



Application of a digital PCR method for *WT1* to myeloid neoplasms in CR and deep ELN *WT1* molecular response (< 10 copies)

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Abstract

Bone marrow *WT1* mRNA levels assessed by the ELN method are useful to establish prognostic correlations in myeloid malignancies treated with chemotherapy or hematopoietic stem cell transplantation (HCT). Those patients with *WT1* levels below ten copies have a good outcome. However, some of these patients relapse. To further characterize this group of cases, we applied a new and sensitive digital (ddPCR) *WT1* method. A consecutive series of 49 patients with treated myeloid malignancies and with an ELN *WT1* quantitation of < 10 copies were included in the study. All cases (47 AML and 2 MDS) have received intensive chemotherapy or HCT. One to four micrograms of total RNA were retrotranscribed to obtain $\geq 10,000$ *ABL1* copies using the ELN protocol. Only those cases with a good quality cDNA were used in the ddPCR *WT1* test. The ddPCR Gene Expression *WT1* Assay of Bio-Rad© was used to perform the PCR amplification, and the microdroplets were quantified in the Bio-Rad's QX200 droplet reader. Eighteen patients showed a negative *WT1* ddPCR assay (0 copies/ μ l), whereas 31 cases were positive (results ranged from 1 to 15.2 copies/ μ l). Survival analysis showed statistically significant differences in terms of OS between both groups, $83 \pm 8\%$ vs. $46 \pm 9\%$ ($p = 0.024$). A statistically significant correlation was also found between ddPCR $WT1$ results and CD123+ cell number detected by flow cytometry ($p = 0.024$). Larger series of patients tested with the current ddPCR $WT1$ method will solve whether it could be used to stratify patients with myeloid malignancies achieving deep *WT1* molecular response (< 10 copies).

Keywords Leukemia · *WT1* · Molecular diagnostics · Minimal residual disease · Digital PCR

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Introduction

Minimal or measurable residual disease (MRD) has been used to predict relapse in patients with myeloid malignancies [1–4]. Chimeric fusion genes (*PML-RAR α* , *RUNX1-RUNX1T1*, *CBFb-MYH11*) or small insertions (*NPM1* mutations) analyzed by sensitive real-time PCR methods are the most widely used targets based on its reliability, sensitivity, and specificity. However, a large proportion of AML cases lack such genetic alterations, and alternative methods should be used. Standardized multiparametric flow cytometry and sensitive strategies of next-generation sequencing (NGS) are gaining acceptance in most laboratories. Despite the potential advantages of these techniques, other less complex alternatives based on PCR methods are also employed. *WT1* has been used as a surrogate marker of the leukemic clone size based on the overexpression of *WT1* mRNA in immature myeloid progenitors [5–8]. *WT1* quantitation has been standardized, and it could be assayed both in peripheral blood and bone marrow [9]. It also could provide meaningful results in cases with low bone marrow cellularity in which multiparametric flow cytometry may be suboptimal. It has been shown that *WT1* mRNA levels are increased in the outgrowing subclone irrespective of the genetic background. Furthermore, this technique is simple, cheap, and rapid, and it does not require specialized software or technical expertise. It has been suggested that the best results are obtained with the peripheral blood given the very low number of *WT1* expressing cells in this sample. However, about 10% of AML and other myeloid malignancies show no increases in *WT1* mRNA levels, and when compared face to face with core-binding factor gene rearrangements or *NPM1* mutation assays, this technique showed less sensitivity. Based on these limiting facts, some experts claim that *WT1* overexpression should only be used as MRD marker in the absence of other more suitable targets [3]. The accurate detection of *WT1* expression ranges from 10 to 1,000,000 copies based on the plasmid levels used to perform the quantitation. In an earlier report using the ELN standardized method, we have shown that those AML cases with less than ten copies at the end of the chemotherapy showed an improved outcome [10, 11]. Thus, in this range of values (< 10 copies), plasmid-based quantification is far from being precise. To further analyze those cases, we applied a new digital PCR (ddPCR $WT1$) method, and we tried to establish clinical correlations.

Patients and methods

Patients' characteristics and basic molecular methods

Bone marrow samples (49 patients) at the end of the chemotherapy treatment or after HCT were obtained in each

participating institution and sent to the CETLAM repository center at the Hospital de la Santa Creu i Sant Pau in Barcelona for immunophenotype and molecular analyses. Samples at diagnosis were also tested for mutations in the *NPM1*, *FLT3*, *CEBPA*, *KMT2A*, and *WT1* genes using well-established protocols [4, 10]. For *WT1* and *ABL1* quantification, the ELN method was used as previously reported [10, 11]. In cases with *NPM1* mutation, a quantitative real-time PCR method was employed to assess the molecular remission [12]. Most patients were treated in accordance with the AML CETLAM 2003 trial (32 patients). Two samples were obtained from pediatric patients with AML at the end of the chemotherapy. Three samples were from patients with a previous HCT. The remaining 12 samples corresponded to AML patients enrolled in different CETLAM trials (Table 1).

