



Minimal residual disease monitoring in childhood Philadelphia chromosome-positive acute lymphoblastic leukemia: prognostic significance and correlation between multiparameter flow cytometry and real-time quantitative polymerase chain reaction

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**Minimal residual disease monitoring in childhood Philadelphia
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and real-time quantitative polymerase chain reaction**

Running Title: MRD monitoring in childhood Ph-positive ALL

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Author Contributions

XZ and JL designed the study. JL, AL, YYG and JW collected data. JL analyzed data and drafted the manuscript. XZ, WY, YW and CYZ made critical revisions to the manuscript for important intellectual content. TH, JZ, PW, LZ, XC, YZ, YC, YG and CL provided guidance on statistical analysis and presentation of results. All authors approved the final manuscript and agreed to submit for publication.

Competing Interest

The authors declare no conflict of interest.

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Ethics Approval and Statement

This study complied with the principles of the Declaration of Helsinki. Approval was obtained from the Ethics Committee and Institutional Review Board of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences (IRB number: IIT-2015010-EC-1).

Data Availability Statement

The data that support the findings of this study are available upon reasonable request from the corresponding author.

Clinical trial registration

This trial is registered with Chinese Clinical Trial Registry (ChiCTR-IPR-14005706).

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With the addition of tyrosine kinase inhibitors (TKIs) to conventional chemotherapy, remarkable improvement had been demonstrated in the outcomes of pediatric Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph-positive ALL).¹⁻³ Dynamic risk stratification directed by minimal residual disease (MRD) plays a critical role in the treatment optimization. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) for *BCR::ABL1* and multiparameter flow cytometry (MFC) are the commonest assays of MRD monitoring in childhood Ph-positive ALL, of which the prognostic significance are still under debate.^{4,5} Meanwhile, different MRD assays sometimes yield discrepancies and pose challenges in clinical decision-making. Zuna et al⁶ reported a subset of patients exhibiting a “chronic myelogenous leukemia (CML)-like” biology characterized by *BCR::ABL1* expression in non-leukemic cells and an unparallel MRD reduction pattern. In the present study, we reviewed the MRD levels and outcomes of Ph-positive ALL enrolled in Chinese Children's Cancer Group (CCCG)-ALL-2015. Our analyses revealed an overall discordance rate of 25.6% between RT-qPCR and MFC, with 27.1% of patients identified as CML-like. Furthermore, only MRD by MFC, rather than RT-qPCR was informative of relapse.

Children under 18 years old with Ph-positive ALL and no previous treatment were enrolled in CCCG-ALL-2015 trial which was detailed in previous study.⁷ Bone marrow (BM) evaluations were done at day 19 (D19) and day 46 (TP1) of induction, end of consolidation (TP2), before re-induction (TP3), completion of 5 cycles of subsequent continuation (TP4) and treatment completion (TP5). RT-qPCR was conducted in accordance with the guidelines by the Europe Against Cancer program.⁸ Immunophenotype analyses were performed on at least 5×10^6 nucleated cells by FACS Cantoll flow cytometer (BD Biosciences). Both assays achieved a detection sensitivity of 10^{-4} (0.01%). Non-quantifiable positive and negative results were given the value of 10^{-5} and 10^{-6} , respectively. We considered two samples discordant if the MRD levels differed by >1 log. Patients with more than one discordant MRD sample were classified as CML-like. BM relapse was defined as BM blasts $\geq 5\%$ by morphology after achieving complete response (CR) and confirmed by MFC and molecular testing. Central nervous system (CNS) relapse was defined as recurrence of CNS leukemia detected by MFC. Molecular relapse was defined as a 2-log or greater increase in MRD measured by RT-qPCR. Cumulative incidence of

relapse (CIR) was measured from CR to relapse of any site. The competing event for CIR was death in remission. Event-free survival (EFS) was measured from diagnosis to relapse, or death from any cause, whichever came the first. Overall survival (OS) was measured from diagnosis to death from any cause. Continuous and categorical variables were compared by Mann-Whitney U test and Fisher's exact test, respectively. Uni- and multivariate regression analyses of EFS and CIR were performed using the Cox and Fine-Gray regression models. We adopted two strategies to derive multivariate model, (1) full model incorporating all variables; (2) stepwise regression model by backward selection using Akaike information criterion. Analyses were primarily based on as-treated, but secondary analyses for intention-to-treat were performed as well. Two-sided P values < 0.05 were considered statistically significant. All statistical analyses were performed by R statistical software version 4.2.2 (www.r-project.org). This study complied with the principles of the Declaration of Helsinki. Approval was obtained from the Ethics Committee and Institutional Review Board of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences (IRB number: IIT-2015010-EC-1).

