Digital droplet PCR for T315I BCR::ABL1 KD mutation assessment in adult Ph-positive acute lymphoblastic leukemia with a minimal residual disease increase

Digital droplet PCR T315I BCR::ABL1 KD mutation assessment in adult Ph-positive acute lymphoblastic leukemia with a minimal residual disease increase

Journal:	*Leukemia and Lymphoma*
Manuscript ID	GLAL-2023-0262
Manuscript Type:	Letter to the Editor
Date Submitted by the Author:	27-Apr-2023
Complete List of Authors:	Cardinali, Deborah; University of Rome La Sapienza, Department of Translational and Precision Medicine Beldinanzi, Marco; University of Rome La Sapienza, Department of Translational and Precision Medicine Ansuinelli, Michela; University of Rome La Sapienza, Department of Translational and Precision Medicine Elia, Loredana; University of Rome La Sapienza, Department of Translational and Precision Medicine Della Starza, Irene; University of Rome La Sapienza, Department of Translational and Precision Medicine; Fondazione Gimema Onlus Bellomarino, Vittorio; University of Rome La Sapienza, Department of Translational and Precision Medicine Matarazzo, Mabel; University of Rome La Sapienza, Department of Translational and Precision Medicine Di Trani, Mariangela; University of Rome La Sapienza, Department of Translational and Precision Medicine Cola, Mattia; University of Rome La Sapienza, Department of Translational and Precision Medicine Salutari, Prassede; Ospedale Civile Pescara, Department of Hematology Cedrone, Michele; San Giovanni-Addolorata Hospital, UOC of Hematology Bassan, Renato; Ospedale dell'Angelo, Hematology De Gobbi, Marco; Universita degli Studi di Torino, Department of Clinical and Biological Sciences Della Porta, Matteo Giovanni; IRCCS Humanitas Research Hospital, Rozzano, Milan De Simone, Mariacarla ; Division of Hematology, Cardarelli Hospital Alati, Caterina; Hematology Unit, Azienda Ospedaliera "Bianchi-Melacrino-Morelli", Fracchiolla, Nicola Stefano; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Lunghi, Monia; University of Eastern Piedmont, Devision of Haematology Intermesoli, Tamara; Department of Oncology and Hematology University of Milan and Azienda Socio-Sanitaria Territoriale Papa Giovanni XXIII, Bergamo Cardinali, Valeria; University of Perugia, Department of Medicine and Surgery Mulè, Antonino; UOC Ospedali Riuniti, Villa Sofia-Cervello Guarini, Anna; University of Rome La Sapienza, Department of Translational and Precision Medicine Foà, Robin; University of Rome La Sapienza, Department of Translational and Precision Medicine

	Chiaretti, Sabina; University of Rome La Sapienza, Department of Translational and Precision Medicine
Keywords:	T315I, DdPCR, Minimal residual disease, BCR::ABL1, Acute lymphoblastic leukemia

Digital droplet PCR T315I BCR::ABL1 KD mutation assessment in adult Ph-positive acute lymphoblastic leukemia with a minimal residual disease increase

Deborah Cardinali[1*], Marco Beldinanzi[1*], Michela Ansuinelli[1], Loredana Elia[1], Irene Della Starza[1,2], Vittorio Bellomarino[1], Mabel Matarazzo[1], Mariangela Di Trani[1], Mattia Cola[1], Prassede Salutari[3], Michele Cedrone[4], Renato Bassan[5], Marco De Gobbi[6], Matteo Giovanni Della Porta[7], Mariacarla De Simone[8], Caterina Alati[9], Nicola Stefano Fracchiolla[10], Monia Lunghi[11], Tamara Intermesoli[12], Valeria Cardinali[13], Antonino Mulè[14], Anna Guarini[1], Robin Foà[1*], Sabina Chiaretti[1*]

