Digital gene expression profiling analysis of childhood acute lymphoblastic leukemia

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Abstract. Acute lymphoblastic leukemia (ALL) is the most commonly diagnosed malignancy in children. It is a heterogeneous disease, and is determined by multiple gene alterations and chromosomal rearrangements. To improve current understanding of the underlying molecular mechanisms of ALL, the present study profiled genome-wide digital gene expression (DGE) in a population of children with ALL in China. Using second-generation sequencing technology, the profiling revealed that 2,825 genes were upregulated and 1,952 were downregulated in the ALL group. Based on the DGE profiling data, the present study further investigated seven genes (*WT1, RPS26, MSX1, CD70, HOXC4, HOXA5* and *HOXC6*) using reverse transcription-quantitative polymerase chain reaction analysis. Gene Ontology analysis suggested that the differentially expressed genes were predominantly involved in immune cell differentiation, metabolic processes and programmed cell death. The results of the present study provided novel insights into the gene expression patterns in children with ALL.

Introduction

Acute lymphoblastic leukemia (ALL) is the most commonly diagnosed malignancy in adolescents and young adults, and represents almost one third of all cases of cancer in children (1,2). ALL is a heterogeneous disease, and multiple subtypes have been identified based on recurrent copy number alterations and structural chromosomal rearrangements (3-5). Due to genetic variations, the incidence of ALL in children varies across regions, and is determined by ethnicity (1). In China, the incidence of childhood ALL is 4/100,000 children aged <10 years (6). Although the 5-year event-free survival rate of children and adolescents with ALL is ~80%, the remaining 20% of patients relapse, the outcome of which remains poor (7-9). Therefore, identifying ALL-associated differentially expressed genes is important for improving therapeutic methods and extending patient survival rates in childhood ALL.

Multiple technologies have been used to identify the differentially expressed genes associated with childhood ALL. For example, gene expression microarray analyses of differentially expressed genes in ALL subtypes (10) have identified 80-300 genes as marker genes, which are necessary for discriminating the subtypes. In addition, sequencing-based methods, including serial analysis of gene expression (SAGE), have been used to measure absolute gene expression levels in ALL subtypes (11). However, as hybridization-based methods are subject to technical limitations, certain genes may be overlooked during the hybridization process in gene expression microarray analysis. In addition, the cost of sequencing technology hinders the widespread usage of SAGE for identifying differentially expressed genes (12). Advances in second-generation DNA sequencing technologies has enabled digital gene expression (DGE) profiling to overcome the drawbacks of the microarray analysis hybridization process and allow the detection of differential expression of low-abundance transcripts on a genome-wide scale (13). In the present study, 3' tag DGE, Gene Ontology (GO) analysis, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were used to analyze the transcriptional profiles of bone marrow mononuclear cells (BMMCs) from Chinese children with ALL and those without ALL. The results of the analysis revealed numerous gene expression changes attributable to pathogenesis. By investigating changes in the expression of genes, various novel candidate genes required for ALL were identified.

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Materials and methods

Patient samples. A total of 10 bone marrow (BM) tissue samples were obtained from children [median age at diagnosis, 8.1 years (range, 3-13.4 years); 5 males and 5 females] newly diagnosed with childhood ALL at Xiangya Hospital (Changsha, China) between May and August 2010. In addition, 10 non-ALL BM samples were obtained from patients who did not have leukemia or other malignancies, but who were undergoing BM aspiration as part of their clinical care at Xiangya Hospital [median follow-up time, 6.75 years (range 4-13.9 years)]. ALL diagnosis was based on the French-American-British classification standards, and the morphology, immunology, cytogenetics and molecular biology classification (14). Complete remission, refractory disease and BM relapse were defined, according to the National Cancer Institute (15). The patient clinical characteristics are listed in Table I. The primary BMMCs from the patients with ALL were analyzed using flow cytometry. The cell-surface antigen staining was performed using a PerCP-conjugated anti-CD45 antibody (cat. no. 304028; BioLegend, Inc., San Diego, CA, USA). For FACS analysis, $5x10^5$ cells were acquired and scored using flow cytometer (Gallios™; Beckman Coulter, Inc., Brea, CA, USA), and the data were analyzed using FlowJo software (version 8.7; Tree Star, Inc., Ashland, OR, USA). The BMMCs were isolated by Ficoll density gradient centrifugation at 671 x g for 20 min at room temperature (Ficoll-Paque was obtained from GE Healthcare Life Sciences; Uppsala, Sweden). The cells at the interface were removed and washed twice with 30 ml sterile phosphate-buffered saline (Well-Biology Co., Ltd., Changsha, China) at 671 x g for 5 min. The isolated cells were counted using the trypan blue (Well-Biology Co., Ltd.,) exclusion method and an inverted phase contrast microscope (IMT-2; Olympus Corporation, Tokyo, Japan). The cells were pelleted and maintained at -80˚C until RNA extraction. The RNA was extracted from frozen cell pellets using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. In accordance with the Xiangya Hospital Committee on Human Research Review Ethics Committee, written informed consent was obtained from the patients, or from the parents or guardians, as appropriate.

