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Original Research

Effect of *BCR::ABL1* transcript type and droplet digital polymerase chain reaction on successful treatment-free remission in chronic myeloid leukemia patients who discontinued tyrosine kinase inhibitor

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Abstract

Background: Droplet digital polymerase chain reaction (ddPCR) is an exact method of measurement.

Objectives: We conducted this study to identify the prognostic factors for successful treatment-free remission in patients with chronic-phase chronic myeloid leukemia who discontinued tyrosine kinase inhibitors (TKIs). We also aimed to validate ddPCR for predicting molecular relapse.

Design: This is a prospective, multicenter study.

Methods: We enrolled patients treated with TKIs for at least 3 years with a confirmed sustained deep molecular response (DMR) for at least 1 year. TKI was re-administered in patients who experienced the loss of major molecular response (MMR).

Results: A total of 66 patients from five institutions in South Korea were enrolled. During a median follow-up period of 16.5 months, 29/66 (43.9%) patients experienced molecular relapse; the probability of molecular relapse-free survival (RFS) at 6 or 12 months after TKI discontinuation was 65.6% or 57.8%, respectively, with most molecular relapses occurring within the first 7 months. All patients who lost MMR were re-treated with TKI, and all re-achieved MMR at a median of 2.8 months. E14a2 transcript type ($p = 0.005$) and longer DMR duration (⩾48 months) prior to TKI discontinuation ($p = 0.002$) were associated with prolonged molecular RFS and with sustained DMR. Patients with both e13a2 transcript type and detectable *BCR::ABL1* (⩾$MR^{5.0}$) by ddPCR at the time of TKI discontinuation showed shorter duration of molecular RFS ($p = 0.015$).

Conclusion: Our data suggest that transcript type and *BCR::ABL1* transcript levels on ddPCR should be taken into consideration when deciding whether to discontinue TKI therapy.

Keywords: chronic myeloid leukemia, droplet digital polymerase chain reaction, transcript type, treatment-free remission, tyrosine kinase inhibitor

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Introduction

Due to the development of tyrosine kinase inhibitors (TKIs), chronic myeloid leukemia (CML) patients can expect near-normal life expectancy.[1] However, ongoing lifelong treatment with TKIs can significantly reduce quality of life, lead to off-target effects, such as life-threatening cardiovascular

or liver toxicity, and represent a significant financial burden.[2,3] Therefore, successful treatment-free remission (TFR) has become a new therapeutic goal, and large, prospective data has shown encouraging results. In the Stop Imatinib (STIM) study, molecular recurrence-free survival (RFS) at 60 months was 38% [95% confidence interval (CI): 29–47%] with no disease progression among patients in whom *BCR::ABL1* transcripts were not detected for >2 years.[4] Multiple subsequent clinical trials have demonstrated the possibility of successful TFR in patients with a deep molecular response (DMR) sustained for several years, and approximately 40–60% of patients could successfully discontinue TKIs, regardless of the type of TKI.[5] Recent European LeukemiaNet guideline recommended that chronic-phase CML patients with typical *BCR::ABL1* transcripts who have been treated > 5 years and sustained a DMR for more than 2 years could attempt the discontinuation of TKIs.[6] However, the prognostic factors for selecting appropriate candidates for TKI cessation remain controversial. The results of the large clinical trials identified longer duration of TKI treatment or DMR as important prognostic factors for successful discontinuation of TKI treatment.[7,8] Some studies also suggest that a low Sokal risk score or interferon pre-treatment is associated with a higher TFR rate.[7–9] Experimental studies emphasized immunologic profiles, including increases in natural killer cells and decreases in regulatory T cells, as having significant roles in successful TFR.[10,11]

Droplet digital polymerase chain reaction (ddPCR) is a sensitive method for quantifying *BCR::ABL1* transcript levels. ddPCR has been used for measuring gene expression and detecting mutations and minimal residual disease in various hematologic malignancies.[12] Limited data showed that CML patients with undetectable or lower levels of *BCR::ABL1* by ddPCR at the time of TKI discontinuation achieved successful TFR.[13–15] However, further evidence is needed to prove the role of ddPCR in identifying suitable candidates for TFR.

Here, we prospectively examined the prognostic factors for successful TFR in patients with Philadelphia chromosome-positive, chronic-phase CML who had been treated with TKIs. We also validated the role of ddPCR in predicting successful TFR.

Methods

Patient characteristics

This prospective, multicenter trial enrolled patients with Philadelphia chromosome-positive, chronic-phase CML in five Korean centers from August 2019 to August 2020. The eligible patients were 18 years or older and had received any TKI as first, second, or higher lines of treatment. Patients were treated with standard doses of TKIs for at least 3 years and had confirmed, sustained DMR for at least 1 year as measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To be included, the patients needed at least three RT-qPCR results confirming a sustained DMR during the 1 year before study entry. Other inclusion criteria were Eastern Cooperative Oncology Group performance status ⩾2 and absence of severe dysfunction in any primary organ.

We excluded patients with a history of allogeneic stem-cell transplant, prior attempt(s) to discontinue TKI treatment, reduced TKI dose due to abnormal laboratory findings, participation in other clinical trials, or diagnosis indicating active concomitant malignancy.

Study procedures

Molecular response was assessed by RT-qPCR using peripheral blood samples obtained at the time of TKI discontinuation, 1 month later, and then every 2 months up to 1 year. ddPCR was performed at the time of TKI discontinuation, 1 month, and 5 months later after TKI discontinuation. RT-qPCR assays with 4.5-log sensitivity and ddPCR assays with 5.0-log sensitivity were performed in the central laboratory (LAS, Gimpo, Korea). The loss of major molecular response (MMR), which indicates molecular recurrence, was determined after two consecutive assessments within 1 month. TKI was re-started in cases of MMR loss. Patients were re-administered the same TKI that had been taken at the time of TKI discontinuation. For these patients, molecular response was assessed every 2 months by RT-qPCR until MMR was re-established.

ddPCR procedure

RNA (1 µg) was reverse-transcribed using QXDx iScript Advanced Reverse Transcriptase in QXDx *BCR::ABL1* %^{IS} Kit (Bio-Rad Pleasanton, CA, USA). After reverse transcription, 16 µL of cDNA was transferred to a ddPCR plate containing ddPCR Master Mix. The samples were then split into two wells per sample (data to be merged during analysis), and the plate was sealed for droplet generation. After droplet generation, ddPCR amplification was performed using the *BCR::ABL1* ddPCR Amplification Thermal Cycling Protocol. After the polymerase chain reaction (PCR) was complete, the PCR plate was transferred to a QX200 Droplet Reader (Bio-Rad). For reading, the QuantaSoft Software 1.7.4 (Bio-Rad) was set to RED (rare events detection) for experiments, FAM signal for *BCR::ABL1* copies (target gene) and HEX signal for *ABL1* copies (internal control gene). Raw data were extracted using QuantaSoft Software 1.7.4 (Bio-Rad), then analyzed using QXDx *BCR::ABL1* Reporter (V1.02). QXDx *BCR::ABL1* %^{IS} ddPCR assay kit inserts include a correlation factor, which is applied to the assay (correlation factor = 1.14). During the entire experimental procedure, from cDNA synthesis to ddPCR, we used reagents only from the QXDx *BCR::ABL1* %^{IS} ddPCR assay kit and performed experiments following the manufacturer's instructions.