Between May 13, 2015, and September 30, 2020, 59 children under 18 years old with newly-diagnosed Philadelphia chromosome-positive B-cell ALL were included in this study. The trial diagram was shown in Supplemental Figure 1. All patients achieved CR after induction treatment and were stratified as intermediate-risk. No patients except for one proceeded to allogeneic stem cell transplant (allo-SCT) in CR1 at other medical center due to persistent MRD positivity by RT-qPCR. There were 21 relapses, mostly BM (n=13, 61.9%), followed by isolated CNS (n=4, 19.1%), molecular (n=2, 9.5%) and combined molecular and CNS (n=2, 9.5%). The median interval from diagnosis to relapse was 30.5 (interquartile range [IQR], 19.3-42.9) months, with 5 very early relapses (<18 months, 23.8%), 9 early relapses (18-36 months, 42.9%) and 7 late relapses (>36 months, 33.3%). Two patients died in remission due to severe pneumonia, at 6.6 and 21.2 months post-diagnosis. TKI treatment was discontinued concurrently with other therapies. Participants who completed treatment had a median treatment duration of 33.1 (IQR, 31.7-34.7) months. With a median follow-up of 48.3 (IQR, 34.6-64.6) months, the 5-year CIR, EFS, OS and incidence of death in remission of the cohort were 45.8% (95% confidence interval [CI], 29.3%-60.9%), 51.2% (95% CI, 37.7%-69.5%), 82.1% (95% CI, 70.3%-95.9%) and 3.4% (95% CI,

0.6%-10.5%), respectively.

We included 219 paired MRD samples and all patients had MRD assessment at D19 and TP1. We observed a decline of MRD positivity by both assays and all available samples were negative by MFC after TP3, whereas there were still 37.0%, 33.3% and 25.9% patients remaining positive by RT-qPCR at TP3, TP4 and TP5 (Figure 1A). The overall discordant rate of the two methods was 25.6% (56/219), reaching the highest at D19 (45.8%, 27/59) and decreased as treatment proceeded (Figure 1B). All discordant samples fell into two patterns: (1) negative MFC and positive RT-qPCR higher than 10^{-3} (0.1%); (2) both positive, but MRD by RT-qPCR significantly higher than MFC, ranging from 2.5 logs to 8.2 logs (median 3.3 logs, Figure 1C).

CML-like accounted for 27.1% (16/59) of the cohort. We identified metamyelocytes carrying *BCR::ABL1* by fluorescence in situ hybridization in a patient with *BCR::ABL1* p190, indicating involvement of the myeloid lineage (Supplemental Figure 2A-B). The positivity rate between the CML-like and typical ALL was similar when evaluating MRD by MFC, but significantly higher in CML-like group by RT-qPCR at all time points except for TP5 (Figure 1D). Meanwhile, no statistical differences were found in clinical features between CML-like and typical ALL (Table 1). Furthermore, the two subtypes shared similar 5-year CIR (typical ALL versus CML-like, 48.9% [95% CI, 29.6%-65.7%] versus 38.0% [8.1%-68.9%], $P=0.31$) and EFS rates (typical ALL versus CML-like, 49.2% [95% CI, 34.3%-70.7%] versus 55.7% [31.1%-99.7%], $P=0.50$) as well. In both full and stepwise multivariate model, MRD at TP1 by MFC, rather than RT-qPCR, was found to be independently associated with CIR and EFS (Table 2), which remained stable when we implemented intention-to-treat analysis by including TKI group as initial allocation (Supplemental Table 1). Of note, no event occurred in patients with *BCR::ABL1* p210, thus type of fusion transcript cannot be included in the models. By log-rank test, p210 group exhibited more favorable 5-year CIR (0% versus 54.2% [34.9%-70.0%], $P=0.018$) and 5-year EFS (100% versus 42.4% [28.4%-63.3%], $P=0.018$).

