Application of a chemiluminescent methodology for detection of minimal residual disease in childhood acute lymphoblastic leukemia

Analysis of minimal residual disease (MRD) can predict outcome in childhood acute lymphoblastic leukemia (ALL). We applied a chemiluminescent methodology in 20 children with ALL. We detected MRD at different time-points throughout the follow-up of our patients, concluding that chemiluminescent detection of MRD is a reliable, safe and sensitive method.

Recent prospective studies clearly demonstrate the prognostic value of MRD in children with ALL.[1,2] Several methodologies are available for MRD analyses in ALL patients.[1-5] In order to test a safe, sensitive and reliable method for those laboratories in which neither radioactive analysis nor the TaqMan strategy can be carried out,[3] we present here a report on application of digoxigenin (DIG)-labeled patient-specific probes for a chemiluminescent detection of MRD in children with ALL.

Twenty children with ALL diagnosed at our institution and treated according to the ongoing protocol of the Associazione Italiana di Ematologia ed Oncologia Pediatrica (AIEOP-ALL 95) were included in this study. The childrens' characteristics are listed in Table 1. We collected samples at 5 time-points (TP): after 43 days (TP1), after three months (TP2), five (TP3), seven (TP4) and 24 months (TP5) of therapy. T-cell receptor (TCR) γ and TCR δ gene rearrangements were identified and characterized by performing diagnostic polymerase chain reactions (PCR), heteroduplex and sequencing analyses using standardized techniques.[6,7] PCR of follow-up were performed using different protocols in order to amplify TCRγ and TCRδ rearrangements. [7] Seven microliters of the products were spotted onto positively charged nylon membranes (NYTRAN N+, Roche Boehringer). Next, 200 pmol of each oligonucleotide were 3'-end labeled with DIG-ddUTP using a DIG oligonucleotide 3'-end labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's instructions. The membranes were prehybridized at 68°C for 2 hours using 25 mL hybridization solution (Roche Molecular Biochemicals) and then hybridized in a sealed plastic bag with 3 mL hybridization solution and 20 μL of the labeling probe (200 pmol). The membranes were incubated overnight at 54°C and then washed twice in 2 × SSC, 0.1% SDS at room temperature for 5 min. The membranes were soaked with blocking solution (Roche Molecular Biochemicals) for 30 min at room temperature. Twenty-four milliliters of blocking solution containing 2.4 μL of anti-DIG-alkaline phosphatase Fab fragments (Roche Molecular Biochemicals) were added, followed by incubation for 30 min. Finally, the membranes were washed twice with washing buffer (Roche Molecular Biochemicals) for 15 min and incubated with detection buffer (Roche Molecular Biochemicals) for 5 min. A chemiluminescent substrate (CSPD, Roche Molecular Biochemicals) was added to the surface of the membrane. The hybridization bags were sealed and incubated at room temperature for 5 min in the dark. Finally the membranes were air-dried, placed in a plastic hybridization bag and incubated at 37°C for 15 min. We exposed each membrane to an X-OMAT AR film (Kodak) for a period ranging from 15 min to 4 hours, in order to increase signal detection.

The sensitivities for respective PCR targets using the chemiluminescent method are summarized in Table 1. Among our cases we designed probes which reached different sensitivities ranging from 10^{-3} to 10^{-6}. Figure 1 shows representative results regarding three paradigmatic situations of MRD analysis which might influence clinical decisions. Figure 1A shows persistence of a leukemic clone in a child with T-cell acute lymphoblastic

