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Multiplex-polymerase chain reaction assay for the detection of prognostically significant translocations in acute lymphoblastic leukemia

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Background and Objectives. The presence of specific chromosomal translocations in acute lymphoblastic leukemias (ALL) plays an important role in determining the prognosis of the patients. Our aim is to develop a highly sensitive and specific method to screen simultaneously for the four most frequent translocations in ALL: t(9;22), t(1;19), t(4;11), t(12;21).

Design and Methods. Our approach uses a multiplex-polymerase chain reaction (PCR) method, which involves two rounds of PCR using fluorescence-labeled nested primers. The chimeric transcripts resulting from these translocations can be identified by agarose gel electrophoresis or by fluorescence analysis. To validate this method we carried out the analysis in 42 pediatric ALL samples previously studied by cytogenetic and fluorescent *in situ* hybridization (FISH) techniques.

Results. In all samples with a known translocation detected by cytogenetic or FISH techniques, the same translocation was identified by the multiplex-PCR assay. Moreover, with this method we detected rearrangements in five patients in clinical remission and in two patients at diagnosis for whom karyotypes were normal and rearrangements had not been detected. The application of this multiplex-PCR assay was also useful in cases without cytogenetic results.

Interpretation and Conclusions. These results show that the multiplex-PCR method allows reliable, sensitive and rapid detection of the prognostically significant translocations in ALL. We believe that this assay combined with cytogenetic analysis should be the strategy of choice for the initial diagnostic phase of acute lymphoblastic leukemia, and that it could be used not only at diagnosis but also to follow-up these alterations in remission samples without previous controls.

Key words: multiplex-PCR, translocations, ALL, Genescan analysis.

Acute lymphoblastic leukemia (ALL), the most common subtype of childhood cancer is associated with the presence of specific chromosomal translocations allowing the identification of prognostically relevant subgroups.[1]

One of the translocations shown to have prognostic significance is the t(9;22)(q34;q11) translocation, in which the *cABL* gene, located on chromosome 9, is joined to the *BCR* gene on chromosome 22, resulting in a chimeric *BCR/ABL* gene. This translocation is found in 3-5% of pediatric ALL patients and is associated with a very poor prognosis.[2,3] For this reason, patients whose leukemia blasts contain the t(9;22) translocation are typically offered the option of bone marrow transplantation after remission is achieved.

A second translocation that identifies a high-risk group of patients is t(1;19)(q23;p13), found in 5-7% of all ALL cases but in 30% of pre-B ALL patients.[4,5] This translocation results in the juxtaposition of the *E2A* gene on chromosome 19p13 and the *PBX1* gene on 1q23. This rearrangement is associated with poor prognosis in patients treated with standard chemotherapy, but such patients show an improved outcome when more intensive therapies are used.[6]

A third translocation known to confer poor prognosis in pediatric ALL patients is t(4;11)(q21;q23),

Table 1. Cytogenetics, FISH and multiplex-PCR data from 42 ALL patients.

Sample	Karyotype/FISH	Multiplex PCR
	Diagnosis	
1	46,XX t(9;22)(q34;q11)	t(9;22) (e1a2)
2	46,XY, t(9;22)(q34;q11)	t(9;22) (e1a2)
3	46,XX(3)/46,XX, t(9;22)(q34;q11)(6)	t(9;22) (e1a2)
4	46,XX (25)/46,XX, der(1) dup(1)(q?),+del(9)(p21),-11(25)	t(1;19) (q23:p13) t(1;19)
5	46,XY(8)/47,XY, t(4;11)(q21;q23),+22(16)	t(4;11)
6	46,XY(23)/46,XY, t(4;11)(q21;q23)(7)	t(4;11)
7	46,XY(21)/46,XY, t(4;11)(q21;q23)(9)	t(4;11)
8	46,XY(3)/47,XY, t(4;11)(q21;q23),+13(12), 46,XY,add(1)(p36), t(4;11)(15)	t(4;11)
9	46, XY/ 47-50, complex karyotype FISH TEL/AML1 (+) 173/231	t(12;21)
10	46,XY FISH TEL/AML1 (+) 271/300	t(12;21)
11	46,XX	t(12;21)[a]
12	46,XY	t(12;21)[b]
13	46,XX	negative
14	46,XX	negative
15	46,XY	negative
16	46,XY	negative
17	46,XY	negative
18	46,XY	negative
19	46,XY	negative
20	46,XX	negative
21	46,XY	negative
22	46,XX	negative
23	46,XY	negative
24	46,XX	negative
25	NR	t(9;22) (e1a2)[b]
26	NR	negative
27	NR	negative
28	NR	negative
29	NR	negative
30	NR	negative
31	NR	negative
32	NR	negative
33	NR	negative
34	NR	negative
35	NR	negative
	Remission	
36	46,XY* / FISH TEL/AML1 (-) 0/300	negative
37	46,XY* / FISH TEL/AML1 (-) 0/300	negative
38	46,XX° / FISH BCR/ABL (-) 0/300	t(9;22) (e1a2)
39	46,XY° / FISH BCR/ABL (-) 0/300	t(9;22) (e1a2)
40	46,XX°	t(9;22) (e1a2)
41	46,XY°	t(9;22) (e1a2)
42	46,XY°	t(9;22) (e1a2)