Preparation of sequencing libraries and sequencing. The total RNA sample was digested using DNaseI (M0303S; New England Biolabs, Inc., Ipswich, MA, USA) and purified using oligo-dT beads (Invitrogen Dynabeads mRNA Purification kit; Thermo Fisher Scientific, Inc.), then poly(A)-containing mRNA was fragmented into 130 bp using First-Strand buffer (Thermo Fisher Scientific, Inc.). First-strand cDNA was generated using N6 primer, First Strand Master mix and Super Script II Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) with the following reaction conditions: 25˚C for 10 min, 42˚C for 40 min and 70˚C for 15 min. The Invitrogen Second Strand Master mix (Thermo Fisher Scientific, Inc.) was then added to synthesize the second-strand cDNA (at 16˚C for 1 h). The cDNA was purified with Agencourt AMPure XP beads (Beckman Coulter, Inc.), combined with Invitrogen End Repair mix (Thermo Fisher Scientific, Inc.) and incubated at 20˚C for 30 min. Invitrogen A-Tailing mix (Thermo Fisher Scientific, Inc.) was added, followed by incubation at 37˚C for 30 min. The Adenylate 3' Ends DNA (Agilent Technologies, Inc., Santa Clara, CA, USA), Illumina adapter (sequence, CATGIAAAAA; Illumina, Inc., San Diego, CA, USA) and ligation mix (Invitrogen; Thermo Fisher Scientific, Inc.) were combined and the ligate reaction was incubated at 20˚C for 20 min. Fifteen rounds of PCR amplification were performed with PCR Primer Cocktail and PCR Master mix (both Takara Bio, Inc., Shiga, Japan) to enrich the cDNA fragments. Then the PCR products were purified with Agencourt AMPure XP beads. Sequencing libraries were prepared from 6 μg total RNA using the *Nla*III Digital Gene Expression Tag Profiling kit (Illumina, Inc.). The bead-bound cDNA was digested with *Nla*III and ligated with the Illumina adaptor (sequence, CATGIAAAAA) containing an *Mme*I recognition site. The adapter-ligated cDNA was digested with *Mme*I to release the cDNA from the bead, retaining a 17-bp sequence in the fragment. Following removal of the 3' fragments via magnetic bead precipitation, a second Illumina adaptor was ligated to the 3' end of the fragment to form a tagged library. Following 15 cycles of linear PCR amplification, 95-bp fragments were purified by 6% Tris-borate-EDTA polyacrylamide gel electrophoresis (Thermo Fisher Scientific, Inc.). Following denaturation, the single-chain molecules were adhered onto an Illumina Sequencing Chip (Illumina, Inc.). *In situ* amplification was performed to expand each molecule into a single-molecule cluster sequencing template. Subsequently, four color-labeled nucleotides (Thermo Fisher Scientific, Inc.) were added, and sequencing was performed using the sequencing by synthesis method (16,17). Each tunnel generated millions of raw reads with 35-bp sequencing lengths.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from the BM using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The ensuing RT-qPCR was performed using an Access RT-PCR system (ISOGEN; Nippon Gene, Tokyo, Japan), according to the manufacturer's protocol. qPCR was performed using a 25-μl reaction mixture containing 12.5 μl 2X Premix Ex Taq™ (Takara Bio, Inc.), 5 pmol primer and 6.5 μl cDNA, obtained as described above. Amplification was performed on an Applied Biosystem PRISM 7700 system (Thermo Fisher Scientific, Inc.) and the PCR conditions were as follows: 50˚C for 2 min, 95˚C for 15 min, and 45 cycles at 95˚C for 30 sec and 60˚C for 1 min, followed by 25˚C for 2 min. Quantification was determined by the standard curve and the $2^{-\Delta\Delta Cq}$ method (4). All primer sets were designed, as described previously, synthesized by Biosearch Technologies (Novato, CA, USA) and are presented in Table II.

