Genome-wide profiling of lncRNA expression patterns in patients with acute promyelocytic leukemia with differentiation therapy

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Abstract. Long non-coding RNAs (lncRNAs) are crucial factors in acute promyelocytic leukemia (APL) cell differentiation. However, their expression patterns and regulatory functions during all-*trans*-retinoic acid (ATRA)-induced APL differentiation remain to be fully elucidated. The profile of dysregulated lncRNAs between three bone marrow (BM) samples from patients with APL post-induction and three BM samples from untreated matched controls was examined with the Human Transcriptome Array 2.0. The dysregulated lncRNA expression of an additional 27 APL BM samples was validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The lncRNA functions were predicted through co-expressed messenger RNA (mRNA) annotations. Co-expressed lncRNA-mRNA networks were constructed to analyze the functional pathways. In total, 825 lncRNAs and 1,218 mRNAs were dysregulated in the treated APL BM group, compared with the untreated APL BM group. The expression of 10 selected lncRNAs was verified by RT-qPCR analysis. During APL differentiation, NONHSAT076891 was the most upregulated lncRNA, whereas TCONS_00022632-XLOC_010933 was the most downregulated. Functional analysis revealed that several lncRNAs may exert activities in biological pathways associated with ATRA-induced APL differentiation through *cis* and/or *trans* regulation of mRNAs. The findings of the present study assist in explaining the contributions of lncRNAs in APL myeloid differentiation and improve current knowledge on the potential mechanisms regarding dysregulated lncRNA expression in ATRA-induced APL differentiation.

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Abbreviations: APL, acute promyelocytic leukemia; lncRNAs, long non-coding RNAs; ATRA, all-*trans*-retinoic acid; mRNA, messenger RNA; BM, bone marrow; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ATO, arsenic trioxide; AML, acute myeloid leukemia; MBMCs, mononuclear bone marrow cells; TFs, transcription factors; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; EEA1, early endosome antigen 1; ZNF564, zinc finger protein 564; ZNF44, zinc finger protein 44; E2F1, E2F transcription factor 1; EBF1, early B cell factor 1; E2F6, E2F transcription factor 6

Key words: acute promyelocytic leukemia, long non-coding RNAs, differentiation therapy, expression pattern

Introduction

Acute promyelocytic leukemia (APL) is distinguished by a specific t(15;17) chromosomal translocation, contributing to the expression of the oncoprotein PML-RARα, which counteracts myeloid differentiation and facilitates APL-initiating cell self-renewal (1,2). The dominant-negative effect of the PML-RARα oncoprotein, which antagonizes the myeloid differentiation process, can be reversed by pharmacological doses of all-*trans*-retinoic acid (ATRA) (3). The combined therapy of ATRA and arsenic trioxide (ATO), which eliminates APL by activating PML-RARα degradation, demonstrates efficacy as an APL therapy and markedly improves the prognosis of patients with APL (4). However, ATO and ATRA induce irreversible resistance, and certain patients with APL ultimately succumb to treatment-resistant diseases (5,6). Therefore, the unifying mechanisms required for myeloid differentiation and response to therapy in APL require further investigation.

Long non-coding RNAs (lncRNAs), which are in excess of 200 nucleotides, are involved in diverse biological

processes including epigenetic regulation, chromosome imprinting, transcription, splicing, translation, cell-cycle control, and differentiation (7). They have been functionally coupled to cancer and cellular differentiation. The investigation of lncRNA expression and function may contribute to the current understanding of leukemogenesis, and the identification of novel therapeutic targets and seminal posttranscriptional factors associated with resistance to chemotherapy. Consequently, the global aberrant expression of lncRNAs that occurs during myeloid differentiation can assist in enhancing the efficacy of current APL therapies and identifying novel therapeutic targets for chemotherapies. lncRNAs have been considered as prognostic and diagnostic molecular biomarkers for acute myeloid leukemia (AML) (8,9). Previous investigations have demonstrated that lncRNAs are crucial in myeloid differentiation and APL therapy (10-12). HOTAIRM1, a myeloid-specific lncRNA, has been defined as a key factor in ATRA-induced APL differentiation (11,13). Furthermore, HOTAIRM1 can promote the degradation of PML-RARα via a pathway associated with autophagy during myeloid cell differentiation or ATRA-induced APL differentiation (10). This suggests that aberrant lncRNA expression may be underlying targets for APL therapy and indicators for response to APL therapy. Several lncRNAs have been identified in APL-associated myeloid differentiation (10-13); however, additional key lncRNAs and their functions in regulating myeloid maturation require characterization in the APL-associated myeloid differentiation transcriptome.

NB4 cells represent a suitable cell model to investigate changes in lncRNA expression between APL cells and their terminally differentiated counterparts. System analysis of the transcriptome has been beneficial for the identification of various pathways or cascades at the transcriptome level associated with ATRA/ATO-induced cell differentiation (14). These are common in the absence of the complicated and dynamic intracorporeal synergy between ATRA and ATO in APL therapy, which may affect cell survival and response to therapy. Although it has been suggested that lncRNAs are involved in ATRA/ATO-induced extracorporeal APL differentiation, further understanding of the lncRNA landscape in ATRA/ATO-induced intracorporeal APL differentiation is required.

The aim of the present study was to examine lncRNA profiles and regulatory functions in ATRA-based targeted therapy for APL differentiation. The lncRNA and messenger RNA (mRNA) profiles were compared in three post-induction bone marrow (BM) samples from patients with APL and pre-induction (untreated) matched controls via whole transcriptome microarray. Subsequently, 10 dysregulated lncRNAs were selected and verified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis in another 27 APL BM samples. In addition, the functions of dysregulated lncRNAs were predicted via their co-expressed mRNAs. The findings identified the lncRNA landscape for myeloid differentiation in APL and revealed potential mechanisms occurring due to dysregulated lncRNA expression in ATRA-induced APL differentiation; this may provide underlying targets for APL therapy and lncRNA biomarkers for APL responses to therapy.

Table I. Primary characteristics of patients with APL.

Characteristic	n (%)
Patients	30
Sex	
Male	18 (60.0)
Female	12 (40.0)
Age (years)	
Median (range)	40 (19-67)
WHO classification	
APL with t(15;17)(q22;q12); PML-RARa	30 (100.0)
Transcripts of PML-RARa	30 (100.0)
PML-RARa bcr1	18 (60.0)
PML-RARa bcr2	1 (3.3)
PML-RARa bcr3	11 (36.7)
Received ATRA-based therapy	30 (100.0)
Paired BM post-induction obtained	30 (100.0)

APL, acute promyelocytic leukemia; BM, bone marrow; WHO, World Health Organization; lncRNA, long non-coding RNA.

Materials and methods

Patient profiles. A total of 30 patients with APL who received ATRA-based targeted therapy at the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (Wenzhou, China) between January 2014 and December 2016 were recruited for the present study. None of the patients received chemotherapy prior to targeted therapy. Written informed consent was collected from all the patients in conformity with the Declaration of Helsinki, and the present study obtained permission from the Ethics Committee of Wenzhou Medical University. The BM samples from the patients with APL at diagnosis and corresponding BM samples from patients with APL post-induction were collected in BD Vacutainer Heparin tubes (BD Biosciences, San Diego, CA, USA). Patient diagnoses were determined according to the 2016 World Health Organization criteria (15). The patients with APL were treated according to the International APL guidelines (15). Among the 60 samples, three paired samples (comprising three post-induction samples and three pre-induction samples) were used for lncRNA microarray analysis and the other samples were used for RT-qPCR analysis. The primary characteristics of the patients with APL are listed in Table I.