[*]Equal contribution

[1]Hematology, Department of Translational and Precision Medicine, "Sapienza" University of Rome, Roma; [2]Fondazione GIMEMA Onlus, Rome; [3]Department of Hematology Ospedale Civile, Pescara; [4]UOC Ematologia, Ospedale San Giovanni Addolorata, Rome; [5]Hematology Unit, Ospedale dell'Angelo and Ospedale Ss Giovanni e Paolo, Mestre Venezia; [6]Department of Clinical and Biological Sciences, Haematopoietic Stem Cell Transplant Unit, University of Turin, San Luigi Gonzaga Hospital, Orbassano; [7] IRCCS Humanitas Research Hospital, Rozzano, Milan; [8]Division of Hematology, Cardarelli Hospital, Napoli; [9]Division of Hematology, Azienda Ospedaliera 'Bianchi Melacrino Morelli', Reggio Calabria; [10]UOC Oncoematologia, Fondazione IRCCS Ca' Grande Ospedale Maggiore Policlinico di Milano, Università degli Studi di Milano, Milano; [11]Division of Hematology, Department of Translational Medicine, University of Eastern Piedmont, Novara; [12]Department of Oncology and Hematology University of Milan and Azienda Socio-Sanitaria Territoriale Papa Giovanni XXIII, Bergamo; [13]Department of Medicine and Surgery, Institute of Hematology, Centro di Ricerca Emato-Oncologica (CREO), University of Perugia, Perugia; [14]UOC Ospedali Riuniti, Villa Sofia-Cervello, Palermo, Italy.

Running head: ddPCR for T315I mutation detection
Abstract count: 143 words
Text count: 1370 words
Table: 1**; Figure:** 1
References: 14

Philadelphia chromosome-positive (Ph+) [1] acute lymphoblastic leukemia (ALL) is characterized by the reciprocal translocation between chromosomes 9q34 and 22q11. The frequency of the Ph+ ALL increases with age (2-5% in children/adolescents, ~20% among young adults and more than 50% in patients over 50 years), representing the most common genetic subset among adults and elderly ALL [2,3]. The advent of tyrosine kinase inhibitors (TKIs) has markedly improved the outcome of patients with Ph+ ALL and indeed changed the natural history of the disease. At present, TKIs represent the gold standard treatment for patients with Ph+ ALL, with or without chemotherapy (reviewed in Foà & Chiaretti, 2022) [4]. While TKIs represented a milestone in the treatment of these patients and the more recent combination with immunotherapy represents a further step towards cure [5,6], the occurrence of *ABL1* mutations is a relatively common event which occurs prevalently with first and second generation TKIs. Among the commonest, the

most deleterious is represented by T315I, which induces resistance to all TKIs with the exception of the third generation TKI ponatinib and is sustained by the substitution of threonine with isoleucine at residue 315 (T315I) [7].

Sanger sequencing (SS) has, so far, been considered the gold standard tool for *BCR::ABL1* KD mutational screening, but it has limited sensitivity allowing the detection of mutations only when present in ≥20% of cells [8]. The digital droplet PCR (ddPCR) technology, a third generation PCR, may represent a valid alternative to SS for the detection of *BCR::ABL1* kinase domain (KD) mutations. This technique is highly sensitive, accurate and rapid, and given its affordable costs it allows routine testing if compared with next generation sequencing (NGS) [9].

In this study, we sought to evaluate if ddPCR is at least as sensitive as SS for the detection of the T315I mutation, and if ddPCR can detect the mutation also at very low levels of minimal residual disease (MRD) when SS is not applicable, ultimately helping to anticipate relapse.

To detect the *ABL1* T315I mutation on the *BCR::ABL1* p190 or p210 mRNA fusion by ddPCR we used the 20X expert designed assay (Bio-Rad, Hercules, CA). For cDNA synthesis, 1µg of RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen™, CA). Experiments were performed according to the manufacturer's instructions and ddPCR was performed as described (Bio-Rad Assay ID dHsaEXD39032815). A standard volume of 20 µl of reaction mix with 3 µl of cDNA template was used in ddPCR with the Quantalife QX200 ddPCR system (Bio-Rad Laboratories, CA) under the following conditions: 95°C for 5 min, 95°C for 30 s, 60°C for 1 min (40 cycles) with a 10 min hold at the temperature of 98°C, and a final hold at 4°C. Only experiments with at least 9000 droplets/replicate were considered acceptable, and each sample was tested in triplicate to increase sensitivity. Moreover, in each experiment we included negative controls, i.e. no template controls (NTCs) and a Ph+ ALL sample negative for the T315I mutation. The results were analyzed using the QuantaSoft software version 1.7.4 (Bio-Rad Laboratories, CA), according to the manufacturer's recommendations. Furthermore, variant allele frequency (VAF) was calculated as the number of *BCR::ABL1*^{T315I} copies/(number of *BCR::ABL1*^{wt} copies + number of *BCR::ABL1*^{T315I} copies) × 100.