**t(12;21) at diagnosis. °t(9;22) at diagnosis. [a]confirmed by FISH; [b]confirmed by specific RT-PCR. NR: no results. e1a2: minor region of t(9;22).*

which is detected in 5% of ALL cases. This translocation is one of the multiple rearrangements involving chromosome 11q23. More than 70% of infant leukemias exhibit 11q23 rearrangements where the *MLL* gene is located.[7] In t(4;11) the *MLL* gene is fused to the *AF4* gene on chromosome 4q21 creating the *MLL/AF4* chimeric gene. Patients with this translocation present more aggressive features and have a high probability of failure in standard treatment protocols.

In contrast to the above mentioned translocations associated with poor prognosis in pediatric ALL, the t(12;21)(p13;q22) translocation confers a favorable prognosis. This translocation is the most frequent genetic lesion in childhood ALL, occurring in approximately 27% of cases. The t(12;21) translocation involves the *TEL* gene, a member of the ETS transcription factor family, located on chromosome 12p13 and the *AML1* gene on chromosome 21q22 and results in the formation of the chimeric *TEL/AML1* gene. ALL patients with the t(12;21) translocation show excellent treatment response with absence of relapse after complete remission is achieved.[8]

The detection of these translocations by conventional cytogenetic methods is not always possible because of lack of adequate metaphases, poor chromosome morphology or the existence of cryptic translocations only visible using the new molecular cytogenetic techniques, as occurs with the t(12;21) translocation. So, fluorescence *in situ* hybridization (FISH) or molecular techniques to detect specific translocations can be very useful to the clinical management of the patients.[9,10,11] However, molecular cytogenetic detection of these translocations in individual samples is not very efficient.

Based on these considerations, we have developed a multiplex-polymerase chain reaction (PCR) that allows the simultaneous detection of the rearrangements t(9;22) (*BCR/ABL* minor breakpoint (m) and BCR/ABL major breakpoint (M)); t(1;19) (*PBX1/E2A*); t(4;11) (*MLL/AF4*) and t(12;21) (*TEL/AML1*) in a single assay. Our results show that molecular screening is a sensitive, specific, and rapid tool for the diagnosis of B-ALL in pediatric patients.

Design and Methods

For the combined purpose of optimizing the PCR primers and obtaining unlimited amounts of material for positive controls for the multiplex assay, we used cell lines containing each of the four translocations. The cell lines used were SD1, 697, MV4-11 and REH (all supplied by DSMZ) which constitute positive controls for t(9;22) minor region, t(1;19), t(4;11) and t(12;21) respectively. In addition, in all reactions we included RNA from a normal tonsil as a negative control.

Validation of the multiplex assay was performed using leukemic blasts from 42 patients with a diag-

nosis of ALL, which had previously been studied by cytogenetics and in some cases also by FISH analysis (Table 1). Cytogenetic studies were performed according to standard procedures. Between 5 and 20 metaphases were usually analyzed. FISH analysis was carried out using locus specific probes for the t(12;21) *TEL/AML1* and the t(9;22) *BCR/ABL* fusion genes (Vysis, Downers Grove, IL, USA). At least two hundred nuclei per probe were analyzed. Cases without FISH analysis correspond to old cases in which material was not available.

