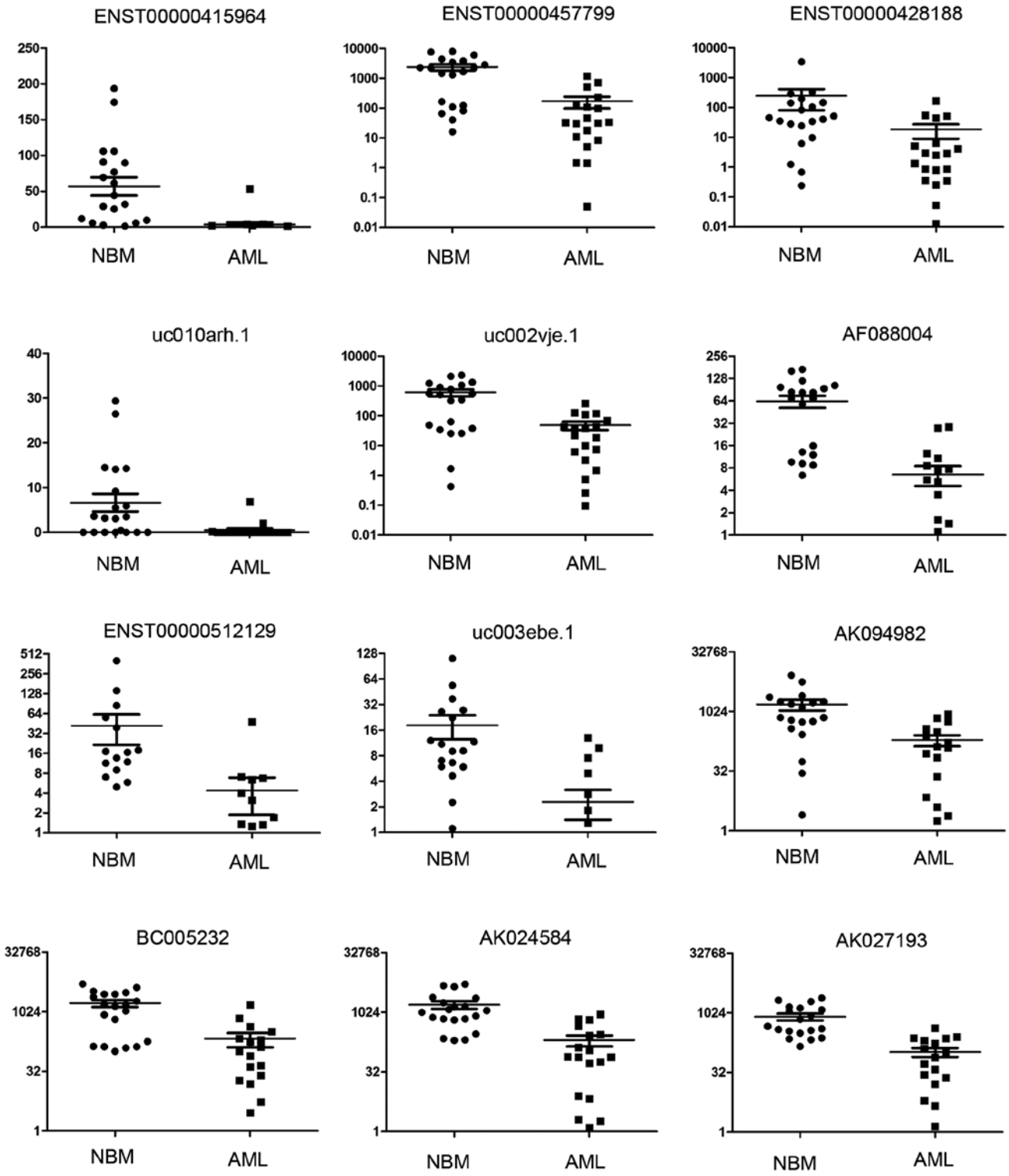

Figure 7. Verification of downregulated lncRNA expression in pediatric acute myeloid leukemia (AML). Expression of pediatric AML samples vs. control samples were analyzed using qRT-PCR, and summarized as mean ± standard error (SE). P<0.05 was considered statistically significant.

2. Sanz GF, Sanz MA, Vallespí T, Cañizo MC, Torrabadella M, García S, Irriguible D and San Miguel JF: Two regression models and a scoring system for predicting survival and planning treatment in myelodysplastic syndromes: A multivariate analysis of prognostic factors in 370 patients. Blood 74: 395-408, 1989.
3. Hope KJ, Jin L and Dick JE: Human acute myeloid leukemia stem cells. Arch Med Res 34: 507-514, 2003.
4. Hasle H, Niemeyer CM, Chessells JM, Baumann I, Bennett JM, Kerndrup G and Head DR: A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leukemia 17: 277-282, 2003.
5. Song X, Wang X, Arai S and Kurokawa R: Promoter-associated noncoding RNA from the CCND1 promoter. Methods Mol Biol 809: 609-622, 2012.
6. Zhang X, Lian Z, Padden C, Gerstein MB, Rozowsky J, Snyder M, Gingeras TR, Kapranov P, Weissman SM and Newburger PE: A myelopoiesis-associated regulatory intergenic noncoding RNA transcript within the human HOXA cluster. Blood 113: 2526-2534, 2009.
7. Rinn JL: lncRNAs: Linking RNA to chromatin. Cold Spring Harb Perspect Biol 6: 6, 2014.

