Four-color flow cytometry bypasses limitations of IG/TCR polymerase chain reaction for minimal residual disease detection in certain subsets of children with acute lymphoblastic leukemia.

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Background and Objectives. Competitive immunoglobulin/T-cell receptor polymerase-chain reaction (PCR) analysis with fluorescent detection is a rapid, cheap and reproducible method for quantifying minimal residual disease (MRD), which is well adapted to the recognition of high-risk childhood acute lymphoblastic leukemia (ALL). We aimed at defining whether flow cytometry (FC) techniques can bypass limitations of PCR for MRD determination.

Design and Methods. We analyzed 140 remission samples from 91 patients using both competitive PCR amplification of antigen-receptor genes and four-color FC identification of leukemia immunophenotype. These methods were chosen with the aim of detecting at least 0.1% blasts.

Results. MRD was measured using both PCR and FC methods in 123 samples and the two methods provided concordant results in 119 of them (97%). Moreover, three out of the four discordant results appeared minor since MRD was detectable by both methods, but at different levels. In 12 of 13 samples from nine patients, mainly infants with early CD10$^-$ and/or t(4;11) B-cell ALL and children with immature T-cell ALL, MRD could be determined using FC whereas PCR failed. Conversely, FC methods were unfeasible due to inappropriate leukemia immunophenotype in three additional children (including two with T-cell ALL) for whom PCR successfully provided MRD results.

Interpretation and Conclusions. The MRD results provided by FC techniques were highly concordant with those of competitive PCR. Moreover, the applicability of FC appeared higher in certain ALL subsets, although the appropriateness of this technique in terms of outcome prediction remains to be demonstrated.

Key words: acute lymphoblastic leukemia, flow cytometry, PCR, minimal residual disease.

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The level of minimal residual disease (MRD) has emerged as a powerful prognostic indicator in the monitoring of childhood acute lymphoblastic leukemia (ALL) and is currently incorporated to refine risk-assignment in most treatment protocols.[1-4] MRD studies conducted so far have shown that patients with MRD $\geq 10^{-2}$ after completion of induction therapy have a very high risk of relapse. On the other hand, patients with very low MRD levels ($<10^{-4}$) at the end of induction were found to have a very good prognosis following some,[5] but not all,[6] treatment protocols. For this reason, patients with high MRD levels receive a rapid intensification of chemotherapy while no change in treatment has been introduced for patients with low levels of MRD in currently ongoing French trials. This risk-adapted stratification requires MRD assays that have a sensitivity of at least 10^{-3} and that can yield results reasonably quickly (within 40-50 days from diagnosis). To fulfil these objectives, a rapid assay for quantifying MRD, based on immunoglobulin (*IG*)/T-cell receptor (*TCR*) genes rearrangement amplification using competitive polymerase chain reaction (PCR) and Genescan analysis[7] was chosen for routine analysis, instead of the more sensitive allele-specific real-time PCR, taking advantage of the simplicity, rapidity, reliability and low-cost effectiveness of the former. Moreover, the results of this method are strongly correlated with those obtained in our previous studies (hybridization to clono-specific probes).[6,7] Flow cytometry (FC) techniques also offer the advantage of speed.[8] Indeed, PCR and FC have been shown to be equally suitable methods for detecting MRD,

although neither is always applicable and both suffer technical limitations.[2,3,8–10] However the question of whether there is any advantage from combining both techniques for the determination of MRD remains unclear. Although several studies combining both methods reported comparable MRD results, their conclusion was usually that both methods should be coupled.[8,11–14] However, in routine laboratory practice, remission samples are sometimes too small to allow both techniques to be conducted. Moreover, such a strategy may appear rather expensive. Therefore, we compared multi-color FC methods and *IG/TCR* competitive PCR with fluorescent detection for MRD determination in childhood ALL. We conducted a pilot study of MRD assessment using *IGH/TCR*-PCR and FC assays in a series of children with ALL in whom treatment was rapidly intensified in those with high levels of MRD (i.e. more than 1% bone marrow leukemic cells) after completion of induction chemotherapy, but not reduced in those with the lowest levels of MRD.