Gene annotation. The human transcriptome (Ensembl version 58; http://asia.ensembl.org/) was used as the reference sequence for sequence read alignment and identification. The DGE tags were annotated by mapping the reads to the sequence-flanking *Nla*III restriction sites on the coding strands. Alignment and candidate gene identification were performed, as reported previously (18). The total expression

Table I. Clinical characteristics of patients with ALL.

Sample ID	Immunophenotype	Genetic subtype	Age at diagnosis (years)	WBC count at diagnosis (10^9 cells/l)	Event
Patient 1	B-ALL	t(12;21)	3.0	114.2	CR1
Patient 2	B-ALL	t(12;21)	13.0	23.3	CR1
Patient 3	B-ALL	t(12;21)	4.2	6.4	CR1
Patient 4	B-ALL	HeH	13.4	78.3	Remission
Patient 5	B-ALL	HeH	3.7	3.5	CR1
Patient 6	B-ALL	t(9;22)	10.2	33.6	CR1
Patient 7	T-ALL	T-ALL	6.0	121.5	CR1
Patient 8	T-ALL	T-ALL	13.2	67.8	DCR1
Patient 9	T-ALL	T-ALL	2.5	42.5	CR1
Patient 10	T-ALL	T-ALL	10.8	23.6	Remission

ALL diagnosis was established by analyses of morphology, immunophenotype and cytogenetics of leukemic cells. Fluorescence *in situ* hybridization and/or reverse transcription-polymerase chain reaction analysis were applied to identify t(12;21). ALL, acute lymphoblastic leukemia; CR1, continuous first remission; DCR1, dead in CR1; B-ALL, B-cell lineage ALL; T-ALL, T-cell lineage ALL; t(12;21), translocation between chromosomes 12 and 21 (p13; q22; ETV6/RUNX1); HeH, high hyperdiploidy; t(9;22), translocation between chromosomes 9 and 22) (q11;q34; BCR/ABL1); WBC, white blood cell.

Table II. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (˚C)	Product size (bp)
WT1	CTATTCGCAATCAGGGTTA	AGGTGGCTCCTAAGTTCAT	55	312
RPS26	TCCGTGCCTCCAAGATGA	ACGCATCGGGCACAGTTA	54	103
MSX1	CACAAGACGAACCGTAAGCC	ACCATATCTTCACCTGCGTCT	56	154
CD70	TCTCCCGCCTCCCGTAGCAT	TGTCCTGCCACCACTACGC	56	310
HOXC4	CAGACCTCCAGAAATGACG	GGGTAGACTATGGGTTGCTT	57	518
HOXA5	GCAGCACCCACATCAGCA	CTTCTGCGGGTCAGGTAA	58	274
HOXC6	TTCCTACTTCACTAACCCTTCC	TGCCCTGCTCAGAACTAAA	54	323
β-actin	GGGTCAGAAGGATTCCTGTG	GGTCTCAAACATGATCTGGG	54	219

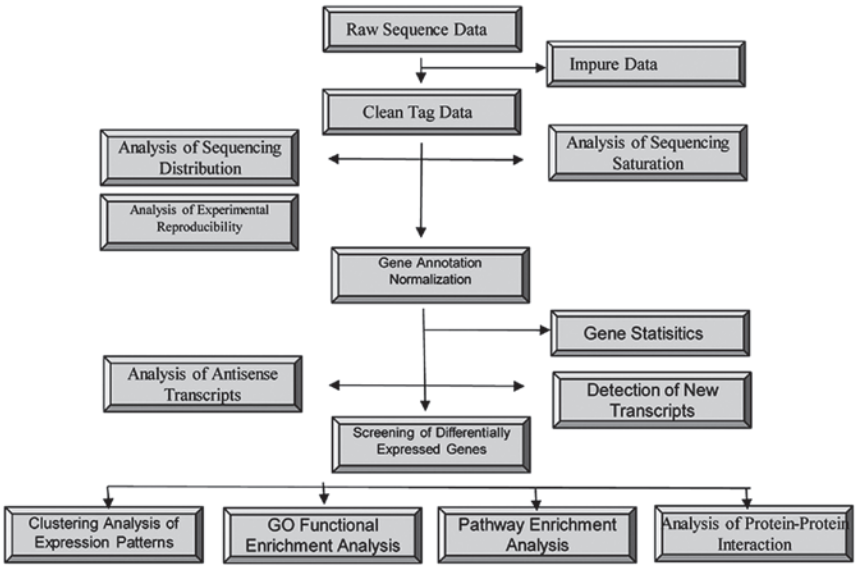

Figure 1. Flow chart of digital gene expression profiling analysis for childhood acute lymphoblastic leukemia. GO, Gene Ontology.