PCR for BCR::ABL1 *transcript type*

To determine the *BCR::ABL1* transcript type, we used the method described by Goh *et al.*[16] For the detection of e14a2 or e13a2, primers specific for e11 of the *BCR* gene and a3 of the *ABL1* gene were chosen. PCR was performed according to the manufacturer's instructions. The PCR reaction consisted of 1 µL cDNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5 U Taq DNA polymerase, and primers in a final volume of 25 µL. PCR was performed in duplicate under the following cycling conditions: pre-denaturation at 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. Amplified products were visualized by ethidium bromide-stained agarose gel electrophoresis. We used the following amounts of cDNA for each technique.

	Total RNA used (µg) for cDNA synthesis	Final volume of cDNA synthesis reaction mixture (µL)	Volume used from cDNA synthesis reaction mixture for PCR (µL)	Final volume of PCR reaction mixture (µL)
RT-qPCR	1.0	20.0	2.0	15.0
ddPCR	1.0	25.0	8.0	45.0

ddPCR, droplet digital polymerase chain reaction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; PCR, polymerase chain reaction.

Outcomes

The primary endpoint was to identify molecular RFS, which is the length of time from the date of TKI discontinuation to the date at which loss of MMR, death, or censoring was recorded. The secondary endpoint was to find prognostic factors affecting molecular recurrence and evaluate safety and disease progression after TKI discontinuation. We also assessed the time to re-achieve MMR after restarting TKI in patients who lost MMR.

Statistical analysis

Previously, STIM and Korean KID trials reported that CML patients who discontinued TKI achieved approximately 40% and 60% molecular RFS at 12 months, respectively.[7,17] Thus, we assumed that the null proportion was 0.4 according to the STIM trial and that the alternative proportion was 0.6 according to the KID study. According to the Cancer Research and Biostatistics SWOG statistical tools using these values (null proportion = 0.4, alternative proportion = 0.6, α error = 5%, and β error = 10%), 53 patients were required to analyze TFR.

The paired *T*-test was used to compare the *BCR::ABL1* values measured by RT-qPCR and ddPCR, and the Kaplan-Meier method was used to analyze molecular RFS. The duration of MMR before stopping TKI was defined as the period of *BCR::ABL1*^{IS} $\leq$0.1%, based on the RT-qPCR results before TKI discontinuation. The duration of DMR before stopping TKI was defined as the period of *BCR::ABL1*^{IS} $\leq$0.01% (molecular response at 4.0-log reduction, $MR^{4.0}$) before TKI discontinuation. The duration of sustained DMR was defined as the date of TKI discontinuation to the date of the loss of $MR^{4.0}$. Median values for

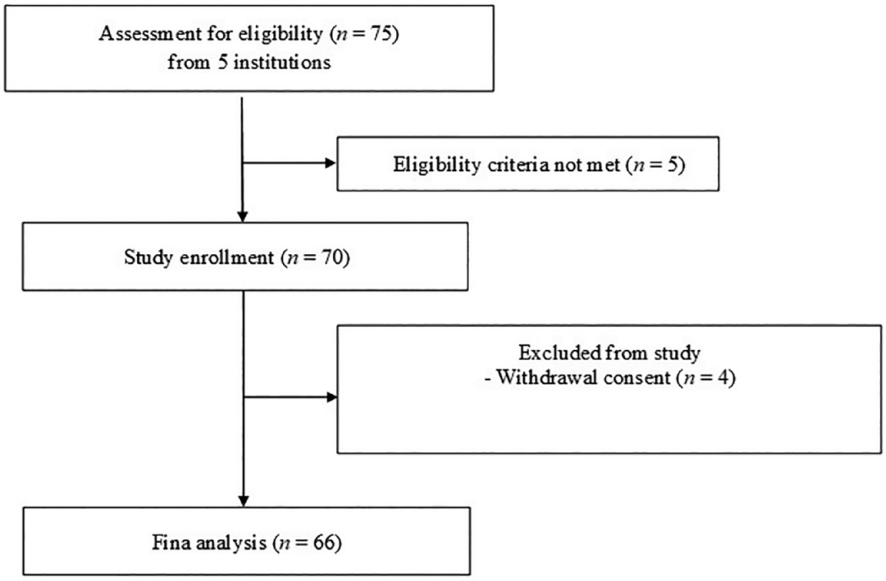

Figure 1. CONSORT flow diagram.

age, TKI treatment duration, the duration of MMR before stopping TKI, and the duration of DMR before stopping TKI were used as cut-off values for each variable. Statistical analyses were performed using IBM SPSS, version 23.0 (IBM, Armonk, NY, USA). The reporting of this study conforms to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement.[18]

Results

Patient characteristics

Sixty-six patients from five institutions in South Korea were finally enrolled in this study between August 2019 and August 2020 (Figure 1). The median follow-up period was 16.5 months (range, 9.8–22.5 months), and the median age of the patients was 49 years. The number of patients who were treated with first-line TKI and second or higher lines at the time of TKI discontinuation were 44/66 (66.7%) and 20/66 (30.3%), respectively. The TKI that most patients had received at the time of study enrollment was nilotinib (57.6%). All patients had been treated with standard doses of TKIs (imatinib 400 mg once daily, dasatinib 100 mg once daily, nilotinib 300 mg twice daily, or radotinib 300 mg twice daily). The patients' characteristics are shown in Table 1.

Outcomes of TKI discontinuation

During the follow-up period, 29/66 (43.9%) patients experienced molecular relapse, and 35/66 (53%) patients maintained MMR (two patients were not evaluated). The median duration of molecular relapse after TKI discontinuation was 3.4 months (range, 1.0–21.3 months), mostly within the first 7 months (86.2%). One patient experienced late molecular relapse, occurring 21.3 months after radotinib was stopped. Thus, the probability of molecular RFS was 65.6% at 6 months and 57.8% at 12 months [Figure 2(a)]. The probability of sustained DMR after TKI discontinuation was 46.0% at 6 months and 33.3% at 12 months [Figure 2(b)].

