A genomic DNA-based NGS method for the simultaneous detection of multiple fusion genes in pediatric leukemia

RONG LIU[1*], SHUNQIAO FENG[1*], YANCHUN LI[2*], HONGCHAO PAN[3], CHAO LIANG[2], YANHUI SU[3], JIAHAO DONG[2], BENSHANG LI[4*], ZHONG CHEN[2,3] and XIAODAI CUI[5]

[1]Department of Hematology, Children's Hospital of Capital Institute of Pediatrics, Beijing 100020;
[2]Beijing Hightrust Diagnostics, Co., Kindstar Globalgene Technology, Inc., Beijing 100176;
[3]Shanghai Simplegene Clinical Laboratory, Co., Kindstar Globalgene Technology, Inc., Shanghai 200025;
[4]Key Laboratory of Pediatric Hematology and Oncology Ministry of Health, Department of Hematology and Oncology, Shanghai Children's Medical Center, Shanghai Jiao Tong University School of Medicine, Shanghai 200127;
[5]Department of Key Laboratory, Children's Hospital of Capital Institute of Pediatrics, Beijing 100020, P.R. China

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Abstract. Fusion genes are products of chromosomal translocations that generate either a dysregulated partner gene or a chimeric fusion protein with new properties, and contribute significantly to leukemia development and clinical risk stratification. However, simultaneous detection of several hundreds of fusion genes has always been a challenge in a clinical laboratory setting. In the present study, a total of 182 pediatric patients with leukemia were screened for fusion genes by employing a novel genomic DNA-, instead of RNA-, based next-generation sequencing (NGS) method. This involved the comparison of the multiply targeted capture sequencing method with a detection panel of 270 fusion genes (MTCS-270) with an RNA-based multiplex reverse transcription-PCR technique with a detection panel of 57 fusion genes (MRTP-57). MRTP-57 has been well established in the clinical lab at Beijing Hightrust Diagnostics, Co. (Beijing, China) for an up-front leukemia diagnosis and served as the control technique in the present study. In the series, MTCS-270 and MRTP-57 yielded a positive fusion gene detection rate of 50.0% (91/182) and 41.8% (76/182), respectively, indicating an advantage of MTCS-270 over MRTP-57 in overall detection sensitivity. Specifically, all the fusion genes detected by MRTP-57 were also identified by MTCS-270, clearly signifying the respectable detection accuracy of MTCS-270. Notably, across the patients screened, MTCS-270 identified more samples with fusion genes than MRTP-57, illustrating a broader fusion gene detection coverage by MTCS-270. The present study provides solid evidence that this DNA-based NGS approach can be used as a potential detection tool together with other well-established molecular cytogenetic methods for leukemia management, and to the best of our knowledge, represents the largest leukemia fusion gene identification analysis by genomic NGS.

Correspondence to: Dr Zhong Chen, Beijing Hightrust Diagnostics, Co., Kindstar Globalgene Technology, Inc., Building 3, 1 Disheng North Street, Beijing 100176, P.R. China
E-mail: chenzhong@kindstar.com.cn

Dr Xiaodai Cui, Department of Key Laboratory, Children's Hospital of Capital Institute of Pediatrics, 2 Yabao Road, Chaoyang, Beijing 100020, P.R. China
E-mail: xdcui61@sina.com

[*]Contributed equally

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Introduction

Leukemia is a heterogeneous clonal malignancy that develops from hematopoietic stem cells. All the different molecular events involved in the development of leukemia result from genetic or chromosomal lesions in blood-forming cells, commonly including duplication of genetic material, loss of genetic information, inactivation of genes that normally suppress tumor development, chromosomal translocations and the release of abnormal fusion proteins. Leukemia is classified by the dominant cell type, and by duration from onset to death (1,2). The 2016 revision to the World Health Organization (WHO) Classification of myeloid neoplasms and acute leukemia mainly divides leukemia into acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL) and other types of hematological neoplasms (1). The 2016 WHO Classification also categorizes AML into 11 genetic subtypes and ALL into nine genetic subtypes, with each subtype containing relatively unique clinical and prognostic characteristics. During diagnosis, molecular cytogenetic abnormalities are the primary indicators that must be investigated for patients with leukemia for the purpose of assessing risk stratification. Notably, chromosomal translocations resulting in fusion genes account for the most important genetic events and are specific for some particular subtypes of leukemia,

such as t(8;21)(q22;q22), inv(16)(p13q22), t(16;16)(p13;q22) and t(15;17)(q22;q12) in AML, t(12;21)(p13;q22), t(9;22)(q34;q11.2), t(1;19)(q23;p13) and 11q23/MLL-rearrangements in ALL, and t(9;22)(q34;q11.2) in chronic myelocytic leukemia (CML) (1,2). Importantly, fusion genes have also been reported in association with 40-50% of childhood AML and ALL cases (3,4). Although not all the fusion genes identified have clinical relevance, a number of them do play a significant role in leukemia diagnosis, risk stratification or minimal residual disease detection (1,2). Therefore, efficient screening and detection of multiple fusion genes plays a significant role in managing patients with leukemia.

Detection of fusion genes can be achieved at RNA (transcripts of fusion genes) or genomic DNA levels. With the development of an RNA-based multiplex reverse transcription-PCR (RT-PCR) technique, simultaneous screening of several tens of fusion genes has become feasible (5). This has provided great convenience over standard molecular cytogenetic tests [traditional chromosome and fluorescence *in situ* hybridization (FISH) analysis] for the clinical diagnosis and treatment of patients with leukemia. However, although RT-PCR allows for the highly sensitive detection of fusion gene transcripts at RNA levels, it requires primer pairs specific for the fusion gene breakpoints. In consideration of the great number of fusion genes and breakpoint variants reported in leukemia, it is difficult and not practically feasible to simultaneously analyze several hundreds of fusion genes and breakpoint variants (5).

With the progress of molecular biology technology, especially following the widespread applications of next-generation sequencing (NGS), NGS has allowed systematic identification of molecular features associated with leukemia (6-8). In the present study, a novel genomic DNA-, instead of RNA-, based NGS method was adopted to simultaneously detect >200 fusion genes in a group of pediatric patients with leukemia. In particular, the DNA-based NGS method involving the multiply targeted capture sequencing method with a detection panel of 270 fusion genes (MTCS-270; Table I) was compared with an RNA-based RT-PCR technique involving multiplex RT-PCR with a detection panel of 57 fusion genes (MRTP-57; Table I) for parallel analysis of fusion genes. The MTCS-270 method followed the same technical principles reported previously by our group (9). MRTP-57 has been well established in the clinical lab at Beijing Hightrust Diagnostics, Co. (Beijing, China) for an up-front leukemia diagnosis and served as the control technique in the present study. To the best of our knowledge, the present study presents the largest fusion gene identification analysis by DNA-based NGS in leukemia.

