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

With the addition of tyrosine kinase inhibitors (TKI) to conventional chemotherapy, remarkable improvement has 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 most common 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 al*.[6] reported a subset of patients exhibiting "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 the Chinese Children's Cancer Group (CCCG)-ALL-2015 (Chinese Clinical Trial Registry: ChiCTR-IPR-14005706). 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 the CCCG-ALL-2015 trial which was detailed in a 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 five 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 CantoII 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 ≥5% by morphology after achieving complete response (CR) and confirmed by MFC and molecular testing. Central nervous system 1 (CNS1) was defined as absence of blasts in cerebral spinal fluid (CSF). CNS2 was defined as presence of blasts in CSF but white blood cell count <5/microliter. 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 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 a multivariate model: i) full model incorporating all variables and ii) 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 Ph-positive B-cell ALL were included in this study. The trial diagram is shown in the *Online Supplementary Figure S1*. 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

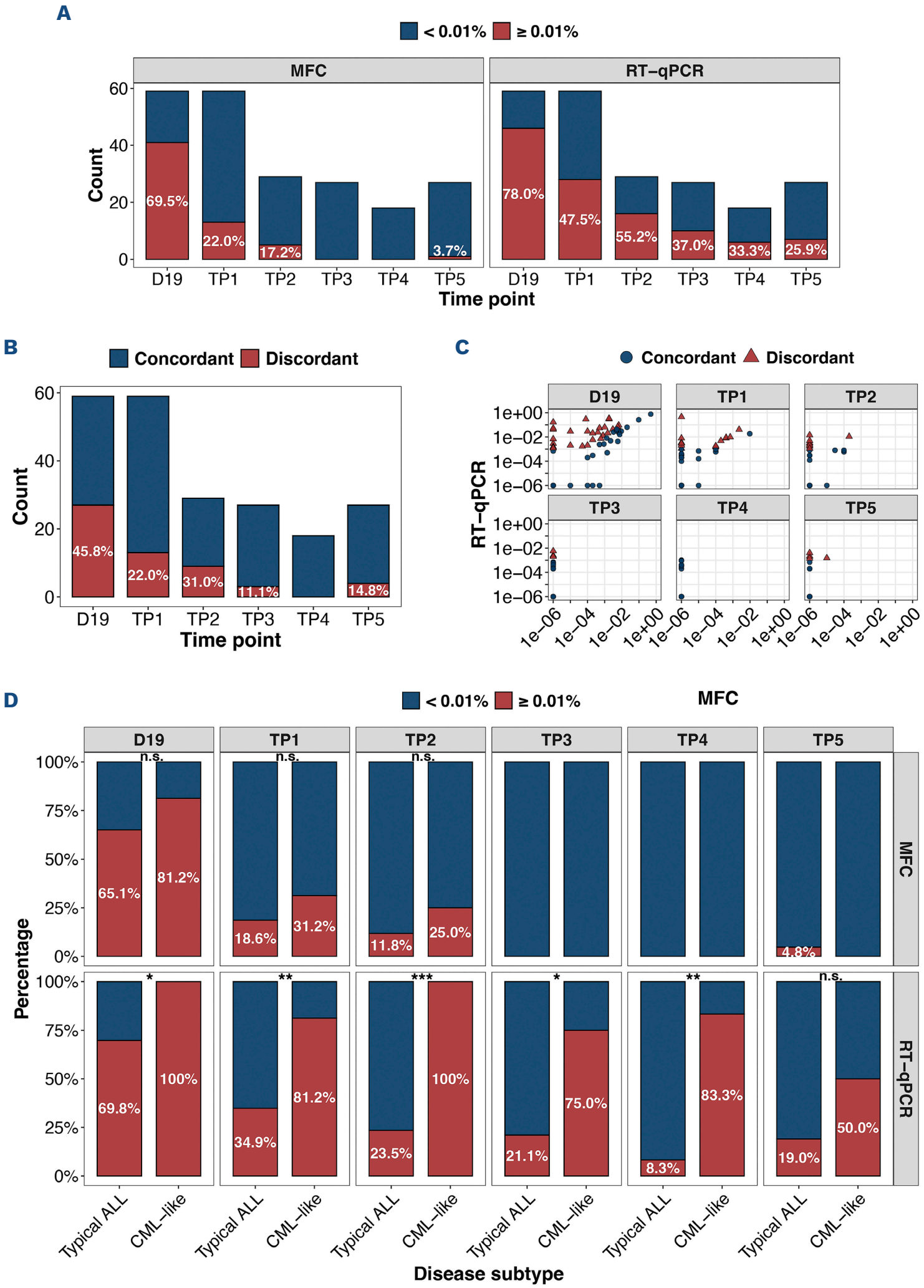

Figure 1. Minimal residual disease levels of different time points and disease subtypes. (A) Minimal residual disease (MRD) positivity by multiparameter flow cytometry (MFC) and real-time quantitative polymerase chain reaction (RT-qPCR) at different time points. (B) Discordance rate of MFC and RT-qPCR at different time points. (C) Scattered points plot showing comparison of MRD levels by MFC and RT-qPCR. All discordant samples fell into 2 patterns: i) negative MFC and positive RT-qPCR higher than 10^{-3}; ii) both positive, but MRD by RT-qPCR significantly higher than MFC. (D) Comparison of positivity rate between disease subtypes by MFC and RT-qPCR. ***P<0.001; **P<0.01; *P<0.05; NS: not significant. Non-quantifiable positive and negative results were given the value of 10^{-5} and 10^{-6}, respectively. D19: day; TP1: day 46 of induction, TP2: end of consolidation; TP3: before re-induction; TP4: completion of 5 cycles of subsequent continuation; TP5 treatment completion; ALL: acute lymphoblastic leukemia; CML: chronic mylogenous leukemia.

range [IQR], 19.3-42.9) months, with five very early relapses (<18 months, 23.8%), nine early relapses (18-36 months, 42.9%%) and seven 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: i) negative MFC and positive RT-qPCR higher than 10^{-3} (0.1%); ii) 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 (*Online Supplementary Figure S2A, 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 *vs*. CML-like, 48.9% [95% CI: 29.6-65.7] *vs*. 38.0% [95% CI: 8.1-68.9]; *P*=0.31) and EFS rates (typical ALL *vs*. CML-like, 49.2% [95% CI: 34.3-70.7%] *vs*. 55.7% [95% CI: 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 (*Online Supplementary Table S1*). 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% *vs*. 54.2% [95% CI: 34.9-70.0]; *P*=0.018) and 5-year EFS (100% *vs*. 42.4% [95% CI: 28.4-63.3]; *P*=0.018).

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