The prognostic indicators for prolonged molecular RFS in univariate analysis were decreased initial white blood cell count ($p = 0.042$), e14a2 transcript type [$p = 0.002$; Figure 2(c)], longer MMR duration before stopping TKI ($p = 0.012$), undetectable transcripts on ddPCR [$<MR^{5.0}$ (molecular response at 5.0-log reduction)] at the time of TKI discontinuation ($p = 0.041$), and longer DMR duration before stopping TKI

Table 1. Baseline characteristics of the patients.

Patient characteristics	All patients (*N* = 66)
Median age at diagnosis, years (range)	49 (20–70)
Sex, *n* (%)	
Male	33 (50.0)
Female	31 (47.0)
Unknown	2 (3.0)
ECOG, *n* (%)	
0	45 (68.2)
1	4 (6.0)
Unknown	17 (25.8)
Median white blood cell count at diagnosis (range), /μL	67,400 (4200–293,000)
The type of *BCR::ABL1*, *n* (%)	
E14a2	38 (57.6)
E13a2	17 (25.8)
Unknown	11 (16.7)
Line of treatment at the time of stopping TKI, *n* (%)	
First line	44 (66.7)
Second or higher lines	20 (30.3)
Unknown	2 (3.0)
First-line treatment, *n* (%)	
Imatinib	31 (47.0)
Dasatinib	7 (10.6)
Nilotinib	25 (37.9)
Radotinib	1 (1.5)
Unknown	2 (3.0)
Treatment at the time of stopping TKI, *n* (%)	
Imatinib	14 (21.2)
Dasatinib	10 (15.2)
Nilotinib	38 (57.6)
Radotinib	2 (3.0)
Unknown	2 (3.0)

ECOG, Eastern Cooperative Oncology Group performance status; TKI, tyrosine kinase inhibitor.

[$p = 0.012$, Figure 2(d); Table 2]. In multivariate analysis, e14a2 transcript type and longer DMR duration before TKI discontinuation (≥48 months) were prognostic factors for prolonged molecular RFS [hazard ratio (HR) = 3.569, 95% CI: 1.462–8.717, $p = 0.005$ for transcript type; HR = 2.867, 95% CI: 1.177–6.983, $p = 0.002$ for DMR duration; Table 2]. The favorable factors in multivariate analysis for sustained DMR after TKI discontinuation were also e14a2 type and longer DMR duration before TKI discontinuation (HR = 4.164, 95% CI: 2.057–8.429, $p < 0.001$ for transcript type; HR = 3.091, 95% CI: 1.530–6.240, $p = 0.002$ for DMR duration; Table 3).

TKI treatment was re-initiated in patients who lost MMR and MMR was re-achieved in all of these patients within a median time after TKI re-administration of 2.8 months (range, 1.0–7.1 months). No patients progressed to the accelerated or blast phase.

Outcomes of ddPCR

Undetectable *BCR::ABL1* transcript (<MR$^{5.0}$) on ddPCR at the time of TKI discontinuation was a favorable prognostic factor for molecular RFS in univariate analysis (1-year molecular RFS: 70.8% for undetectable *BCR::ABL1 versus* 50.0% for detectable *BCR::ABL1*, $p = 0.041$); however, this association was not observed in multivariate analysis (Table 2). When considering the combination of transcript type and detection of *BCR::ABL1* by ddPCR, patients who were both e13a2 type and had detectable transcripts on ddPCR (≥MR$^{5.0}$) at the time of TKI discontinuation showed significantly worse probability for molecular RFS than other groups [1-year molecular RFS: 21.4% for e13a2 type and detectable transcripts on ddPCR *versus* 62.5% for e14a2

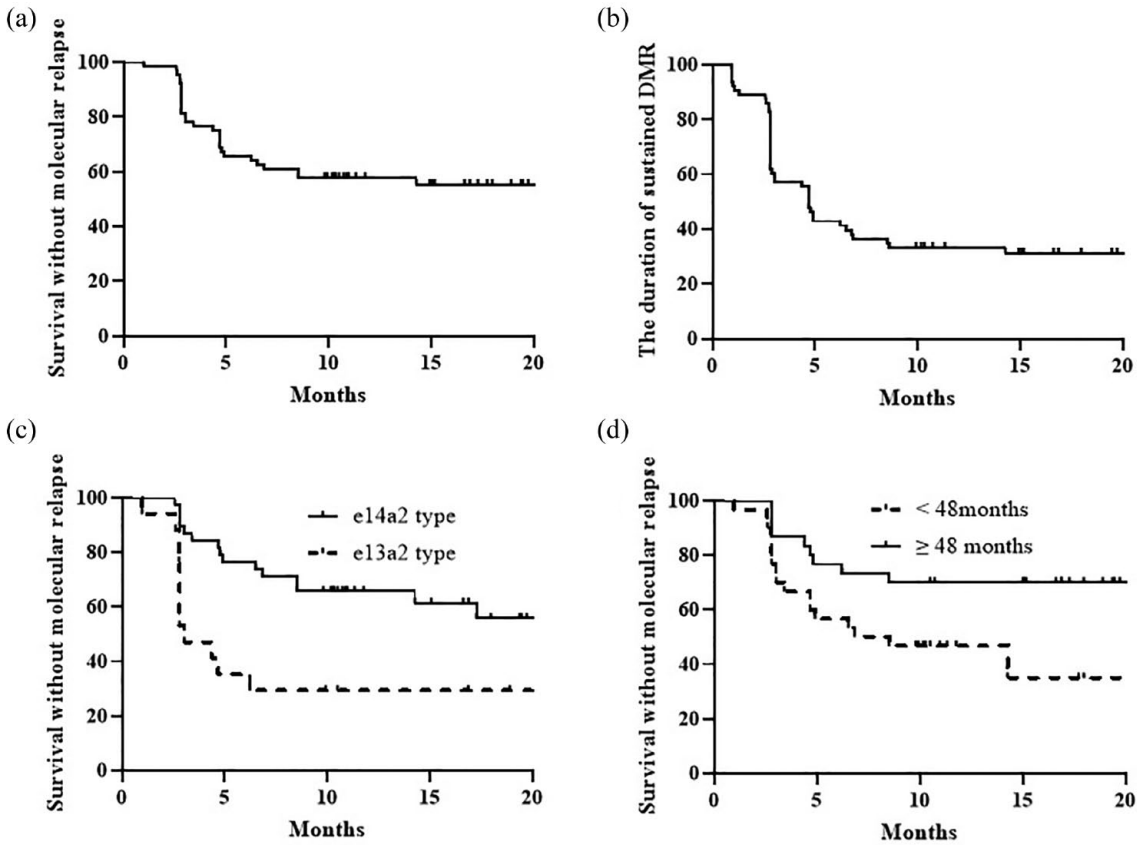

Figure 2. The probability of molecular relapse (a) and sustained DMR after TKI discontinuation (b). The probability of molecular relapse according to *BCR::ABL1* transcript type (c) and duration of DMR (d).
DMR, deep molecular response; TKI, tyrosine kinase inhibitor.