One potential cause of the discordance and why MRD by RT-qPCR did not show prognostic value, could be attributed to multilineage involvement of *BCR::ABL1* resembling CML as we

demonstrated. Despite the underlying mechanism and clinical relevance of CML-like is still under investigated, it should be noted that CML-like is distinct from what has historically been recognized as CML in blast phase. Since both studies by us and Zuna et al⁶ demonstrated that CML-like took up around one-fourth of all Ph-positive ALL with no differences in clinical characteristics or overall outcomes comparing with typical ALL, which also posed challenges to distinguish them clinically. Furthermore, the fact that the positivity rates of MRD by MFC were similar at all time points between the two disease subtypes indicated equivalent sensitivity of blast cells to chemotherapy, and patients could achieve long-term survival treated by traditional ALL-based regimen without allo-SCT. These results are on the contrary of previous knowledge on CML in lymphoid blast crisis.⁹ Nevertheless, it raises further questions-should CML-like receive prolonged TKI therapy even when treatment for ALL had discontinued? Will the Ph-positive, non-ALL cells cause relapse? Could they benefit from allo-SCT in CR1? We still need longer follow-up and advanced techniques to answer these questions.

Interestingly, we did not confirm the superiority of dasatinib over imatinib as the multicenter study did⁷. This could be attributed to following factors: (1) the duration of follow-up in our study was significantly extended (median, 48.3 versus 26.4 months), allowing for detection of late relapses. (2) the discontinuation of randomization caused imbalance between the two groups. (3) dasatinib may exhibit better efficacy in preventing CNS relapse.¹⁰ But limited number of CNS events restricted us from performing such analysis, underscoring the need for larger cohorts with an extended follow-up periods to draw a more solid conclusion.

Surprisingly, no event was observed in patients with *BCR::ABL1* p210, which was contrast to adult studies where p210 was associated with adverse outcomes comparing to p190.^{11,12} Though derived from a limited sample size, this discrepancy highlighted substantial heterogeneity that remained to be elucidated between different fusion transcripts and age groups.

In summary, our findings suggested it is feasible to distinguish CML-like by the discrepancy between MFC and RT-qPCR, echoing previous results reported by Zuna et al⁶ where quantification of clonal immunoglobulin and T-cell receptor genes rearrangements was used

instead of MFC. MFC is more reliable when two assays yield conflicting conclusions, which contributes to enhance MRD-driven risk stratification. More integrated studies are warranted to confirm our conclusions and unravel the underlying mechanisms of Ph-positive ALL with CML-like features.

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Table 1. Demographic and clinical characteristics of included patients and comparison between typical acute lymphoblastic leukemia and chronic myelogenous leukemia-like.

Characteristic	Level	Overall (N=59)	Typical ALL (N=43)	CML-like (N=16)	P value
Gender (%)	Female	25 (42.4)	17 (39.5)	8 (50.0)	0.56
	Male	34 (57.6)	26 (60.5)	8 (50.0)	
Age at diagnosis (median [IQR])		9 [6, 12]	9 [6, 11]	10 [6, 14]	0.13
Age at diagnosis (years, %)	<10	34 (57.6)	26 (60.5)	8 (50.0)	0.56
	≥10	25 (42.4)	17 (39.5)	8 (50.0)	
WBC at diagnosis ($\times 10^9/L$, median [IQR])		74.11 [27.92, 178.26]	59.80 [27.92, 139.82]	108.34 [66.44, 204.59]	0.28
WBC at diagnosis ($\times 10^9/L$, %)	<50	21 (35.6)	17 (39.5)	4 (25.0)	0.37
	≥50	38 (64.4)	26 (60.5)	12 (75.0)	
Fusion transcript (%)	p190	50 (84.7)	37 (86.0)	13 (81.2)	0.69
	p210	9 (15.3)	6 (14.0)	3 (18.8)	
CNS status (%)	CNS1	55 (93.2)	41 (95.3)	14 (87.5)	0.30
	CNS2 or traumatic lumbar puncture	4 (6.8)	2 (4.7)	2 (12.5)	
Initial TKI group (%)	Dasatinib	44 (74.6)	33 (76.7)	11 (68.8)	0.52
	Imatinib	15 (25.4)	10 (23.3)	5 (31.2)	
Final TKI group (%)	Dasatinib	51 (86.4)	36 (83.7)	15 (93.8)	0.43
	Imatinib	8 (13.6)	7 (16.3)	1 (6.2)	

IQR, interquartile range; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; WBC, white blood cell count; CNS, central nervous system; TKI, tyrosine kinase inhibitor.