Digital PCR of *WT1*

Mononuclear cells were separated using Lymphoprep (Alere Technologies, Oslo, Norway). The total RNA was extracted with AllPrep DNA/RNA kit (Qiagen, Hilden, Germany) and Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions and quantified by spectrophotometry and input amount was optimized for retrotranscription. In all cases, enough RNA was retrotranscribed to obtain a minimum *ABL1* of 10^4 copies, in a total reaction volume of 20 μ l. RNA, Random Hex 15 μ M, and dNTP (1 mM) were incubated for 65 °C 5 min and quickly chilled on ice. Collection of the content of the tube was done by brief centrifugation, and then we added 1 \times First-Strand Buffer, 10 mM DTT, Rnasin 20 units, and 200 units of M-MLV RT enzyme (Life Technologies, Carlsbad, USA), and incubated at 37 °C for 2 min, 10 min at 25 °C, 50 min at 37 °C, 15 min at 70 °C, and finally at 4 °C.

After the cDNA synthesis, an aliquot was employed to test the *ABL1* copy number using the standard Biomed protocol. The ddPCR mix was prepared as follows: 10 μ l of 2 \times ddPCR Supermix for Probes (Bio-Rad Laboratories, Inc., USA), 1 μ l of 20 \times ddPCR Assay *WT1*Hsa (HEX) #10031255 (Bio-Rad Laboratories, Inc., USA), 4 μ l of DNAase-free water, and 5 μ l of cDNA. The 20- μ l droplet digital PCR (ddPCR) reaction mixture was then loaded into a sample well of a DG8 Cartridge for QX200 Droplet Generator (Bio-Rad Laboratories, Inc., USA) followed by 70 μ l of Droplet Generation oil for probes into the oil wells, according to the QX200 Droplet Generator instruction manual.

The generated droplets were transferred into a clean 96-well plate. The plate was heat-sealed with pierceable foil seal (# 1814040 Bio-Rad) from PX1 PCR Plate Sealer (Bio-Rad Laboratories, Inc., USA) and then placed on a thermal cycler and amplified to the endpoint. Thermal-cycling conditions are indicated in Suppl table 1.