Table III. Candidate genes upregulated 10-fold in acute lymphoblastic leukemia.

Gene	log 2 ratio	Description	Function
HOXA11	12.69349	Homeobox A11	Endometrial cancer
CCL1	12.50705	Chemokine (C-C motif) ligand 1	CC chemokine receptors, airway inflammation
WT1	12.05494	Wilms' tumor 1	Wilms' tumor, Leukemia, uterine tumors
TWIST1	11.80292	Twist homolog 1 (Drosophila)	Hepatocellular carcinoma
SLITRK6	10.98513	SLIT and NTRK-like family, member 6	Leading factor of nerve growth
HLA-DRB4	10.78627	Major histocompatibility complex, class II, DR β4	Hepatitis, leukocyte antigen, acute lymphoblastic leukemia
HOXC6	10.75322	Homeobox C6	Stem cell differentiation for lymphocytes
ELN	10.49685	Elastin	Williams syndrome
WIT1	10.20945	Wilms' tumor upstream neighbor 1	Wilms' sarcoma downstream factor
SYN1	10.00000	synapsin I	Depression neuroblastoma
CD70	9.911392	CD70 molecule	Lymphocyte antigen
CTHRC1	9.850187	Collagen triple helix repeat containing 1	Gastric cancer, liver cancer, colon cancer
GGT5	9.850187	γ-glutamyltransferase 5	Liver cancer
PROCR	9.575539	Protein C receptor, endothelial	Liver cancer
H19	9.370687	H19, imprinted maternally expressed transcript (non-protein coding)	Breast cancer, cervical cancer, choriocarcinoma
HHIP	9.326429	Hedgehog interacting protein	Pancreatic cancer
LIF	9.184875	Leukemia inhibitory factor (cholinergic differentiation factor)	Leukemia inhibitory factor
HOXC4	9.184875	Homeobox C4	Stem cell differentiation for lymphocytes
NCR3	9.134426	Natural cytotoxicity triggering receptor 3	Multiple myeloma
MSX1	9.134426	Msh homeobox 1	Apoptosis
ERBB2	9.027906	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma-derived oncogene homolog (avian)	Breast, stomach and endometrial cancer, non-small cell lung cancer
LZTS1	8.851749	leucine zipper, putative tumor suppressor 1	Primary esophageal cancer
USP27X	8.787903	Ubiquitin specific peptidase 27, X-linked	Ubiquitination
HERV-FRD	8.721099	HERV-FRD provirus ancestral Env polyprotein	Retrovirus
PMS2L1	8.573647	Postmeiotic segregation increased 2-like 1 pseudogene	Nasopharyngeal carcinoma
IRX5	8.495855	Iroquois homeobox 5	Ovarian cancer
MKRN3	8.413628	Makorin ring finger protein 3	Osteocarcinoma
IGFBP6	8.134426	Insulin-like growth factor binding protein 6	Breast cancer
TRIM2	8.027906	Tripartite motif-containing 2	Cancer cell development
FZD7	7.912889	Frizzled homolog 7 (*Drosophila*)	Wnt signal
NTRK1	7.912889	Neurotrophic tyrosine kinase, receptor, type 1	Thyroid cancer
RPS26	7.912889	Ribosomal protein S26	Non-Hodgkin's lymphoma
MYCN	6.665077	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	Neuroblastoma
SPINK2	6.427893	Serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	Liver cancer
OLIG1	5.786659	Oligodendrocyte transcription factor 1	Oligodendrocyte tumor
HOXA7	5.578747	Homeobox A7	Epithelial ovarian cancer
NAT14	5.022015	N-acetyltransferase 14 (GCN5-related, putative)	Lung cancer