The samples were retrospectively collected from the University of Navarra (Department of Genetics) and from the Centro National de Investigaciones Oncológicas Carlos III (CNIO). The selected series included 42 cases; 7 cases in remission and 35 cases at diagnosis (Table 1). Cases 36-42 presented at remission with normal karyotypes while they had shown one of the specific translocations at diagnosis, either the t(9;22) or the t(12;21). The karyotype data for 24 out of the 35 samples taken at diagnosis were: eight cases (cases 1-8) presented specific translocations detected by conventional cytogenetic techniques: 3 cases of t(9;22), 1 case of t(1;19) and 4 cases of t(4;11); two cases (cases 9 and 10) presented a t(12;21) identified by FISH, and fourteen cases (cases 11-24) had a normal karyotype. The remaining samples (cases 25-35) did not yield metaphases for the cytogenetic analysis to be carried out.

RNA preparation

Total RNA was prepared either by the guanidinium thiocyanate phenol-chloroform extraction method or by using a TRI REAGENT Kit (Molecular Research Centre, INC) according to the manufacturer's recommendations. One microgram of cell line RNA or 1-2 mg of patient RNA were reverse transcribed with 1.5 units of AMV reverse transcriptase (Promega, Madison, USA). Following denaturation at 80ºC for 5 min, the cDNA synthesis was carried out at 42ºC for 60 min using random hexamers (Amersham Pharmacia Biotech Inc.) in a total volume of 40 µL. Subsequently, the cDNA was heated to 94ºC for 10 min to inactivate the reverse transcriptase and was then stored at -20ºC.

Multiplex PCR analysis

After cDNA synthesis a multiplex-PCR to detect chimeric transcripts derived from the four translocations was performed. This multiplex reaction was carried out using nested primers yielding maximum sensitivity and specificity. To verify the integrity of the isolated RNA and the correct synthesis of the cDNA, the ubiquitously expressed ABL gene was amplified in a separate PCR reaction. Primer sequences for the multiplex PCR assay and the amplification of ABL are given in Table 2. The multiplex-PCR was carried out in two steps. The first round of the PCR was performed with the external primers, and the second one with reverse internal primers marked with a characteristic fluorescence dye at their 5'-end (Table 2). Amplification was performed with a 9700 Perkin Elmer Thermocycler (Perkin Elmer, Germany).

In the first round of PCR, 2 µL of cDNA were used for the multiplex assay. The PCR was carried out in a final volume of 20 µL with 1× PCR-Buffer (Reaction Buffer 10×-Biotools B&M Laboratories, S.A., Spain), 1.5 mM $MgCl_2$ (Mg_2Cl 50 mM-Biotools B&M Laboratories, S.A Spain), 0.2 mM of each dNTP (Promega Madison USA), 5 pmol of each primer

Table 2. List of primers used in the multiplex-PCR.

	Abl control primers (5'-3')	
ABL-F	TGT TGA CTG GCG TGA TGT AGT TGC TTG G	
ABL- R	TTC AGC GGC CAG TAG CAT CTG ACT T	
	External primers (5'-3')	
BCR-P1-F	CGC TCT CCC TCG CAG AAC T	
BCR-P2-F	GAG TCA CTG CTG CTG CTT ATG TC	
ABL-P1-R	TTT TGG TTT GGG CTT CAC AC	
PBX1- P-R	GCC ACG CCT TCC GCT AAC	
E2A- P1-F	TTC TCG TCC AGC CCT TCT ACC	
MLL-P1-F	CCG CCT CAG CCA CCT ACT AC	
MLL-P2-F	AGC ACT CTC TCC AAT GGC AAT AGT	
AF4-P1-R	GAA TTT GAG TGA GTT TTT GAA GAT GTA TC	
TEL-P-S	CAC TCC GTG GAT TTC AAA CAG TC	
AML1-P-R	AGC CGA GTA GTT TTC ATC ATT GC	
	Internal primers (5'-3')	*Size of PCR fragments (bp) **
BCR-N1-F	ACT GCC CGG TTG TCG TGT C	320
BCR-N2-F	CAC GTT CCT GAT CTC CTC TGA C	397
ABL-N1-R	**6-FAM**-ACA CCA TTC CCC ATT GTG ATT AT	472
E2A-N1-F	CTA CGA CGG GGG TCT CCA C	376
PBX1- N-R	**TET**-CAT GTT GTC CAG CCG CAT CAG	403
MLL-N1-F	GGA CCG CCA AGA AAA GAA GT	
MLL-N2-F	AGC AGA TGG AGT CCA CAG GAT CAG	72 – 475
AF4-N1-R	**HEX**-GTT TTT GGT TTT GGG TTA CAG AAC T	
TEL-N-F	CTC ATC GGG AAG ACC TGG CTT AC	293
AML1-N-R	TET-AGC ACG GAG CAG AGG AAG TTG	332