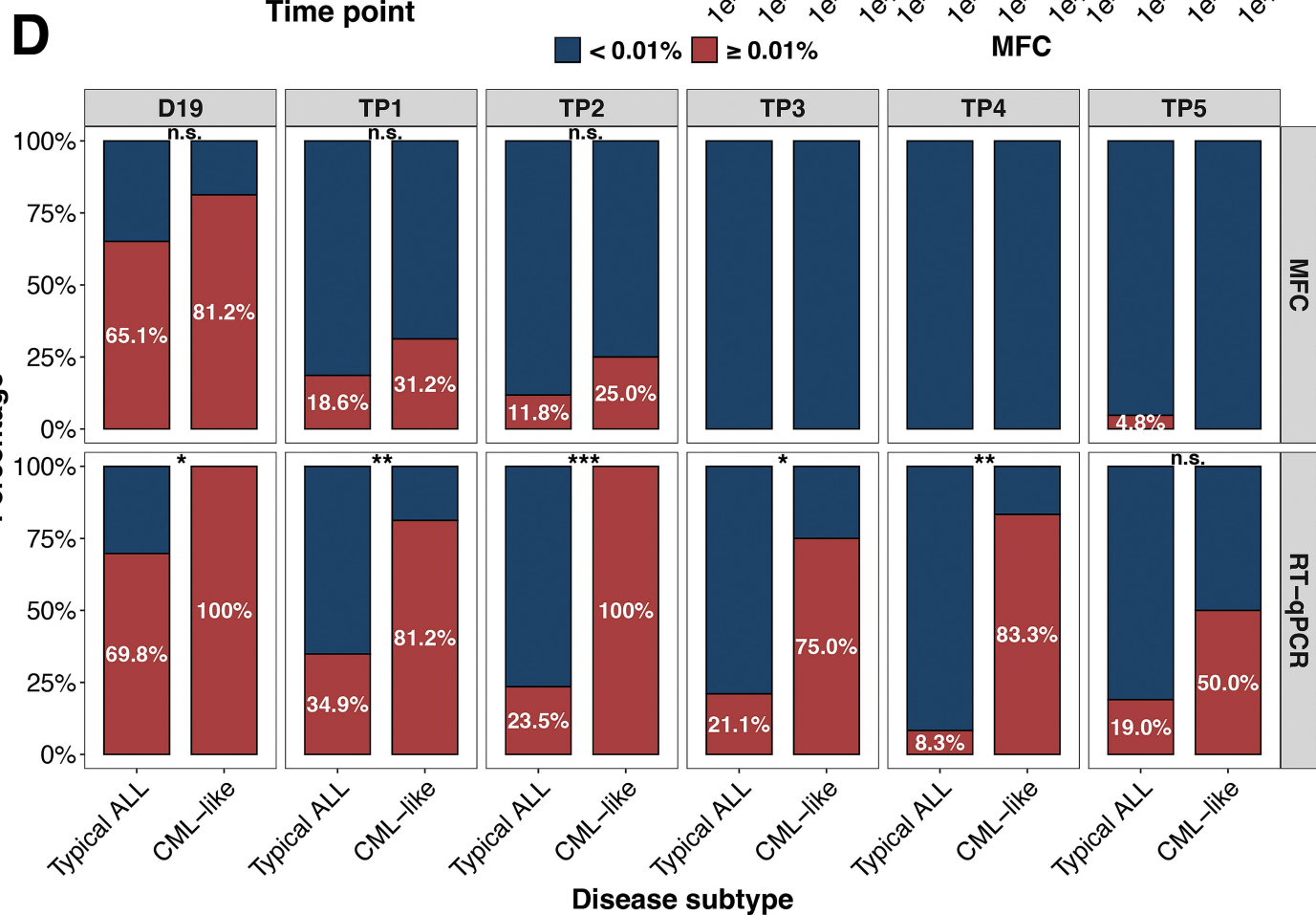
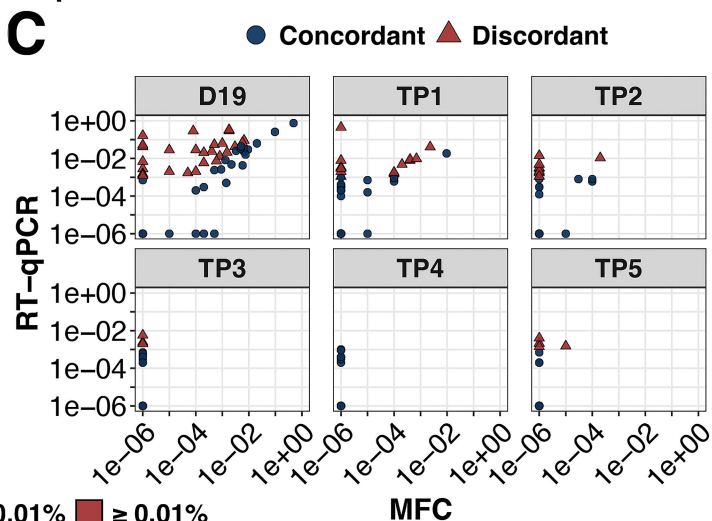
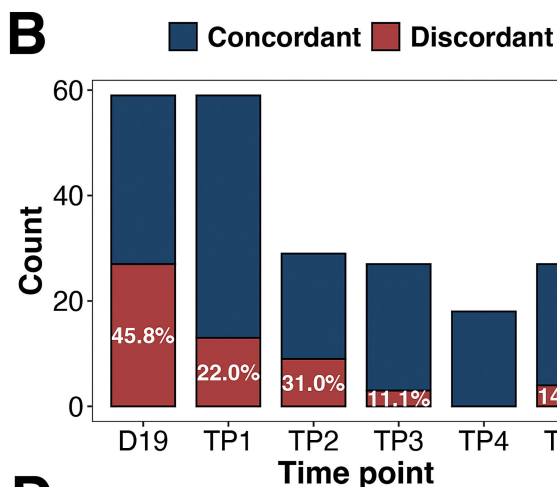
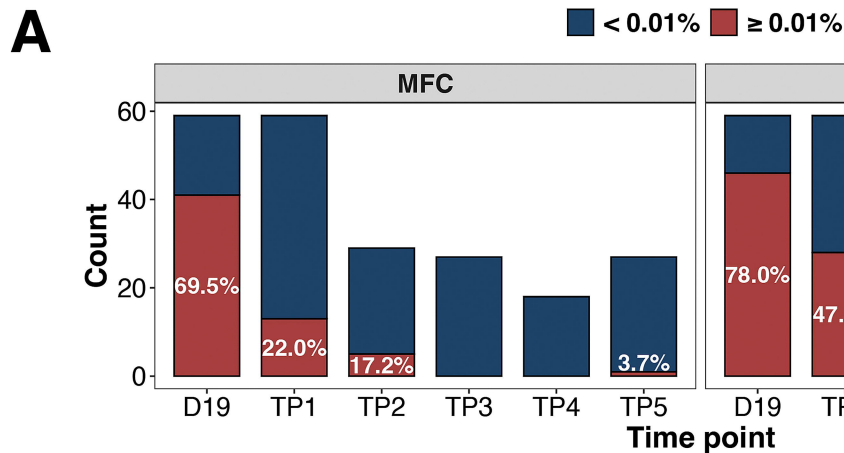
Table 2. Uni- and multivariate analyses of cumulative incidence of relapse and event-free survival of as-treated patients.