Design and Methods

Patients and treatment

We studied 136 remission bone marrow samples containing enough cells for dual PCR and FC analysis obtained from 88 consecutive unselected children, including 7 infants (<12 months old), with ALL diagnosed between 2000 and 2003 in Nantes (n=84) and in 2003 in Besançon (n=4) University Hospitals. According to the immunophenotype, there were 76 cases of B-cell ALL (including 7 with negative CD10 expression and 5 with t(4;11) translocation) and 12 of T-cell ALL. Four additional samples from three children with T-cell ALL (including one infant) from Robert-Debré Hospital (Paris) in whom PCR-MRD measurements had been unfeasible were selectively analyzed with FC methods in the Nantes laboratory. All of the patients were enrolled in three Berlin-Frankfurt-Munster (BFM)-derived treatment protocols. MRD was assessed at the end of induction (day 35±5) (time-point 1) and consolidation (day 85±25) (time-point 2) -therapy in 85 and 55 patients, respectively. MRD was measured at the second time-point in all those cases in which MRD at the first time-point was > 1% according to the protocol recommendations, as well as in a subset of unselected patients with a lower MRD level at the first time-point. Informed consent was obtained from the parents in every case. All the patients were in complete remission at the time of each MRD determination, and had less than 5% blasts in bone marrow smears.

Sample collection and preparation

Bone marrow aspirates were collected at diagnosis and during the follow-up. Ficoll-separated mononuclear cells were then separated into two fractions, one being processed immediately by FC methods in Nantes or Besançon, the other being frozen and sent to Paris, in CO_2 ice, for PCR analysis.

PCR assessment of MRD

PCR reactions allowing detection of the most common gene combinations of IG heavy chain (*FRIII-JH*), TCR δ (*TCRD*) (all combinations involving VD1, VD2, DD2, DD3, and J1 were amplified in a multiplex assay) and TCR γ (*TCRG*) (all combinations involving VGI, VG9, VG10, VG11, J1, J2, JP1, and JP2 were amplified) were performed on each initial sample at presentation.[15,16] When no proper marker was available in B-lineage ALL, rearrangements of the immunoglobulin κ (*IGK*) locus was also investigated.[16] MRD was quantified using a competitive PCR assay identifying the clonal gene rearrangements of leukemic blasts by their size without sequencing, as previously described.[7]

Briefly, the PCR product corresponding to the blast rearrangement was analyzed by capillary electrophoresis, detected by fluorescence and visualized as a peak of separated size using an ABI-PRISM 3100 genetic analyzer (Applied Biosystems, Roissy, France) and a Genscan 672 program (Applied Biosystems). The blast level was determined using a competitive PCR assay based on the co-amplification of an internal standard homologous to the marker to be quantified. The result was eventually obtained by comparing the remission (*test*) sample to a *reference* sample with a blast concentration of 0.1%, 0.5%, or 1% (mixture of polyclonal mononuclear bone marrow cells and known amounts of the patient's blasts) and containing the same amount of the internal standard.

MRD was evaluated with the use of a single marker in 13 cases of B-cell ALL and 2 of T-cell ALL and with at least two markers in 59 B-cell ALL and 7 T-cell ALL cases. *TCRD* and *TCRG* markers were used, alone or combined, in 35 and 32 B-cell ALL and in 2 and 7 T-cell ALL patients, respectively. In addition, *IG* markers were used alone in 14 cases of B-cell ALL.

FC assessment of MRD

The FC strategy that we used for determination of MRD derived from the method extensively described by Campana and Coustan-Smith[9] and involved an identical technical procedure, monoclonal antibody panel and flow cytometer in the laboratories at Nantes and Besançon. Mononuclear cells (1×10^6/tube) were stained with four-color combina-

Table 1. Number and type of leukemia-associated markers (LAP) tested for the FC determination of MRD.

Number of markers	*B-cell ALL (n= 76)*	*Number of markers*	*T-cell ALL (n= 15)*
<2	2 (3%)	<2	1 (7%)
2	17 (22%)	2	6 (40%)
≥3	57 (75%)	≥3	8 (53%)
Median (limits)	3 (1-7)	Median (limits)	3 (1-5)
Type of markers		Type of markers	
$CD10^{+bright}$	40 (53%)	$CD45^{+low}$	9 (60%)
$CD34^{+bright}$	39 (51%)	$CD34^{+}$	6 (40%)
$CD20^{-}$	37 (49%)	$CD7^{+bright}$	5 (33%)
$CD22^{+bright}$	36 (47%)	$CD1a^{+}$	4 (27%)
$CD38^{+low}$	29 (38%)	$CD13^{+}/CD15^{+}/CD33^{+}/CD117^{+}$	4 (27%)
$CD45^{-}/^{+low}$	26 (34%)	TdT^{+}	3 (20%)
$CD58^{+bright}$	24 (32%)	$CD3^{-}$ $CD4^{+bright}$ $CD8^{+bright}$	3 (20%)
$CD13^{+}/CD15^{+}/CD33^{+}/CD65^{+}$	23 (30%)	$CD3^{+}$ $TCR\gamma/\delta^{-}$ $CD4^{-}$ $CD8^{-}$	3 (20%)
$CD10^{-}$	7 (9%)	$CD2^{-}$ $CD3^{+}$ $TCR\gamma/\delta^{+}$	3 (20%)
$CD2^{+}/CD4^{+}/CD7^{+}$	3 (4%)	$cCD3^{+}$ $TCR\alpha/\beta^{-}$ $TCR\gamma/\delta^{-}$	2 (13%)
$CD79b^{+}$	2 (3%)	$CD2^{-}$ $CD3^{+}$ $CD4^{-}$ $CD8^{-}$	2 (13%)
Surface Ig λ light chain^{+}	1 (1%)	$CD38^{-}$	1 (7%)