*6-FAM, TET and HEX correspond to the fluorescent dye used to label the reverse primers of the nested PCR; * alternative breakpoints and/or splice variants generate different sizes of the fragments amplified.*

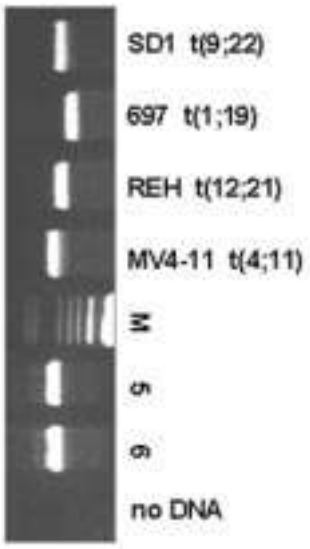

Figure 1. Agarose gel visualization of the four translocations after the multiplex-PCR in cell lines and patient samples. The first four lines correspond to the cell lines used as positive controls for each of the translocations. M: 1 Kb ladder molecular weight marker (Gibco, BRL). Patients 5 and 6 showed positive t(4;11). A control without c-DNA was included.

(Gibco BRL), and 1 unit of DNA polymerase (Biotools B&M Laboratories S.A., Spain). After an initial melting step (5 min at 94ºC), 30 amplification cycles of 30 s at 94ºC, 30 s at 60ºC and 50 s at 72ºC were performed, followed by an extension step of 7 min at 72ºC. One microliter of the first round PCR product was subjected to the second round of PCR, differing in the final volume (25 µL) and in the extension time (1 min at 72ºC). Twenty microliters of the final PCR products were analyzed on a 3% agarose gel (MetaPhor agarose) and visualized by ethidium bromide staining.

Individual detection of the translocations included in the multiplex assay can also be performed separately using the specific primers for each one of them. In this case, the same PCR conditions are used for both the first round and the nested reaction.

Genescan analyses

All amplified products were subsequently characterized by Genescan analysis in a 310 Perkin Elmer automatic DNA sequencer. Depending on the concentration, the final PCR product was diluted with formamide and mixed with 0.4 µL of the TAMRA Genescan standard to reach a final volume of 15 µL. Then the samples were denatured at 95ºC for 5 min before they were subjected to electrophoresis.

Results

We designed a multiplex-PCR assay to detect the most frequent translocations occurring in childhood ALL, t(9;22), t(1;19), t(4;11) and t(12;21), which constitute prognostically significant rearrangements.[3,6-8]

To test the performance of this multiplex-PCR assay we first tested the detection of these four translocations in the cell lines SD1 (*BCR/ABL* e1a2), 697 (*PBX1/E2A*), MV4-11 (*MLL/AF4*), REH (*TEL/AML1*) used as positive controls. All four translocations and their characteristic splicing variants or molecular breakpoints were detected (Figure 1). PCR products could be discriminated by their fragment size on an agarose gel or by Genescan with the same results.

Once the PCR conditions had been established, 42 selected ALL samples were analyzed. We compared previous cytogenetic or FISH results in ALL patients and the new results from the multiplex-PCR to know the concordance between these methods. The selection of cases was made according to the objectives of this work, i.e. trying to have a representation of the different situations that one can find in the cytogenetic analysis. First, we analyzed a group of 24 samples with cytogenetic results. Cases 1-10 showed one of the translocations identified by cytogenetic or FISH techniques. In all these cases we could detect the same translocation with our primer mix. Patients 11-24 presented a normal karyotype at diagnosis. In these cases the multiplex-PCR was negative for most of them, but positive for t(12;21) in cases 11 and 12. To confirm these results we performed FISH in case 11, and specific PCR for this translocation in case 12. Both cases were confirmed to be positive for t(12;21). The detected fusion genes for each sample are shown in Table 1.

We also studied 11 cases without cytogenetic results at diagnosis (cases 25-35). All except one were negative for any of the four translocations using the multiplex-PCR. Case 25 was positive for t(9;22). The presence of this translocation was also confirmed by individual PCR using specific primers for this translocation.

Finally, we selected a group of patients in clinical remission and with a normal karyotype, but who had presented some of the translocations at diagnosis. Cases 36 and 37 had a t(12;21) at diagnosis, but at remission, FISH analyses in 300 nuclei from both cases were negative, concordantly with our multiplex-PCR results. Cases 38 and 39, with a t(9;22) at diagnosis, showed at remission normal karyotypes and negative FISH, but they were positive with our primer mix. Moreover, all the remaining samples at remission (cases 40-42) resulted positive for the t(9;22) with the molecular assay.