Sample collection and RNA extraction. The mononuclear BM cells (MBMCs) were isolated from ~2 ml heparinized BM samples using density gradient medium centrifugation (800 x g, for 20 min at room temperature). Subsequently, $1x10^7$ cells were resuspended in TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and temporarily stored at -80°C until further analysis. Total RNA was extracted from the MBMCs according to the manufacturer's protocol and dissolved in 100 μl nuclease-free water. The RNA yield was measured using a NanoDrop ND-2000

Table II. Reverse transcription-quantitative polymerase chain reaction primers for lncRNAs.

lncRNA primary ID	Primer sequence (5'-3')	Product (bp)
NONHSAT121385	Forward: TACATGTTCCTGGTGAAAT Reverse: GGCATCAAGTATGTCTCT	108
ENST00000469418	Forward: CAATTTACGGCTGGACGTTT Reverse: GAAAGGAATGCTGGGAAACA	128
ENST00000424415	Forward: ATATTGAGATAGGAGGATGG Reverse: GGCTTCTTCTAGGATAAGT	107
NR_003186	Forward: TACATGTTCCTGGTGAAAT Reverse: ATCTTTGGGCATCAAGTA	115
ENST00000536425	Forward: TTGAATAATCCTAAATTATACATAC Reverse: TCATAGTGACTAAATTGAATAAGTACCAAA	78
NONHSAT061249	Forward: AGGATCGCTTGAGATGCAGT Reverse: GCTACCGCTCTCAAGTTTGG	110
TCONS_00017553-XLOC_008249	Forward: GTGTCTGTGTGTACAGAA Reverse: ACATTCCATACACACAAAC	187
TCONS_l2_00030950-XLOC_l2_015963	Forward: GTTGGAAGATGAAGGAAC Reverse: ATCACTGTGTAAAGGACTA	114
NONHSAT076891	Forward: GGATCTCCCCTGTGTTCTCA Reverse: GACCAGGTAGTGGGGGAAGT	146
TCONS_00022632-XLOC_010933	Forward: TCTTCCACGTAACAACCA Reverse: CTGACAGTGTCTTCCATA	124

lncRNA, long non-coding RNA.

spectrophotometer from Thermo Fisher Scientific, Inc. and the RNA integrity was assessed using an Agilent 2100 bioanalyzer system (Agilent Technologies, Inc., Santa Clara, CA, USA). When the 28S:18S ratio was ascertained and the RNA integrity number (RIN) of each sample was assigned; RNA samples with a 28S:18S ratio ≥0.7 and RIN ≥7.0 were further analyzed.

lncRNA and mRNA microarray expression profiling. RNA from each sample (~200 ng) was applied for lncRNA and mRNA microarray analyses using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). Gene expression was analyzed using an Affymetrix GeneChip® Human Transcriptome Array 2.0 (Affymetrix, Inc., Santa Clara, CA, USA). The microarray contained 67,539 probes for 22,829 human lncRNAs and 44,710 human mRNAs, which were derived from eight authoritative databases, including RefSeq, Ensembl, UCSC, MGC, nocode, lncRNAdb, Broad Institute, TUCP catalogue and Human Body Map lincRNAs. Additionally, the microarray contained probes for small non-coding RNAs, but not microRNAs, and the majority of these were small nuclear RNAs and small nucleolar RNAs. The array experiments and computational analysis were performed according to the manufacturer's protocol (Affymetrix, Inc.). The raw data were extracted and standardized using the GeneChip Command Console software 4.0 and Expression Console software 1.3.1 from Affymetrix, Inc. Additional data processing was performed with GeneSpring software 12.5

(Agilent Technologies, Inc.). Dysregulated lncRNAs or mRNAs defined by an absolute value of fold change (FC) ≥2.0 and P≤0.05 (Student's t-test) were selected for further analysis. The dysregulated mRNAs were categorized into different Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation groups. The lncRNA chip experiments were performed in the laboratory at Shanghai OE Biotech Co., Ltd. (Shanghai, China).

RT-qPCR verification of 10 dysregulated lncRNAs. The lncRNA and mRNA expression were analyzed using FastStart Universal SYBR-Green Master Mix (Rox) from Roche Diagnostics (Indianapolis, IN, USA) and an ABI ViiA™ 7 Real-Time PCR system from Applied Biosystems (Thermo Fisher Scientific, Inc.). Briefly, the total RNA was transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) as per the manufacturer's protocol. PCR amplification was performed in a 25 μl reaction containing 1 μl of cDNA template (~10 ng), 12.5 μl of FastStart Universal SYBR-Green Master Mix (Rox), 10.5 μl of nuclease-free water, and 0.5 μl of each pair of primers (Shanghai GeneCore BioTechnologies Co., Ltd., Shanghai, China). The RT-qPCR primers for the lncRNAs are listed in Table II. The reaction conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 45 sec. All experiments were repeated three times in parallel. The expression of lncRNAs was further standardized to the *GAPDH* gene and calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Microarray results analysis and functional prediction of selected dysregulated lncRNAs. The identification of the overall functional distributions for the dysregulated lncRNAs identified in the experiment were performed as follows. Briefly, the co-expressed mRNAs for each dysregulated lncRNA were first calculated, and functional enrichment analysis for the set of co-expressed mRNAs was then performed. The enriched functional terms served as the predicted functional term for a given lncRNA. Furthermore, the co-expressed mRNAs of lncRNAs were identified by calculating the Pearson's correlation (P<0.05). The functional enrichment terms for annotating the co-expressed mRNAs were determined using the hypergeometric cumulative distribution function (17,18). The top 200 reliable prediction associations between the lncRNAs and the predicted functional terms were selected to reflect the functional distribution of the dysregulated lncRNAs. The frequency of each predicted functional term for these associations was determined, following which the GO term and KEGG term with more functional annotations were statistically identified (19).

Identification of cis-regulated mRNAs for the dysregulated lncRNAs. The present study further examined how the dysregulated lncRNAs may exert activities through *cis*- and/or *trans*-regulated mRNAs. The regions of *cis*-regulation were identified as follows: Gene locations for different lncRNAs on the chromosome were determined; for each dysregulated lncRNA, the mRNAs were identified as *cis*-regulated mRNAs when the co-expressed mRNA loci were within 300 kbp downstream or upstream of the given lncRNA and the Pearson's correlation of lncRNA-mRNA expression was significant at the P<0.05 level. When the mRNAs did not conform to the *cis*-regulated mRNA rules, they were identified as possible *trans*-regulated mRNAs.

Identification of transcription factors associated with dysregulated lncRNAs. It has been documented that specific lncRNAs may be involved in certain biological processes, including transcriptional regulation, through key transcription factors (TFs) (17). Therefore, the TF/chromatin regulation complexes that may have critical co-regulatory roles with lncRNAs were identified (18,20). Briefly, the set of co-expressed mRNAs for lncRNA and TF/chromatin regulation complex target genes was determined, the enrichment level of which was analyzed using the hypergeometric distribution. Therefore, the TFs prominently associated with dysregulated lncRNAs were finally determined. The lncRNA-TF network was established using the hypergeometric distribution, and a graph showing the associations between TFs and lncRNAs was drawn using Cytoscape software (version 3.6.1; http://www.cytoscape.org/), an open source software platform. In the network, the core TF with the highest degree of expression was considered the centre of highest importance.