Table 1 Patients' characteristics

Sample number	Dx	Gender	Age	Leukocyte count × 10 ⁹ /L	BM blast %	Cytogenetics	FL3-ITD	NPM1	NPM1 copies	CEBPA	WT1 copies by digital PCR	WT1 copies by ELN
1	AML	M	54	12.66	87	46,XY [20]	MUT	MUT	0.74	NA	0	1.4
2	AML	M	26	194.8	97	46,XY,t(1;2)(p32;p13) [18]/46,XY [2].	WT	WT	NA	NA	1.7	5
3	AML	F	34	14.53	25	46,XX [20]	WT	WT	WT	WT	5.8	4.1
4	AML	F	21	43	93	46,XX [20]	WT	MUT	1	WT	3	6.6
5	AML	F	53	7.3	67	46,XX [20]	WT	MUT	1.88	NA	0	9.7
6	AML	F	38	20.5	20	46,XX [20]	WT	MUT	1.5	WT	3.3	2.2
7	AML	F	65	10.33	79	46,XX [20]	WT	WT	WT	WT	0	2.5
8	AML	F	44	1.7	95	46,XX [20]	WT	WT	WT	WT	0	7
9	AML	F	65	32.1	44	46,XX [20]	MUT	MUT	0.52	WT	1.6	2
10	AML	F	50	159	93	46,XX [20]	MUT	NA	WT	WT	11.5	2.9
11	AML	F	64	4.55	98	Hyperploidy	NA	NA	NA	NA	2.8	7
12	AML	F	72	0.16	70	NA	NA	NA	NA	NA	3.5	9
13	AML	M	50	90.9	94	46,XY [4]	WT	WT	WT	WT	0	4
14	AML	M	45	4.8	70	46,XY,t(6;9)(p23;q34) [18]/46,XY [2]	MUT	WT	WT	WT	3.9	5.5
15	AML	F	60	0.6	70	47,XX,+8[20]	NA	NA	NA	NA	6.1	7
16	AML with MDS related changes	F	61	85	75	50,XX,+5,-6,+8,+13,+22,+mar/46,XX,-6,+mar/46,xx	MUT	NA	NA	NA	1.6	8
17	AML	M	40	5.6	88	94,XXYY,+13[14]/47,XY,+13[3]/46,XY	WT	WT	WT	WT	6.8	8
18	AML	M	48	11.5	95	46,XY [20]	WT	MUT	0.42	MA	6.5	8
19	AML	M	61	29	87	47,XY,iso(8)(q10) [16]	WT	NA	WT	WT	2.5	3
20	MDS EB2	M	59	7.8	10	46,XY/47,XY,+8	NA	NA	NA	NA	9	5.2
21	AML	M	51	1.9	51	47,XY,+11[16]/46,XY [3]	MUT	WT	WT	WT	0	5
22	AML	F	53	1.5	64	46,XX,t(1;12)(p36;p13) [6]/46,XX,-3,+mar4 [3]/46,XX [13]	WT	WT	WT	WT	0	8.13
23	AML	M	24	8.1	50	46,XX,del(7) [15]/46,XY [5]	WT	WT	MUT	MUT	0	0.1
24	AML	M	31	50	100	47,XY,+8,inv.(16)(p13q22) [20]	WT	NA	WT	WT	2.1	8.4
25	AML	M	60	81	92	NA	WT	NA	WT	WT	0	3.2
26	AML	M	44	4.89	88	46,XY [20]	WT	WT	WT	WT	1.4	3.2
27	AML	F	48	75	77	46,XX,del(9q)/46,XX	NA	NA	NA	NA	1.8	3.2
28	MDS EB2	F	76	2.6	18	46,XX [20]	NA	NA	NA	NA	2.7	0.68
29	AML	M	29	89	46	46,XY	NA	NA	NA	NA	2.2	2
30	AML	F	49	23	70	46,XX [20]	MUT	MUT	2.3	WT	3	9
31	AML	F	42	2.4	21	46,XX [20]	MUT	MUT	20	WT	0	8
32	MDS	F	54	3.4	NA	45,XX,-7[20]	NA	NA	NA	NA	0	9.47
33	AML	M	34	14.5	94	NA	WT	NA	WT	WT	15.2	5.5
34	AML	F	60	130	97	46,XY [20]	MUT	MUT	2	WT	1.3	8
35	AML	F	2	5.44	19	NA	NA	NA	NA	NA	0	4
36	AML	M	61	44	98	NA	WT	NA	WT	WT	2.6	5.2
37	AML	M	8	2.85	45	46,XY [20]	WT	WT	WT	WT	2	9.5
38	AML	F	37	410	85	NA	MUT	NA	NA	NA	0	6
39	AML	M	32	79	80	46,XX [20]	WT	WT	WT	WT	1	0.5
40	AML	M	53	24	54	46,XY [20]	WT	WT	WT	WT	0	1.5
41	AML	F	69	16.7	80	46,XY [20]	MUT	NA	NA	NA	0	3.2
42	AML	M	56	79.86	50	46,XY [20]	MUT	MUT	NA	WT	1.4	8
43	AML	F	29	54.4	90	46,XX,-1,-10,11q+,-12,-15,+4mar	WT	WT	WT	WT	2	3.7
44	AML	M	20	16.1	87	46,XY [20]	WT	WT	MUT	MUT	3	6.5
45	AML	M	66	42.82	85	46,XY [20]	WT	MUT	1.26	WT	0	1

Table 1 (continued)

Sample number	Dx	Gender	Age	Leukocyte count $\times 10^9/L$	BM blast %	Cytogenetics	FL3-ITD	NPM1 copies	CEBPA	WT1 copies by digital PCR	WT1 copies by ELN
46	AML	F	36	NA	NA	NA	NA	NA	NA	0	3.45
47	AML	M	52	4.64	45	46,XY [20]	WT	2.68	WT	0	6.8
48	AML	M	66	8	27	45-47,XY,del(5)(q11q22),del(7)(q22),-17,add(21)(q21),+1-2mar [8]/44-46,XY,idem,-de(5)(q11q22),-5[8]	NA	NA	NA	4.9	7.5
49	AML	M	26	11	52	46,XY [20]	MUT	NA	NA	3.4	9

2 refers to NPM1 copies assessed by real-time PCR

After PCR, the 96-well PCR plate was loaded on QX200 Droplet Reader (Bio-Rad Laboratories, Inc., USA), which reads the droplets from each well of the plate. Analysis of the ddPCR data was performed with QuantaSoft analysis software version 1.7.4 that accompanied the QX200 droplet reader.

Flow cytometry immunophenotyping

Combinations of four monoclonal antibodies were used to assess the MRD in myeloid neoplasms (CD15/CD34/HLA-Dr/CD45, CD15/CD117/CD45/HLA-Dr, CD34/CD123/HLA-Dr/CD45, CD34/CD33/CD19/CD45) using the clones and fluorochromes previously reported with minor modifications [12]. The percentage of viable CD45+ cells was recorded in every case. A two-step protocol on viable cellularity was employed to assess the most immature myeloid populations and a minimum of 200,000 events were analyzed to establish the percentage of positive cells for each antigen. All the analyses were performed by the same person (JN) in a FACS-Canto flow cytometer (Becton Dickinson, CA) and using the PAINT-A-GATE software (BD).