Table 2. Prognostic factors for molecular RFS after TKI discontinuation.

Variables	Univariate analysis		Multivariate analysis		
	1-year RFS ± SE (%)	*p*-Value	HR	95% CI	*p*-Value
Age					
⩾49 years	65.6 (±8.4)	0.106			
<49 years	50.0 (±8.8)				
Gender					
Male	57.6 (±8.6)	0.684			
Female	58.1 (±8.9)				
Initial WBC count					
⩾100,000/μL	40.0 (±11.0)	0.042	2.109	0.906–4.910	0.084
<100,000/μL	68.4 (±7.5)		1		

(Continued)

Table 2. (Continued)

Variables	Univariate analysis		Multivariate analysis		
	1-year RFS ± SE (%)	*p*-Value	HR	95% CI	*p*-Value
Type of *BCR::ABL1*					
E14a2	63.2 (±7.8)	0.002	1		0.005
E13a2	29.4 (±11.1)		3.569	1.462–8.717	
BCR::ABL1 at 3 months					
⩾10%^{IS}	44.4 (±16.6)	0.632			
<10%^{IS}	60.0 (±7.7)				
Line of TKI					
First-line therapy	55.2 (±7.9)	0.850			
Second-line therapy	55.0 (±11.1)				
Type of TKI at TKI discontinuation					
First generation	57.1 (±13.2)	0.741			
Second generation	58.0 (±7.0)				
Duration of TKI treatment					
⩾78 months	65.6 (±8.4)	0.070			
<78 months	50.0 (±8.8)				
Duration of MMR before stopping TKI*					
⩾66 months	68.8 (±8.2)	0.012			
<66 months	46.7 (±9.1)				
Duration of DMR before stopping TKI					
⩾48 months	70.0 (±8.4)	0.012	1		0.002
<48 months	46.7 (±9.1)		2.867	1.177–6.983	
RT-qPCR values at TKI discontinuation					
⩾MR$^{4.5}$–<MR$^{4.0}$	50.0 (±15.8)	0.342			
<MR$^{4.5}$	59.3 (±6.7)				
ddPCR values at TKI discontinuation					
⩾MR$^{5.0}$	50.0 (±7.9)	0.041	1.137	0.436–2.968	0.792
<MR$^{5.0}$	70.8 (±9.3)		1		

*Due to the correlation between the duration of MMR and DMR before stopping TKI, only duration of DMR was included in final multivariable analysis.
CI, confidence interval; ddPCR, droplet digital polymerase chain reaction; DMR, deep molecular response; HR, hazard ratio; IS, International Scale; MMR, major molecular response; MR$^{4.5}$, molecular response at 4.5-log reduction; MR$^{4.0}$, molecular response at 4.0-log reduction; MR$^{5.0}$, molecular response at 5.0-log reduction; RFS, relapse-free survival; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SE, standard error; TKI, tyrosine kinase inhibitor; WBC, white blood cell.

Table 3. Prognostic factors for a sustained DMR after TKI discontinuation.

Variables	Univariate analysis		Multivariate analysis		
	1-year sustained DMR ± SE (%)	*p*-Value	HR	95% CI	*p*-Value
Age					
⩾49 years	35.5 (±8.6)	0.448			
<49 years	31.3 (±8.2)				
Gender					
Male	37.5 (±8.6)	0.831			
Female	29.0 (±8.2)				
Initial WBC count					
⩾100,000/μL	26.3 (±10.1)	0.350			
<100,000/μL	39.5 (±7.9)				
Type of *BCR::ABL1*					
E14a2	45.9 (±8.2)	<0.001	1		<0.001
E13a2	5.9 (±5.7)		4.164	2.057–8.429	
BCR::ABL1 at 3 months					
⩾10%^{IS}	35.9 (±7.7)	0.745			
<10%^{IS}	33.3 (±15.7)				
Line of TKI					
First-line therapy	30.2 (±7.0)	0.738			
Second-line therapy	40.0 (±11.0)				
Type of TKI at TKI discontinuation					
First generation	28.6 (±12.1)	0.870			
Second generation	34.7 (±6.8)				
Duration of TKI treatment					
⩾78 months	37.5 (±8.6)	0.323			
<78 months	29.0 (±8.2)				
Duration of MMR before stopping TKI					
⩾66 months	40.6 (±8.7)	0.075			
<66 months	24.1 (±7.9)				

(Continued)

Table 3. (Continued)

Variables	Univariate analysis		Multivariate analysis		
	1-year sustained DMR ± SE (%)	*p*-Value	HR	95% CI	*p*-Value
Duration of DMR before stopping TKI					
⩾48 months	46.7 (±9.1)	0.006	1		0.002
<48 months	20.7 (±7.5)		3.091	1.530–6.240	
RT-qPCR values at TKI discontinuation					
⩾$MR^{4.5}$–<$MR^{4.0}$	20.0 (±12.6)	0.140			
<$MR^{4.5}$	35.8 (±6.6)				
ddPCR values at TKI discontinuation					
⩾$MR^{5.0}$	30.8 (±7.4)	0.484			
<$MR^{5.0}$	37.5 (±9.9)				

CI, confidence interval; ddPCR, droplet digital polymerase chain reaction; DMR, deep molecular response; HR, hazard ratio; IS, International Scale; MMR, major molecular response; $MR^{4.0}$, molecular response at 4.0-log reduction; $MR^{4.5}$, molecular response at 4.5-log reduction; $MR^{5.0}$, molecular response at 5.0-log reduction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SE, standard error; TKI, tyrosine kinase inhibitor; WBC, white blood cell.

Table 4. Effect of *BCR::ABL1* type and ddPCR value at the time of TKI discontinuation on molecular RFS after TKI discontinuation.