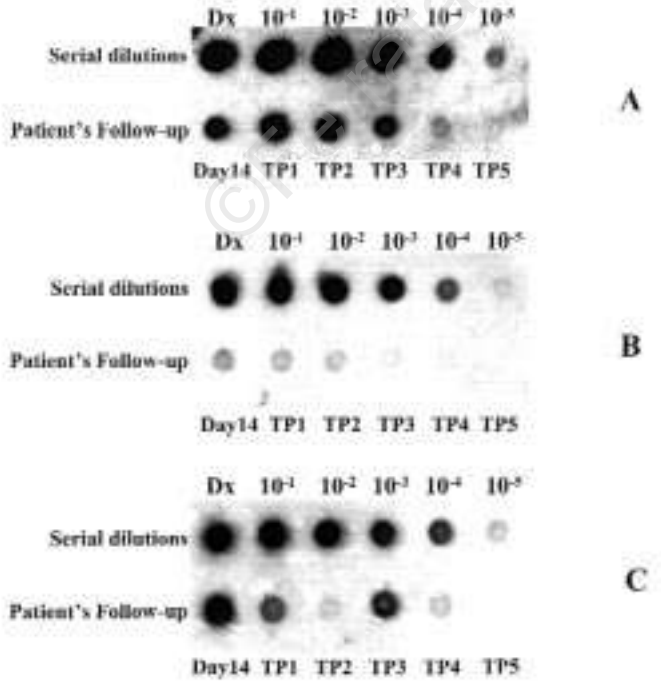

Figure 1. Chemiluminescent dot-blot hybridization of follow-up samples for cases LG (1A), SR (1B) and FD (1C), respectively; the patient-specific probes of all three cases resulted in a sensitivity of 10^{-4}, as shown in serial dilutions prepared with leukemic DNA into normal healthy donor peripheral blood DNA. Minimal residual disease was detected at different time-points in the three cases: a persistence of leukemic clone was noted in LG (1A) until the patient underwent allogeneic bone marrow transplantation; there was a progressive eradication in patient SR (1B), as well as in the majority of the other cases; the clone re-emerged at the beginning of the re-induction phase (TP3) in case FD (1C).

Table 1. Characteristics of children with ALL regarding rearrangements used for the chemiluminescent methodology.

Pts	Sex/Age at Dx	Immuno-phenotype	Rearrangement at Dx	Junctional region	Patient-Specific Probes (5'_3')	Probe Sensitivity
AG	M/3ys	Common	Vδ2/Dδ3	-3/6/0	ctgtgacTCCCGTactgggg	10^{-3}
AD	M/4ys	Common	Vδ2/Dδ3	-3/4/-2	cctgtgactTCTTTtgggggata	10^{-3}
CA	M/7ys	Common	Vδ2/Dδ3	-5/11/0	tgcctgtgTTCACAGGGGAactg	10^{-4}
FD	F/3ys	Pre-B	Vδ2/Dδ3	-1/del 80bp/Hept	tgtgacacTCTCCTacagtgctac	10^{-4}
GA	F/11ys	Common	Vδ2/Dδ3	0/3/0	ctgtgacaccTTTactggggg	10^{-3}
MM	F/4ys	Pre-B	Vδ2/Dδ3	-8/6/0	tactgtgcctCTTTCCactgggg	10^{-4}
SS	M/3ys	Common	Vδ2/Dδ3	-5/3/-2	tgtgcctgtgTTTtgggggat	10^{-3}
CS	F/10ys	Common	Vδ2/Dδ3	-3/del 100bp	tgtgcctgtacCCTTCAGGGCCTGT	10^{-4}
CM	M/4ys	Common	Vδ2/Dδ3	-5/4/0	tgtgcctgtgGCGTactGGGGG	10^{-4}
FL	F/2ys	Common	Vγ2	-4/7/-1	cctgggaCAGGCGAaattatt	10^{-4}
SR	F/3ys	Common	Vδ2/δd3	0/4/-3	cgtgacaccCCGAggggaata	10^{-4}
SA	M/2ys	Common	Vδ2/δd3	-1/6/0	cctgtgacacGGGCGTactggg	10^{-4}
MG	M/12ys	T-ALL	Vγ4	-2/18/-6	ggatgCCCCGAAGTAGATTATTctt	10^{-4}
BS	M/7ys	T-ALL	Vγ4	-4/8/-6	ccacctgggaTCTTTCTGgttataaga	10^{-4}
DSE	F/13ys	T-ALL	Vδ1/Jδ1	-2/25*/0	tttGAgggatCCCACCCCATacac	10^{-6}
LG	M/2ys	T-ALL	Vδ1/Jδ1	-1/37^/-1	CCGCTccttcctGGAAtggg	10^{-4}
SE	F/7ys	T-ALL	Vδ1/Jδ1	-4/14^/0	gggTcttTTAGgggggTacacc	10^{-4}
SR	F/3ys	T-ALL	Vδ1/Jδ1	-6/44*/-5	CCAagtGGTTGGGGAAACACGT	10^{-4}
TD	M/3ys	T-ALL	Vδ1/Jδ1	-2/18*/0	gggTTAGGTATTTATacac	10^{-4}
LG	M/13ys	T-ALL	Vδ2/δd3	-3/13/0	gacACCGGGAGAAATGact	10^{-4}