Results are expressed as number (percentage) of cases with each type of LAP. LAP include over- (bright fluorescence) or under- (low fluorescence) expressed antigens by leukemic cells in comparison with their normal counterparts, as well as illegitimate markers (i.e. markers not expressed by normal cells of the same lineage). "cCD3": cytoplasmic CD3. "/": or

tions of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridinin chlorophyll protein (PerCP) (or phycoerythrin cyanin 5 [PC5]). Quantitative or qualitative (illegitimate expression) antigen aberrations of leukemic cells (leukemia-associated phenotype) (LAP) were determined at diagnosis in comparison with normal bone marrow cells from 12 healthy donors or from 15 patients with expansion of normal precursor B-cells (hematogones) studied simultaneously and according to the literature.[9,17-24] The LAP used for MRD assessment in our study are listed in Table 1. The monoclonal antibody combinations were chosen in each case according to the type and number of LAP. In 118/140 samples, at least two combinations (maximum five) were employed. However 18 samples were tested with only one monoclonal antibody quadruplet.

In 69 $CD10^{+}$ B-cell ALL cases, each combination included three constant monoclonal antibodies, CD45-PerCP/CD19-APC/CD10-FITC, and a variable fourth PE-conjugated monoclonal antibody against CD20, CD22, CD34, CD38, CD58, CD13 or CD33 antigen. Occasionally, the latter was an anti-CD2, CD4, CD7, CD15, CD65, CD79b, CD117 or an anti-Ig λ light-chain monoclonal antibody. In seven $CD10^{-}$ B-cell ALL cases, a CD20-FITC monoclonal antibody was added to all of the combinations as a fifth antibody since CD20 was found to be negative in all of them.

The antibody combinations used for T-cell ALL cases (n=15) were more variable from one case to another but predominantly included anti-CD3-APC and CD7-FITC (or CD5-PE) together with CD1a, CD4, CD8, CD13, CD15, CD33, CD34, CD38, CD45, CD56, CD117, anti-TCRAB, anti-TCRGD and/or anti-terminal deoxynucleotidyl transferase (TdT). All antibodies were purchased from Becton-Dickinson (San Jose, CA, USA) and Beckman-Coulter (Miami, FL, USA). Cytoplasmic expression of CD3 (cCD3) and TdT were studied using a two-step procedure with surface staining by a first set of monoclonal antibodies followed by a second incubation of the cells with a permeabilization reagent (IntraPrep™, Beckman-Coulter, Miami, FL, USA) and anti-CD3 and -TdT monoclonal antibodies. Analyses were performed with a dual-laser FASCalibur™ flow cytometer, equipped with Cell Quest Pro™ and PAINT-A-GATE Pro™ software (Becton-Dickinson, San Josè, CA, USA).

To increase sensitivity and accuracy of the analysis, acquisition was performed in two consecutive steps. First, 10000 events were analyzed to determine the amount of B- (or T-) cells. In the second step, acquisition through a *live gate* drawn on the $CD19^{+}$/side scatter $(SSC)^{low}$ or $CD7^{+}$ (or $CD3^{+}$ or $CD5^{+}$)/SSC^{low} cell fraction was performed to allow a selective acquisition (7000 to 100000 events) of B-cells (in B-cell ALL) or T-cells (in T-cell ALL). Abnormal cell subsets were then searched and quantified in subsequent gates drawn on the basis of the LAP characteristics. A cluster of more than 10 dots with aberrant marker expression was considered as MRD.