Variables	Univariate analysis		Multivariate analysis		
	1-year RFS ± SE (%)	*p*-Value	HR	95% CI	*p*-Value
Age					
⩾49 years	65.6 (±8.4)	0.106			
<49 years	50.0 (±8.8)				
Gender					
Male	57.6 (±8.6)	0.684			
Female	58.1 (±8.9)				
Initial WBC count					
⩾100,000/μL	40.0 (±11.0)	0.042	1.897	0.839–4.289	0.124
<100,000/μL	68.4 (±7.5)		1		
Type of *BCR::ABL1* and transcript on ddPCR					
E13a2 and ⩾$MR^{5.0}$	21.4 (±11.0)	0.001	1		0.015
E14a2 and <$MR^{5.0}$	62.5 (±12.1)		0.331	0.119–0.924	0.035
Others	64.0 (±9.6)		0.275	0.108–0.699	0.007

(Continued)

Table 4. (Continued)

Variables	Univariate analysis		Multivariate analysis		
	1-year RFS ± SE (%)	*p*-Value	HR	95% CI	*p*-Value
BCR::ABL1 at 3 months					
⩾10%^{IS}	44.4 (±16.6)	0.632			
<10%^{IS}	60.0 (±7.7)				
Line of TKI					
First-line therapy	55.2 (±7.9)	0.850			
Second-line therapy	55.0 (±11.1)				
Type of TKI at TKI discontinuation					
First generation	57.1 (±13.2)	0.741			
Second generation	58.0 (±7.0)				
Duration of TKI treatment					
⩾78 months	65.6 (±8.4)	0.070			
<78 months	50.0 (±8.8)				
Duration of MMR before stopping TKI*					
⩾66 months	68.8 (±8.2)	0.012			
<66 months	46.7 (±9.1)				
Duration of DMR before stopping TKI					
⩾48 months	70.0 (±8.4)	0.012	1		0.037
<48 months	46.7 (±9.1)		2.478	1.058–5.803	
RT-qPCR values at TKI discontinuation					
⩾MR$^{4.5}$–<MR$^{4.0}$	50.0 (±15.8)	0.342			
<MR$^{4.5}$	59.3 (±6.7)				

*Due to the correlation between the duration of MMR and DMR before stopping TKI, only duration of DMR was included in final multivariable analysis.
CI, confidence interval; ddPCR, droplet digital polymerase chain reaction; DMR, deep molecular response; HR, hazard ratio; IS, International Scale; MMR, major molecular response; MR^{4}, molecular response at 4.0-log reduction; MR$^{4.5}$, molecular response at 4.5-log reduction; MR$^{5.0}$, molecular response at 5.0-log reduction; RFS, relapse-free survival; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SE, standard error; TKI, tyrosine kinase inhibitor; WBC, white blood cell.

type and undetectable transcript on ddPCR *versus* 64.0% for others; Table 4 and Figure 3(a)]. In multivariate analysis, both e13a2 type and detectable transcript on ddPCR (⩾MR$^{5.0}$) were statistically significant factors for shorter molecular RFS (HR = 0.331, 95% CI: 0.119–0.924, $p = 0.035$ for e14a2 and undetectable transcript on ddPCR; HR = 0.275, 95% CI: 0.108 – 0.699, $p = 0.007$ for others). Shorter DMR duration before TKI discontinuation (<48 months) was also a significant factor for shorter molecular RFS (HR = 2.478, 95% CI: 1.058–5.803, $p = 0.037$) (Table 4).

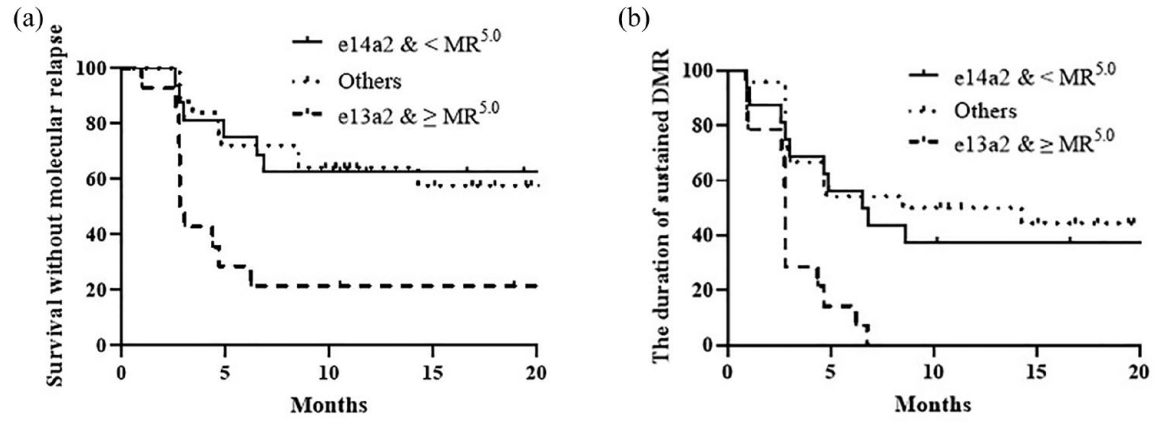

Figure 3. The probability of molecular relapse (a) and sustained DMR (b) according to transcript type and detectability by ddPCR at time of TKI discontinuation.
ddPCR, droplet digital polymerase chain reaction; DMR, deep molecular response; TKI, tyrosine kinase inhibitor.

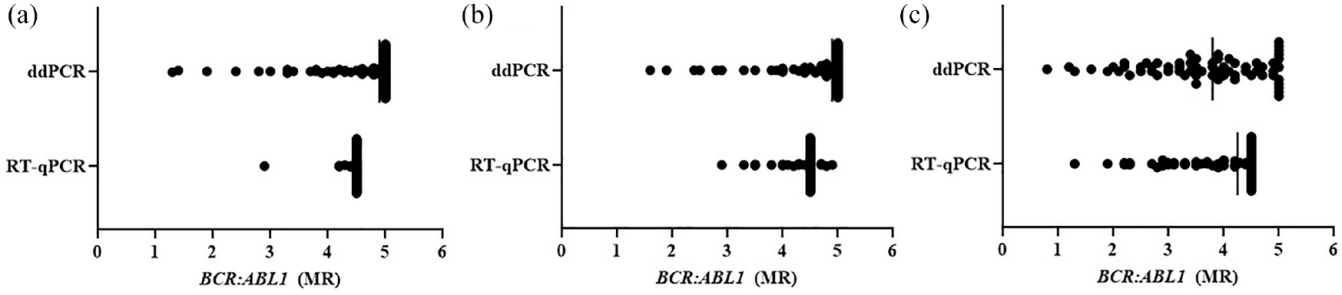

Figure 4. Comparison between RT-qPCR and ddPCR. (a) At the time of TKI discontinuation (mean $MR^{4.5}$ for RT-qPCR *versus* $MR^{4.5}$ for ddPCR, $p = 0.931$), (b) 1 month after TKI discontinuation (mean $MR^{4.4}$ for RT-qPCR *versus* $MR^{4.5}$ for ddPCR, $p = 0.246$), and (c) 5 months after TKI discontinuation (mean $MR^{3.8}$ for RT-qPCR *versus* $MR^{3.6}$ for ddPCR, $p = 0.017$).
ddPCR, droplet digital polymerase chain reaction; $MR^{3.6}$, molecular response at 3.6-log reduction; $MR^{3.8}$, molecular response at 3.8-log reduction; $MR^{4.4}$, molecular response at 4.4-log reduction; $MR^{4.5}$, molecular response at 4.5-log reduction; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TKI, tyrosine kinase inhibitor.