Statistical methods

Characteristics between groups were compared using the χ^2 test and Fisher's exact test for categorical variables and the Mann-Whitney *U* test for continuous variables. The median value of continuous variables was used to dichotomize them for prognostic analyses. Overall survival (OS) was measured, from the date of enrollment until the date of death, and was estimated by the Kaplan-Meier method; comparisons between groups were performed by the log-rank test. The threshold values were established by selecting the most accurate values by a nonparametric receiver-operating characteristics (ROC) analysis, taking into account the maximum ratio sensitivity-specificity. The endpoint for the threshold was death and relapse. This approach was applied following the REMARK guidelines [13].

The statistical packages used were the IBM® SPSS® Statistics (version 25.0, USA) and the Rversion 3.3.2 from The R Foundation for Statistical Computing.

Results

ddPCRWT1

One microgram of total RNA was retrotranscribed following the recommendations of the ELN group to perform the molecular diagnostic of the most common chimeric gene rearrangements. A quality requirement for the preanalytical phase was established: *ABL1* copy number should be greater than

10,000 copies. Previous experiments performed in cases with lower values showed a lack of reproducibility, i.e., repeated experiments showed marked discrepancies in the number of positive droplets (data not shown). For those cases failing to achieve this requirement, we increased the amount of total RNA in the retrotranscription reaction until four micrograms. The ddPCR volume mix was run following the recommendations of the manufacturer, and the hands-on time was 6 h. The experiments were performed in duplicate on two different days. All samples showed adequate droplet counts (> 11,000) (Table 2). Blank controls (H₂O) did not show positive droplets. We established two groups of patients: those without *WT1* copies in the ddPCR assay (negative) and the second group of cases with any positive result (positive). The quartile distribution of all the series is depicted in Fig. 1 and Table 2. The interpretation of ddPCR is very easy: a negative case shows no positive droplets, whereas positive cases showed any level of positivity (provided that more than 11,000 droplets have been rightly included in the analysis).

When we investigated the overall survival of both groups, and despite the heterogeneity and the low number of cases included in this series, we detected a statistically significant advantage for those cases being ddPCR*WT1* negative ($p = 0.024$) (Fig. 2). This improved outcome did not reach statistic meaning for leukemia-free survival and cumulative incidence of relapse.

In 10 out of 11 cases with type *A-B-D NPM1* mutations, the copy number was below three copies, whereas one case (UPN31) showed 20 copies (Table 1).

Correlation between bone marrow ddPCR*WT1* results with myeloid cell populations assessed by flow cytometry

We investigated the number of viable CD45+ cells and immature cell compartments in all the cases (CD34+, CD117+, CD123+). There were no differences in the percentage of CD45+ cases in both groups. In negative ddPCR*WT1* samples, the percentage of immature cells was lower than in ddPCR*WT1*+ patients. This difference reached statistical significance for CD123+ cells, suggesting that the number of

stem cells in this group of patients (neoplastic or normal) is lower than in ddPCR*WT1*+ cases (Table 3).

Discussion

In this work, we applied a ddPCR*WT1* method, which makes full use of widely available reagents, and we also established the technical conditions of amplification and results interpretation. As a quality requirement for each retrotranscription, the product was tested using the European Leukemia Net method of real-time PCR amplification for the *ABL1* gene, which was used as a control. Only those samples with more than 10,000 *ABL1* copies were employed in the ddPCR*WT1* assay. Given that some of those samples came from other centers, showed poor cellularity, or are obtained in the post-HCT phase, we retrotranscribed more than 1 µg as needed to reach the 10,000 *ABL1* threshold (until 4 µg). The limited number of available samples with deep *WT1* molecular response (< 10 copies) and the technical constraints for the amplification procedure restricted our study to only 49 cases.

Using this approach and despite all the above-mentioned limitations, our preliminary results showed that ddPCR*WT1* might be informative to establish prognosis stratifications further. Irrespective of the diagnosis or the previous therapy, patients with a negative ddPCR*WT1* result showed a statistically significant advantage in terms of overall survival. We also detected a positive correlation between ddPCR*WT1* status and CD123+ cell number, suggesting that *WT1* levels may be linked to the size of the leukemic stem cell compartment. Given the low number of cases analyzed, we were not able to detect statistically significant associations with the number of positive cells for other immature markers (CD34, CD117). Samples from patients with the lowest quantity of CD123+ cells are those with a negative ddPCR*WT1* result. CD123 (IL3 receptor α chain) is expressed in immature precursors with stem cell behavior and it is also a marker present in most AML and B cell lineage ALL, being constantly present in the leukemic stem cell fraction. Based on these findings, immunotherapeutic strategies to deplete CD123+ cells are currently being investigated to treat AML [14–17].