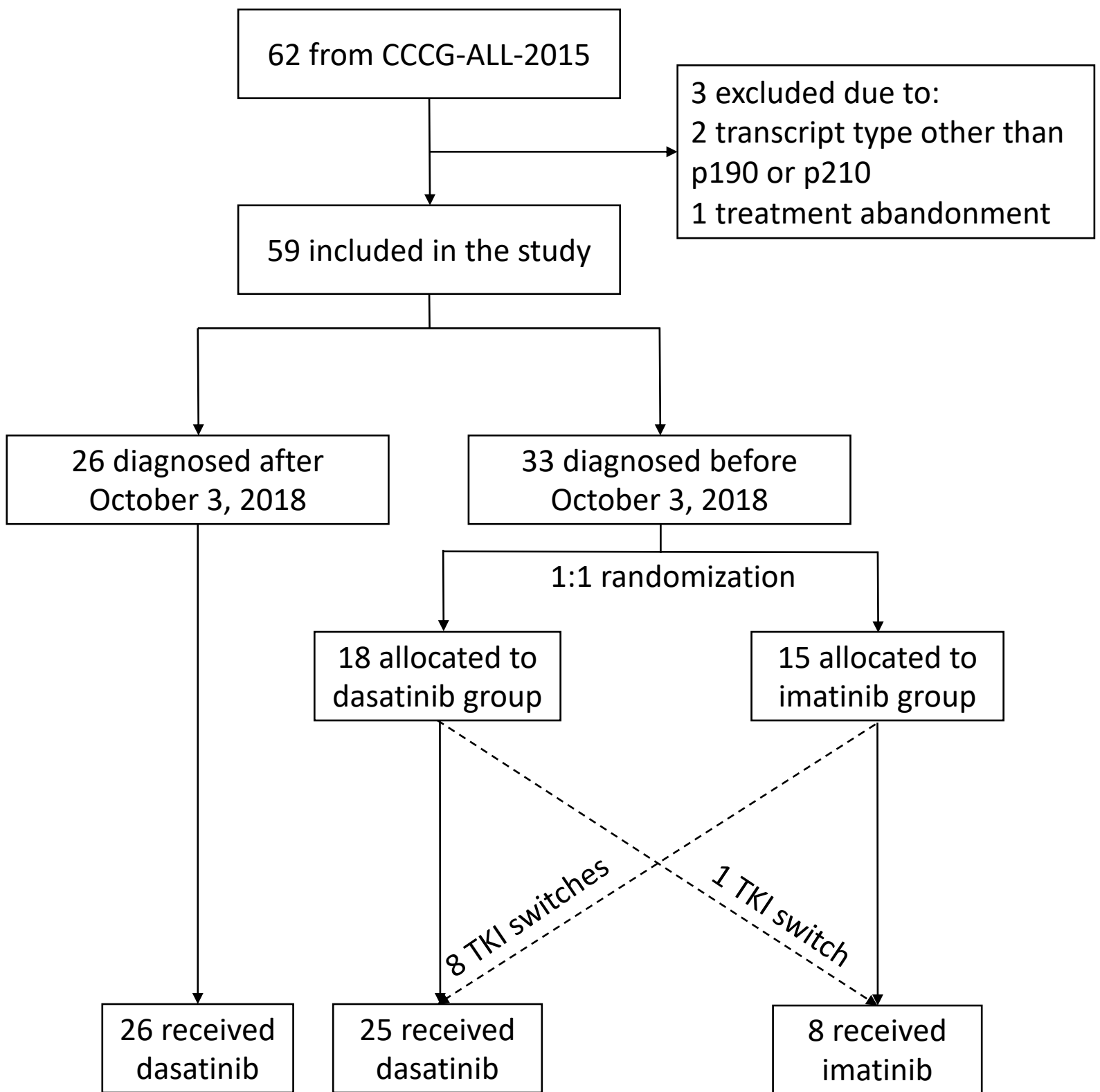
Characteristic	Level	N	CIR						EFS					
			Univariate model		Full multivariate model		Stepwise multivariate model		Univariate model		Full multivariate model		Stepwise multivariate model	
			HR (95% CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value
Gender	Female	25												
	Male	34	2.26(0.92-5.57)	0.076	3.63(1.15-11.5)	0.028	3.23(1.17-8.91)	0.023	2.03(0.83-4.95)	0.12	2.97(1.10-8.01)	0.031	3.24(1.21-8.66)	0.019
Age at diagnosis (years)	<10	34												
	≥10	25	0.89(0.38-2.11)	0.79	1.44(0.39-5.38)	0.59			0.92(0.40-2.13)	0.85	1.11(0.38-3.24)	0.85		
WBC at diagnosis ($\times 10^9/L$)	<50	21												
	≥50	38	3.06(1.11-8.45)	0.031	6.14(1.77-21.3)	0.004	5.34(1.67-17.1)	0.005	3.68(1.25-10.84)	0.018	5.27(1.56-17.83)	0.008	5.65(1.76-18.18)	0.004
CNS status	CNS1	55												
	CNS2 or traumatic lumbar puncture	4	0.58(0.2-1.66)	0.31	0.7(0.09-5.74)	0.74			3.48(1.02-11.82)	0.046	1.56(0.31-7.75)	0.59		
Disease subtype	Typical ALL	43												
	CML-like	16	1.68(0.3-9.37)	0.56	0.29(0.04-1.93)	0.20	0.30(0.09-1.03)	0.055	0.71(0.26-1.92)	0.50	0.42(0.11-1.58)	0.20	0.44(0.15-1.31)	0.14
Final TKI group	Dasatinib	51												
	Imatinib	8	1.43(0.48-4.26)	0.52	2.81(0.65-12.0)	0.16			1.92(0.71-5.19)	0.33	2.71(0.76-9.68)	0.12	2.66(0.89-7.97)	0.08
TP1 MRD by MFC	<0.01%	50												
	≥0.01%	9	2.17(0.68-6.91)	0.19	8.03(1.77-36.4)	0.007	4.23(1.47-12.2)	0.008	1.87(0.69-5.05)	0.22	4.74(1.03-21.75)	0.045	3.87(1.25-11.97)	0.019
TP1 MRD by RT-qPCR	<0.01%	31												
	≥0.01%	28	0.82(0.35-1.93)	0.65	0.60(0.15-2.38)	0.47			0.83(0.37-1.90)	0.67	0.65(0.19-2.21)	0.49		