We first evaluated if the assay was adequate for the detection of the T315I *BCR::ABL1* KD mutation at low MRD levels. To this end, we diluted the cDNA of a T315I positive Ph+ ALL patient, with a MRD value of 139.4 expressed as *BCR::ABL1/ABL1* x 100 to obtain a starting dilution of 13.94 transcript level and then we tested serial dilutions up to 10^{-4}. As shown in Figure 1, this assay was capable of detecting the T315I mutation at a MRD value as low as 10^{-3} (0.1394 *BCR::ABL1/ABL1* x 100), confirming the reproducible sensitivity of the assay. A maximum sensitivity of 10^{-4} (0.01394 *BCR::ABL1/ABL1* x 100) was observed, but it did not prove reproducible in all cases. The analysis of the positive droplets (*BCR::ABL1*^{T315I}) was carried out setting the threshold at a >6000 FAM (Carboxyfluorescein) amplitude value to avoid the detection of non-specific targets.

We then evaluated samples from 16 adult patients enrolled in the phase II GIMEMA LAL2116 protocol, designed for newly diagnosed Ph+ ALL and based on a chemotherapy-free induction/consoldation strategy combining the second-generation TKI dasatinib with the bispecific monoclonal antibody blinatumomab [5].

The *BCR::ABL1* KD mutational screening by SS was carried out in all patients at the time of a MRD increase, and in 1 patient who had an overt relapse during the induction phase with dasatinib. Overall, of the 16 patients with a MRD increase (10 during the induction phase, 6 in the consolidation phase), 8 resulted wild type (WT) while mutations were detected in 8 patients (7 harboring the T315I mutation and 1 the E255K). In the T315I positive patients, the evaluation by ddPCR of the same time-point (TP) samples indeed confirmed the presence of the mutation in all cases. More importantly, the analysis of the previous TPs – where MRD levels were low ranging from positive-not-quantifiable (PNQ) to 0.94 *BCR::ABL1/ABL1* x 100 – showed that ddPCR was capable of detecting the T315I *BCR::ABL1* KD mutation in 6/7 cases; 1 PNQ case, proved WT. The median VAF of these patients was 15.7% (range, 4.6-32%). To determine if ddPCR could anticipate the detection of mutations compared to SS, the previous TPs were also evaluated by SS in 4 cases with available material. In 2 cases the mutation was detected also by SS, while in 2 it could not be found (Table 1). Thus, ddPCR proved as reliable as SS, but more sensitive in some cases where SS failed to detect the T315I mutation at low MRD levels.

DdPCR for T315I mutation was also performed in cases that showed a MRD increase but proved negative by SS, and the absence of mutations was confirmed in all patients; this is also corroborated by the clinical follow-up of patients (manuscript in preparation), since only 1 patient, at a median follow-up of 47 months, has experienced a relapse (Table 1).

DdPCR is emerging as a sensitive tool for MRD refinement both in Ph- ALL [10] and in Ph+ ALL [11], showing in both settings that this assay is at least as sensitive as RQ-PCR and that it can refine MRD quantifiability, ultimately anticipating relapse. As a step further, ddPCR can find other applications. The detection of *ABL1* mutations before the occurrence of an overt hematologic relapse and the rapid switch to another TKIs is pivotal for an optimal clinical management of Ph+ ALL patients. Thus, it is fundamental to develop and standardize sensitive molecular methods capable of identifying mutations as soon as possible, in the setting of a MRD status, in order to avoid therapeutic failures. Our results suggest that the ddPCR *BCR::ABL1* KD mutation assay shows a good sensitivity and accuracy to detect mutations in the *BCR::ABL1* KD, with a high rate of reproducibility. Furthermore, ddPCR appears useful to predict molecular relapse before the increase in MRD at levels for which SS is not sufficiently sensitive.

In support of our observations, in several studies [12-14] the ddPCR assay has been used to detect a wide spectrum of mutations (i.e., *MYD88 L265P, JAK V617F, NOTCH1*) in different hematologic malignancies. Moreover, Soverini and colleagues [9] have shown that in chronic myeloid leukemia ddPCR was able to detect and quantify *BCR::ABL1* fusion transcripts harboring mutations conferring resistance to second-generation TKIs; mutations could be detected up to 0.5% and across 3-log *BCR::ABL1* levels. The authors defined the ddPCR method as a sensitive tool for mutation analysis that provides higher sensitivity compared to the standard approaches [9].