Results

General expression profiles of dysregulated lncRNAs and mRNAs. To investigate the lncRNA landscape involved in ATRA-induced APL differentiation, lncRNA and mRNA microarray analyses were performed on BMMCs from patients with APL. The microarray data were filtered through a volcano plot to determine the dysregulated lncRNAs and mRNAs in BM samples from patients with APL post-induction and corresponding BM samples from patients with APL at diagnosis (Fig. 1A). The lncRNA and mRNA expression data were clustered using Cluster 3.0 (Fig. 1B). Based on the similar expression patterns, the samples were further classified into two groups using dendrogram-based methods for clustering. It was found that 825 lncRNAs were dysregulated between the patients with APL post-induction and the matched controls at diagnosis, with 410 upregulated and 415 downregulated lncRNAs (Fig. 1C). Among the dysregulated lncRNAs, NONHSAT076891 was upregulated the most, with an FC of 304.00, whereas TCONS_00022632-XLOC_010933 was downregulated the most, with an FC of 447.09. It was also found that 1,218 mRNAs were dysregulated, with 660 upregulated and 558 downregulated mRNAs (Fig. 1C). The most upregulated and downregulated mRNAs were MPEG1 and CYTL1, with FCs of 257.13 and 1,169.37, respectively. The top 20 upregulated and downregulated lncRNAs and mRNAs are listed in Table III.

Verification of dysregulated expression of lncRNAs and mRNAs. To verify the dysregulated expression of lncRNAs and mRNAs associated with ATRA-induced APL differentiation, RT-qPCR analysis was performed to examine the upregulation or downregulation of the genes. A total of 10 dysregulated lncRNA transcripts were selected for RT-qPCR analysis. These 10 lncRNAs consisted of four randomly selected lncRNAs, including NONHSAT121385, ENST00000469418, ENST00000424415, and NR_003186, and six specifically selected lncRNAs, including ENST00000536425, NONHSAT061249, TCONS_00017553-XLOC_008249, TCONS_l2_00030950-XLOC_l2_015963, NONHSAT076891, and TCONS_00022632-XLOC_010933. ENST00000536425 and NONHSAT061249 were selected as a result of their predicted *cis*-regulating potential. TCONS_00017553-XLOC_008249 and TCONS_l2_00030950-XLOC_l2_015963 were selected due to their predicted *trans*-regulating potential and their presence among the top 100 lncRNA-TF pairs. NONHSAT076891 and TCONS_00022632-XLOC_010933 were selected as they were the most upregulated and downregulated, respectively (Table III). The RT-qPCR results were in agreement with the results obtained from the microarray chip analysis (Fig. 2). As shown in Fig. 2, all selected lncRNAs were dysregulated and exhibited the same trend of upregulation or downregulation (P<0.05 for each lncRNA, Student's t-test).

lncRNA and mRNA co-expression profiles and lncRNA function prediction. Several hundred lncRNAs were co-expressed with hundreds of mRNAs. For example, ENST00000419668 was co-expressed with 515 mRNAs and TCONS_00022632-XLOC_010933 with 488 mRNAs. The GO and KEGG pathway annotations of the co-expressed mRNAs were used to predict the functions of the dysregulated lncRNAs. The lncRNAs were clustered into hundreds of GO and KEGG terms with more functional annotations. In the corresponding association between the 'lncRNA name'
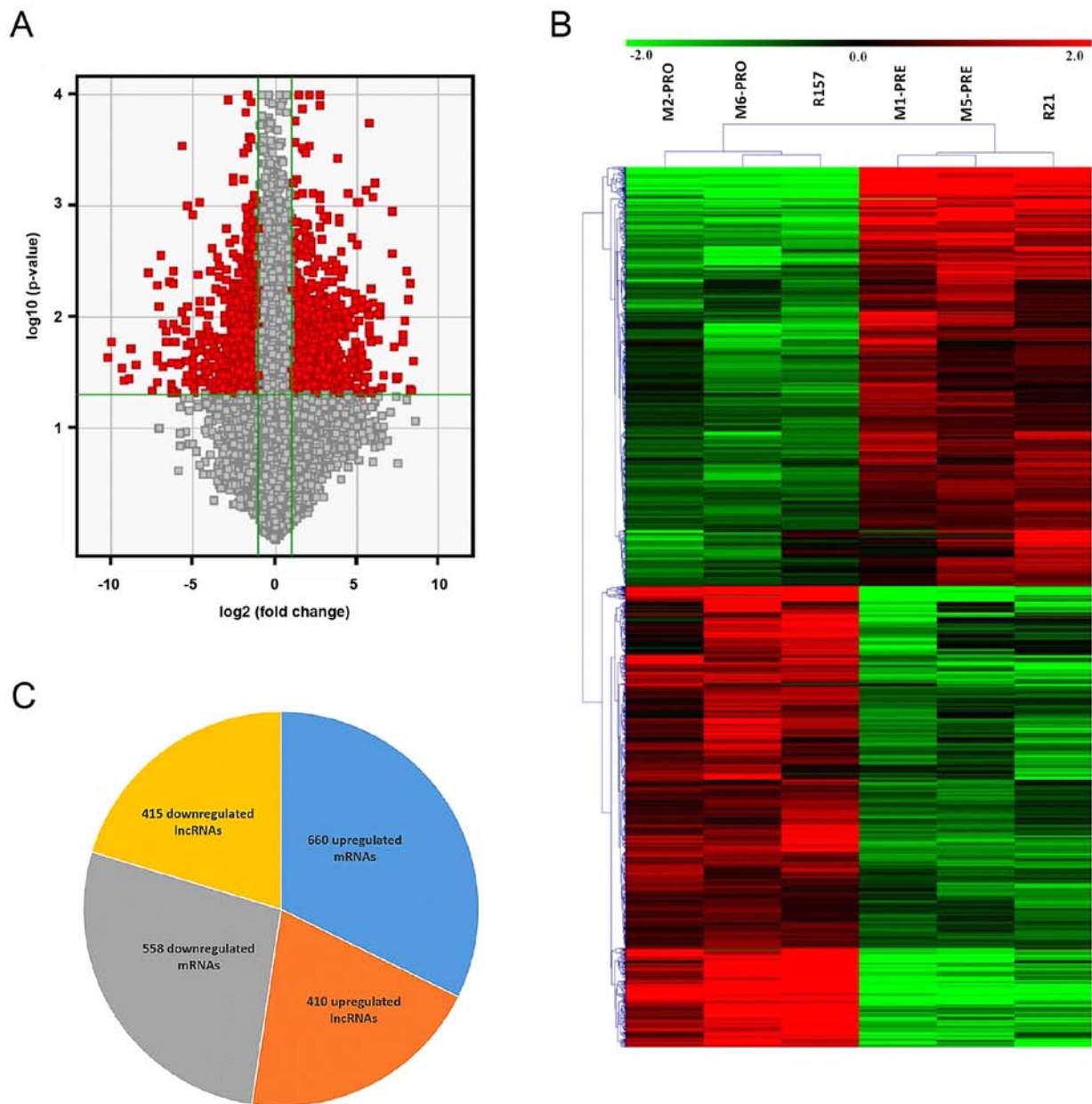
Figure 1. Profiles of dysregulated lncRNAs and mRNAs in BM samples from patients with APL post-induction and corresponding BM samples from patients with APL at diagnosis (pre-induction). (A) Volcano plot of the dysregulated lncRNAs and mRNAs. (B) Heat map of dysregulated lncRNAs and mRNAs. (C) Numbers of common upregulated or downregulated lncRNAs and mRNAs. lncRNAs, long non-coding RNAs; mRNAs, messenger RNAs; BM, bone marrow; APL, acute promyelocytic leukemia.