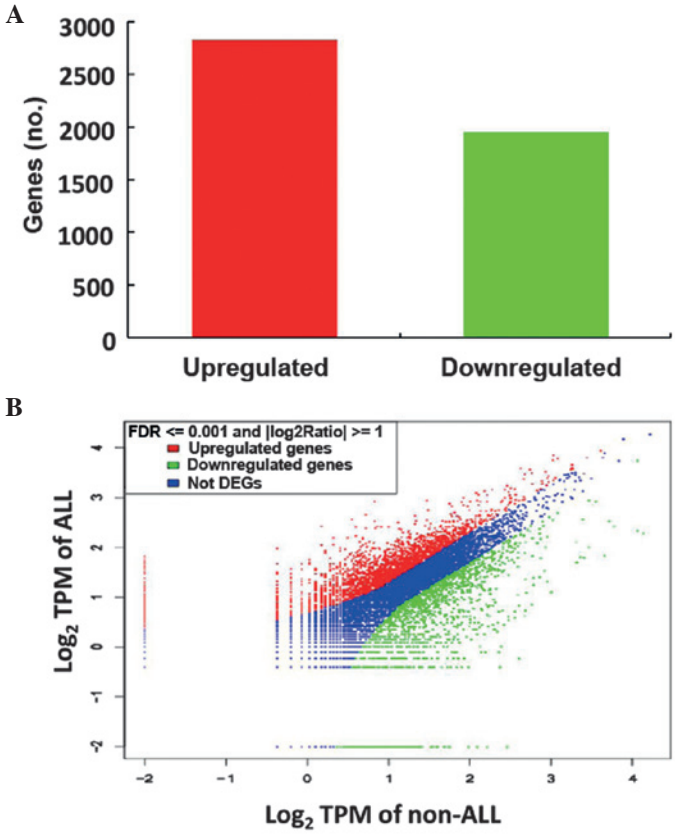

Figure 2. DEGs in patients with childhood ALL and in non-ALL individuals. (A) DEGs in the ALL group. (B) Gene expression levels in the ALL and the non-ALL groups. The DEGs are presented in red (upregulated) and green (downregulated). Genes not demonstrating expression changes are blue. ALL, acute lymphoblastic leukemia; DEGs, differentially expressed genes; TPM; tags per million; FDR, false discovery rate.

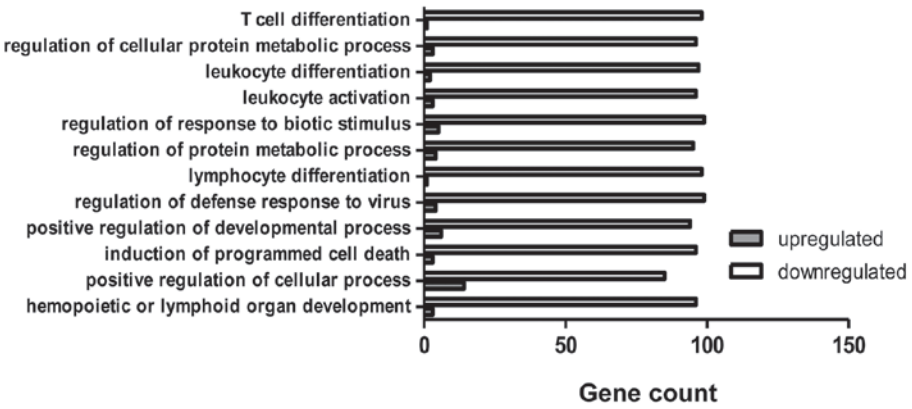

Figure 3. GO analysis of differentially expressed genes in childhood acute lymphoblastic leukemia. Bar plot represents the gene count within each GO category. All processes listed had enrichment P-values <0.05. GO, Gene Ontology.

profile for each gene was then calculated by summing all the tags mapped to the same gene, including intronic tags.