Similarly, multivariate analysis showed that the factors associated with a shorter DMR after TKI discontinuation were both e13a2 transcript type and undetectable *BCR::ABL1* by ddPCR (HR = 0.272, 95% CI: 0.112–0.659, $p = 0.004$ for e14a2 and undetectable transcript on ddPCR; HR = 0.230, 95% CI: 0.102–0.518, $p < 0.001$ for others). In addition, a shorter DMR duration before TKI discontinuation (<48 months) was associated with a shorter DMR (HR = 2.675, 95% CI: 1.330–5.381, $p = 0.006$) [Figure 3(b)].

In subgroup analysis according to the various factors, undetectable *BCR::ABL1* on ddPCR (<$MR^{5.0}$) was an important factor for longer molecular RFS in patients with longer TKI treatment duration (⩾78 months) or long MMR duration before stopping TKI (⩾66 months) (Supplemental Table 1).

When comparing *BCR::ABL1* values detected by ddPCR and RT-qPCR, there was no significant difference at the time of stopping TKI and 1 month after stopping TKI, but there was a difference 5 months after TKI discontinuation (Figure 4; Supplemental Table 2).

Discussion

We conducted this study in hopes of establishing the prognosis of patients who discontinue TKI and the associated factors for successful TFR. We found that (1) the probability of the continuous discontinuation of TKIs was 57.8% at 12 months,

(2) the prognostic factors for successful TKI cessation were e14a2 type and longer DMR duration (⩾48 months), and (3) ddPCR could be a sensitive method for predicting successful TFR. Especially, patients with e13a2 type and detectable *BCR::ABL1* on ddPCR (⩾$MR^{5.0}$) at the time of stopping TKI showed poor TFR outcomes.

The present study showed that 65.6% of the patients sustained MMR at 6 months and 57.8% at 12 months after TKI cessation, with most molecular relapses occurring within the first 7 months. These results confirm the results of previous studies, which showed that 40–60% of chronic-phase CML patients can successfully discontinue TKIs.[5] No patient experienced disease progression, and all patients re-achieved MMR within a median of 2.8 months after restarting TKI. Although our criteria for TKI discontinuation were less stringent than criteria used in previous large clinical trials, our findings suggest that patients who receive first- or second-generation TKIs for at least 3 years and demonstrate DMR for at least 1 year could be considered as candidates for discontinuation of TKI treatment.[7,19]

Prognostic factors for successful TFR were e14a2 type and longer DMR duration before stopping TKI (⩾48 months). This was not related to patient age, the line of treatment, TKI treatment duration, or the type of TKI, which were taken into consideration as clinical parameters (Table 2). The favorable prognosis of the e14a2 transcript type was reported in several earlier studies.[20–25] Previous imatinib data showed that patients expressing the e14a2 type achieved higher and more rapid molecular responses than e13a2-expressing patients.[20,24,25] Additional reports support that the e14a2 transcript correlates positively with sustained DMR and potential to achieve TFR.[21,24] Retrospective studies also found that patients with e14a2 transcripts had higher rates of TFR success compared to those with e13a2 variants.[22,23] Little is known about functional relevance according to transcript type. One possible hypothesis is that the increased tyrosine kinase activity of the e13a2 transcript may have affected the results.[20] Immunological control of minimal residual disease due to different immunogenicity is also proposed for the clinical benefit of the e14a2 variant.[26,27] Another possibility is that RT-qPCR artifacts may influence clinical outcomes. Recently, a study comparing amplification according to the transcript type found that shorter e13a2 transcripts may be amplified more efficiently than e14a2 by RT-qPCR, possibly leading to an underestimation of the quantified values of the e14a2 transcript.[28–31] However, some reports showed better or similar molecular responses in patients expressing the e13a2 transcript than in patients expressing the e14a2 transcript. Thus, more data are needed to understand the significance and role of *BCR::ABL1* transcript type.[32,33]

Based on our experience and accumulated data, we can tentatively conclude that a longer DMR duration (⩾48 months) before TKI discontinuation is a predictive indicator for successful TFR. In the EURO-SKI trial, patients with longer DMR durations with a cut-off of 3.1 years before TKI cessation had a higher probability of TFR ($p = 0.0029$).[8] A retrospective United States trial suggested that the relapse rate for patients with $MR^{4.5}$ >2 years in duration was 32%, whereas for those with $MR^{4.5}$ for <2 years, the relapse rate was 82%.[34] Similarly, Takahashi *et al.*[35] reported that DMR duration >24 months was a relevant factor for sustained DMR.[35] Furthermore, the TRAD trial revealed that patients with $MR^{4.0}$ for more than 7.7 years had a 6-month RFS rate of 89.5%, whereas the rate for the comparison group was 36.3% ($p = 0.003$).[36] Our results were comparable to those reported in previous studies, even though a statistically significant relationship between the duration of TKI treatment and successful TFR was not demonstrated in our study. Other studies also suggested that the duration of TKI treatment was a significant factor for TFR, and the duration of DMR could be a factor dependent on the total treatment period.[8,34,36] However, the EURO-SKI trial placed greater importance on duration of DMR than on total treatment duration.[8] Based on this evidence, a durable, deep response is a significantly relevant indicator for successful TFR. In the present study, we used the median DMR duration cut-off value of 48 months. However, the appropriate cut-off value is expected to be different for different patient cohorts and further research is needed to understand this variation more precisely.

We performed ddPCR with a sensitivity of $MR^{5.0}$, and it proved to be a useful method for detecting minimal residual disease that could not be detected by RT-qPCR. Due to the increased sensitivity of ddPCR, *BCR::ABL1* was detected in numerous samples by ddPCR but not by RT-qPCR in this study (Supplemental Table 2).

In this study, approximately 6% more cDNA was used in ddPCR compared to RT-qPCR. This difference is not expected to have a significant effect on sensitivity. The ddPCR reaction volume was divided into 20,000 droplets, and each droplet became the actual final PCR reaction. Such a nanoscale environment for each droplet will have a relatively higher sensitivity to detect extremely low copy numbers of the target *BCR::ABL1* molecule compared to normal qPCR, with a bulky PCR reaction volume. Some previous studies supported the increased sensitivity of ddPCR. Kim *et al.*[37] also showed that ddPCR is more sensitive than RT-qPCR. Similarly, Bernardi *et al.*[14] suggested that RT-qPCR could not discriminate patients with a higher risk of MR loss after TKI discontinuation, whereas ddPCR could recognize stable DMR. However, Scott *et al.*[38] suggested that the results of ddPCR and RT-qPCR were comparable with no statistically significant difference. Therefore, further studies are needed to confirm ddPCR as a more sensitive tool compared to conventional RT-qPCR.