Materials and methods

Patient samples. All 182 pediatric patients enrolled in the present study were diagnosed at the Department of Hematology, Children's Hospital of Capital Institute of Pediatrics Beijing, China between January 2018 and December 2019. At hospital admission, bone marrow samples were obtained from the patients as part of a diagnosis, from which DNA and RNA were extracted for analysis. This research was approved by the Ethics Committee at the Children's Hospital of Capital Institute of Pediatrics (Beijing, China). All parents/guardians of the minor patients (<18 years old) included in the study signed a written informed consent form.

Genomic DNA library preparation and multiply targeted capture sequencing. A QIAamp DNA Blood Mini Kit (Qiagen GmbH) was used to extract total DNA from bone marrow samples according to the manufacturer's instructions. Each DNA sample was quantified (20-100 ng/μl) with the Qubit™ dsDNA BR Assay kit (Thermo Fisher Scientific, Inc.) and DNA integrity was evaluated by 2% agarose gel electrophoresis. High-quality genomic DNA (OD 260/280 ratio of 1.8-2.0) was fragmented by sonication (cycles, 10; cycle time, 60 sec; temperature, 4˚C) using a Covaris S2 (Covaris LLC). The fragmented DNA was repaired, ligated with Illumina adapters (Illumina, Inc.) and size-selected at 350 to 400 base pairs (bp). Subsequently, the size-selected product was amplified, tagged with a unique index using the Illumina DNA prep kit, and validated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

The amplified DNA product was captured with a customized panel of biotinylated oligoprobes using MyGenostics GenCap Enrichment technology (MyGenostics, Inc.). The capture experiment was performed according to the manufacturer's protocol. For the detection of a fusion gene involving a gene of interest, the oligoprobes were designed to capture the whole region of the gene and to cover the likely break regions of a fusion partner gene. These oligoprobes were projected to identify 270 types of fusion genes found in leukemia (Table I). Finally, the captured DNA library was amplified, quantified with the Qubit™ dsDNA BR Assay kit (Thermo Fisher Scientific, Inc.), and sequenced at ~20 pM using the HiSeq X Reagent Kit on a HiSeq X Ten system (Illumina, Inc.) for paired reads at 150 bp. Fig. 1 shows the location of biotinylated oligoprobes relative to two representatives of commonly detected fusion genes in this study (*BCR/ABL1* and *PML/RARα*).

For bioinformatics analysis, sequence variant detection was performed by using paired-end sequencing data, as previously described [Phrap (http://www.phrap.org/), SAMtools (http://samtools.sourceforge.net/)] (9,10).

RNA collection and multiplex RT-PCR. The MRTP-57 protocol followed a previously reported strategy (11). In brief, bone marrow samples were prepared first by the trizol (ZYMO Research Corp.) homogenization method according to the manufacturer's recommendations. Subsequent total RNA extraction was performed using the Direct-zol™ RNA MiniPrep (ZYMO Research Corp.) assay. Extracted RNA was resuspended in RNase-free water, and the concentration and quality determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The RNA samples were reverse transcribed into high-quality cDNA using optimized primers [oligo (dT)-based primers and random hexamer primers] and other related reagents from Takara Bio, Inc. (RNase inhibitor, dNTPs and moloney murine leukemia virus reverse transcriptase). Using an ABI system (Veriti96; Applied Biosystems; Thermo Fisher Scientific, Inc.), the RT-PCR procedure was then performed with individual primer sets (Table SI), which were specifically designed for the detection of 57 fusion genes

Table I. Fusion genes targeted by the MTCS-270 and MRTP-57 detection panels.