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

IQR: interquartile range; ALL: acute lymphoblastic leukemia; CML: chronic myelogenous leukemia; WBC: white blood cell count; CNS: central nervous system; CNS1: absence of blasts in cerebral spinal fluid; CNS2: presence of blasts in cerebral spinal fluid; 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*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex	Female Male	25 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	<10 years ≥10 years	34 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 ≥50	21 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 CNS2 or traumatic lumbar puncture	55 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 CML-like	43 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 Imatinib	51 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% ≥0.01%	50 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% ≥0.01%	31 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	- -	- -

CI: cumulative incidence of relapse; EFS: event-free survival; CI: confidence interval; HR: hazard ratio; WBC: white blood cell count; CNS: central nervous system; CNS1: absence of blasts in cerebral spinal fluid; CNS2: presence of blasts in cerebral spinal fluid; ALL: acute lymphoblastic leukemia; CML: chronic myelogenous leukemia; TKI: tyrosine kinase inhibitor; MRD: minimal residual disease; MFC: multiparametric flow cytometry; TP1: time point 1; RT-qPCR: quantitative reverse transcriptase polymerase chain reaction.

resembling CML as we demonstrated. Despite the fact that the underlying mechanism and clinical relevance of CML-like is still underinvestigated, 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 al*.[6] 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 contrary to previous knowledge on CML in lymphoid blast crisis.[9] Nevertheless, it raises further questions: i) should CML-like receive prolonged TKI therapy even when treatment for ALL is discontinued?; ii) will Ph-positive, non-ALL cells cause relapse?; and iii) could CML-like patients benefit from allo-SCT in CR1? We still need a 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 did.[7] This could be attributed to the following factors: i) the duration of follow-up in our study was significantly extended (median, 48.3 *vs*. 26.4 months), allowing for detection of late relapses; ii) the discontinuation of randomization caused an imbalance between the two groups; iii) dasatinib may exhibit better efficacy in preventing CNS relapse.[10] However, limited number of CNS events prevented us from performing such analysis, underscoring the need for larger cohorts with extended follow-up periods to draw a more solid conclusion.

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

In summary, our findings suggest that it is feasible to distinguish CML-like by the discrepancy between MFC and RT-qPCR, echoing previous results reported by Zuna *et al*.[6] where quantification of clonal immunoglobulin and T-cell receptor gene 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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Disclosures

No conflicts of interest to disclose.

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 of 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.

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Data-sharing statement

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

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