While these data are preliminary and additional cases are warranted, their clinical value is extremely important since they will allow to change treatment in a biologically-driven manner, being more sensitive than conventional SS screening and also considering that, from a methodologic standpoint, ddPCR allows to obtain a result in a shorter time compared to NGS, thus allowing therapeutic intervention in a timely manner.

As such, ddPCR is being investigated in the context of the ongoing phase III GIMEMA LAL2820 trial (NCT04722848), designed for newly diagnosed adult Ph+ ALL, to conclusively define whether ddPCR may anticipate relapse, ultimately further improving the outcome of Ph+ ALL patients.

References

1. Nowell PC, Hungerford DA. Chromosome studies on normal and leukemic human leukocytes. J Natl Cancer Inst. 1960;25:85-109.
2. Burmeister T, Schwartz S, Bartram CR, et al. Patients' age and BCR-ABL frequency in adult B-precursor ALL: a retrospective analysis from the GMALL study group. Blood. 2008;112:918-919.
3. Chiaretti S, Vitale A, Cazzaniga G, et al. Clinico-biological features of 5202 patients with acute lymphoblastic leukemia enrolled in the Italian AIEOP and GIMEMA protocols and stratified in age cohorts. Haematologica. 2013;98(11):1702-1710.
4. Foà R, Chiaretti S. Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia. N Engl J Med. 2022;386(25):2399-2411.
5. Foà R, Bassan R, Vitale A, et al; GIMEMA Investigators. Dasatinib-Blinatumomab for Ph-Positive Acute Lymphoblastic Leukemia in Adults. N Engl J Med. 2020;383(17):1613-1623.
6. Jabbour E, Short NJ, Jain N, et al. Ponatinib and blinatumomab for Philadelphia chromosome-positive acute lymphoblastic leukaemia: a US, single-centre, single-arm, phase 2 trial. Lancet Haematol. 2023;10(1):e24-e34.
7. Soverini S, De Benedittis C, Papayannidis C, et al. Clinical impact of low-burden BCR-ABL1 mutations detectable by amplicon deep sequencing in Philadelphia-positive acute lymphoblastic leukemia patients. Leukemia. 2016;30(7):1615-1619.
8. Soverini S, Hochhaus A, Nicolini FE, et al. BCR-ABL kinase domain mutation analysis in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors: recommendations from an expert panel on behalf of European LeukemiaNet. Blood 2011;118(5):1208-1215.
9. Soverini S, De Santis S, Martelli M, et al. Droplet digital PCR for the detection of second-generation tyrosine kinase inhibitor-resistant BCR::ABL1 kinase domain mutations in chronic myeloid leukemia. Leukemia. 2022;36(9):2250-2260.
10. Della Starza I, De Novi LA, Santoro A, et al. Digital Droplet PCR Is a Reliable Tool to Improve Minimal Residual Disease Stratification in Adult Philadelphia-Negative Acute Lymphoblastic Leukemia. J Mol Diagn. 2022;24(8):893-900.
11. Ansuinelli M, Della Starza I, Lauretti A, et al. Applicability of droplet digital polymerase chain reaction for minimal residual disease monitoring in Philadelphia-positive acute lymphoblastic leukaemia. Hematol Oncol. 2021;39(5):680-686.
12. Drandi D, Genuardi E, Dogliotti I, et al. Highly sensitive MYD88^{L265P} mutation detection by droplet digital polymerase chain reaction in Waldenström macroglobulinemia. Haematologica. 2018;103(6):1029-1037.

13. Nystrand CF, Ghanima W, Waage A, et al. JAK2 V617F mutation can be reliably detected in serum using droplet digital PCR. Int J Lab Hematol. 2018;40(2):181-186.
14. Skórka K, Chojnacki M, Masternak M, et al. The Predominant Prognostic Significance of *NOTCH1* Mutation Defined by Emulsion PCR in Chronic Lymphocytic Leukemia. Cancer Manag Res. 2021;13:3663-3674.

Authors contribution

DC, MB, MA performed experiments, and wrote the manuscript, LE, IDS, VB, MM, MDT, MaC performed experiments, PS, MiC, RB, MDG, MGDP, MDS, CA, NSF, ML, TI, VC, AM provided clinical samples, AG, RF and SC designed research and critically revised the manuscript.

Disclosures

The authors have no potential conflict of interest.

Acknowledgments

The authors wish to thank Associazione Italiana per la Ricerca sul Cancro (AIRC) 5x1000, Special Program Metastases (21198), Milan (Italy) to RF; Finanziamento Medi Progetti Universitari 2015 to SC (Sapienza University of Rome) and PRIN 2017 (2017PPS2X4_002) to SC.