Statistical analysis. Data were analyzed using GraphPad Prism Software (version 5.0; GraphPad Software, Inc, San Diego, CA, USA). Data are presented as means ± standard deviation or means ± standard error of the mean. Statistical analyses were performed using two-tailed Student's t-tests for the differences between two groups and two-way analysis of variance for the difference between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

mRNA sample preparation and Illumina genome analyses. In the present study, BMMC samples were collected from pediatric patients with ALL (Table I) and from non-ALL individuals. The cellular mRNA was extracted and DGE sequencing libraries were prepared for sequencing in an Illumina Genome Analyzer, which obtained 15,200,000-20,400,000 quality-filtered sequence reads (tags) per sample. Any tags with an abundance of <2 tags per million (TPM), those that mapped to >1 gene, or those that did not match the Ensembl

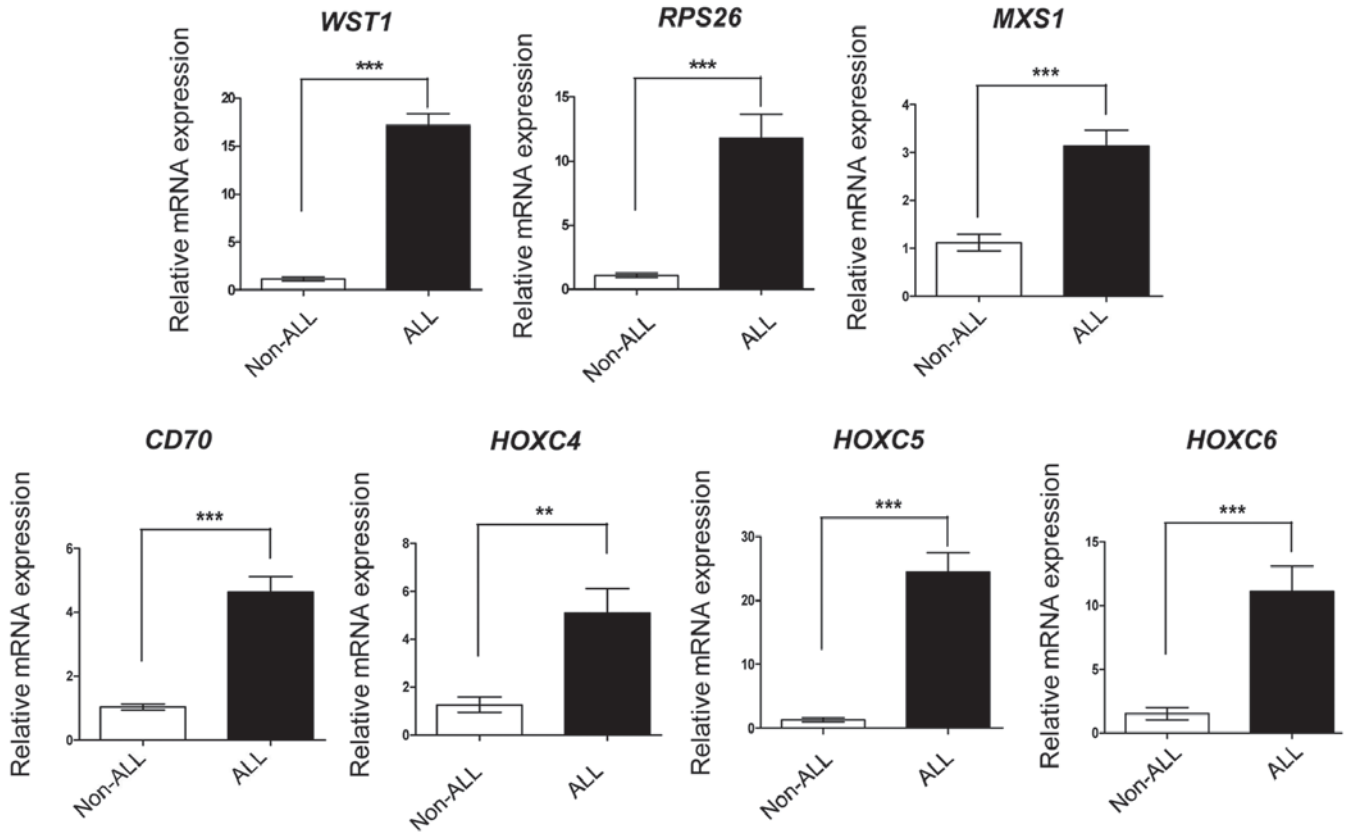

Figure 4. RT-qPCR analysis of upregulated genes in ALL. The seven genes in ALL with the highest expression levels, identified by digital gene expression profiling, were selected for further RT-qPCR analysis. Images shown are representative Western blot (top) and RT-qPCR results (bottom) for the seven genes in 10 ALL and non-ALL samples. β-actin was used as the loading control. Data are expressed as means ± standard deviation (n=3); **P<0.01 and ***P<0.001. ALL, acute lymphoblastic leukemia; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

database reference sequence were omitted. Following filtering, 25,000-53,000 unique nucleotide sequence tags were obtained per library, which were mapped to the transcriptome (Fig. 1).