Results

PCR and FC provided highly concordant MRD results

MRD was successfully determined by both PCR and FC methods in 123 samples and the two methods provided highly concordant results in 119 of them (97%). Thus, as shown in Table 2, MRD was found to be below 0.1% in 86 samples with both techniques while 20 (17%) results were concordantly positive, including 14 with more than 1% residual leukemic cells. Thirteen additional MRD determinations were considered negative with both the PCR and FC techniques although the sensitivity of the markers used with the former was limited to 0.5%. According to the follow-up time-point, MRD levels were identical with both methods in 76/79 (96%) post-induction and 43/44 (98%) post-consolidation

Table 2. Tandem analysis of MRD with PCR and FC techniques.

Levels	*FC-MRD: <0.1%*	*FC-MRD: 0.1-1.0%*	*FC-MRD: >1.0%*	*FC-MRD: failure*
CD10⁻ B-cell ALL (13 samples from 7 patients (including 7 TP1 and 6 TP2 samples)				
PCR-MRD: <0.1	4	0	0	0
PCR-MRD:0.1-1.0%	0	1*	0	0
PCR-MRD: >1.0%	0	0	4	0
PCR-MRD: failure	3	1	0	0
CD10⁺ B-cell ALL (106 samples from 69 patients) (including 67 TP1 and 39 TP2 samples)				
PCR-MRD: <0.1	79+10*	0	0	1
PCR-MRD:0.1-1.0%	1	5	0	0
PCR-MRD: >1.0%	0	2	7	0
PCR-MRD: failure	0	0	0	1
T-cell ALL (17+4$ samples from 12+3$ patients) (including 10+1$ TP1 and 7+3$ TP2 samples)				
PCR-MRD: <0,1	3+3*	0	0	1
PCR-MRD:0.1-1.0%	0	0	0	1
PCR-MRD: >1.0%	0	1	3	0
PCR-MRD: failure	2+3$	1+1$	2	0
all ALL (136+4$ samples from 88+3$ patients) (including 84+1$ TP1 and 52+3$ TP2 samples)				
PCR-MRD: <0.1	86+13*	0	0	2
PCR-MRD:0.1-1.0%	1	5+1*	0	1
PCR-MRD: >1.0%	0	3	14	0
PCR-MRD: failure	5+3$	2+1$	2	1

*All results are expressed as number of samples from consecutive patients except for the three cases selectively studied with FC techniques because of failure of PCR methods (the results of the latter are marked with " $ ") . TP1: time-point 1 (post-induction), TP2: time-point 2 (post-consolidation). Failure: failure of MRD determination (details in Table 3). *Samples with PCR sensitivity limited to 0.5.*

analyses, including 14 (18%) and 6 (14%) with similarly positive (i.e., >0.1%) MRD. The percentage of concordant MRD results was similar in samples from CD10⁻ (9/9, 100%), CD10⁺ (101/104, 98%) B-cell ALL and T-cell ALL (9/10, 90%) patients' subgroups.

We observed four discordances between PCR and FC MRD results. Only one of them (MRD at the second time-point) was major, since MRD was found to be positive (0.5-1.0%) with PCR whereas it was undetectable by FC. Interestingly, MRD was positive with both methods in the other three samples (all at the first time-point, but at a lower positivity level with FC (0.25%, 0.3% and 0.7%) than with PCR (>1.0%). The former and one of the latter samples were from the same patient with only one LAP suitable for FC analysis.

PCR and FC are alternative techniques for MRD detection

Considering only the results obtained from unselected, consecutive cases (n= 88), MRD could not be determined by PCR in 10/136 (7%) samples from six patients, because of absence (three cases: #38, #39, #59) or oligoclonality (two cases: #47, #48) of *IG/TCR* gene rearrangements and because of a very unusual oligoclonal aspect of the bone marrow taken at the second time-point from the last patient (#28), as shown in Tables 3 and 4. In addition, FC was unfeasible in four (3%) samples from four patients due to the lack of a suitable LAP. Interestingly, among these 13 samples for which FC or PCR methods were unfeasible, MRD measurement was successful with at least one technique in 12 and unsuccessful with both in only one sample (at the second time-point) from an infant with a common pre-B-cell ALL (#28) and an unusual oligoclonal regenerating bone marrow so that the distinction between leukemic and normal cells was impossible with both techniques.