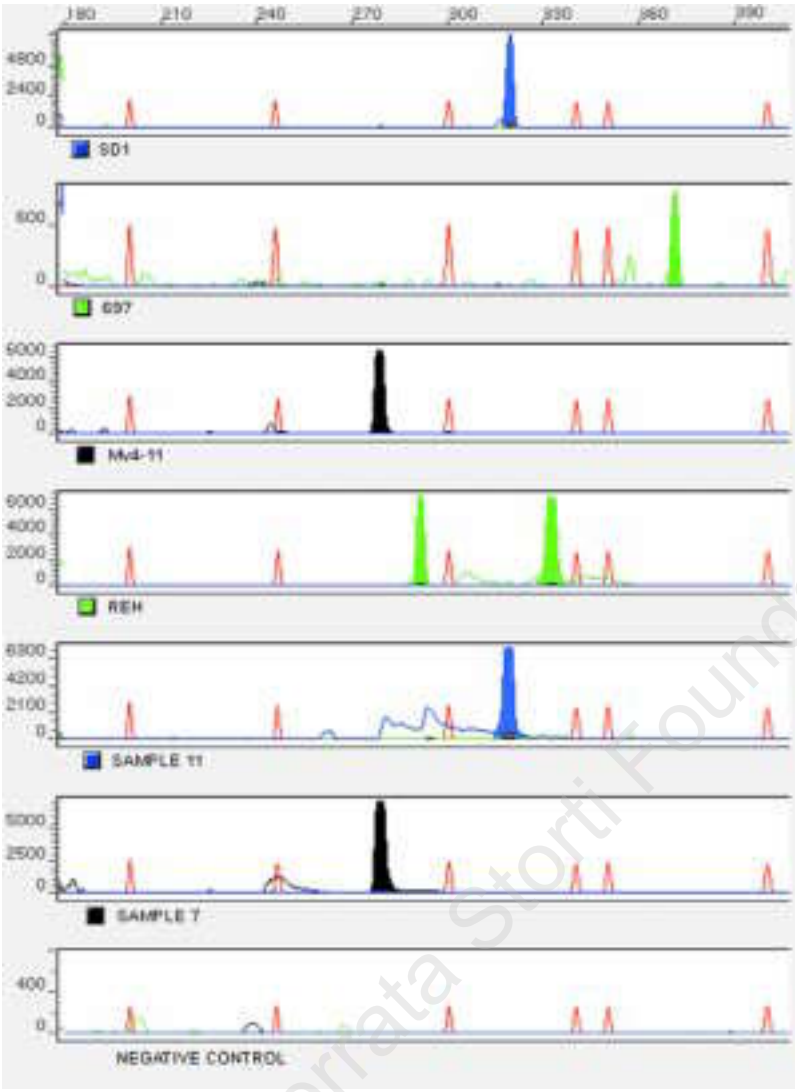

Figure 2. Example of the detection of the four translocations by Genescan analysis: t(9;22) in blue, t(1;19) in green, t(4;11) in black and t(12;21) in green of the cell lines SD1, 697, Mv4-11 and REH, respectively. The two peaks shown in the REH cell line correspond to alternative splicing. Detection of t(9;22) in sample 11 and t(4;11) in sample 7 is shown. The size of the products is calculated by means of the Genescan standard TAMRA labeled in red. The numbers at the top represent the base pairs scale.

Discussion

Identification of specific chromosomal aberrations is an important tool for diagnosis and risk-stratification in childhood ALL, because they confer different prognoses and have potential use in the follow-up of minimal residual disease. Conventional cytogenetic analysis has allowed the identification of some of these translocations and other chromosomal aberrations. However, this technique is time consuming, requires expertise, and sometimes it is not possible to obtain reliable results because of the quality of the chromosomes. FISH analysis has improved the detection of some of these translocations, such as the t(12;21) which is not possible to detect by conventional cytogenetics.[12] However, probes to detect all translocations are not yet available. Moreover, FISH in remission cases requires a large number of nuclei to be analyzed and for this reason its sensitivity is low, when compared to other molecular methods.