Table 2 Raw ddPCR*WT1* results

	ddPCR <i>WT1</i> + ($n = 31$)	ddPCR <i>WT1</i> - ($n = 18$)
Droplets	15,566 (12835–19,062)	16,176 (11269–19,146)
Positive droplets	50 (14–182)	0
DNA concentration (copies/µl)	3.9 (1–15.2)	0
Quartile distribution* (DNA concentration/ptn)	1.65(6) /3.07(25)	0 (18)
<i>WT1</i> ELN copies	5.6 (0.5–9.5)	4.4 (0.1–9.7)
<i>ABL1</i> copies	32,708 (10128–105,183)	24,484 (10755–46,066)

*Positive cases were distributed in quartiles and the number belonging to each group is shown (3 + 4 merged)

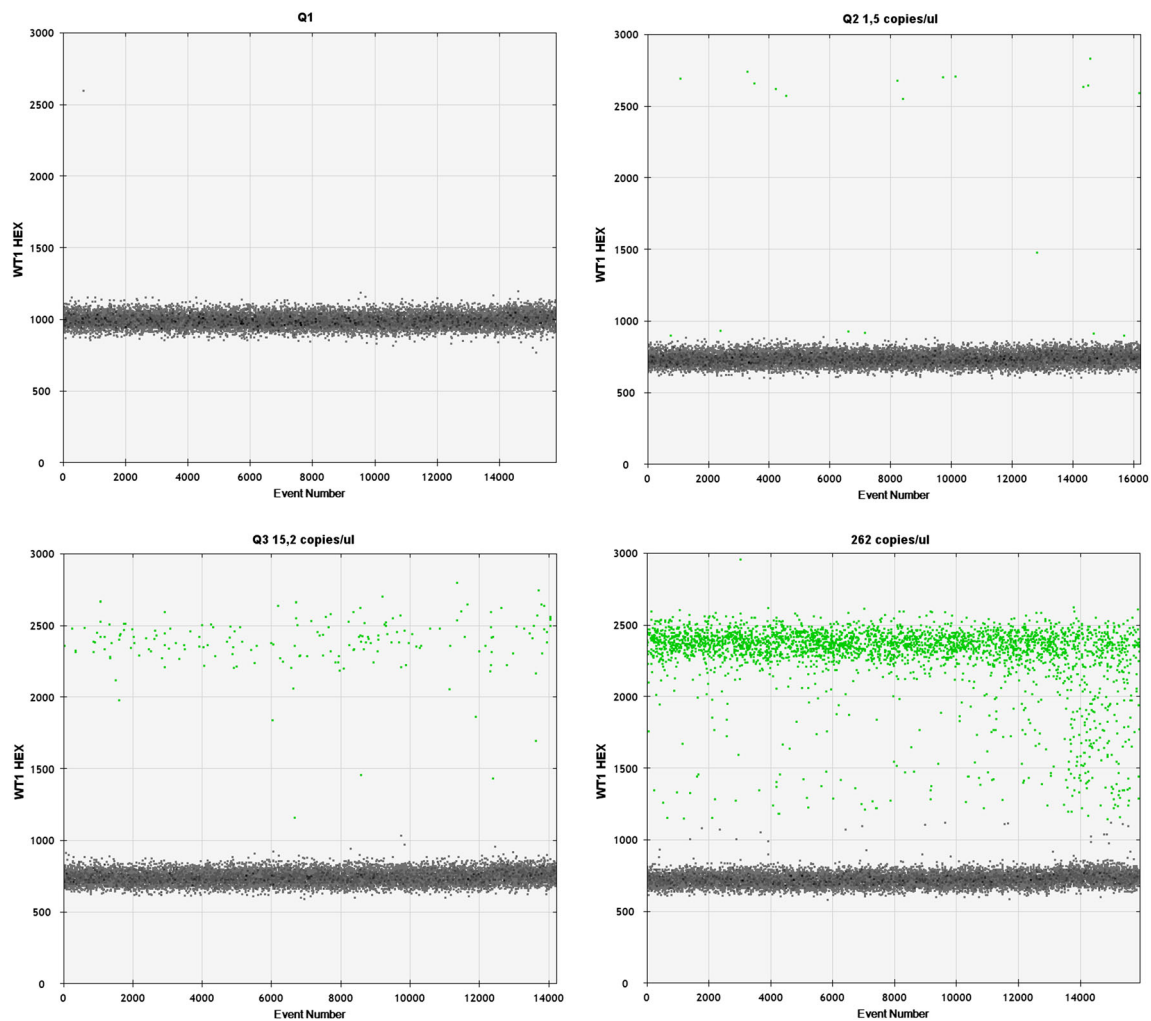


Fig. 1 Representative dot plots of ddPCRWT1. Top left: no positive droplets were detected (0 copies/ml, negative). Top right and bottom left: increasing numbers of positive droplets were detected in these cases (1.5 and 15.2 copies/ μ l, respectively). All two samples had *WT1* ELN values of less than ten copies but were considered as positives in the

ddPCRWT1 assay. Bottom right, ddPCRWT1 dot plot diagram in a case with more than ten copies in the *WT1* ELN test and ddPCRWT1 strong positivity (262 copies/ μ l). Positive droplets in green and negative droplets in gray