*Pts: patents; Dx: diagnosis; ys: years; del: deletion; bp: base pair; hept: heptamer; *D_1/D_2/D_3 gene segments are included; ^:D_2/D_3 gene segments are included; capital letters represent N-region inserted nucleotides.*

leukemia (case LG) who subsequently received an allogeneic bone marrow transplantation. Figure 1B (case SR) demonstrates evident progressive decrement of the chemiluminescent signal indicating eradication of the leukemic clone, whereas Figure 1C (case FD) shows how reappearance of a leukemic clone at the beginning of the reinduction phase (TP3) has not been followed by a subsequent relapse (TP4).

There are several advantages in using the chemiluminescent method for detection of MRD.[5] Firstly, the procedure is not radioactive and can, therefore, be performed in any laboratory where radioactive products are not allowed or the TaqMan strategy is too expensive or not yet applied. Secondly, because of the short exposure time (2-10 min), the whole procedure can be shortened in the future. However, end labeling of the oligonucleotide can be done at any time and the labeled probes can be stored at –20°C and used up to four times. In addition, the hybridization solution of DIG-labeled probes can be reused several times in order to analyze all time-points. We decided to use TCRδ and TCRγ gene junctional regions as patient-specific targets, because these rearrangements represent stable markers of disease throughout the duration of treatment and between diagnosis and relapse, as previously demonstrated.[8,9] Our data confirm that the chemiluminescent methodology can be performed in any laboratory and provides information important for clinical and therapeutic decisions. This procedure is faster and safer than the radioactive dot-blot technique, reducing biological hazards for the operator.

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References

1. Cave H, van der Werff ten Bosch J, Suciu S, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer. Childhood Leukemia Cooperative Group. N Engl J Med 1998; 339:591-8.
2. Van Dongen JJ, Seriu T, Panzer-Grümayer ER, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet 1998; 352:1731-8.
3. Pongers-Willemse MJ, Verhagen OJ, Tibbe GJ, et al. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. Leukemia 1998; 12: 2006-14.
4. Dibenedetto SP, Lo Nigro L, Di Cataldo A, Schiliro' G. Detection of minimal residual disease: methods and relationship to outcome in T-lineage acute lymphoblastic leukemia. Leuk Lymphoma 1998; 32:65-75.
5. Nakao M, Janssen JW, Schäfer M, Bartram CR. Digoxigenin-end labeled oligonucleotides facilitate the detection of

minimal residual disease in acute lymphoblastic leukemia. Leukemia 2000; 14:767-8.
6. Langerak AW, Szczepanski T, van der Burg M, Wolvers-Tettero IL, van Dongen JJ. Heteroduplex PCR analysis of rearranged T cell receptor genes for clonality assessment in suspect T cell proliferations. Leukemia 1997; 11:2192-9.
7. Pongers-Willemse MJ, Seriu T, Stolz F, et al. Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the BIOMED-1 CONCERTED ACTION: investigation of minimal residual disease in acute leukemia. Leukemia 1999; 13:110-8.
8. Dibenedetto SP, Lo Nigro L, Mayer SP, Rovera G, Schilirò G. Detectable molecular residual disease at the beginning of maintenance therapy indicates poor outcome in childhood T-cell acute lymphoblastic leukemia. Blood 1997; 90:1226-32.
9. Lo Nigro L, Cazzaniga G, Di Cataldo A, et al. Clonal stability in children with acute lymphoblastic leukemia (ALL) who relapsed five or more years after diagnosis. Leukemia 1999; 13:190-5.