FC methods would bypass PCR limitations for the detection of MRD especially in infants, in B-cell ALL with CD10⁻ phenotype or t(4;11) translocation and in immature T-cell ALL

Lack of suitable *IG/TCR*-PCR targets for MRD monitoring was significantly more frequent in infants than in older children (3/7, 43% versus 3/81, 4%, respectively, only unselected cases included)($p<0.01$, χ^2 test). As a consequence, MRD determination was unfeasible with PCR methods in 5/12 (42%) versus only 5/124 (4%) samples from infants and children, respectively ($p<0.001$). Interestingly, FC methods successfully determined MRD in all of these samples in which PCR failed except one from an infant (#28) (Table 3).

According to immunophenotype, failure of PCR-MRD determination was significantly more frequent with samples from CD10⁻ B-cell ALL (4/13, 31%) than those from CD10⁺ B-cell ALL (1/106, 1%) ($p<0.01$%, χ^2 test). In all of the patients with CD10⁻ B-cell ALL, MRD measurements were easily obtained using FC, given the high number of LAP (3-7, median: 4). Notably, 5/7 (71%) CD10⁻ B-cell ALL cases were infants versus only 2/69 (3%) CD10⁺ B-cell ALL cases ($p<0.001$). Moreover, all of the 7 (100%) CD10⁻ B-cell ALL cases but only 1/69 (2%) CD10⁺ B-cell ALL were associated with a t(11q23)/*MLL* translocation ($p<0.001$). Among the former group, five cases presented with a t(4;11)/*MLL-AF4* translocation and a pro-B-cell (CD10⁻ cIg⁻, sIg⁻) immunophenotype. Although two of them (40%) showed no detectable antigen-receptor gene rearrangement, MRD determination was efficiently obtained with FC techniques in all of their nine follow-up samples (Table 3).

Among 12 consecutive children (17 samples) with T-ALL, PCR techniques failed in three cases (five samples). However, FC-MRD determination was successful in all of them. In contrast, FC methods

Table 3. PCR and FC MRD levels in infants (<1 year old) or in cases with CD10⁻ B-cell ALL.

PN	Age (years)	Immunophenotype/ Cytogenetics	PCR			Flow cytometry		
			targets	TP1-MRD	TP2-MRD	LAP	TP1-MRD	TP2-MRD
9	<1	pro-B/CD10⁻ t(4;11)/MLL-AF4	Vγ10-JP1JP2	<0.5%£	Not done	CD45⁻ˡᵒʷ, CD10⁻, CD20⁻, CD38⁺ˡᵒʷ	0.15%	Not done
39	<1	pro-B/CD10⁻ t(4;11)/MLL-AF4	No detectable IG/TCR gene rearrangements			CD10⁻, CD15⁺, CD20⁻, CD33⁺, CD34⁺ᵇʳⁱᵍʰᵗ, CD38⁺ˡᵒʷ , CD65⁺	<0.1%	<0.1%
49	<1	pro-B/CD10⁻ t(4;11)/MLL-AF4	Vδ2- Dδ 2-(I,II)	>1.0%	>1.0%	CD10⁻, CD20⁻, CD34⁺ᵇʳⁱᵍʰᵗ, CD38⁺ˡᵒʷ	3.5%	5.0%
59	<1	pro-B/CD10⁻ t(4;11)/MLL-AF4	No detectable IG/TCR gene rearrangements			CD10⁻, CD20⁻, CD34⁺ᵇʳⁱᵍʰᵗ, CD58⁺ᵇʳⁱᵍʰᵗ	0.25%	<0.1%
83	9	pro-B/CD10⁻ t(4;11)/MLL-AF4	Vδ2- Dδ 2- (I,II)	>1.0%	>1.0%	CD10⁻, CD15⁺, CD20⁻, CD58⁺ ᵇʳⁱᵍʰᵗ, CD65⁺	1.2%	2.2%
4	1	pre-B /cμ⁺ CD10⁺ t(9;11)/MLL-AF9	FRIII-JH (I,II)	<0.1%	<0.1%	CD45⁺ˡᵒʷ CD10⁻, CD20⁻, CD22⁺ᵇʳⁱᵍʰᵗ		
28	<1	common pre-B /CD10⁺ t(11;19)/MLL-ENL	FRIII-JH (I,II)	<0.1%	Oligo°	CD20⁻	<0.1%	insufficient sensitivity#
16	<1	mature B/CD10- sIg⁺ t(9;11)/MLL-AF9	FRIII-JH (I,II)	<0.1%	<0.1%	CD10⁻, CD20⁻, sIgL⁺	<0.1%	<0.1%
53	<1	mature Tγδ (CD3⁺Tγδ⁺)	Vγ-JP1JP2 (I,II)	>1.0%	Not done	(CD2⁻ CD3⁺ CD4⁻CD8⁻) (CD2⁻ CD3⁺ Tγδ⁺ CD4⁻ CD8⁻)	4.3%	0.26%
72*	<1	mature Tγδ (CD3⁺Tγδ⁺)	Oligoclonality of TCR gene markers			(CD45⁺ᵈⁱᵐ CD2⁻ CD3⁺ CD4⁻CD8⁻) (CD2⁻ CD3⁺ Tγδ⁺ CD4⁻ CD8⁻) (CD3⁺ CD4⁻CD8⁻ CD15⁺)	Not done	0.22%