WT1 is expressed in a small population of myeloid committed immature progenitors. In some experimental models, this population could be the target of the myeloid transforming events [18]. Ye et al. [19] have shown that myeloid differentiation is required for acquiring a leukemia stem cell (LSC) phenotype and AML initiation and that blocking GMP formation abrogates leukemic transformation. This critical step in myeloid cell development could be indirectly identified by raised *WT1* levels and CD123 expression. Real-time PCR findings in one *NPM1* mutated case (UPN31, 20 copies) showing negative digital *WT1* results may suggest that both measurements may provide complementary information in selected cases.

Digital PCR techniques rely on the partition of the PCR mix in compartments (droplets in the most commonly used formats, although they could also be obtained in solid cells) to determine the concentration of the target sequence without a

need of a calibration curve. Efficient partitioning methods allow obtaining homogeneous, numerous, and of the same size droplets. Most clinical instruments show a volume variation of the droplets of less than 3%. Each partition could contain either a few or no target sequences. After the PCR reaction, a fraction of positive partitions are used to quantify the concentration of the target sequence using Poisson's statistics. It has been assumed that digital PCR is less prone to inhibition than real-time PCR [20–22]. Digital PCR has been employed to detect point mutations in plasma DNA from patients with solid neoplasms [23] and to perform MRD studies in core-binding factor AML with *KIT* mutations. Despite the promising results obtained in such studies, some uncertainties remain and we should be cautious to wrongly qualify dropout samples, which in our study means a negative ddPCRWT1 result. The increased number of partitions (more than 11,000 in our design) could increase the change of PCR inhibition.

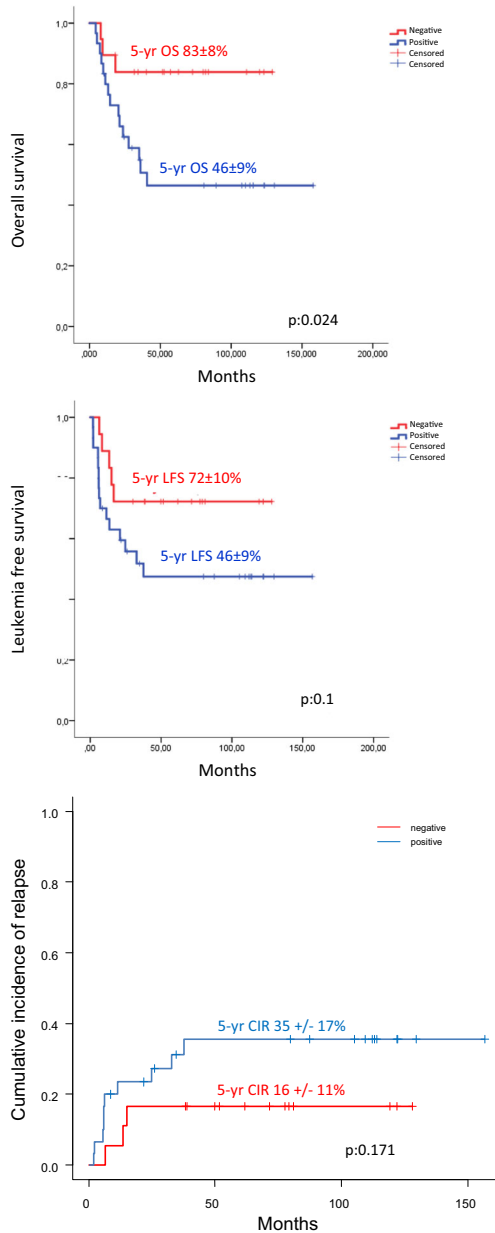


Fig. 2 Survival analysis in myeloid malignancies in deep ELN molecular response (<10 *WT1* copies) based on the results of the ddPCR*WT1* assay. **a** Overall survival. **b** Leukemia-free survival. **c** Cumulative incidence of relapse. ddPCR*WT1* positive cases in blue and negative cases in red

Furthermore, single-molecule amplification is often less efficient than amplification with a higher number of molecules,

Table 3 Correlation between cell number assessed by flow cytometry and ddPCR*WT1* results