Because we demonstrated a correlation between detectable *BCR::ABL1* by ddPCR at the time of stopping TKI and failure of TFR by univariate analysis, but not multivariate analysis due to the small cohort (Table 2), we suggest a potential role for ddPCR in selecting appropriate candidates for TFR. More specifically, patients expressing e13a2 transcript type and detectable transcript on ddPCR ($\geqslant MR^{5.0}$) at TKI discontinuation showed a significantly higher probability of molecular relapse than other patients. Therefore, patients who express the e13a2 type and detectable *BCR::ABL1* on ddPCR ($\geqslant MR^{5.0}$) may be poorer candidates for TKI discontinuation. Similarly, the ISAV and KID trials showed that patients with positive *BCR::ABL1* values on ddPCR at the time of imatinib discontinuation were more likely to experience molecular recurrence.[13,17] Other groups suggested different cut-off values for *BCR::ABL1* on ddPCR that might predict successful TFR. An Italian group proposed a cut-off of <0.468 *BCR::ABL1* copies/μL, and a French group proposed $<0.0023\%^{IS}$ at TKI discontinuation for predicting significantly lower probability of molecular relapse.[14,15] A method to standardize the quantitative measurement of *BCR::ABL1* by ddPCR has not been widely established. Therefore, different proposed cut-off values for cohorts may depend on technical differences and pre-analytic steps.[15] Recently, Fava *et al.*[39] suggested that results obtained by different ddPCR platforms may be aligned. In the future, rigorous standardization will be needed before ddPCR is widely used as a prognostic tool for various hematologic diseases.

Limitations

This study had several limitations. First, the sample size was small despite being a multi-institution study. Second, Sokal and EUTOS risk scores were not analyzed, because of difficulties in obtaining consistent spleen size measurements in a multicenter study. Third, some clinical data was lost or omitted when patients were transferred to a different hospital during treatment. Fourth, we did not measure the *BCR::ABL1* level using ddPCR at the time of loss of MMR or DMR. Previous studies found that ddPCR is a more stable method than RT-qPCR to evaluate molecular response.[14,40] Therefore, monitoring of *BCR::ABL1* by ddPCR may detect more precisely molecular relapse after TKI cessation. Nevertheless, this study contributes significantly to our understanding of prognostic factors for successful TFR, especially in Asian populations.

Conclusion

In conclusion, this study demonstrates that more than half of CML patients could successfully discontinue TKI. E14a2 transcript type and longer DMR duration were predictive indicators for successful TFR, while e13a2 transcript type and detectable *BCR::ABL1* on ddPCR are predictors of poor outcome after TKI discontinuation, emphasizing the need for careful consideration when deciding whether to discontinue TKI therapy. Therefore, in the future, ddPCR is expected to become a useful tool for predicting prognosis for CML patients in the era of TKI discontinuation.

Declarations

Ethics approval and consent to participate

This trial was approved by the ethics committees of each hospital. Written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Author contributions

Hyunkyung Park: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Writing – original draft; Writing – review & editing.

Hyeong-Joon Kim: Data curation; Investigation; Writing – review & editing.

Sang-Kyun Sohn: Data curation; Investigation; Writing – review & editing.

Yoonsuk Baik: Formal analysis; Writing – review & editing.

Dongho Kim: Formal analysis; Writing – review & editing.

Sung-Yeoun Lee: Formal analysis; Writing – review & editing.

Jee Hyun Kong: Data curation; Investigation; Writing – review & editing.

Hawk Kim: Data curation; Investigation; Writing – review & editing.

Dong-Yeop Shin: Data curation; Investigation; Writing – review & editing.

Jae-Sook Ahn: Data curation; Investigation; Writing – review & editing.

Jinny Park: Data curation; Investigation; Writing – review & editing.

Seonyang Park: Data curation; Investigation; Writing – review & editing.

Inho Kim: Conceptualization; Data curation; Funding acquisition; Investigation; Supervision; Writing – review & editing.

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

The data of this study are available from the corresponding author on reasonable request.

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Supplemental material

Supplemental material for this article is available online.

References

1. Cortes J, Pavlovsky C and Saußele S. Chronic myeloid leukaemia. *Lancet (London, England)* 2021; 398: 1914–1926.
2. Moslehi JJ and Deininger M. Tyrosine kinase inhibitor-associated cardiovascular toxicity in chronic myeloid leukemia. *J Clin Oncol* 2015; 33: 4210–4218.
3. Stuckey R, López Rodríguez JF and Gómez-Casares MT. Discontinuation of tyrosine kinase inhibitors in patients with chronic myeloid leukemia: a review of the biological factors associated with treatment-free remission. *Curr Oncol Rep* 2022; 24: 415–426.
4. Etienne G, Guilhot J, Rea D, *et al.* Long-term follow-up of the French Stop Imatinib (STIM1) study in patients with chronic myeloid leukemia. *J Clin Oncol* 2017; 35: 298–305.
5. Chen KK, Du TF, Xiong PS, *et al.* Discontinuation of tyrosine kinase inhibitors in chronic myeloid leukemia with losing major molecular response as a definition for molecular relapse: a systematic review and meta-analysis. *Front Oncol* 2019; 9: 372.
6. Hochhaus A, Baccarani M, Silver RT, *et al.* European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia. *Leukemia* 2020; 34: 966–984.
7. Mahon FX, Réa D, Guilhot J, *et al.* Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective, multicentre Stop Imatinib (STIM) trial. *Lancet Oncol* 2010; 11: 1029–1035.
8. Saussele S, Richter J, Guilhot J, *et al.* Discontinuation of tyrosine kinase inhibitor therapy in chronic myeloid leukaemia (EURO-SKI): a prespecified interim analysis of a prospective, multicentre, non-randomised, trial. *The Lancet Oncology* 2018; 19: 747–757.