Technique	Fusion gene
MTCS-270	*ABL1/PRRC2B, ACIN1/NUTM1, ACTB/GLI1, AKAP9/BRAF, ALK/PTPN3, ALPHA/TFEB, AML1/CLCA2, AML1/ETO, AML1/ERG, AML1/LRP16AML1/MDS1, AML1/MTG16, AML1/PRDM16, AML1/PRDX4, AML1/ZFPM2, ANKRD11/SPG7, ARID1B/ZNF384, API2/MALT1, ATF7IP/JAK2, ATIC/ALK, BCOR/RARα, BCR/ABL1, BCR/EXOSC2, BCR/FGFR1, BCR/JAK2, BCR/PDGFRα, BMP2K/ZNF384, BMP6/MYC, BTL/ETV6, CALM/AF10, CARS/ALK, CBFA2T3/GLIS2, CBFβ/MYH11, CBP/MORF, CCDC6/ROS1, CCND1/MYC, CCND3/MYC, CEV14/PDGFRβ, CHD4/EP300, CIC/DUX4, CLTC/ALK, CLTC/TFE3, CREBBP/ZNF384, CSF2RA/CRLF2, DEK/CAN, DGKH/ZFAND3, DUX4/FRG2B, E2A/HLF, E2A/PBX1, E2A/TFPT, ELF1/FOXO1, EMILIN2/PAX5, EML1/ABL1, EML4/ALK, EP300/ZNF384, EPC1/PHF1, ERBB2IP/MAST4, ERG/DUX4, ETV6/ABL1, ETV6/CDX2, ETV6/FLT3, ETV6/MDS1, ETV6/MDS2, ETV6/NID1, ETV6/NTRK3, ETV6/PDGFRα, ETV6/PDGFRβ, ETV6/RUNX1, EWS/CREB1, EWS/ERG, EWS/ETV1, EWS/ETV4, EWS/FEV, EWS/FLI1, EWSR1/ELF5, EWSR1/ZNF384, FGFR1/RANBP2, FGFR2/CIT, FIP1L1/PDGFRα, FIP1L1/RARα, FN1/ALK, FOXK2/CLIP2, FOXO3/MYC, FOXP1/ABL1, FUS/ATF1, FUS/CHOP, FUS/CREB3L1, FUS/CREB3L2, HOXA11/MIR181A1HG, HOXA11AS/PBX1, IGH/BCL10, IGH/BCL2, IGH/BCL6, IGH/BCL9, IGH/CCND1, IGH/CRLF2, IGH/CEBPA, IGH/CEBPB, IGH/CEBPD, IGH/CEBPE, IGH/EPOR, IGH/MAFB, IGH/MALT1, IGH/MYC, IGK/MYC, IGL/MYC, IKZF1/CDK2, IKZF1/NUTM1, JAK2/SNX29, KIF5B/ALK, KIF5B/PDGFRα, KIF5B/RET, MEF2D/BCL9, MEF2D/CSF1R, MEF2D/DAZAP1, MEF2D/FOXJ2, MEF2D/HNRNPUL1, MEF2D/SS18, MLL/AF1P, MLL/AF1Q, MLL/AF4, MLL/AF6, MLL/AF9.MLL/AF10.MLL/AF17.MLL/AFX, MLL/ARHGEF12, MLL/CASC5, MLL/CBL, MLL/CREBBP, MLL/DIAPH2, MLL/ELL, MLL/ENL, MLL/FLNA, MLL/GAS7, MLL/KIAA0284, MLL/MEF2C, MLL-PTD, MLL/SEPT5, MLL/SEPT6, MLL/SEPT9, MLL/TET1, MLL/TIRAP, MLL/USP2, MOZ/CBP, MOZ/EP300, MOZ/TIF2, MSL2/TBL1XR1, MSN/ALK, MUM1/IRF4, MYH9/ALK, MYH9/IL2RB, MYO1F/KMT2A, MYST3/CREBBP, NCOR1/LYN, NDE1/PDGFRβ, NDUFA4/SCIN, NFIA/CBFA2T1, NFIA/CBFA2T3, NIN/PDGFRβ, NOC4L/FBRSL1, NONO/TFE3, NPM/ALK, NPM/MLF1, NPM/RARα, NT5C2/MLL, NUMA1/RARα, NUP214/ABL1, NUP98/ADD3, NUP98/BPTF, NUP98/CCDC28A, NUP98/DDX10, NUP98/FN1, NUP98/HOXA, NUP98/HOXA9, NUP98/HOXA10, NUP98/HOXA11, NUP98/HOXA13, NUP98/HOXC11, NUP98/HOXC13, NUP98/HOXD11, NUP98/HOXD13, NUP98/JARID1A, NUP98/LEDGF, NUP98/NSD1, NUP98/NSD3, NUP98/PHF23, NUP98/PMX1, NUP98/POU1F1, NUP98/PRRX2, NUP98/RAP1GDS1, NUP98/RARG, NUP98/SETBP1, NUP98/TOP1, P2RY8/CRLF2, P2RY8/PAX5, P2RY8/ZBED1, PAG1/ABL2, PALM/NCLN, PAX3/FKHR, PAX3/MLLT7, PAX3/NCOA1, PAX5/ASXL1, PAX5/WDR5, PAX5/AUTS2, PAX5/CBFA2T3, PAX5/ESRRB, PAX5/IGH, PAX5/JAK2, PAX5/KIF3B, PAX5/MLLT3, PAX5/RNF38, PAX5/TEL, PAX5/ZCCHC7, PAX5/ZNF521, PAX7/FKHR, PCM1/JAK2, PDE4DIP/PDGFRβ, PDZD7/ETV6, PICALM/BRAF, PIM3/BRD1, PIM3/METRNL, PIM3/SLC7A5, PLEKHA6/NTRK3, PLK5/ATN1, PLZF/RARα, PML/RARα, PPF1BP1/JAK2, PPFIBP1/ALK, PRC1/KIF7, PRCC/TFE3, PRDM16/RPN1, PRKAR1A/RARα, PRKG2/PDGFRβ, PRRC2B/BCR, RAEP1/PDGFRβ, RUNX1/EVX1, SET/CAN, SIL/TAL1, SSBP2/PDGFRβ, STAT5b/RARα, STRN/ALK, STRN3/JAK2, STRN/PDGFRα, SYNRG/ZNF384, TAF15/ZNF384, TCF3/ZNF384, TCRB/HOX11, TCRB/HOXA1, TCRB/LMO2, TCRB/TAL1, TCRB/TAL2, TCRD/HOX11, TCRD/LMO1, TCRD/LMO2, TCRD/RANBP17, TCRD/TAL1, TCTA/TAL1, TFG/ALK, TFG/CHN, TFG/NTRK1, TERF2/JAK2, TLS/ERG, TMPRSS2/ETV6, XBP1/MYC, ZEB2/PDGFRβ, ZEB2/RUNX1, ZMYND8/RELA, ZNF124/PUS3, ZNF384/RNF180*
MRTP-57	*AML1/ETO, AML1/ERG, AML1/MDS1, ARID1B/ZNF384, BCR/ABL1 (P190, P210, P230), BMP2K/ZNF384, CBFβ/MYH11, CREBBP/ZNF384, DEK/CAN, E2A/HLF, E2A/PBX1, EP300/ZNF384, ETV6/ABL1, ETV6/PDGFRα, ETV6/PDGFRβ, ETV6/RUNX1, EWSR1/ZNF384, FIP1L1/PDGFRα, FIP1L1/RARA, MEF2D/BCL9, MEF2D/CSF1R, MEF2D/DAZAP1, MEF2D/FOXJ2, MEF2D/HNRNPUL1, MEF2D/SS18, MLL/AF1P, MLL/AF1Q, MLL/AF4, MLL/AF6, MLL/AF9, MLL/AF10, MLL/AF17, MLL/AFX, MLL/ENL, MLL/ELL, MLL-PTD, NPM/ALK, NPM/MLF1, NPM/RARA, NUMA1/RARA, NUP98/HOXA9, NUP98/HOXA11, NUP98/HOXA13, NUP98/HOXC11, NUP98/HOXD13, NUP98/PMX1, NUP98/RARG, PLZF/RARA, PML/RARA(L,V,S), PRKARIA/RARA, SET/CAN, SIL/TAL1, STAT5B/RARA, SYNRG/ZNF384, TAF15/ZNF384, TCF3/ZNF384, TLS/ERG*

MTCS-270, multiply targeted capture sequencing method with a detection panel of 270 fusion genes. MRTP-57, RNA-based multiplex reverse transcription-PCR technique with a detection panel of 57 fusion genes; L, (gene) long-type; S, (gene) short-type; V, (gene) variant-type.