Marker	ddPCR <i>WT1</i> + (<i>n</i> = 31)	ddPCR <i>WT1</i> - (<i>n</i> = 18)	<i>p</i>
CD45	0.819039 (0.132469–0.977081)	0.858990 (0.497457–0.941481)	ns
CD117	0.134484 (0.000351–0.030796)	0.009733 (0–0.111318)	ns
CD34	0.008838(0.000798–0.043241)	0.004984 (0.000911–0.027932)	ns
CD123	0.002916 (0.000739–0.015281)	0.001799 (0.001204–0.052821)	0.024

Expressed as percentage of viable events (1 = 100% of viable events)

and the readout may be, in some instances, dependent on the source of nucleic acid purification (bone marrow in our series). Bearing all these factors in mind, we tried to establish a simple and reproducible method of ddPCR*WT1*. We focused on those samples in which the precision of conventional real-time PCR is low. In recent times, other groups have tried a similar approach.

Waterhouse et al. [24] used a digital PCR platform (Bio-Rad) to assess the chimerism status and other molecular abnormalities (*NPM1* mutations, core-binding factor chimeric fusions, and *WT1* expression levels) in bone marrow samples obtained after HCT. Fourteen patients showed raised levels of *WT1* and mixed chimerism, which was predictive of clinical relapse. However, the method of digital amplification of *WT1* is not currently available. Koizumi et al. [25] analyzed 40 samples from 32 patients using the Quantstudio 3D Digital PCR System. These authors established a comparison between the *WT1* levels obtained using this method and a real-time PCR technique. The correlation between both methods was strong (*r* = 0.99). The mean *ABL1* expression for the real-time PCR group was 15,238 copies and 5947 copies for the digital PCR samples. However, in this paper, *WT1* expression was not detected in 20 healthy volunteers suggesting that the sensitivity of both methods was low.

In contrast with these reports, we provide a simple and reproducible protocol that could be applied to samples previously tested for real-time PCR using the ELN method. In more than one-third of cases (18/49) analyzed, the digital PCR was negative (0 copies/ml). We also can assume that our limit of detection was higher than our limit of the blank. The validity of this approach could be supported by the survival advantage.

In conclusion, the digital *WT1* PCR method could be easily applied to the study of myeloid malignancies. The main limitation, as in conventional real-time approaches, is the obtention of good quality material in heavily treated patients. The application of this technique to cases with myeloid malignancies achieving *WT1* deep molecular response (ELN levels below ten copies) may be helpful in establishing prognostic predictions. The interpretation of negative ddPCR*WT1* results needs to be fully elucidated in future prospective trials. This measurement could be combined with the assessment of the number CD123+ by flow cytometry, specific chimeric transcripts, or insertion mutations (*NPM1*). This approach may be potentially used to define a high-quality remission

status after chemotherapy and/or HCT in myeloid neoplasms. The analysis of the efficacy of CD123-targeted therapies by ddPCR $WT1$ also warrants future studies.

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Compliance with ethical standards

Conflict of interest Josep Nomdedeu (JN) received honoraria from Novartis. The rest of the authors (EB, MP, MC, LS, MAR, AM, AB, IB, JE, MA, CT, MT, AG, FV, XO, CP, JB, SB, and JS) had nothing to disclose.

Ethical approval All procedures were following the ethical standards of the Hospital de Sant Pau research committee, followed the SEHH recommendations, and were in accordance with the 1964 Helsinki declaration and its later amendments.

Statement of informed consent Informed consent was obtained from all individual participants included in the study.