and 'functional prediction term', the top 200 predicted associations were selected to reflect the functional distribution of the dysregulated lncRNAs. Among the GO terms, the most common biological processes for the dysregulated lncRNAs were cell cycle phase transition, regulation of spindle checkpoint, negative regulation of viral genome replication, DNA damage checkpoint, and attachment of spindle microtubules to kinetochore (Fig. 3A). The most common KEGG terms were DNA replication, spliceosome, NF-κB signalling pathway, mismatch repair, primary immunodeficiency, nucleotide excision repair, and cell cycle (Fig. 3B). Therefore, according to the enrichment, cell cycle phase transition was the most enriched GO term and DNA replication was the most enriched KEGG term (Fig. 3).

Analysis of 'cis' lncRNAs and their adjacent co-expressed mRNAs. Evidence shows that a number of lncRNAs may *cis*-regulate the transcription of themselves and their adjacent mRNAs by recruiting remodelling factors to local chromatin (21). To determine the potential '*cis*' mRNAs associated with a specific lncRNA, the co-expressed mRNAs 300 kb upstream and downstream of the dysregulated lncRNAs were analyzed. In total, 48 lncRNAs and their predictively *cis*-regulated mRNAs were identified using accurate genomic mapping based on the criteria mentioned above. The lncRNAs and their underlying *cis*-regulated mRNAs are listed in Table IV. The results suggested that lncRNA ENST00000536425 *cis*-regulates one mRNA, early endosome antigen 1 (EEA1) (Fig. 4A), whereas

Table III. Top 20 upregulated and downregulated lncRNAs and mRNAs in patients with APL with induction therapy.

Upregulated lncRNAs	FC (abs)	Downregulated lncRNAs	FC (abs)	Upregulated mRNAs	FC (abs)	Downregulated mRNAs	FC (abs)
NONHSAT076891	304.001	TCONS_00022632-XLOC_010933	447.093	MPEG1	257.128	CYTL1	1,169.366
NONHSAT066562	301.188	ENST00000419668	145.949	LRRK2	235.480	HGF	357.526
NONHSAT104550	272.525	NONHSAT032682	141.184	PLBD1	223.158	CPA3	206.801
NONHSAT121385	86.674	TCONS_00022633-XLOC_010934	79.771	P2RY13	220.328	FLT3	119.563
NR_003186	85.927	ENST00000429159	63.192	CLEC7A	165.126	CFD	82.293
NR_003187	63.356	NR_034105	52.276	VCAN	163.269	KIT	77.132
NR_028324	53.106	NR_003611	44.230	FGL2	157.751	PRSS57	75.924
TCONS_12_00031106-XLOC_12_016008	47.907	NONHSAT021421	43.840	RAB31	146.390	CCNA1	75.130
NR_039983	46.826	TCONS_00008717-XLOC_004231	43.062	IFI30	138.929	ITM2A	73.741
ENST00000469418	42.355	ENST00000545120	40.173	VNN2	101.935	MAMDC2	40.283
TCONS_00028171-XLOC_013531	41.054	ENST00000579458	36.424	S100A12	92.761	STAB1	35.361
NONHSAT144652	38.665	ENST00000535351	34.801	C5AR1	81.921	GTSF1	33.527
NONHSAT060114	38.653	ENST00000423708	32.984	SAMHD1	65.141	RFX8	31.113
NONHSAT097386	35.242	TCONS_12_00004167-XLOC_12_001953	30.059	HLA-DRA	63.702	MMP2	26.319
NONHSAT051747	32.846	ENST00000413504	24.286	NCF2	59.800	TDRD9	24.590
NONHSAT150916	32.737	ENST00000451267	24.286	GLT1D1	57.236	UGT3A2	24.494
NONHSAT120680	27.076	ENST00000426063	21.941	STX11	55.971	ZNF711	20.705
NONHSAT098146	21.665	TCONS_12_00003557-XLOC_12_001971	21.569	IGF2R	54.459	BLID	16.816
TCONS_00013664-XLOC_006324	19.262	TCONS_12_00003558-XLOC_12_001972	19.349	FLJ45445	53.106	CALR	16.644
NONHSAT102906	17.430	ENST00000514473	19.153	CEACAM1	50.279	GABRE	15.980

FC (abs), absolute fold change (APL pre-induction therapy set as 1); lncRNAs, long non-coding RNAs; APL, acute promyelocytic leukemia.
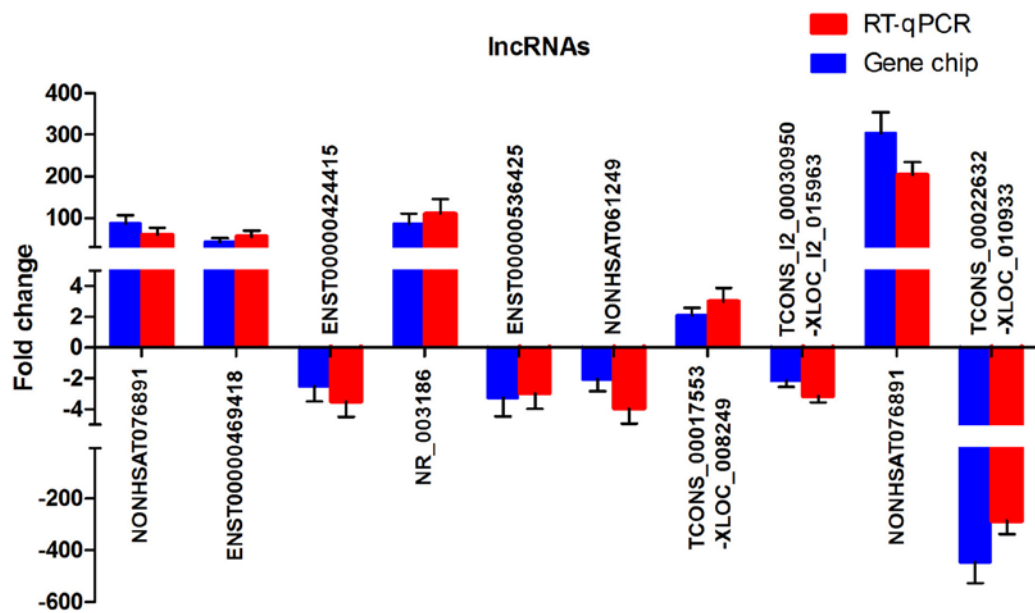
Figure 2. RT-qPCR verification of 10 selected lncRNAs in an additional 27 samples from patients with APL, including 27 post-induction BM samples and 27 pre-induction BM samples. lncRNA expression was consistent with the microarray data (P<0.05, Student's t-test). lncRNAs, long non-coding RNAs; APL, acute promyelocytic leukemia; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; BM, bone marrow.
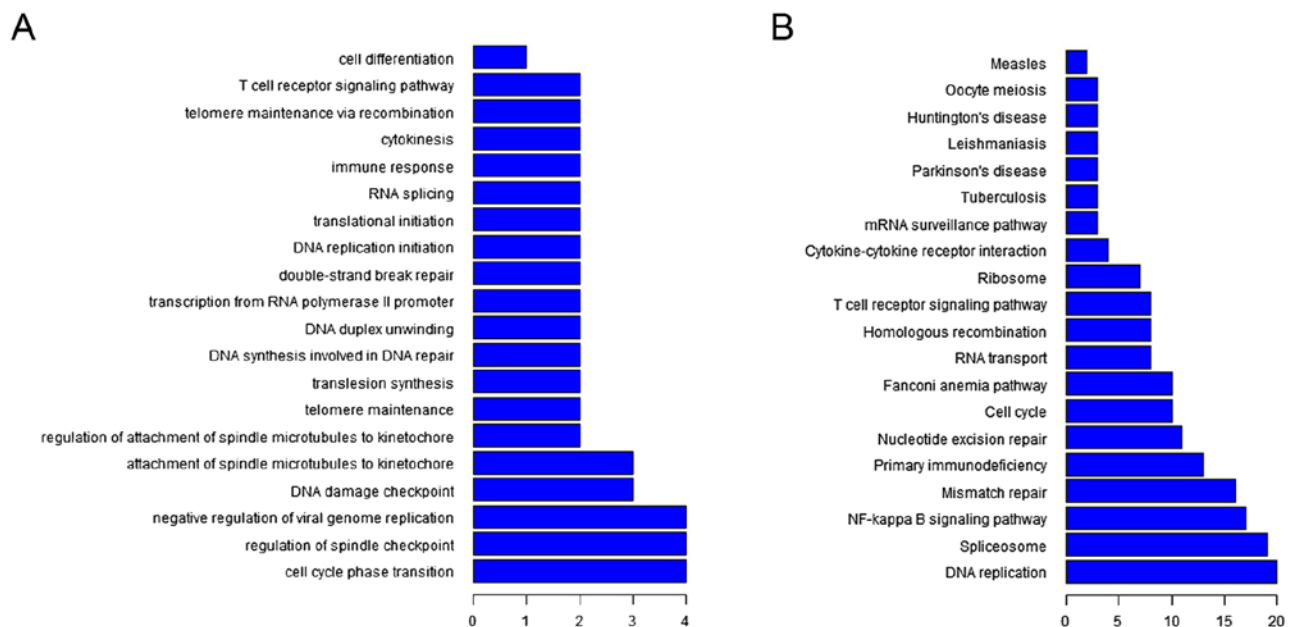