Figure legend

Figure 1: 2D plots representative results for the T315I *BCR::ABL1* KD mutation obtained with serial dilutions of the transcript (expressed as *BCR::ABL1/ABL1* x 100) from the cDNA of a Ph+ ALL patient with T315I mutation. Circles in each box highlight the T315I mutation.

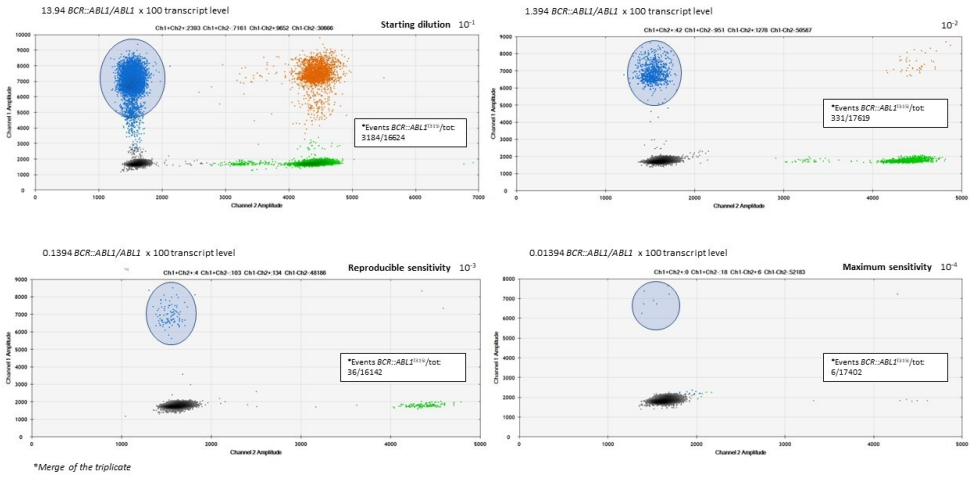

Figure 1. 2D plots representative results for the T315I *BCR::ABL1* KD mutation obtained with serial dilutions of the transcript (expressed as *BCR::ABL1/ABL1* x 100) from the cDNA of a Ph+ ALL patient with T315I mutation. Circles in each box highlight the T315I mutation.

Figure 1: 2D plots representative results for the T315I BCR::ABL1 KD mutation obtained with serial dilutions of the transcript (expressed as BCR::ABL1/ABL1 x 100) from the cDNA of a Ph+ ALL patient with T315I mutation. Circles in each box highlight the T315I mutation

108x60mm (300 x 300 DPI)

Table 1. Comparison of SS and ddPCR to detect the T315I *BCR::ABL1* KD mutation at MRD increase and at a previous time-point.

	BCR::ABL1 isoform	MRD values^	T315I mutation by SS	T315I mutation by ddPCR	MRD values^ at previous time-point	T315I mutation by SS at previous time-point	T315I mutation by ddPCR at previous time-point	Last follow-up
1	P210	0.93	WT	WT	0.29	/	/	Alive in CCR
2	P210	11.37	WT	WT	10.9	/	/	Alive in second CR
3	P210	4.3	WT	WT	0.05	/	/	Transplant related death in CCR
4	P190	3.41	WT	WT	1.18	/	/	Alive in CCR
5	P190	0.86	WT	WT	0.14	/	/	Alive in CCR
6	P190	0.28	WT	WT	0.17	/	/	Alive in CCR
7	P190	8.6	WT	WT	3.4	/	/	Alive in CCR
8	P190	0.06	WT	WT	PNQ*	/	/	Alive in CCR
9	P190	73.11	T315I	T315I	0.01	T315I	T315I	Death for relapse
10	P190	0.22	T315I	T315I	0.02	WT	T315I	Death for relapse
11	P190	7.63	T315I	T315I	0.12	Not available	T315I	Alive in CCR
12	P190	1.6	T315I	T315I	0.05	Not available	T315I	Death for relapse
13	P210	1.82	T315I	T315I	0.11	WT	T315I	Death for relapse
14	P190	112.3	T315I	T315I	0.94	T315I	T315I	Death for relapse
15	P190	0.19	T315I	T315I	PNQ*	Not detected	WT	Alive in second CR

*PNQ: positive-not-quantifiable; ^expressed as *BCR::ABL1/ABL1* x 100