*LAP : Leukemia-associated immunophenotype. TP1/TP2-MRD: minimum residual disease level at time-point 1 (or 2). sIgL: surface immunoglobulin λ light chain. *patients from other institutions selected for FC-MRD determination because of failure of PCR methods. £sensitivity of PCR limited to 0.5%; °Oligo: oligoclonality of IGH markers in TP2 sample, only; #impossibility to distinguish leukemic and normal precursor B-cells (hematogones: 10% of bone marrow mononuclear cells).*

were inefficient for MRD analysis in two out of four samples from two other consecutive patients (impossibility to discriminate between normal and leukemic γ/δ T-cells), whereas PCR techniques allowed MRD determination in both samples (Table 4).

Failure of PCR-MRD determination was significantly more frequent in T-cell ALL (5/17, 29%) than in CD10⁺ B-cell ALL (1/106, 1%) (p<0.02%, χ^2 test) (Table 2). Interestingly, four of these samples in which *TCR*-PCR was unsuccessful were from two T-cell ALL cases with a CD3⁻ CD34⁺ immature phenotype (Table 4). Moreover, two out of three additional, selected patients with unsuccessful *TCR*-PCR (and successful FC-MRD determination), also had an immature T-cell phenotype (Table 4).

Discussion

Our comparative study of MRD monitoring of childhood ALL confirmed that *IGH/TCR*-PCR and FC methods provided highly concordant results. Thus, the two methods provided 97% concordance of MRD measurements in our series. This high percentage of concordance is comparable to that reported in some recent series,[8,11,14] although the level of MRD detection used to define MRD positivity was one log lower in the latter.

Analysis of the four discordant results in our study shows that in all cases FC techniques yielded lower MRD values than PCR but only in one case was MRD undetectable using FC. These results are in line with another series by Neale *et al.*[11] who examined 62 samples and found only two discordant results (weakly positive with PCR but negative with FC). In a new study extended to 1375 samples from 227 children, the same authors reported a variation of estimated MRD level between both techniques in a few cases.[14] Similarly, Malec *et al.*[8] compared the highly sensitive real-time quantitative-PCR and multicolor FC methods in 71 childhood ALL follow-up samples and observed some discordant results, mostly due to the lower sensitivity of FC. Alternatively, PCR can overestimate MRD due to DNA from apoptotic cells.[2,25] Another reason for discrepant results is the

Table 4. PCR and FC MRD levels of T-cell ALL.