Figure 3. Top 200 GO terms and KEGG pathway terms for dysregulated lncRNAs in BM samples from patients with APL post-induction and BM samples from patients with APL at diagnosis. (A) Top 200 GO term hits for dysregulated lncRNAs. (B) Top 200 KEGG pathway term hits for dysregulated lncRNAs. The x-axis represents the number of annotated lncRNAs, the y-axis represents the GO or KEGG pathway annotations. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNAs, long non-coding RNAs; BM, bone marrow; APL, acute promyelocytic leukemia.

lncRNA NONHSAT061249 *cis*-regulates two mRNAs, including zinc finger protein 564 (ZNF564) and zinc finger protein 44 (ZNF44) (Fig. 4B).

Dysregulated lncRNA 'trans' mechanism and construction of the TF-lncRNA-target gene network. As numerous dysregulated lncRNAs were involved in mRNA regulation, a 'TF-lncRNA' network is likely to be large and complex. Consequently, the top 100 associations were selected to generate a core TF-lncRNA network, visualized by hypergeometric

distribution analysis (Fig. 5). The core TF-lncRNA network map for patients with APL post-induction, vs. matched controls at diagnosis is shown in Fig. 5A. The majority of potential *trans*-regulation lncRNAs were found to be involved in pathways regulated by three TFs, including E2F transcription factor 1 (E2F1), E2F transcription factor 6 (E2F6), and early B cell factor 1 (EBF1) (Fig. 5B). In the core network of lncRNA-TF pairs, E2F1, E2F6, and EBF1 regulated the expression of 16 lncRNAs, 13 lncRNAs and 10 lncRNAs, respectively.
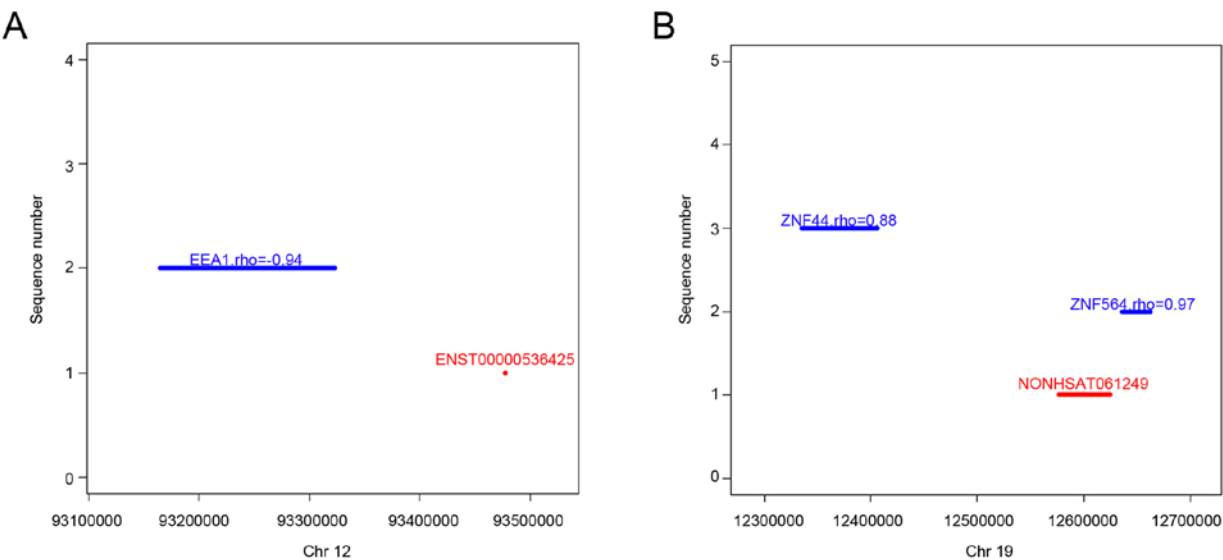
Figure 4. Representative lncRNAs, their '*cis*' mRNAs, and their positions on chromosomes. (A) Co-expression of ENST00000536425 and downstream EEA1 mRNA on chromosome 12. (B) Co-expression of NONHSAT061249 and downstream ZNF564 or ZNF44 mRNA on chromosome 19. The abscissa shows the genomic position, the red line/point indicates lncRNA genomic position, the blue line indicates the mRNA location, the rho value represents the correlation coefficients between the lncRNA and its '*cis*' mRNA. lncRNAs, long non-coding RNAs; mRNAs, messenger RNAs; EEA1, early endosome antigen 1; ZNF564, zinc finger protein 564; ZNF44, zinc finger protein 44.
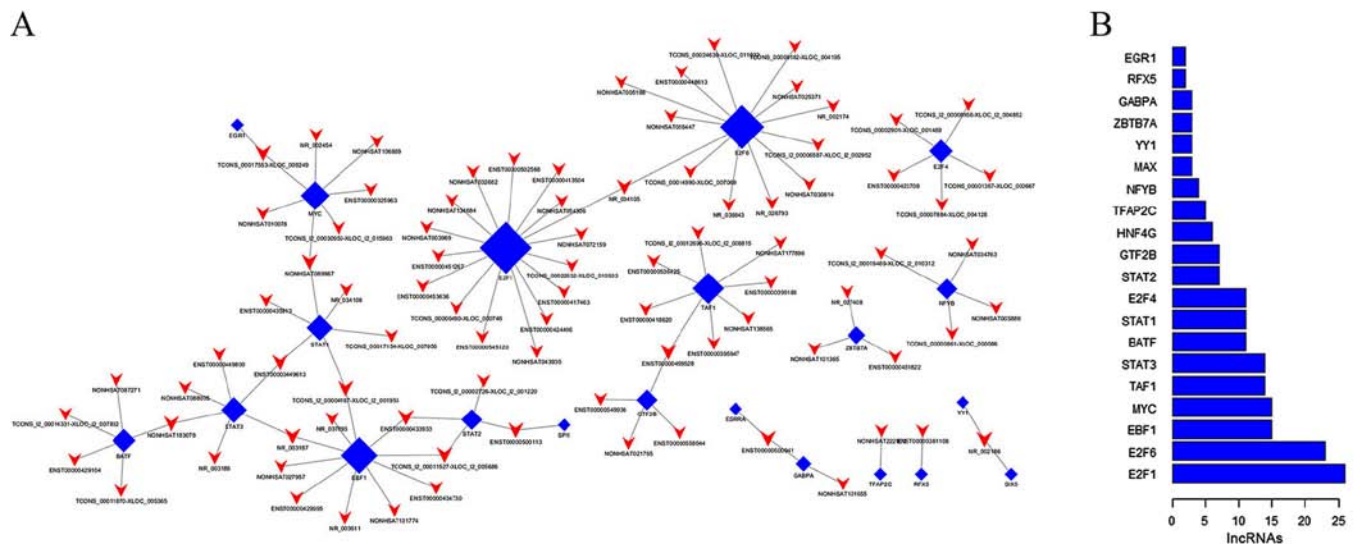

