Single Monochrome Real-Time RT-PCR Assay for Identification, Quantification, and Breakpoint Cluster Region Determination of t(9;22) Transcripts

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t(9;22) generates the *BCR-ABL* fusion gene, the hallmark of chronic myeloid leukemia (CML) but also found in acute lymphoblastic leukemia (ALL). Multiple chimeric transcripts translate to proteins of 190 or 210 kd and, rarely, 230 kd. CML typically carries p210 *BCR-ABL* while ALL is most often associated with p190. Detection and quantification of these fusion transcripts is useful in clinical management. We have exploited the unique melting profiles of these transcripts to design a new, simple, and cost-effective assay based on monochrome multiplex real-time RT-PCR for identification and quantification of each of these transcripts (b3-a2, b2-a2, and e1-a2) without further manipulation. The sensitivity of this assay was 10⁻⁴ for e1-a2 and 10⁻⁵ for b3-a2/b2-a2, which is appropriate for detection of minimal residual disease (MRD). Inter- and intra-assay variation was minimal. We applied this assay to assess the distribution of p190 and p210 in 260 childhood ALL samples from India. *BCR-ABL* was detected in 19 (7.3%), including one T-ALL. Eight patients (3.1%) demonstrated *mBCR-ABL* (p190) and 11 (4.2%) had *MBCR-ABL* (p210). Transcript levels varied markedly (up to 3000-fold) but e1-a2 were generally expressed at higher levels than b3/b2-a2 (*P* = 0.05). This simple real-time multiplex assay can thus be easily applied to monitor patients with ALL as well as CML. (*J Mol Diagn* 2005, 7:40-47)

Present day treatment protocols for leukemia are based on prognostic factors that allow stratification of therapy. The typical risk factors identified over the years include age, WBC counts, immunophenotype, and gene rearrangements, most commonly, chromosomal translocations.¹⁻⁴ The t(9;22) which juxtaposes the *ABL* proto-oncogene to the *BCR* gene generating a chimeric gene, *BCR-ABL*, is the hallmark of chronic myeloid leukemia (CML).⁵ It is also well-established that a fraction of acute lymphoblastic leu-

kemias (ALL) carry this chromosomal translocation.⁶ The t(9;22) is more frequent in ALL in adults (20 to 40%) than in ALL in children (<5%).³ Furthermore, the presence of a *BCR-ABL* fusion gene in ALL is associated with poor prognosis and is indicative of bone marrow transplantation in certain treatment protocols.^{7,8}

At the genomic level, different breakpoints have been defined that will, in turn, generate different transcripts and somewhat different proteins.⁹ The most common *BCR-ABL* rearrangement defines the major breakpoint cluster region (*MBCR-ABL*) and encodes for a protein of 210 kd (p210). This chimeric protein is generated by the juxtaposition b2-a2 (exon 13 of *BCR* - exon 2 of *ABL*) or b3-a2 (exon 14 of *BCR* - exon 2 of *ABL*). Another fusion transcript is due to a rearrangement in the minor breakpoint region (*mBCR-ABL*) that causes an e1-a2 (exon 1 of *BCR* - exon 2 of *ABL*) fusion that encodes for the smaller p190 protein. A third, very rare transcript (e19-a2) that is translated into a p230 was also described in CML.¹⁰

Although the vast majority of CMLs express p210, sporadic cases of CML expressing p190 were reported. Furthermore, CMLs patients at diagnosis may co-express very low amounts of p190 by alternative splicing events.¹¹ Similarly, ALL cells typically express p190 but p210 expression has been observed, especially in adult patients.¹² All *BCR-ABL* chimeric proteins have tyrosine kinase activity. However, transgenic mice demonstrated that p190 is associated with a shorter latency to develop leukemia than p210. Moreover, p190 transgenic develop B-lineage leukemia while p210 transgenic develop B, T, or myeloid leukemia.¹³ These animal models suggest that p190 and p210 associate with clinically different conditions. In a study on CML, it was suggested that patients with b3-a2 have a longer survival than patients expressing b2-a2.¹⁴ However, very little information is available on the distribution of these variants and their clinical relevance, especially in childhood ALL.