References

- Maurillo L, Buccisano F, Del Principe MI et al (2008) Toward optimization of postremission therapy for residual disease-positive patients with acute myeloid leukemia. *J Clin Oncol* 26:4944–4951
- Buccisano F, Maurillo L, Del Principe MI et al (2012) Prognostic and therapeutic implications of minimal residual disease detection in acute myeloid leukemia. *Blood* 119:332–341
- Schuurhuis GJ, Heuser M, Freeman S et al (2018) Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood* 13:1275–1291
- Perea G, Lasa A, Aventin A et al (2006) Prognostic value of minimal residual disease (MRD) in acute myeloid leukemia (AML) with favorable cytogenetics [t(8:21) and inv(16)]. *Leukemia* 20:87–94
- Hohenstein P, Hastie ND (2006) The many facets of the Wilms' tumour gene, WT1. *Hum Mol Genet* 15(2):R196–R201
- Huff V (2011) Wilms' tumours: about tumour suppressor genes, an oncogene and a chameleon gene. *Nat Rev Cancer* 11:111–121
- Chau YY, Hastie ND (2012) The role of Wt1 in regulating mesenchyme in cancer, development, and tissue homeostasis. *Trends Genet* 28:515–524
- Ostergaard M, Olesen LH, Hasle H et al (2004) WT1 gene expression: an excellent tool for monitoring minimal residual disease in 70% of acute myeloid leukaemia patients - results from a single-centre study. *Br J Haematol* 125:590–600
- Cilloni D, Renneville A, Hermitte F, Hills RK, Daly S, Jovanovic JV, Gottardi E, Fava M, Schnittger S, Weiss T, Izzo B, Nomdedeu J, van der Heijden A, van der Reijden B, Jansen JH, van der Velden V, Ommen H, Preudhomme C, Saglio G, Grimwade D (2009) Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet study. *J Clin Oncol* 27:5195–5201
- Nomdedeu JF, Hoyos M, Carricondo M, Bussaglia E, Estivill C, Esteve J, Tormo M, Duarte R, Salamero O, de Llano MP, Garcia A, Bargay J, Heras I, Martí-Tutusa JM, Llorente A, Ribera JM, Gallardo D, Aventin A, Brunet S, Sierra J, CETLAM Group (2013) Bone marrow WT1 levels at diagnosis, post-induction and post-intensification in adult de novo AML. *Leukemia* 27:2157–2164
- Nomdedeu JF, Esquirol A, Carricondo M, Pratorcorona M, Hoyos M, Garrido A, Rubio M, Bussaglia E, Garcia-Cadenas I, Estivill C, Brunet S, Martino R, Sierra J (2018) Bone marrow WT1 levels in allogeneic hematopoietic stem cell transplantation for acute myelogenous leukemia and myelodysplasia: clinically relevant time points and 100 copies threshold value. *Biol Blood Marrow Transplant* 24:55–63
- Nomdedeu J, Bussaglia E, Villamor N et al (2010) Immunophenotype of acute myeloid leukemia with NPM mutations: prognostic impact of the leukemic compartment size. *Leuk Res* 35:163–168
- Mallett S, Timmer A, Sauerbrei W et al (2009) Reporting of prognostic studies of tumour markers: a review of published articles in relation to REMARK guidelines. *Br J Cancer* 102:173–180
- Jordan CT, Upchurch D, Szilvassy SJ et al (2000) The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* 14:1777–1784
- Muñoz L, Nomdedeu JF, López O, Carnicer MJ, Bellido M, Aventin A, Brunet S, Sierra J (2001) Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies. *Haematologica* 86:1261–1269
- Stevens BM, Khan N, D'Alessandro A et al (2018) Characterization and targeting of malignant stem cells in patients with advanced myelodysplastic syndromes. *Nat Commun* 12(9):3694
- Lin J, Lee EM, Ramshaw H et al (2009) Monoclonal antibody-mediated targeting of CD123, IL-3 receptor α chain, eliminates acute myeloid leukemic stem cells. *Cell Stem Cell* 5:31–42
- Hosen N, Shirakata T, Nishida S, Yanagihara M, Tsuboi A, Kawakami M, Oji Y, Oka Y, Okabe M, Tan B, Sugiyama H, Weissman IL (2007) The Wilms' tumor gene WT1-GFP knock-in mouse reveals the dynamic regulation of WT1 expression in normal and leukemic hematopoiesis. *Leukemia* 21:1783–1791
- Ye M, Zhang H, Yang H, Koche R, Staber PB, Cusan M, Levantini E, Welner RS, Bach CS, Zhang J, Krivtsov AV, Armstrong SA, Tenen DG (2015) Hematopoietic differentiation is required for initiation of acute myeloid leukemia. *Cell Stem Cell* 17:611–623
- Pinheiro L, Emslie KR (2018) Basics concepts and validation of digital PCR measurements. In: Karlin-Neumann G, Bizouarn F (eds) *Digital PCR: Methods and Protocols, Methods in Molecular Biology*, vol. 1768. Springer Nature
- Quan PL, Sauzade M, Brouzes E (2018) dPCR: a technology review. *Sensors* 18:1271
- Chen Y, George AM, Olsson E, Saal LH (2018) Identification and use of personalized genomic markers for monitoring circulating tumor DNA. In: Karlin-Neumann G, Bizouarn F (eds) *Digital PCR: Methods and Protocols, Methods in Molecular Biology*, vol. 1768. Springer Nature
- Ono Y, Sugitani A, Karasaki H, Ogata M et al (2017) An improved digital polymerase chain reaction protocol to capture low-copy KRAS mutations in plasma cell-free DNA by resolving 'subsampling' issues. *Mol Oncol* 11:1448–1458
- Waterhouse M, Pfeifer D, Duque-Afonso J et al (2019) Droplet digital PCR for the simultaneous analysis of minimal residual disease and hematopoietic chimerism after allogeneic cell transplantation. *Clin Chem Lab Med* 57:641–647
- Koizumi Y, Furuya D, Endo T, Asanuma K, Yanagihara N, Takahashi S (2018) Quantification of Wilms' tumor 1 mRNA by digital polymerase chain reaction. *Int J Hematol* 107:230–234

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