UPN	age (years)	Immunophenotype	PCR targets	PCR TP1-MRD	PCR TP2-MRD	Flow cytometry LAP	Flow cytometry TP1-MRD	Flow cytometry TP2-MRD
22*	10	immature T (cCD3$^+$ CD3-)	No detectable *IGH/TCR* gene rearrangements			(cCD3$^+$ Tαβ-Tδγ-CD7$^+$) (CD3$^+$ CD7$^+$ CD33$^-$ CD56$^-$)	Not done	<0.1%
31*	11	immature T (cCD3$^+$ CD3- CD34$^+$)	No detectable *IGH/TCR* gene rearrangements			(CD45^{+dim} cCD3$^+$ CD7$^+$ CD34$^+$) (CD45^{+dim} cCD3$^+$ CD7$^+$ CD117$^+$)	<0.1%	<0.1%
36	6	immature T (cCD3$^+$ CD3- CD34$^+$)	Vδ2-Dδ3 (I,II) Vδ2-Jδ1	>1.0%	not done	(CD45^{+dim} CD7$^{+bright}$ CD3$^-$ CD34$^+$) (CD45^{+dim} CD7$^{+bright}$ CD3$^-$ CD33$^+$)	0.32%	not done
48	12	immature T (cCD3$^+$ CD3$^-$ CD34+)	Oligoclonality of *TCR* gene markers			(CD45^{+dim} cCD3$^+$ TdT$^+$) (CD45^{+dim} CD7$^{+bright}$ CD3- CD34$^+$)	3.5%	5.0%
38	14	immature T (cCD3$^+$ CD3- CD34$^+$)	No detectable *IGH/TCR* gene rearrangements			(CD3- CD4$^{+bright}$ CD8$^{+bright}$) (CD3- CD4$^{+bright}$ CD34+)	<0.1%	<0.1%
47	15	corticothymocytic T (CD1a$^+$ CD3$^-$)	Oligoclonality of *TCR* gene markers			(CD45^{+dim} CD1a$^+$ CD7$^{+bright}$ CD3-) (CD2$^+$ CD3- CD4$^{+bright}$)	not done	0.3%
51	13	corticothymocytic T (CD1a$^+$ CD3$^-$)	Vγl-J1J2 (I,II)	<0.5%$^£$	not done	(cCD3$^+$ Tαβ- Tδγ- TdT$^+$) (CD45^{+dim} CD7$^{+bright}$ CD3- CD34$^+$) (CD45^{+dim} CD7$^{+bright}$CD13+ DR$^+$)	<0.1%	<0.1%
54	13	corticothymocytic T (CD1a$^+$ CD3$^-$)	Vγl-J1J2 (I,II)	<0.1%	not done	(CD45^{+dim} CD1a$^+$ CD7+ CD3-) (CD45^{+dim} CD3- CD4$^{+bright}$ CD8$^{+bright}$)	<0.1%	<0.1%
71	12	corticothymocytic T (CD1a$^+$ CD3$^-$)	Vγl-J1J2 (I,II)	<0.5%$^£$	<0.5%$^£$	(cCD3$^+$ TdT$^+$) (CD1a$^+$ CD3-CD4- CD8$^{+bright}$)	<0.1%	<0.1%
69	8	corticothymocytic T (CD1a$^+$ CD3$^+$)	Vγl-J1J2 Vγ9-J1J2	<0.1%	<0.1%	(CD45^{+dim} CD1a$^+$ CD3$^+$ Tαβ-)	<0.1%	<0.1%
24	4	mature Tδγ (CD3$^+$Tδγ$^+$)	Vγ9-J1J2	>1.0%	<0.1%	(CD2- CD3$^+$ CD7$^{+bright}$ Tδγ$^+$) (CD2- CD3$^+$ CD5$^+$ Tδγ$^+$)	16.00°	insufficient sensitivity$^\#$
45	4	mature Tδγ (CD3$^+$Tδγ$^+$)	Vγl-J1J2	>1.0%	0.1%	(CD3$^+$ T δγ$^+$ CD4$^-$ CD8$^-$)	2.6%	insufficient sensitivity$^\#$
53	<1	mature Tδγ (CD3$^+$Tδγ$^+$)	Vγl-JP1JP2 (I,II)	>1.0%	Not done	(CD2$^-$ CD3$^+$ CD4$^-$ CD8$^-$) (CD2$^-$ CD3$^+$Tδγ$^+$ CD4- CD8$^-$)	4.3%	0.26%
72*	<1	mature Tδγ (CD3$^+$Tδγ$^+$)	Oligoclonality of *TCR* genes markers			(CD45^{+dim} CD2$^-$ CD3$^+$ CD4$^-$CD8$^-$) (CD2$^-$ CD3$^+$ Tδγ$^+$ CD4- CD8$^-$) (CD3$^+$ CD4-CD8- CD15+)	not done	0.22%
73	15	mature Tδγ (CD3$^+$Tδγ$^+$)	Vδ2-Jδ3	0.1-0.5%	Not done	(CD45^{+dim} CD3^{+dim} CD34$^+$CD38$^-$) (CD45^{+dim} CD2$^+$ CD3^{+dim} CD56$^-$)	not done	<0.1%

**Patients from other institutions selected for FC-MRD determination because of failure of PCR methods. LAP: leukemia-associated immunophenotype. TP1/TP2-MRD: minimum residual disease level at time-point 1 (or 2). £sensitivity of PCR limited to 0.5%. °Absence of blast cells on May-Grünwald-Giemsa-stained bone marrow smears. #Sensitivity of FC markers limited to 1% (LAP difficult to distinguish from normal Tγδ cell phenotype).*

way quantification is performed. In the FC assay, the proportion of blast cells among total mononuclear cells in the same sample is measured. In the PCR assay, the quantity of MRD is derived from a comparison to a standard curve made of serial dilutions of the blasts obtained at diagnosis. This relies on the assumption that 100% of them contain the marker. However, when the presence of the marker is restricted to a subset of blasts (oligoclonality) displaying treatment resistance, the quantity will be overestimated. We can assume that this has no clinical importance since the bad prognosis is linked to a slow decrease of the blast population rather than to the absolute number of residual leukemic cells. However, in these cases, the MRD result will be higher than that measured using FC.