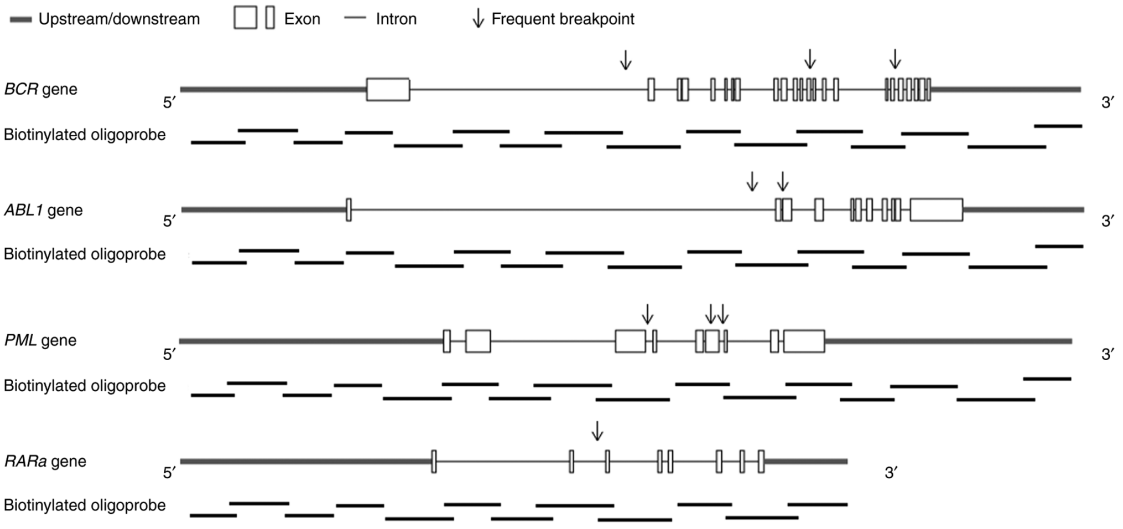

Figure 1. Location of biotinylated oligoprobes relative to two representative fusion gene partners (*BCR/ABL1* and *PML/RARα*).

commonly reported as primary genetic events involved in leukemogenesis (Table I).

Dependent on the breakpoints involved, some fusion genes may need only one pair of primers to cover the hot-breakpoint regions, while other may need two or more pairs of primers to do so, as illustrated in Table SI.

A multiplex touchdown RT-PCR strategy was used to detect the fusion genes. In brief, the samples were initially heated at 94˚C for 2 min, followed by 20 cycles of amplification each at 94˚C for 30 sec, 68˚C (decreasing incrementally by 0.5˚C per cycle) for 30 sec and 72˚C for 1 min, followed by another 20 cycles of amplification each at 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 1 min, and culminating with a final cycle at 72˚C for 10 min.

Results

Bone marrow samples were obtained from 182 newly diagnosed pediatric patients (102 men and 80 women; <18 years; median age, 4 years), including 46 patients with AML (median age, 6 years), 116 with B-cell ALL (B-ALL; median age, 5 years), 13 with T-cell ALL (T-ALL; median age, 5 years), 5 with CML (median age, 8 years) and 2 with juvenile myelomonocytic leukemia (JMML; ages, 3 years and 10 months). These samples were analyzed using MTCS-270 in parallel with MRTP-57. The clinical information and detection results are shown in Tables II-IV.

In the 46 AML samples screened (Table II), 28 (60.9%) and 21 (45.7%) were identified as containing fusion genes using MTCS-270 and MRTP-57, respectively (Fig. 2). All 21 samples with fusion genes detected by MRTP-57 were also identified by MTCS-270, signifying the accuracy and effectiveness of MTCS-270 in detecting these fusion genes. Due to the technical constraints of MRTP-57 in experimental design, numerous leukemia-related fusion genes could not be included in the detection panel for analysis; by contrast, MTCS-270 had a much broader fusion gene detection range and was able to identify 7 additional fusion genes in the AML samples screened. Each additional fusion gene detected was different (i.e., *CBFA2T3/GLIS2, NIFA/CBFA2T1, ZEB2/RUNX1, MYO1F/KMT2A, NUP98/NSD1, HOXA11AS/PBX1* and *EWSR/ELF5*), demonstrating the more comprehensive capability of MTCS-270 in fusion gene detection. Notably, by combining both the MTCS-270 and MRTP-57 detection approaches, the frequently observed fusion genes in AML, such as the *AML1/ETO*, *PML/RARA*, *CBFβ/MYH11* and *MLL*-related fusion genes, were detected in 5/46 (10.9%), 4/46 (8.7%), 2/46 (4.3%) and 9/46 (19.6%) samples, respectively, which was generally consistent with the findings reported in the literature (12,13). Furthermore, MTCS-270 was able to differentiate isoforms of affected fusion genes, which was beyond the detection capability of MRTP-57. For example, in the positively identified AML cases, 2 isoforms of the *AML1/ETO* fusion gene (i.e., *AML1* exons 1-5 to *ETO* exons 2-12 and *AML1* exons 1-5 to *ETO* exons 3-12), 3 isoforms of *PML/RARA* (i.e., *PML* exons 1-6 to *RARA* exons 3-9, *PML* exons 1-3 to *RARA* exons 3-9 and *PML* exons 1-6+ to *RARA* exons 3-9), 2 isoforms of *CBFβ/MYH11* (i.e., *CBFβ* exons 1-4 to *MYH11* exons 34-41 and *CBFβ* exons 1-5 to *MYH11* exons 33-41) and 2 isoforms of *MLL/AF9* (i.e., *MLL* exons 1-9 to *AF9* exons 6-11 and *MLL* exons 1-10 to *AF9* exons 6-11) were observed, further illustrating the precision of MTCS-270 in fusion gene analysis.

In the 116 B-ALL samples screened (Table III), 55 (47.4%) and 49 (42.2%) were identified as containing fusion genes using MTCS-270 and MRTP-57, respectively (Fig. 3). Similar to the results from the AML group, all the 49 samples with fusion genes identified by MRTP-57 were also included in the positively detected samples by MTCS-270; furthermore, 6 additional samples were found to carry fusion genes by MTCS-270, but not by MRTP-57, including 3 samples with *IGH/MYC*, 1 with *IGH/CRLF2*, 1 with *PAX5/WDR5* and 1 with *HOXA11/MIR181A1HG*. The analyses of MTCS-270 together with MRTP-57 revealed that the commonly observed fusion genes in the B-ALL samples screened were *ETV6/RUNX1* in 22/116 (19.0%), *BCR/ABL1* (P190) in 7/116 (6.0%), *ZNF384*-related fusion genes in 6/116 (5.2%), *MLL*-related fusion genes in 5/116 (4.3%), *E2A/PBX1* in 4/116 (3.4%)

Table II. Clinical information and detection results for patients with AML.