9. Radich JP, Hochhaus A, Masszi T, *et al.* Treatment-free remission following frontline nilotinib in patients with chronic phase chronic myeloid leukemia: 5-year update of the ENESTfreedom trial. *Leukemia* 2021; 35: 1344–1355.
10. Irani YD, Hughes A, Clarson J, *et al.* Successful treatment-free remission in chronic myeloid leukaemia and its association with reduced immune suppressors and increased natural killer cells. *Br J Haematol* 2020; 191: 433–441.
11. Imagawa J, Tanaka H, Okada M, *et al.* Discontinuation of dasatinib in patients with chronic myeloid leukaemia who have maintained deep molecular response for longer than 1 year (DADI trial): a multicentre phase 2 trial. *Lancet Haematol* 2015; 2: e528–e535.
12. Galimberti S, Balducci S, Guerrini F, *et al.* Digital droplet PCR in hematologic malignancies: a new useful molecular tool. *Diagnostics (Basel, Switzerland)* 2022; 12: 1305.
13. Mori S, Vagge E, le Coutre P, *et al.* Age and dPCR can predict relapse in CML patients who discontinued imatinib: the ISAV study. *Am J Hematol* 2015; 90: 910–914.
14. Bernardi S, Malagola M, Zanaglio C, *et al.* Digital PCR improves the quantitation of DMR and the selection of CML candidates to TKIs discontinuation. *Cancer Med* 2019; 8: 2041–2055.
15. Nicolini FE, Dulucq S, Boureau L, *et al.* Evaluation of residual disease and TKI duration are critical predictive factors for molecular recurrence after stopping imatinib first-line in chronic phase CML patients. *Clin Cancer Res* 2019; 25: 6606–6613.
16. Goh HG, Hwang JY, Kim SH, *et al.* Comprehensive analysis of BCR-ABL transcript types in Korean CML patients using a newly developed multiplex RT-PCR. *Transl Res* 2006; 148: 249–256.
17. Lee SE, Choi SY, Song HY, *et al.* Imatinib withdrawal syndrome and longer duration of imatinib have a close association with a lower molecular relapse after treatment discontinuation: the KID study. *Haematologica* 2016; 101: 717–723.
18. von Elm E, Altman DG, Egger M, *et al.* The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *Lancet (London, England)* 2007; 370: 1453–1457.
19. Ross DM, Branford S, Seymour JF, *et al.* Safety and efficacy of imatinib cessation for CML patients with stable undetectable minimal residual disease: results from the TWISTER study. *Blood* 2013; 122: 515–522.
20. Lucas CM, Harris RJ, Giannoudis A, *et al.* Chronic myeloid leukemia patients with the e13a2 BCR-ABL fusion transcript have inferior responses to imatinib compared to patients with the e14a2 transcript. *Haematologica* 2009; 94: 1362–1367.
21. D'Adda M, Farina M, Schieppati F, *et al.* The e13a2 BCR-ABL transcript negatively affects sustained deep molecular response and the achievement of treatment-free remission in patients with chronic myeloid leukemia who receive tyrosine kinase inhibitors. *Cancer* 2019; 125: 1674–1682.
22. Shanmuganathan N, Branford S, Yong ASM, *et al.* The e13a2 BCR-ABL1 transcript is associated with higher rates of molecular recurrence after treatment-free remission attempts: retrospective analysis of the Adelaide cohort. *Blood* 2018; 132: 1731.
23. Claudiani S, Apperley JF, Gale RP, *et al.* E14a2 BCR-ABL1 transcript is associated with a higher rate of treatment-free remission in individuals with chronic myeloid leukemia after stopping tyrosine kinase inhibitor therapy. *Haematologica* 2017; 102: e297–e299.
24. Castagnetti F, Gugliotta G, Breccia M, *et al.* The BCR-ABL1 transcript type influences response and outcome in Philadelphia chromosome-positive chronic myeloid leukemia patients treated frontline with imatinib. *Am J Hematol* 2017; 92: 797–805.
25. Hanfstein B, Lauseker M, Hehlmann R, *et al.* Distinct characteristics of e13a2 versus e14a2 BCR-ABL1 driven chronic myeloid leukemia under first-line therapy with imatinib. *Haematologica* 2014; 99: 1441–1447.
26. Baccarani M, Rosti G and Soverini S. Chronic myeloid leukemia: the concepts of resistance and persistence and the relationship with the BCR-ABL1 transcript type. *Leukemia* 2019; 33: 2358–2364.
27. Baccarani M, Castagnetti F, Gugliotta G, *et al.* The proportion of different BCR-ABL1 transcript types in chronic myeloid leukemia: an international overview. *Leukemia* 2019; 33: 1173–1183.
28. Kjaer L, Skov V, Andersen MT, *et al.* Variant-specific discrepancy when quantitating BCR-ABL1 e13a2 and e14a2 transcripts using the Europe Against Cancer qPCR assay. *Eur J Haematol* 2019; 103: 26–34.

29. Dominy KM, Claudiani S, O'Hare M, *et al.* Assessment of quantitative polymerase chain reaction for BCR-ABL1 transcripts in chronic myeloid leukaemia: are improved outcomes in patients with e14a2 transcripts an artefact of technology? *Br J Haematol* 2022; 197: 52–62.

30. Salmon M, White HE, Zizkova H, *et al.* Impact of *BCR::ABL1* transcript type on RT-qPCR amplification performance and molecular response to therapy. *Leukemia* 2022; 36: 1879–1886.

31. Bernardi S, Bonifacio M, Iurlo A, *et al.* "Variant-specific discrepancy when quantitating BCR-ABL1 e13a2 and e14a2 transcripts using the Europe Against Cancer qPCR assay". Is dPCR the key? *Eur J Haematol* 2019; 103: 272–273.

32. de Lemos JA, de Oliveira CM, Scerni AC, *et al.* Differential molecular response of the transcripts B2A2 and B3A2 to imatinib mesylate in chronic myeloid leukemia. *Genet Mol Res* 2005; 4: 803–811.

33. Polampalli S, Choughule A, Negi N, *et al.* Analysis and comparison of clinicohematological parameters and molecular and cytogenetic response of two Bcr/Abl fusion transcripts. *Genet Mol Res* 2008; 7: 1138–1149.

34. Chamoun K, Kantarjian HM, Rios MB, *et al.* CML patients outcome after TKI discontinuation: a single institution experience in the US. *Blood* 2016; 128: 1923–1923.

35. Takahashi N, Kyo T, Maeda Y, *et al.* Discontinuation of imatinib in Japanese patients with chronic myeloid leukemia. *Haematologica* 2012; 97: 903–906.

36. Kim DDH, Bence-Bruckler I, Forrest DL, *et al.* Treatment-free remission accomplished by dasatinib (TRAD): preliminary results of the pan-canadian tyrosine kinase inhibitor discontinuation trial. *Blood* 2016; 128: 1922–1922.

37. Kim Y, Kim S, Lee JM, *et al.* Discontinuation of tyrosine kinase inhibitors based on BCR-ABL1 monitoring by digital droplet PCR in pediatric chronic myeloid leukemia. *Front Pediatr* 2022; 10: 928136.

38. Scott S, Cartwright A, Francis S, *et al.* Assessment of droplet digital polymerase chain reaction for measuring BCR-ABL1 in chronic myeloid leukaemia in an international interlaboratory study. *Br J Haematol* 2021; 194: 53–60.

39. Fava C, Bernardi S, Gottardi EM, *et al.* Alignment of Qx100/Qx200 Droplet Digital (Bio-Rad) and QuantStudio 3D (Thermofisher) digital PCR for quantification of BCR-ABL1 in Ph+ chronic myeloid leukemia. *Diseases (Basel, Switzerland)* 2021; 9: 35.

40. Zanaglio C, Bernardi S, Gandolfi L, *et al.* RT-qPCR versus digital PCR: how do they impact differently on clinical management of chronic myeloid leukemia patients? *Case Rep Oncol* 2020; 13: 1263–1269.