8. Rinn JL and Chang HY: Genome regulation by long noncoding RNAs. Annu Rev Biochem 81: 145-166, 2012.
9. Chen Z, Jia S, Li D, Cai J, Tu J, Geng B, Guan Y, Cui Q and Yang J: Silencing of long noncoding RNA AK139328 attenuates ischemia/reperfusion injury in mouse livers. PLoS One 8: e80817, 2013.
10. Yin Z, Guan D, Fan Q, Su J, Zheng W, Ma W and Ke C: lncRNA expression signatures in response to enterovirus 71 infection. Biochem Biophys Res Commun 430: 629-633, 2013.
11. Kung JT, Colognori D and Lee JT: Long noncoding RNAs: Past, present, and future. Genetics 193: 651-669, 2013.
12. Fu X, Ravindranath L, Tran N, Petrovics G and Srivastava S: Regulation of apoptosis by a prostate-specific and prostate cancer-associated noncoding gene, PCGEM1. DNA Cell Biol 25: 135-141, 2006.
13. Gutschner T, Hämmerle M and Diederichs S: MALAT1 - a paradigm for long noncoding RNA function in cancer. J Mol Med Berl 91: 791-801, 2013.
14. Schmidt LH, Spieker T, Koschmieder S, Schäffers S, Humberg J, Jungen D, Bulk E, Hascher A, Wittmer D, Marra A, *et al*: The long noncoding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth. J Thorac Oncol 6: 1984-1992, 2011.
15. Li G, Zhang H, Wan X, Yang X, Zhu C, Wang A, He L, Miao R, Chen S and Zhao H: Long noncoding RNA plays a key role in metastasis and prognosis of hepatocellular carcinoma. Biomed Res Int 2014: 780521, 2014.
16. He Y, Meng XM, Huang C, Wu BM, Zhang L, Lv XW and Li J: Long noncoding RNAs: Novel insights into hepatocelluar carcinoma. Cancer Lett 344: 20-27, 2014.
17. Lai MC, Yang Z, Zhou L, Zhu QQ, Xie HY, Zhang F, Wu LM, Chen LM and Zheng SS: Long non-coding RNA MALAT-1 overexpression predicts tumor recurrence of hepatocellular carcinoma after liver transplantation. Med Oncol 29: 1810-1816, 2012.
18. Tsang WP, Ng EK, Ng SS, Jin H, Yu J, Sung JJ and Kwok TT: Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer. Carcinogenesis 31: 350-358, 2010.
19. Ariel I, Miao HQ, Ji XR, Schneider T, Roll D, de Groot N, Hochberg A and Ayesh S: Imprinted H19 oncofetal RNA is a candidate tumour marker for hepatocellular carcinoma. Mol Pathol 51: 21-25, 1998.
20. Lottin S, Vercoutter-Edouart AS, Adriaenssens E, Czeszak X, Lemoine J, Roudbaraki M, Coll J, Hondermarck H, Dugimont T and Curgy JJ: Thioredoxin post-transcriptional regulation by H19 provides a new function to mRNA-like non-coding RNA. Oncogene 21: 1625-1631, 2002.
21. Luo M, Li Z, Wang W, Zeng Y, Liu Z and Qiu J: Upregulated H19 contributes to bladder cancer cell proliferation by regulating ID2 expression. FEBS J 280: 1709-1716, 2013.
22. Luo M, Li Z, Wang W, Zeng Y, Liu Z and Qiu J: Long non-coding RNA H19 increases bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression. Cancer Lett 333: 213-221, 2013.
23. Guo G, Kang Q, Chen Q, Chen Z, Wang J, Tan L and Chen JL: High expression of long non-coding RNA H19 is required for efficient tumorigenesis induced by Bcr-Abl oncogene. FEBS Lett 588: 1780-1786, 2014.
24. Emmrich S, Streltsov A, Schmidt F, Thangapandi VR, Reinhardt D and Klusmann JH: LincRNAs MONC and MIR100HG act as oncogenes in acute megakaryoblastic leukemia. Mol Cancer 13: 171, 2014.
25. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR and Sultan C: Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. Br J Haematol 33: 451-458, 1976.
26. Yu G, Yao W, Wang J, Ma X, Xiao W, Li H, Xia D, Yang Y, Deng K, Xiao H, *et al*: LncRNAs expression signatures of renal clear cell carcinoma revealed by microarray. PLoS One 7: e42377, 2012.
27. Prensner JR and Chinnaiyan AM: The emergence of lncRNAs in cancer biology. Cancer Discov 1: 391-407, 2011.
28. Li J, Chen Z, Tian L, Zhou C, He MY, Gao Y, Wang S, Zhou F, Shi S, Feng X, *et al*: LncRNA profile study reveals a three-lncRNA signature associated with the survival of patients with oesophageal squamous cell carcinoma. Gut 63: 1700-1710, 2014.
29. Yang F, Zhang L, Huo XS, Yuan JH, Xu D, Yuan SX, Zhu N, Zhou WP, Yang GS, Wang YZ, *et al*: Long noncoding RNA high expression in hepatocellular carcinoma facilitates tumor growth through enhancer of zeste homolog 2 in humans. Hepatology 54: 1679-1689, 2011.