Clinical information	MTCS-270	MRTP-57
Diagnosis	AML	AML
Age, years		
Median	6	6
Range	0-13	0-13
Sex, n		
Male	25	25
Female	21	21
Fusion gene, n (fusion breakpoint)		
AML1/ETO	1 *(E1-5_E2-12)*[a]	5
	4 *(E1-5_E3-12)*	
PML/RARα	1 *(E1-6_E3-9) L*	4
	2 *(E1-3_E3-9) S*	
	1 *(E1-6+_E3-9) V*	
NPM/RARα	1 *(E1-4_E3-9)*	1
CBFβ/MYH11	1 *(E1-4_E34-41)*	2
	1 *(E1-5_E33-41)*	
MLL/AF9	1 *(E1-9_E6-11)*	5
	4 *(E1-10_E6-11)*	
MLL/AF10	1 *(E1-9_E9-24)*	1
MLL/AF1Q	1 *(E1-8_E2)*	2
	1 *(E1-9_E2)*	
MLL/ENL	1 *(E1-8_E2-12)*	1
CBFA2T3/GLIS2	1 *(E1-11_E5-8)*	
NIFA/CBFA2T1	1 *(E1-4_E3-12)*	
MYO1F/KMT2A	1 *(E1_E9-36)*	
ZEB2/RUNX1	1 *(E1-2_E2-8)*	
NUP98/NSD1	1 *(E1-12_E6-23)*	
HOXA11AS/PBX1	1 *(E1-4_E2-1)*	
EWSR1/ELF5	1 *(E1-8_E2-7)*	
Total patients with fusion genes, n	28	21
Total patients analyzed, n	46	46
Fusion gene detection frequency, %	60.9	45.7

[a](*E1-5_E2-12*) signifies a fusion of *AML1* exons 1-5 to *ETO* exons 2-12. This nomenclature has been followed throughout the table. AML, acute myeloid leukemia; E, exon; L, (gene) long-type; MRTP-57, multiplex RT-PCR with a detection panel of 57 fusion genes; MTCS-270, multiply targeted capture sequencing method with a detection panel of 270 fusion genes; S, (gene) short-type; V, (gene) variant-type.

and *MEF2D/BCL-9* in 4/116 (3.4%), which was generally consistent with observations reported in the literature (14-16). Finally, as with the AML samples, several isoforms of the affected fusion genes were distinguished only by MTCS-270, including 2 isoforms of the *ETV6/RUNX1* fusion gene (i.e., *ETV6* exons 1-5 to *RUNX1* exons 2-8 and *ETV6* exons1-5 to *RUNX1* exons 3-8), 2 isoforms of *MLL/AF4* (i.e., *MLL* exons 1-8 to *AF4* exons 4-20 and *MLL* exons 1-10 to *AF4* exons 4-20), 2 isoforms of *MLL/ENL* (i.e., *MLL* exons 1-10 to *ENL* exons 1-12 and *MLL* exons 1-10 to *ENL* exons 7-12) and 2 isoforms of *MEF2D/BCL9* (i.e., *MEF2D* exons 1-3 to *BCL9* exons 9-10 and *MEF2D* exons 1-6 to *BCL9* exon 10).

In the remaining patients, 1/13 T-ALL (Table III), 4/5 CML (Table IV) and 1/2 JMML (Table IV) samples containing disease-relevant fusion genes were detected by MRTP-57; by contrast, in addition to the aforementioned fusion genes, MTCS-270 also detected that 1 more T-ALL and 1 more CML sample that contained disease-related fusion genes. Notably, in comparison with MRTP-57, MTCS-270 was also able to differentiate 2 isoforms of the *BCR/ABL1* (P210) fusion gene and to disclose the presence of *BCR/EXOSC2*, a variant of the *BCR/ABL1* fusion gene, in CML samples.

Discussion

Clinically, FISH and RT-PCR are the methods commonly employed for detection of fusion genes in leukemia. However, due to various technical constraints, such as intensive labor and reagent costs, as well as some limitations involved in experimental design and execution, it is practically impossible

Table III. Clinical information and detection results for patients with ALL.

Clinical information	MTCS-270		MRTP-57	
Diagnosis	B-ALL	T-ALL	B-ALL	T-ALL
Age, years				
Median	5	5	5	5
Range	0-14	1-9	0-14	1-9
Sex, n				
Male	67	6	67	6
Female	49	7	49	7
Fusion gene, n (fusion breakpoint)				
ETV6/RUNX1	19 *(E1-5_E2-8)*[a]		22	
	3 *(E1-5_E3-8)*			
BCR/ABL1	7 *(E1_E2-11)*		7	
E2A/PBX1	4 *(E1-16_E3-9)*		4	
MLL/AF4	2 *(E1-8_E4-20)*		3	
	1 *(E1-10_E4-20)*			
MLL/ENL	1 *(E1-10_E1-12)*		2	
	1 *(E1-10_E7-12)*			
TCF3/ZNF384	3 *(E1-13_E3-11)*		3	
TAF15/ZNF384	1 *(E1-9_E3-11)*		1	
EP300/ZNF384	2 *(E1-6_E3-11)*		2	
MEF2D/BCL9	3 *(E1-3_E9-10)*		4	
	1 *(E1-6_E10)*			
TLS/ERG	1 *(E1-7_10)*		1	
SIL/TAL1		1 *(E1_E1-5)*		1
IGH/MYC	3 *(IGHJ _E2-3)*			
IGH/CRLF2	1 *(IGHJ_E1-6)*			
RUNX1/EVX1		1 *(E1_E2-3)*		
PAX5/WDR5	*1 (E1-5_E2-14)*			
HOXA11/MIR181A1HG	1 *(E2-1_E3)*			
Total patients with fusion gene, n	55	2	49	1
Total patients analyzed, n	116	13	116	13
Fusion gene detection frequency, %	47.4	15.4	42.2	7.7