Figure 5. lncRNA-TF core network map for patients with APL post-induction, vs. matched controls at diagnosis. (A) Top 100 lncRNA-TF associations were selected to construct a dyad relationship network using the hypergeometric distribution. Red and blue nodes represent the lncRNAs and TFs, respectively; the node sizes vary as the outlet connection. (B) Majority of lncRNAs were predictively regulated by E2F1, E2F6 and EBF1. The x-axis represents the number of enriched lncRNAs to each TF and the y-axis represents the TF name. lncRNA, long non-coding RNA; TF, transcription factor; APL, acute promyelocytic leukemia; E2F1, E2F transcription factor 1; E2F6, E2F transcription factor 6; EBF1, early B cell factor 1.

Based on the lncRNA co-expression results, the target genes were incorporated into a 'TF-lncRNA' network to determine the TF-lncRNA-target network. Due to the large and complex networks, the top 10 associations were selected to produce a core TF-lncRNA-target network map (Fig. 6). The core TF-lncRNA-target gene association for patients with APL post-induction therapy, vs. pre-induction therapy is shown in Fig. 6, and includes 10 dysregulated lncRNAs (TCONS_l2_00006587-XLOC_12_002952, ENST00000536425, NR_002174, TCONS_00002901-XLOC_001489, ENST00000558044,

NONHSAT101365, TCONS_00017553-XLOC_008249, TCONS_00000490-XLOC_000746, NONHSAT034763, and TCONS_l2_00030950-XLOC_l2_015963), 247 target genes, and eight TFs (TAF1, GTF2B, E2F4, ZBTB7A, E2F1, NFYB, MYC, and E2F6) in the core map. As shown in Fig. 6, the core TF *MYC* association regulated two lncRNAs (TCONS_00017553-XLOC_008249 and TCONS_l2_00030950-XLOC_l2_015963) and 97 target genes. As observed for 'MYC-TCONS_00017553-XLOC_008249-RIOK1' in this map, target genes, including *RIOK1*, were co-expressed for TCONS_00017553-XLOC_008249. As observed for

Table IV. All lncRNA transcripts and their potentially *cis*-regulated mRNA transcripts.

Chrom	Strand	Correlation	lncRNA primary ID	txStart	txEnd	mRNA primary ID	txStart	txEnd
10	-	-0.846333103	ENST00000437232	17256238	17271983	CUBN	16865963	17171830
10	-	-0.889863566	ENST00000439671	94428529	94429500	IDE	94211441	94333852
10	-	-0.827414528	ENST00000453853	46798370	46809021	SYT15	46952762	46971400
15	+	-0.844858112	ENST00000499528	64663186	64667537	TRIP4	64679952	64747502
5	+	-0.821398914	ENST00000512856	96120481	96121703	LNPEP	96271098	96373219
11	-	-0.931044512	ENST00000525500	62357121	62357959	UBXN1	62443970	62446562
11	-	0.904455214	ENST00000525500	62357121	62357959	HNRNPUL2	62480097	62494857
11	-	-0.832660059	ENST00000525500	62357121	62357959	AHNAK	62201016	62314332
12	-	-0.936840914	ENST00000536425	93477374	93477451	EEA1	93164413	93323107
14	+	0.933330841	ENST00000555403	50793244	50794627	L2HGDH	50704281	50779266
14	+	0.908030062	ENST00000555460	22886826	22887645	TRAV38-1	22739821	22740446
14	+	0.871856814	ENST00000555460	22886826	22887645	TRAJ61	22944306	22944365
14	+	0.825846372	ENST00000555460	22886826	22887645	TRAV30	22636293	22636884
1	-	-0.833908257	NONHSAT005886	145372088	145373798	POLR3GL	145456236	145470388
1	+	0.83645797	NONHSAT007706	173865073	173871776	DARS2	173793641	173827684
11	-	-0.901219661	NONHSAT021765	62334412	62340225	HNRNPUL2	62480097	62494857
11	-	0.874686208	NONHSAT021765	62334412	62340225	UBXN1	62443970	62446562
11	-	-0.844180413	NONHSAT025134	130434325	130628495	ADAMTS8	130274818	130298888
12	-	0.834550722	NONHSAT028485	54059021	54062993	CALCOCO1	54104902	54121529
12	-	0.904345693	NONHSAT031503	122826057	122839031	ZCCHC8	122956146	122985543
14	+	-0.837693156	NONHSAT035752	22564302	22564896	TRAV41	22788568	22789123
14	+	-0.820822874	NONHSAT035752	22564302	22564896	TRAV12-1	22309321	22309956
16	-	-0.864461252	NONHSAT051747	223162	223620	AXIN1	337440	402676
19	-	0.971374656	NONHSAT061249	12576359	12624647	ZNF564	12636184	12662356
19	-	0.880808619	NONHSAT061249	12576359	12624647	ZNF44	12335501	12405714
5	-	-0.889390591	NONHSAT101116	39105326	39105857	RICTOR	38938021	39074510
5	-	0.827904836	NONHSAT101123	39383148	39393457	DAB2	39371776	39462402
5	+	-0.83173824	NONHSAT102906	96071868	96077284	LNPEP	96271098	96373219
5	-	-0.921818421	NONHSAT104550	149782674	149782885	RBM22	150070352	150080669
7	+	0.833858641	NONHSAT119665	27135713	27139877	OTTHUMG00000065294	27420181	27420825
X	+	0.82095135	NONHSAT137231	54841718	54842362	PAGE2B	55101496	55105342
19	-	0.83717369	NONHSAT180547	52359056	52391189	SIGLEC14	52114781	52150151
22	-	0.859220203	NONHSAT192618	18710726	18711888	CLTCL1	18900087	19279239
1	+	-0.821131915	NR_003133	89873238	89890493	GBP6	89829436	89853719
6	+	-0.865567866	NR_003288	160514114	160517244	SLC22A1	160542805	160579750
19	+	-0.90635912	NR_024247	1285890	1378430	DAZAP1	1407584	1435683

Table IV. Continued.