Identification and functional classification of differentially expressed genes. The differentially expressed genes were compared between the patients with childhood ALL and the non-ALL individuals. The selection criteria for putative differentially expressed genes were as follows: i) Average fold change between ALL and non-ALL groups ≥2; ii) single sample *t*-test false discovery rate <0.1%. In the ALL group, 2,825 genes were upregulated and 1,952 genes were down-regulated (Fig. 2). Of the upregulated genes, 37 that have been associated with the promotion of tumorigenesis, according to Ensembl, were upregulated >10-fold in childhood ALL (Table III).

GO analysis. In the present study, GO analysis was performed by mapping each differentially expressed gene into the GO database (http://www.geneontology.org/). The predominant functional group of upregulated genes was associated with the positive regulation of the cellular process, whereas the down-regulated genes were associated with multiple function groups involved in immune cell differentiation, the metabolic process and programmed cell death (Fig. 3).

RT-qPCR analysis of differentially expressed genes. To further evaluate the DGE profiling reliability in the present study, RT-qPCR analysis was performed for a subset of seven genes, which had been determined by the DGE profiling as upregulated >10-fold in the ALL group. The seven genes were expressed at high levels in the ALL samples, and had the same expression profiles as in the initial DGE profiling (Fig. 4). This result confirmed the reliability of the DGE profiling.

Discussion

ALL is a multigenic disease with multiple subtypes; recurrent copy number alterations and structural chromosomal rearrangements characterize the disease. Although the clinical perspective for the ALL subtypes is well established, the underlying molecular mechanisms in the development of ALL development remain to be fully elucidated Thus, characterizing ALL-associated differentially expressed genes is important for determining the molecular mechanisms of ALL and for early ALL diagnosis. Several genomic-wide expression profiling approaches have been used to identify the ALL-associated differentially expressed genes (19). The development of second-generation sequencing has enabled the use of DGE profiling for determining genome-wide gene expression profiles in ALL cell samples. For example, Nordlund *et al* used DGE profiling to characterize the gene expression patterns in different ALL subtypes (20), and found that antisense tags expressed from the non-coding strand were also expressed as a subtype-specific pattern. Additionally, other

studies have used microarray-based methods to profile ALL gene expression patterns (10,11,21) However, these previous studies were performed in Western countries, and comparable information in Asian populations is limited. To the best of our knowledge, the present study is the first to use DGE profiling to determine gene expression patterns in childhood ALL in a Chinese population.

The development of second-generation sequencing technology has resulted in DGE profiling possessing several advantages, compared with earlier methods of genome-wide expression analysis (22) For example, it requires smaller RNA samples and costs less than other transcriptome sequencing methods, and it overcomes the limitations of the hybridization process present in microarray-based methods. In addition, computational calculation analysis of DGE data is less challenging. In the present study, DGE profiling revealed that 37 genes were upregulated by >10-fold in the childhood ALL group, compared with the non-ALL group. Notably, these genes are important in tumorigenesis. For example, the wild-type *WT1* is expressed in breast cancer, renal cell cancer, ovarian cancer, mesothelioma, lung cancer, melanoma and acute leukemia (23,24). and high levels of *WT1* are associated with poor prognosis in ovarian cancer and leukemia (25,26) Other genes, including *HOXA11*, *HHIP*, *NCR3* and *ERBB2* are involved in the tumorigenesis of several types of solid tumor (27-31). Thus, in addition to characterizing the expression patterns of ALL-associated genes in the present study, novel candidate genes have been identified, which may be associated with ALL. In addition, GO analysis demonstrated that these genes were predominantly involved in immune cell differentiation, the metabolic process and programmed cell death, suggesting the importance of these signaling pathways in ALL. Future investigations to characterize the roles of these genes in childhood ALL experimentally may further understanding.

In conclusion, DGE profiling was conducted to determine the gene expression profile of childhood ALL in a Chinese population, identifying 2,825 upregulated and 1,952 downregulated genes. Of these, 37 of the upregulated genes were upregulated by >10-fold, and were found to be important in tumorigenesis. These findings suggested that DGE profiling can provide novel genome-wide information on gene expression in ALL.

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