[a](*E1-5_E2-8*) signifies a fusion of *ETV6* exons 1-5 to *RUNX1* exons 2-8. This nomenclature has been followed throughout the table. ALL, acute lymphocytic leukemia; B-ALL, B-cell ALL; E, exon; MRTP-57, multiplex RT-PCR with a detection panel of 57 fusion genes; MTCS-270, multiply targeted capture sequencing method with a detection panel of 270 fusion genes; T-ALL, T-cell ALL.

to simultaneously analyze several tens to several hundreds of fusion genes and breakpoint variants by these two methods. RNA-based NGS (RNA-Seq) is the leading technique for characterizing transcriptomes of cells in a patient sample, providing a theoretically ideal tool for simultaneous detection of multiple fusion genes in leukemia. In addition, due to its capability in simultaneously identifying global gene transcripts and diverse RNA species, RNA-Seq has the promise to revolutionize clinical testing for a wide range of disorders (17). However, due to some challenges encountered in translating this technology into clinical practice, including ongoing efforts to establish reference standards, achieving testing results with high reproducibility, accuracy and precision, and several potential procedure-related technical difficulties (such as inappropriate sample handling and preparation resulting in RNA instability, degradation, composition biases and gene expression artifacts, as well as RNA capture inefficiency), at present RNA-Seq is not commonly recommended as an up-front approach for multiple fusion gene detection in the clinical setting (17).

DNA-, but not RNA-, based NGS has been well documented in detecting genomic structural and single-nucleotide variants (6,9,18); in addition, it has been reported to simultaneously analyze some fusion genes and breakpoint variants in leukemic cells (19). This approach identified breakpoints and flanking sequences at the genomic level through the design and synthesis of capture probes spanning genomic regions of fusion gene breakpoints. This method required

Table IV. Clinical information and detection results for the patients with CML and JMML.

Clinical information	MTCS-270		MRTP-57	
Diagnosis	CML	JMML	CML	JMML
Age, years				
Median	8	N/A	8	N/A
Range	5-10	0-3	5-10	0-3
Sex, n				
Male	3	1	3	1
Female	2	1	2	1
Fusion gene, n (fusion breakpoint)				
BCR/ABL1	1 *(E13-22_E2-11)*		4	
	3 *(E14-22_E2-11)*			
BCR/EXOSC2	1 *(E1_E9)*[a]			
MLL/ELL		1 *(E1-9_E2-12)*		1
Total patients with fusion genes, n	5	1	4	1
Total patients analyzed, n	5	2	5	2

[a](*E1_E9*) signifies a fusion of *BCR* exon 1 to *EXOSC2* to exon 9. This nomenclature also applies to *MLL/ELL* (*E1-9_E2-12*). CML, chronic myelocytic leukemia; E, exon; JMML, juvenile myelomonocytic leukemia; MRTP-57, multiplex RT-PCR with a detection panel of 57 fusion genes; MTCS-270, multiply targeted capture sequencing method with a detection panel of 270 fusion genes.

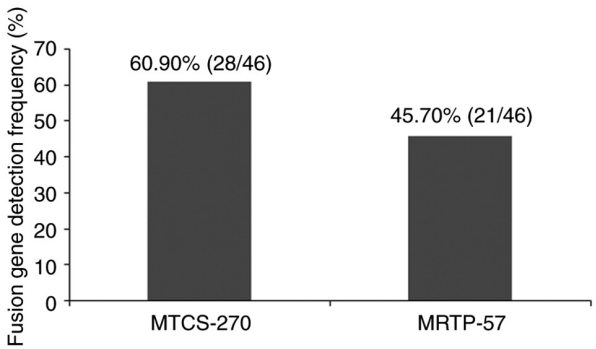

Figure 2. Detection results for patients with acute myeloid leukemia. MTCS-270, multiply targeted capture sequencing method with a detection panel of 270 fusion genes; MRTP-57, RNA-based multiplex reverse transcription-PCR technique with a detection panel of 57 fusion genes.

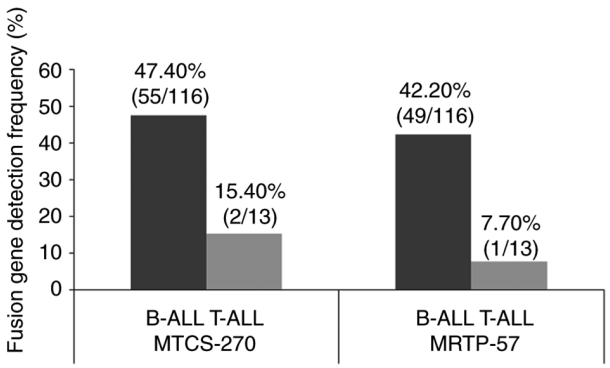

Figure 3. Detection results for patients with ALL. MTCS-270, multiply targeted capture sequencing method with a detection panel of 270 fusion genes; MRTP-57, RNA-based multiplex reverse transcription-PCR technique with a detection panel of 57 fusion genes; T-ALL, T-cell acute lymphocytic leukemia; B-ALL, B-cell acute lymphocytic leukemia.

lower amounts of genomic DNA for analysis, possessed a relatively lower workload for multiple-sample handling and could yield more meaningful information, such as detailed breakpoints and flanking sequences of fusion genes. In order to further appreciate the efficacy of this approach in analyzing genomic aberrations of leukemic cells, the present study focused on fusion genes, one of the most difficult and complex types of genomic aberrations, as the targets for evaluation. The effectiveness of this approach was assessed by simultaneously analyzing >200 fusion genes in patient samples.

In the present study, MTCS-270 and MRTP-57 yielded an overall positive fusion gene detection rate of 50.0% (91/182) and 41.8% (76/182), respectively, indicating an advantage of MTCS-270 over MRTP-57 in overall detection sensitivity. Specifically, all the fusion genes detected by MRTP-57 were also identified by MTCS-270 across all the leukemia samples studied, clearly signifying the respectable detection accuracy and effectiveness of MTCS-270.