Chrom	Strand	Correlation	lncRNA primary ID	txStart	txEnd	mRNA primary ID	txStart	txEnd
4	+	0.882358648	NR_036694	88812995	88815167	DMP1	88571454	88585513
7	+	-0.879682301	TCONS_00012852-XLOC_006277	141870970	141923474	TRBV4-2	142045252	142045816
7	+	-0.852206355	TCONS_00012852-XLOC_006277	141870970	141923474	TRBV2	142000747	142211011
10	-	-0.823174131	TCONS_00017969-XLOC_008805	46798370	46809021	SYT15	46952762	46971400
1	-	-0.880035509	TCONS_l2_00002788-XLOC_l2_001347	228155359	228158853	C1orf148	228351787	228353213
12	+	-0.842080292	TCONS_l2_00005685-XLOC_l2_002994	53547053	53548417	ESPL1	53662083	53778657
12	+	0.933722808	TCONS_l2_00005705-XLOC_l2_003009	56904734	56906923	IL23A	56732663	56734194
15	+	-0.853220916	TCONS_l2_00008528-XLOC_l2_004602	30846790	30848689	ARHGAP11B	30916697	30931023
17	+	-0.948957318	TCONS_l2_00011527-XLOC_l2_005686	18330175	18333940	TRIM16L	18601311	18639432
17	+	0.815854971	TCONS_l2_00011527-XLOC_l2_005686	18330175	18333940	LGALS9C	18380098	18398259
19	+	0.840205468	TCONS_l2_00012696-XLOC_l2_006815	54278778	54279108	MIR516A2	54264387	54264476
2	+	-0.866656281	TCONS_l2_00014331-XLOC_l2_007832	242626503	242633704	ING5	242641450	242668896

lncRNA, long non-coding RNA; mRNA, messenger RNA; chrom, chromosome.

'MYC- TCONS_l2_00030950-XLOC_l2_015963-RPLP2' in the map, target genes, including *RPLP2*, were co-expressed for TCONS_l2_00030950-XLOC_l2_015963-RPLP2. Therefore, these maps provided vital information on the lncRNAs, TFs and target genes.

Discussion

In the present study, the expression patterns of genome-wide lncRNAs were first evaluated in BM samples from patients with APL post-induction and corresponding BM samples from patients with APL at diagnosis using microarray analysis. Their potential functions were then examined by analysing their co-expressed mRNAs. The experimental results showed that 825 lncRNAs and 1,218 mRNAs were dysregulated. Furthermore, 10 selected dysregulated lncRNAs were validated by RT-qPCR analysis. Several hundred lncRNAs were co-expressed with hundreds of mRNAs, and a number of these may contribute to ATRA/ATO-induced intracorporeal myeloid differentiation by affecting these mRNAs in *trans* and/or in *cis*. The present study not only clarified the contributions of lncRNAs to myeloid differentiation in APL and/or intracorporeal therapy response, but also elucidated possible dysregulated lncRNA expression mechanisms associated with ATRA-induced APL differentiation.

APL has shifted from a complex problem in the past into a paradigm with successful targeted therapies, and a series of published randomized clinical trials in patients with APL have all demonstrated efficacy on the frontline of ATRA/ATO association. The identification of a novel class of lncRNAs has attracted attention and may have encouraged investigations focused on characterizing lncRNAs associated with myeloid differentiation and responses to APL therapy. For example, several lncRNAs, including NEAT1 and HOTAIRM1, are indispensable during APL differentiation induced by ATRA (10,12,22). However, numerous lncRNAs and their roles in APL differentiation remain to be fully elucidated. A systematic analysis of the ATRA/ATO-induced intracorporeal myeloid differentiation transcriptome may reflect the complicated and dynamic intracorporeal synergy between ATRA and ATO in patients with APL. lncRNA and mRNA analysis in BM from patients with APL is expected to reflect real changes.

To examine global lncRNA and mRNA profiling in the present study, the Human Transcriptome Array 2.0 was used to screen the dysregulated lncRNAs in three patients with APL post- and pre-induction therapy. The results showed that 825 lncRNAs and 1,218 mRNAs were dysregulated, including 410 upregulated lncRNAs, 415 downregulated lncRNAs, 660 upregulated mRNAs, and 558 downregulated mRNAs, suggesting that these dysregulated lncRNAs may be involved in ATRA-induced myeloid differentiation. Consequently, the present study may assist in determining whether these dysregulated lncRNAs and mRNAs can be applied for the early assessment of APL therapy response or efficacy. The present study also cross-validated the dysregulated gene results from the GeneChip with the results of RT-qPCR analysis; this comparison showed consistency between the microarrays and RT-qPCR results, which supports further predictions.
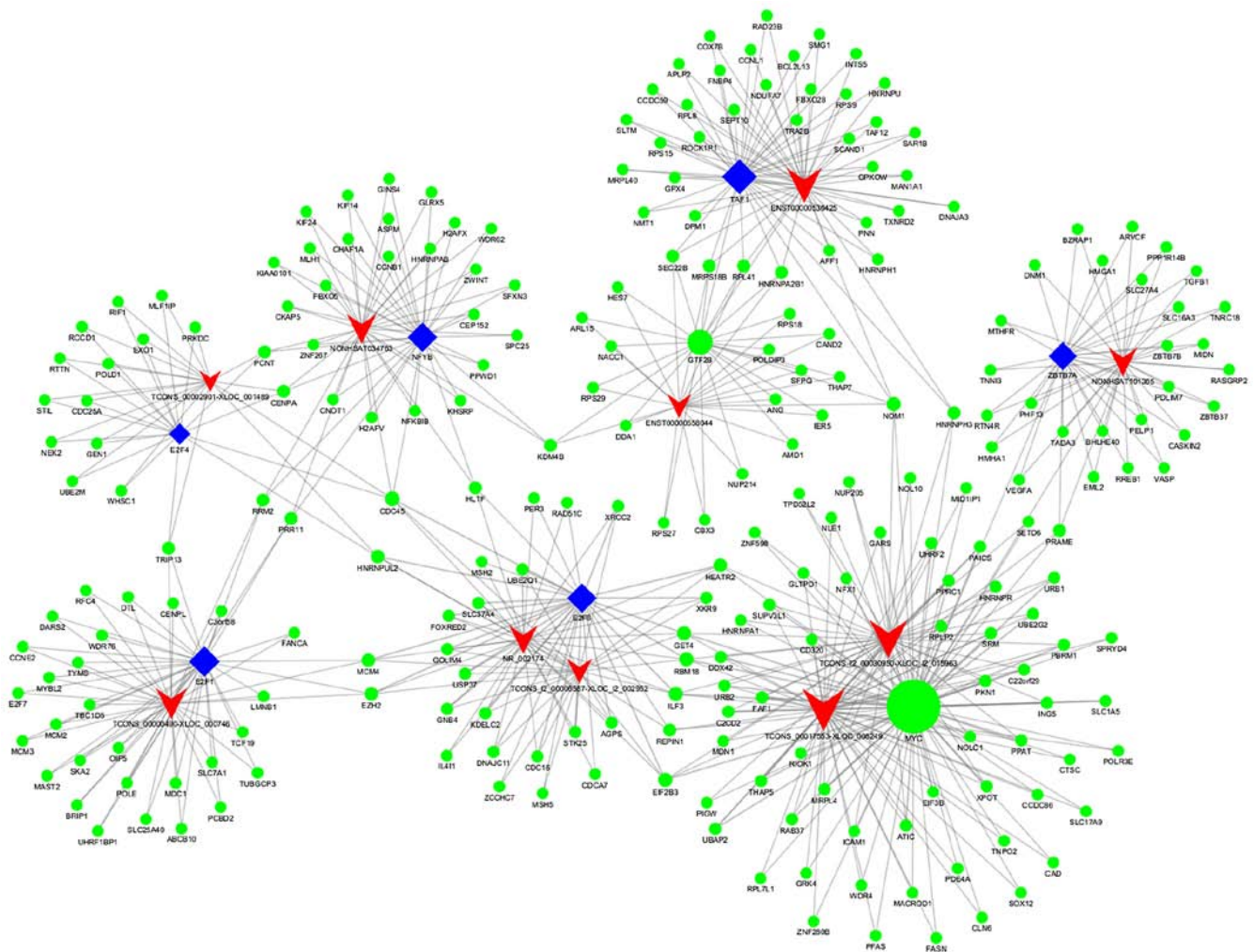
Figure 6. lncRNA-TF-target core network map for patients with APL post-induction, vs. matched controls at diagnosis. The TF-lncRNA-target network comprised 10 lncRNAs, 247 target genes and eight TFs. Blue, red and green nodes represent the TFs, lncRNAs and target genes, respectively; the node sizes vary as the outlet connection. lncRNA, long non-coding RNA; TF, transcription factor; APL, acute promyelocytic leukemia.