CIR, cumulative incidence of relapse; EFS, event-free survival; CI, confidence interval; HR, hazard ratio; WBC, white blood cell count; CNS, central nervous system; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; TKI, tyrosine kinase inhibitor; MRD, minimal residual disease; MFC, multiparametric flow cytometry; RT-qPCR, quantitative reverse transcriptase polymerase chain reaction.

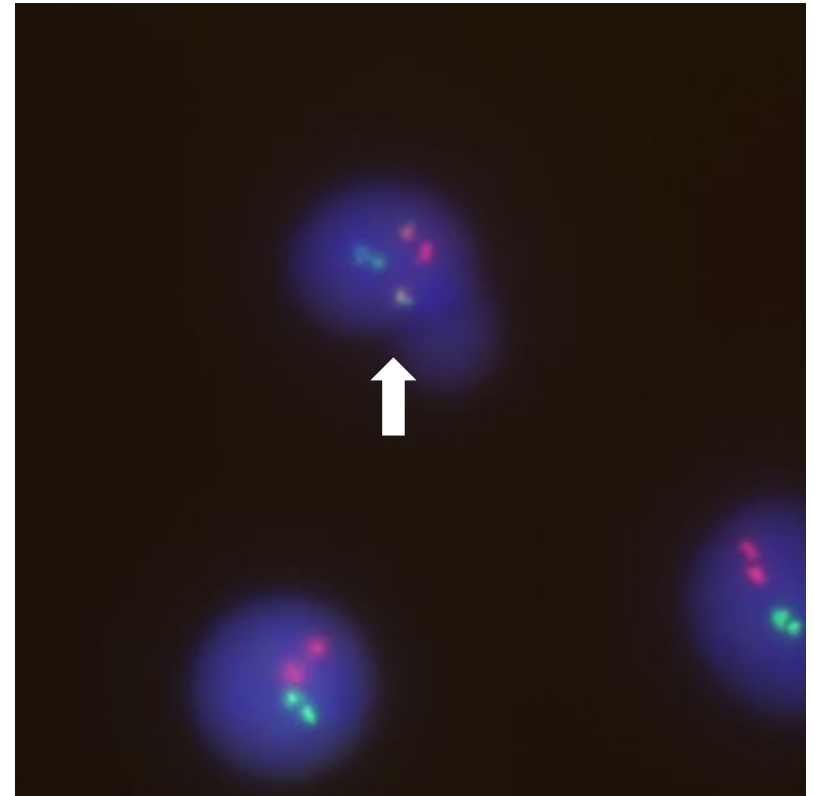
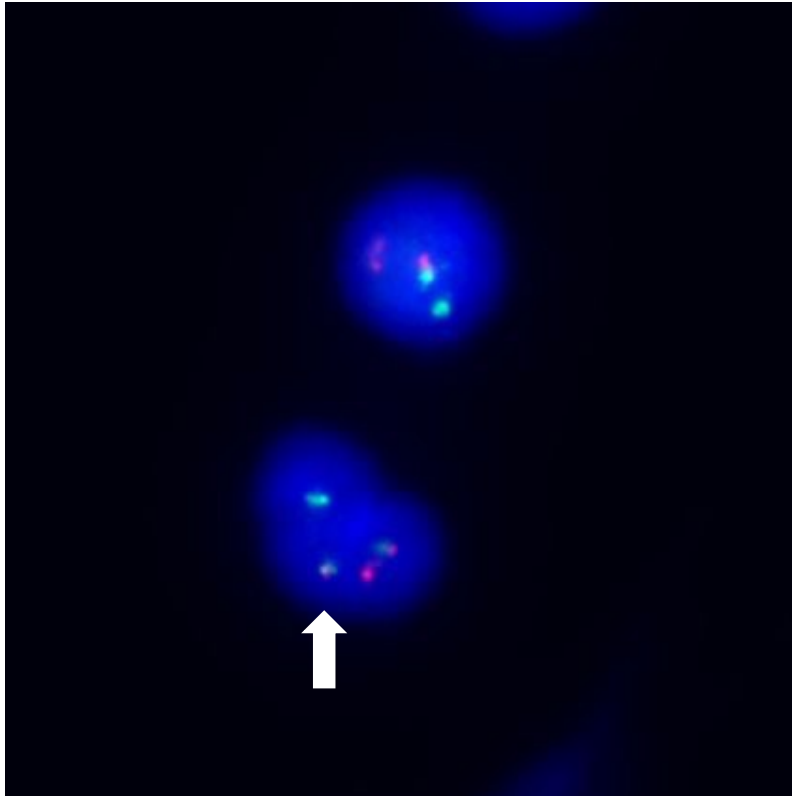
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Figure 1. Minimal residual disease (MRD) levels of different time points and disease subtypes. (A) MRD positivity by multiparameter flow cytometry (MFC) and real-time quantitative polymerase chain reaction (RT-qPCR) at different time points. (B) Discordance rate of MFC and RT-qPCR at different time points. (C) Scattered points plot showing comparison of MRD levels by MFC and RT-qPCR. All discordant samples fell into two patterns: (1) negative MFC and positive RT-qPCR higher than 10^{-3} ; (2) both positive, but MRD by RT-qPCR significantly higher than MFC. (D) Comparison of positivity rate between disease subtypes by MFC and RT-qPCR. ***, <0.001; **, <0.01; *, <0.05; n.s., not significant. Non-quantifiable positive and negative results were given the value of 10^{-5} and 10^{-6} , respectively.