Notably, MTCS-270 identified more samples with disease-related fusion genes (including 7 additional fusion genes in AML, 6 in B-ALL, 1 in T-ALL and 1 in CML) than MRTP-57, reflecting the fact that MTCS-270 was designed to capture more fusion genes than MRTP-57 and thus had a much broader detection coverage. From the additional cases with fusion genes identified exclusively by MTCS-270, the AML sample with *CBFA2T3/GLIS2* and the ALL sample with *PAX5/WDR5* were selected as a case in point, to further verify the detection accuracy of MTCS-270. RT-quantitative PCR with the primers and probes (Table SII) designed according to the breakpoints identified in the MTCS-270 analysis were employed to conduct the verification study on the extracted

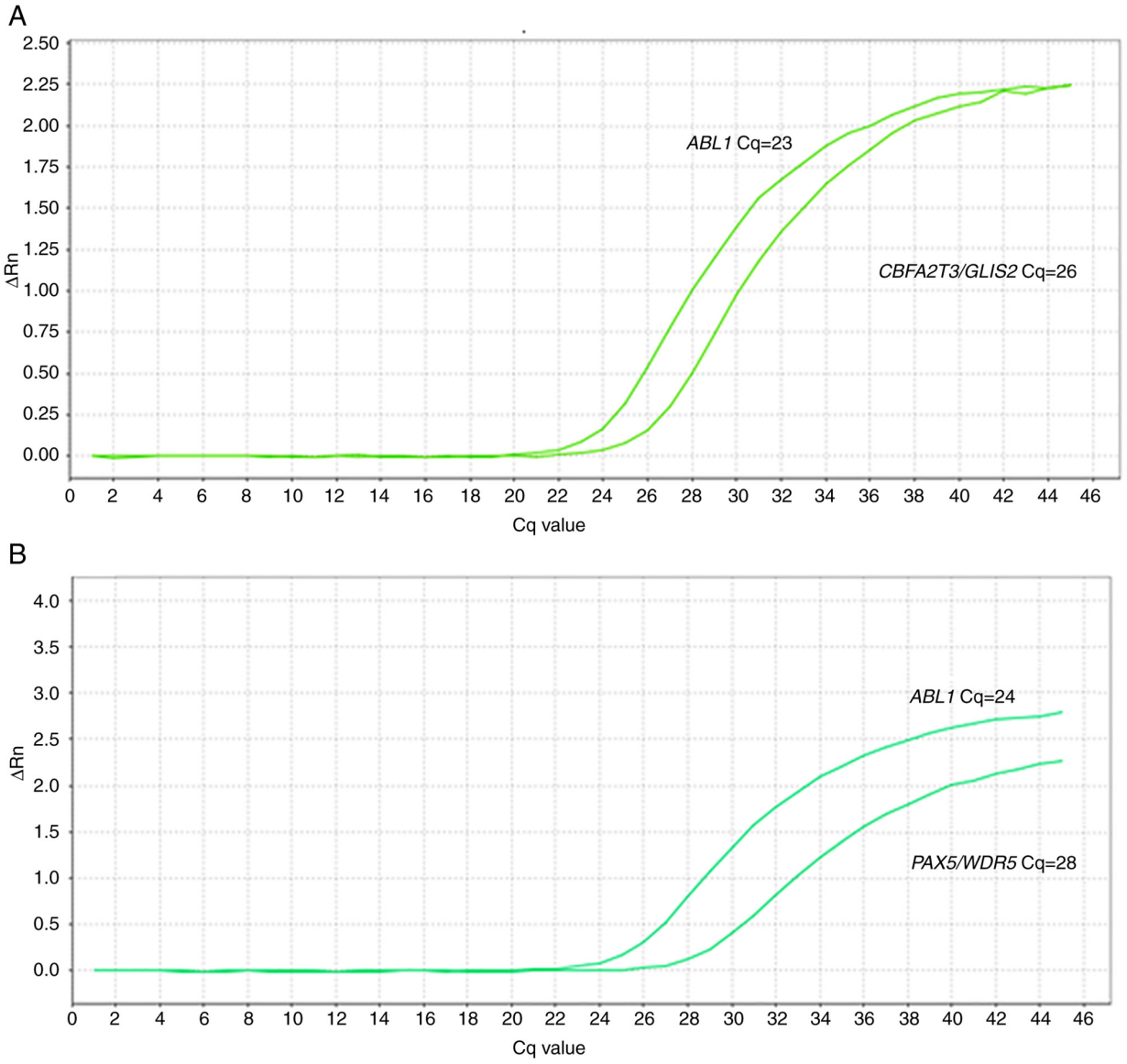

Figure 4. Verification study using reverse transcription-quantitative PCR to confirm the presence of two fusion genes (*CBFA2T3/GLIS2* and *PAX5/WDR5*) in the respective patients. (A) The AML sample with *CBFA2T3/GLIS2*. (B) The ALL sample with *PAX5/WDR5*. Cq value, quantification cycle value; ΔRn, Δ normalized reporter.

RNA samples (Table SII). The *ABL1* gene served as the internal control in this verification study. The results showed that quantification cycle values were 26 for *CBFA2T3/GLIS2* (23 for *ABL1*) and 28 for *PAX5/WDR5* (24 for *ABL1*) (Fig. 4), clearly confirming the presence of these two fusion genes in the patients screened.

Finally, MTCS-270 was capable of distinguishing isoforms of affected fusion genes, such as 2 isoforms of *AML1/ETO*, 3 of *PML/RARA*, 2 of *CBFβ/MYH11* and 2 of *MLL/AF9* in patients with AML, and 2 isoforms of *ETV6/RUNX1*, 2 of *MLL/AF4*, 2 of *MLL/ENL* and 2 of *MEF2D/BCL9* in patients with B-ALL, and finally 2 isoforms of *BCR/ABL1* in patients with CML. This was above the analyzing capability of MRTP-57 and further demonstrated the analytic precision of MTCS-270 in such examinations. Precise differentiation of isoforms of affected fusion genes could be clinically significant. For example, based on various breakpoint cluster regions within the *PML* locus, the *PML/RARα* fusion genes are termed long (L)-type, variant (V)-type and short (S)-type. Acute promyelocytic leukemia patients with S-type *PML/RARA* have a poorer prognosis compared with those with L-type *PML/RARA* (20).

The proposed DNA-based NGS method can be further improved by adding more targeted and clinically significant fusion genes to the detection panel, which would allow for an even broader detection coverage for leukemia. Although the present study highlighted the efficacy of MTCS-270 in detecting leukemia-related fusion genes, it should be considered as a complementary approach to RNA-based multiplex RT-PCR and other well-established molecular cytogenetic methods, as genomic DNA-based NGS has its technical limitations. For example, DNA-based NGS is unable to detect numerous complex chromosomal rearrangements due to a lack of recognizable fusion genes generated. In this event, karyotyping or FISH analysis is often needed to resolve the issue. In prospect, the transcriptional profile provided by RNA-Seq may soon supplant current multiplex RT-PCR

methods for the up-front detection of various fusion genes in leukemia.