Altogether, these data show that the risk of false negative MRD results with PCR or FC techniques is very low. The extension of target panels with markers expressed on minor sub-clones at diagnosis may minimize but not suppress false-negative results.[10] The use of tandem PCR and FC assays may circumvent this risk and ensure MRD monitoring of ALL.[3,11,13,14]

Neither of the two methods can be applied to all patients. In our series, MRD determination was unfeasible in 7% of the patients with PCR-based methods due to the absence or oligoclonality of the *IG*, *TCRG* and *TCRD* rearrangements. Unfortunately, the benefit of studying the *IGK* rearrangement (prevalent in up to 60% of B-cell ALL)[26,27] was weak since a majority of our patients had T-cell ALL. Moreover, the CD10$^-$ subtype, which accounted for two of four B-lineage ALL cases without *IG* heavy chain and *TCR* markers had no *IGK* rearrangement. A study of rearranged *TCRB* genes would have been interesting in T-cell ALL cases. However, the technology (i.e. real-time quantitative PCR with allele-specific oligonucleotides) to do this is complex, expensive and time-consuming.[28] Our study shows that four-color FC is a valuable alternative to PCR. The applicability of FC was extremely high, since MRD could be successfully determined in 97% of the samples, including all but one of those lacking PCR results. This is in agreement with the large study by Neale[14] in which 99.5% of the patients were amenable to MRD evaluation with tandem PCR and FC analysis despite one of the two methods being unsuitable in 15% of them.

Recent studies have shown that the incidence of clonal *TCR* gene rearrangements in B-cell precursor ALL varies with age, immunophenotype and genotype.[27,29-31] In line with these reports, we failed to identify antigen-receptor gene rearrangements suitable for PCR-MRD monitoring more frequently in some ALL subsets including up to 40% of infants, CD10$^-$ B-cell ALL and ALL with t(4;11) translocation (all with pro-B-cell immunophenotype). Interestingly, FC techniques allowed easy measurement of MRD in all but one of these cases. Therefore, FC is a highly efficient alternative to *IGH/TCR*-PCR in these subsets of ALL. When feasible, PCR provided concordant MRD results with FC methods in all of these cases suggesting that *IGH/TCR*-PCR remained reliable even in these subsets of ALL.

In our series, it appeared unfeasible to quantify MRD by PCR in some samples from T-cell ALL cases. Interestingly, two of the three patients in whom PCR failed exhibited an immature CD3$^-$ CD34$^+$ immunophenotype similar to that of two out of three additional cases selectively studied with FC methods because of problematic PCR analysis. This is in agreement with other reports demonstrating that up to 50% of immature T-cell ALL still had all *TCR* genes in a germ-line configuration.[32-34] On the other hand, FC methods failed to identify suitable LAP in two additional cases with *TCRGD*$^+$ T-cell ALL. However, a tandem strategy eventually succeeded at determining MRD in all these cases in which PCR or FC analysis had been unfeasible. An alternative way to reduce the limitations of FC may be related to new markers not studied in the current series.

In this respect, the recent demonstration by Dworzak *et al.*[24] of broad and stable overexpression of CD99 on leukemic T cells is highly interesting. We studied CD99 expression on cryopreserved blasts from our two T-ALL cases who did not have suitable LAP and observed that its expression was at least one log higher than that on normal T-cells from both patients (*data not shown*), making this antigen a new good candidate for FC-MRD studies in T-ALL in the future.

We conclude that the MRD results of FC techniques and competitive PCR are strongly concordant. The applicability of FC appeared higher, especially in certain subsets of ALL, including ALL in infants and ALL with an immature B- or T-cell phenotype, although its appropriateness for outcome prediction remains to be demonstrated. FC is thus a powerful alternative to PCR. Further clinical studies including independent outcome data are required to determine whether FC may be used instead of PCR as a primary method for monitoring MRD, especially in the particular ALL subsets mentioned above.

NR, FM, FG-O, PSR, HA-L: conception, analysis and interpretation of flow cytometry data, revising the article critically, final approval of the version to be published; HC: analysis and interpretation of PCR data, revising the article critically,final approval of the version to be published; HG, RG: conception and design, analysis and interpretation of data, drafting the article, final approval of the version to be published. The authors declare that they have no potential conflicts of interest.

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