An increasing number of lncRNAs have been recognized as critical factors in gene programs to control cell differentiation and function, and serving as scaffolds or decoys at the transcriptional and translational levels (23). Although the number of novel and known lncRNAs is increasing exponentially, only a small subset of these have functional annotations, and the most commonly used method to predict lncRNA function is by investigating their co-expressed mRNAs and associated biological pathways. In the present study, it was found that thousands of mRNAs are co-expressed with the dysregulated lncRNAs. Through functional prediction with the co-expressed mRNAs, the present study identified lncRNAs involved in DNA replication associated with cell cycle phase transition as the most affected by ATRA-induced myeloid differentiation. When stabilized and inhibited via cell cycle phase transitions, lncRNAs function as a vital factor for regulating cell cycle during the course of myeloid maturation in NB4 APL cells (13). Therefore, lncRNAs may be a potential modulator of DNA replication, in addition to certain regulatory TFs.

Compared with mRNAs, lncRNAs have the inherent characteristic of *cis*-regulation (24) and can *cis*-regulate their adjacent mRNAs (25). The epigenetic upregulation of lncRNAs is associated with *cis*-downregulation of a functional gene cluster in leukemia (26). In the present study, 48 lncRNAs were found to potentially promote *cis*-regulation of their adjacent mRNAs. Although the majority of the identified lncRNAs have not been characterized, two sets of lncRNA-mRNAs, including ENST00000536425 and *EEA1* mRNA and NONHSAT061249 and *ZNF564* and *ZNF44* mRNAs, were identified. A previous study revealed that *EEA1* serves as an identifying marker of early endosomes for neddylation of type II receptor, and aberrant neddylation results in the development of leukemia (27). The biological processes associated with *ZNF564* and *ZNF44* are involved in transcription and transcription regulation, respectively. These findings suggest that lncRNA-mRNA networks may contribute to the regulation of cell responses to ATRA-induced APL differentiation.

Certain lncRNAs have been demonstrated to be involved in *cis*-regulation; however, the majority of characterized lncRNAs are functionally *trans*-regulating (18,24). The present study predicted the *trans*-regulatory functions of lncRNAs through TFs. In the central network of lncRNA-TF pairs, the potential *trans*-regulatory lncRNAs were mainly *trans*-regulated by E2F1, E2F6, and EBF1. E2F1 and E2F6 are TFs

that contribute to controlling cell cycle. Several studies have associated the activity of E2F with cell-cycle control (28,29). The E2F1-C/EBPa feedback loop regulates the expression of the oncogene tribbles 2, which is important for AML cell proliferation control (30). E2F6 may transcriptionally regulate cell-cycle G1/S genes via recruiting *BRG1* (29). *EBF1*, a TF that is critical for normal and malignant B-lymphocyte development, controls DNA repair in a dose-dependent manner, which may explain the reason for the frequent loss of the *EBF1* gene in leukemia (31). In the present study, the dysregulated lncRNAs involved in the pathways mainly regulated by E2F1, E2F6, and EBF1 were candidate participants in ATRA-induced myeloid differentiation. Consequently, the '*trans*' analysis provides a method to interpret the functions of lncRNAs and their biological processes in APL therapy.

The lncRNA-TF analysis identified novel lncRNAs and three TFs for enriched dysregulated mRNAs that contributed to APL treatment. The results of the '*cis*' and '*trans*' analyses provided essential clues on the modular regulation of lncRNAs. The data obtained may promote future investigations on therapeutic mechanisms in APL. However, the present study had several limitations. First, the sample size was small; thus, investigations with larger sample sizes are required. Second, as the functions of several of the identified lncRNAs have not been annotated, it was only possible to predict the functions of lncRNAs through network and pathway analyses with their co-expressed mRNAs. Therefore, the biological functions of these lncRNAs require further validation.

In conclusion, the present study offered insight into the genome-wide patterns of lncRNA expression during the course of ATRA-based APL therapy. A set of dysregulated lncRNAs were identified in patients with APL who received ATRA-based therapy compared with untreated matched controls. Several lncRNAs may be involved in biological pathways associated with ATRA-induced myeloid differentiation through the *cis* and/or *trans* regulation of mRNAs. Furthermore, targeting aberrantly activated pathways in APL cells may offer strategies to circumvent or mitigate disease. The present study provides a foundation for future investigations on lncRNAs associated with ATRA-based APL therapy as therapeutic and diagnostic targets by supplying candidate genes. Additionally, the results provide a platform for systematically evaluating a large number of lncRNAs and mRNAs to identify pathways critical for APL elimination using primary APL patient specimens prior to and following targeted therapy.

Availability of data and materials

The datasets used during the present study are available from the corresponding authors upon reasonable request.

Authors' contributions

ZGC, JCR and XQZ designed and supervised the project. YYB and JJY provided the samples and clinical information. JY and XLG performed the experiments. YYB and XQZ provided instructions for the experiments. ZGC and JY analyzed the data and wrote the manuscript. ZGC and XLG edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Written informed consent was collected from all the patients in conformity with the Declaration of Helsinki, and the present study obtained permission from the Ethics Committee of Wenzhou Medical University.

Patient consent for publication

Written informed consent was collected from all the patients.

Competing interests

The authors declare that they have no competing interests.

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