In summary, a genomic DNA-, instead of RNA-, based NGS method was utilized to simultaneously analyze >200 leukemia-related fusion genes. In comparison with the multiplex RT-PCR approach, the NGS method employed in the present study demonstrated an improved fusion gene detection efficacy in terms of technical sensitivity, accuracy, precision and overall comprehensiveness, which provides solid evidence that this genomic NGS method can be used as a potential detection tool, together with other well-established molecular cytogenetic methods, for leukemia management.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in figshare (https://doi.org/10.6084/m9.figshare.21391575.v1).

Authors' contributions

ZC, RL, BL and XC contributed to the conception of the study. HP, CL, YS and JD performed the experiments. ZC, YL and JD contributed significantly to the data analysis and manuscript preparation. SF and BL helped perform the analysis with constructive discussions. RL and ZC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This research was approved by the Ethics Committee of the Children's Hospital of Capital Institute of Pediatrics (Beijing, China). All parents/guardians of the pediatric patients included in the study signed a written informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, Bloomfield CD, Cazzola M and Vardiman JW: The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 127: 2391-2405, 2016.
2. Wiemels J: Chromosomal translocations in childhood leukemia: Natural history, mechanisms, and epidemiology. J Natl Cancer Inst Monogr 39: 87-90, 2008.
3. Conneely SE and Rau RE: The genomics of acute myeloid leukemia in children. Cancer Metastasis Rev 39: 189-209, 2020.
4. Taylor J, Xiao W and Abdel-Wahab O: Diagnosis and classification of hematologic malignancies on the basis of genetics. Blood 130: 410-423, 2017.
5. Xiong FF, Li BS, Zhang CX, Xiong H, Shen SH and Zhang QH: A pipeline with multiplex reverse transcription polymerase chain reaction and microarray for screening of chromosomal translocations in leukemia. Biomed Res Int 2013: 135086, 2013.
6. Wang Q, Xia J, Jia P, Pao W and Zhao Z: Application of next generation sequencing to human gene fusion detection: Computational tools, features and perspectives. Brief Bioinform 14: 506-519, 2013.
7. de Boer EN, Johansson LF, de Lange K, Bosga-Brouwer AG, van den Berg E, Sikkema-Raddatz B and van Diemen CC: Detection of fusion genes to determine minimal residual disease in leukemia using next-generation sequencing. Clin Chem 66: 1084-1092, 2020.
8. Chen B, Jiang L, Zhong ML, Li JF, Li BS, Peng LJ, Dai YT, Cui BW, Yan TQ, Zhang WN, *et al*: Identification of fusion genes and characterization of transcriptome features in T-cell acute lymphoblastic leukemia. Proc Natl Acad Sci USA 115: 373-378, 2018.
9. Jin Y, Wang X, Hu S, Tang J, Li B and Chai Y: Determination of ETV6-RUNX1 genomic breakpoint by next-generation sequencing. Cancer Med 5: 337-351, 2016.
10. Levy MA, Santos S, Kerkhof J, Stuart A, Aref-Eshghi E, Guo F, Hedley B, Wong H, Rauh M, Feilotter H, *et al*: Implementation of an NGS-based sequencing and gene fusion panel for clinical screening of patients with suspected hematologic malignancies. Eur J Haematol 103: 178-189, 2019.
11. Pallisgaard N, Hokland P, Riishøj DC, Pedersen B and Jørgensen P: Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. Blood 92: 574-588, 1998.
12. Quessada J, Cuccuini W, Saultier P, Loosveld M, Harrison CJ and Lafage-Pochitaloff M: Cytogenetics of pediatric acute myeloid leukemia: A review of the current knowledge. Genes (Basel) 12: 924, 2021.
13. Iijima-Yamashita Y, Matsuo H, Yamada M, Deguchi T, Kiyokawa N, Shimada A, Tawa A, Takahashi H, Tomizawa D, Taga T, *et al*: Multiplex fusion gene testing in pediatric acute myeloid leukemia. Pediatr Int 60: 47-51, 2018.
14. Ampatzidou M, Papadhimitriou SI, Paterakis G, Pavlidis D, Tsitsikas K, Kostopoulos IV, Papadakis V, Vassilopoulos G and Polychronopoulou S: ETV6/RUNX1-positive childhood acute lymphoblastic leukemia (ALL): The spectrum of clonal heterogeneity and its impact on prognosis. Cancer Genet 224-225: 1-11, 2018.
15. Moorman AV, Ensor HM, Richards SM, Chilton L, Schwab C, Kinsey SE, Vora A, Mitchell CD and Harrison CJ: Prognostic effect of chromosomal abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: Results from the UK medical research council ALL97/99 randomised trial. Lancet Oncol 11: 429-438, 2010.
16. Gu Z, Churchman M, Roberts K, Li Y, Liu Y, Harvey RC, McCastlain K, Reshmi SC, Payne-Turner D, Iacobucci I, *et al*: Genomic analyses identify recurrent MEF2D fusions in acute lymphoblastic leukaemia. Nat Commun 7: 13331, 2016.
17. Byron SA, Keuren-Jensen KV, Engelthaler DM, Carpten JD and Craig DW: Translating RNA sequencing into clinical diagnostics: Opportunities and challenges. Nat Rev Genet 17: 257-271, 2016.
18. Zhan D, Zhang Y, Xiao P, Zheng X, Ruan M, Zhang J, Chen A, Zou Y, Chen Y, Huang G, *et al*: Whole exome sequencing identifies novel mutations of epigenetic regulators in chemorefractory pediatric acute myeloid leukemia. Leuk Res 65: 20-24, 2018.
19. Zhao J, Liang JW, Xue HL, Shen SH, Chen J, Tang YJ, Yu LS, Liang HH, Gu LJ, Tang JY and Li BS: The genetics and clinical characteristics of children morphologically diagnosed as acute promyelocytic leukemia. Leukemia 33: 1387-1399, 2018.
20. Ruiz-Argüelles GJ, Garcés-Eisele J, Reyes-Núñez V, Gómez-Rangel JD and Ruiz-Delgado GJ: More on geographic hematology: The breakpoint cluster regions of the PML/RARalpha fusion gene in Mexican Mestizo patients with promyelocytic leukemia are different from those in Caucasians. Leuk Lymphoma 45: 1365-1368, 2004.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.