Response to initial therapy provides a further prognostic marker in both acute and chronic leukemias.¹⁵⁻¹⁷ MRD information is today considered an independent prognostic factor in at least three diseases: in childhood

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ALL after induction therapy; in CML after allogeneic stem cell transplantation, imatinib, or interferon treatment; and in promyelocytic leukemia after consolidation therapy. Therefore, detection of minimal residual disease (MRD) by molecular monitoring of the patients has become a major focus of diagnostic laboratories to enable detection of leukemic cells beyond the threshold of cytomorphology or karyotyping. Since quantification is a must, several methodologies are reported and continuously improved for standardized usage in multi-center trials.¹⁸

Some leukemogenic translocations, including BCR-ABL, can be detected in peripheral blood from healthy individuals.¹⁹ Thus, MRD assays should be designed to discriminate such false-positive results. The sensitivity of an assay should be sufficient enough to detect minimal disease but at the same time should indicate only levels of transcripts (above a threshold) that arise from the presence of leukemic cells.

Over time, different methodologies have been applied to determine the presence of fusion genes either for diagnosis or for monitoring disease (MRD). These included cytogenetics, FISH, Southern blot, and RT-PCR.^{20,21} These procedures are time-consuming, cumbersome, or are not appropriate for quantification of the transcript level. More recently, real-time RT-PCR using TaqMan or hybridization probes strategies have also been described for detection of *BCR-ABL*.^{22–30} These strategies have the inherent advantages of allowing quantification, specificity, and high sensitivity. However, all of these real-time RT-PCR assays require using expensive fluorescently labeled oligonucleotides. A single assay that allows simultaneous determination of the breakpoint cluster region and quantification of the transcript is not available. Although several independent reactions with sequence-specific probes can be used, this increases the complexity, workload, and cost. Alternatively, a real-time quantitative RT-PCR followed by capillary electrophoresis has been recently proposed³¹ but it requires additional equipment and manipulation of PCR products. We have previously demonstrated that the melting profile of amplicons can be exploited for reliable and consistent identification of various leukemogenic fusions, without the need for hybridization probes.³² We have now used this to develop a simple monochrome multiplex RT-PCR using the LightCycler technology to identify the type of *BCR-ABL* transcript (b2-a2, b3-a2, and e1-a2) and quantify its level without any further procedures. We have also applied this assay to blood mononuclear cells from healthy individuals and confirmed that the potential background *BCR-ABL* positivity in normal cells does not interfere with the quantification of leukemic cells.

We have reported that the relative distribution of molecular subtypes of pediatric ALL in India differs from USA/Europe.³³ For example, the t(12;21) was present in only 7% of precursor B cell ALL patients. This observation is relevant for designing therapeutic strategies, since the presence of translocations define risk-stratification groups. We have now applied our new monochrome multiplex real-time RT-PCR assay to further characterize the distribution of variant *BCR-ABL* fusions and their relative levels in childhood ALL from India.

Materials and Methods

Biological Samples

We used the leukemia cell lines K562 and SupB15 carrying the t(9;22)(q34;q11) and expressing b3-a2 and e1-a2 *BCR-ABL* transcripts, respectively. The RS4;11 cell line was used as a negative control. Cells were grown at 37°C in a 5% CO₂ atmosphere in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mmol/L glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma, St. Louis, MO).

Samples from 260 children with primary ALL were available from previous studies.³³ The median age of the patients was 7 years (range, 6 months to 18 years). Male to female ratio was 2.9:1. Bone marrow aspirates or peripheral blood samples were collected at the time of routine diagnostic procedures. Written informed consent was obtained and this study was approved by an Institutional Review Board.