Molecular detection methods based on specific PCR of individual translocations are widely used in many laboratories, although several PCR reactions must be done to screen for the presence of these aberrations in a single patient. This method is sensitive, but it is laborious and used routinely only to detect some translocations such as t(9;22) or t(15;17) in M3 variant of acute myeloid leukemia.

In order to screen simultaneously for the most frequent translocations in pediatric ALL we have developed a multiplex-PCR assay. Other works have also described multiplex assays to detect an indi-

vidual translocation,[13,14] specific translocations found in other types of leukemia[15,16] or to detect different translocations in ALL.[17,18,19] However, not all the works trying to identify translocations in ALL included the four translocations known to have prognostic significance in pediatric ALL patients, t(9;22), t(1;19), t(4;11) and t(12;21). Moreover, in this work we improve the identification of these translocations by fluorescent detection using Genescan analysis. Our multiplex-PCR method may be used, first, to detect all these translocations in the initial diagnostic phase of ALL. In this sense, our results showed a good reliability, because in all cell lines and our cases with an already identified translocation by cytogenetics or FISH, the multiplex-PCR was positive for each one of the translocations. Second, this method is also useful to evaluate minimal residual disease in remission samples, mainly in those cases in remission but without a previous study. The high sensitivity of this method, because of the use of nested primers, allowed us to identify some of these translocations in cases in remission. In fact, cases 38 and 39 with absence of t(9;22) by FISH in remission showed the presence of minimal residual disease when the primer mix was used. So, the advantages of the multiplex PCR is that it allows fast, sensitive and specific identification of translocations with clinical significance.

Although faster and more sensitive, the PCR-based methods cannot replace cytogenetic analyses because numerical aberrations, unknown balanced translocations, hyperdiploidy and other abnormalities cannot be detected but could also have prognostic significance. For this reason, cytogenetic analysis is necessary for characterization of genetic aberrations and to make a complete diagnosis of ALL. Nevertheless our multiplex-PCR had great importance in cases without cytogenetic results at diagnosis. Case #25 was a carrier of a t(9;22) and the bad evolution (the patient died in 10 months) could have been controlled with correct identification of its translocation. So, we believe that multiplex-PCR combined with cytogenetic analysis should be the strategy of choice for the initial diagnostic phase of acute lymphoblastic leukemia.

The translocations included in this multiplex-PCR assay have different breakpoints and in some cases alternative splicing has been described.[20,21,22] With the primer set used we can detect all possible transcripts generated from these rearrangements. This heterogeneity sometimes makes difficult the identification of a rearrangement occurring in a sample based only on the size of the fragments in agarose gel. For this reason we improved the detection of these translocations using fluorescent-labeled reverse primers in the nested PCR in order to analyze the final PCR products by Genescan (Figure 2). This method allows the assignment of the exact size of the PCR products as well as the identification of the translocations by their characteristic color. Alternative to the use of Genescan analysis, another possibility to identify which translocation occurs in a sample when the size of the rearrangement is confounding is the use of individual PCRs for any of the four translocations. However, this method is more laborious and time consuming. We think that the performance of the multiplex-PCR followed by Genescan analysis simplifies the study and allows faster definition of which translocation is present in a sample.

Although this multiplex-PCR assay is highly sensitive for detecting the most frequent translocations in childhood ALL, nested PCRs increase the risk of contamination and the occurrence of false positive samples. Consequently, much care must be taken in manipulation to prevent such problems.

In summary, we believe that this multiplex-PCR assay is very useful to detect chimeric transcripts derived from t(9;22), t(1;19), t(4;11) and t(12;21) in ALL samples in an accurate, sensitive and fast way. Thus, it could be used not only at diagnosis but also to follow-up these alterations in remission samples.

Contributions and Acknowledgments

CM, BMD, BM: conception and design, analysis and interpretation of molecular data, drafting the article; MJL, AM, JCC, MJC: conception and design, analysis and interpretation of cytogenetics, cell lines, drafting the article: JB drafting the article, final approval of the version to be submitted. BM and AM-R are fellows of the Comunidad Autónoma de Madrid and Instituto de Salud Carlos III, respectively.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Susana Raimondi, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Prof. Raimondi and the Editors. Manuscript received August 24, 2001; accepted October 25, 2001.

Potential implications for clinical practice

The availability of molecular methods to detect the most prognostic relevant chromosomal translocations in childhood ALL has suggested that genetic features of leukemic cells are used to define risk classification of the individual patient.[23] Multiplex-polymerase chain reaction assay for the detection of prognostically significant translocations may be a very useful tool for this purpose.

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