Supplemental Figure 1. Inclusion of patients and trial profile. All patients diagnosed after October 3, 2018 received dasatinib because the interim analysis showed significantly improved 3-year event-free survival in the dasatinib group. Therefore, the chair of the data and safety monitoring committee recommended that randomization be stopped.



Supplemental Figure 2. Representative pictures of metamyelocyte carrying *BCR::ABL1* fusion gene confirmed by fluorescence in situ hybridization. White arrows denote signals of *BCR::ABL1* fusion, with red signals originating from *ABL1* probes and green signals from *BCR* probes.

Supplemental Table 1. Multivariate analyses of cumulative incidence of relapse and event-free survival of intention-to-treat patients.

		CIR					EFS			
		Full multivariate model			Stepwise multivariate model		Full multivariate model		Stepwise multivariate model	
Characteristic	Level	N	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value
Gender	Female	25								
	Male	34	3.81(1.20-12.1)	0.023	4.18(1.38-12.7)	0.011	3.07(1.12-8.42)	0.03	3.20(1.19-8.59)	0.021
Age at diagnosis (years)	<10	34								
	≥10	25	1.47(0.41-5.36)	0.56			1.15(0.40-3.33)	0.80		
WBC at diagnosis (×10 ⁹ /L)	<50	21								
	≥50	38	8.13(1.98-33.3)	0.004	6.52(1.92-22.2)	0.003	6.21(1.70-22.73)	0.006	6.36(1.95-20.82)	0.002
CNS status	CNS1	55								
	CNS2 or traumatic lumbar puncture	4	0.47(0.06-3.66)	0.47			1.31(0.21-8.02)	0.77		
Disease subtype	Typical ALL	43								
	CML-like	16	0.20(0.04-1.18)	0.076	0.24(0.07-0.79)	0.02	0.30(0.08-1.17)	0.083	0.32(0.10-1.02)	0.054
Initial TKI group	Dasatinib	44								
	Imatinib	15	3.11(1.08-8.95)	0.035	2.43(0.94-6.26)	0.066	2.18(0.71-6.70)	0.17	2.40(0.95-6.09)	0.065
TP1 MRD by MFC	<0.01%	50								
	≥0.01%	9	7.94(2.00-31.5)	0.003	5.14(1.67-15.8)	0.004	3.86(0.90-16.54)	0.069	3.59(1.16-11.10)	0.027
TP1 MRD by RT-qPCR	<0.01%	31								
	≥0.01%	28	0.70(0.21-2.38)	0.57			0.80(0.26-2.46)	0.70		

CIR, cumulative incidence of relapse; EFS, event-free survival; CI, confidence interval; HR, hazard ratio; WBC, white blood cell count; CNS, central nervous system; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; TKI, tyrosine kinase inhibitor; MRD, minimal residual disease; MFC, multiparametric flow cytometry; RT-qPCR, quantitative reverse transcriptase polymerase chain reaction.