Peripheral blood mononuclear cells from 97 healthy individuals were also collected. A single donation of 20-ml peripheral blood was obtained from each individual after informed consent was obtained and the samples were immediately coded to ensure anonymity of the donors.

Patient and donor mononuclear cells were obtained from Ficoll-Hypaque density gradient centrifugation and lysed with Trizol (Invitrogen, Carlsbad, CA) for RNA extraction. Two and one-half µg of total RNA was reverse-transcribed using the Superscript first-strand system for RT-PCR (Invitrogen, Carlsbad, CA) and random hexamers. Aliquots representing 1/100 (ie, 25 ng RNA) were used as templates in the LightCycler (Roche, Mannheim, Germany).

Multiplex Real-Time RT-PCR

We used the Lightcycler instrument and fluorogenic SYBR Green I. The primers were previously designed to identify the most common *BCR-ABL* transcripts. Three primers were used in each reaction, one reverse primer in ABL exon 2 (tcactggccacaaaatcatcacgt) and two forward primers in BCR exon 13 (b2, tcagaagcttctccctgacatccgt) and BCR exon 1 (e1, acctcacctccagcgaggag-gactt) (Figure 1).

The PCR reactions were carried out in 10 µl, including 4 pmoles of each primer, 2.5 mmol/L MgCl₂, and reagents from the FastStart DNA master SYBR Green I kit (Roche, Mannheim, Germany) using 2 µl of the indicated dilution of cDNA. The amplification consisted of 10 minutes at 95°C followed by a touchdown setting of 45 cycles of 10 seconds at 95°C, 5 seconds at 64 to 56°C, and 10 seconds at 72°C. To analyze the melting profile, a final cycle of 20 seconds at 97°C, 15 seconds at 50°C, and a slow increase in the temperature until 98°C was added.

Three independent experiments were conducted to ensure reproducibility always including the appropriate positive, negative, and no-template controls. Fluorescence emission spectra were monitored in real time for amplifica-

Figure 1. The top graph represents the involved area of the *BCR* and *ABL* genes with the indicated location of primers in *e1*, *b2* and *a2*. Exons are numbered. **A:** Melting curve analysis for identification of various *BCR-ABL* transcripts. Discrete melting peak temperatures are obtained for each fusion as indicated. **Dotted lines** represent amplicons from cDNA from the control cell lines, K562 and SUPB15. The other peaks correspond to patient samples. **B:** Conventional RT-PCR analysis demonstrates different size bands. **Lanes:** M is a 100-bp marker and the rest are indicated by the *BCR* exon (*b3*, *e1*, or *b2*) joint to *ABL* exon 2 from independent patient samples.

tion kinetics and melting curve analyses were performed to assess the specificity of the amplified products.

Standard RT-PCR and direct sequencing of RT-PCR products were used to confirm the presence and identity of the translocations. Frequencies were statistically compared by Fisher's exact test using SPSS.10 software.

Quantification of Transcript Levels

PCR products were measured by the threshold cycle (or crossing point, *Cp*) at which fluorescence became detectable above the baseline. The *Cp* was used for kinetic analysis and was proportional to the initial number of target copies in the sample. We calculated the relative expression of *BCR-ABL* transcripts as the ratio between

the level of *BCR-ABL* and the level of *GAPDH*. For this purpose, we generated a standard curve by measuring the *Cp* values for *GAPDH* transcripts from serial dilutions of ALL cell lines cDNA to allow a relative expression value for the target transcript per ng of RNA (normalizer). *GAPDH* primers were cgggaagctgtgcatcaatgg and catgttcacacccatgacg. As per our previous experience, we considered a sample adequate for analysis when the *Cp* value for *GAPDH* was ≤ 30 .³² Therefore, each sample (cell lines, normal controls, and patients) was initially analyzed to determine the *GAPDH* level.

BCR-ABL amplicons were cloned in a TA vector (Invitrogen) and serial 10-fold dilutions of plasmids, corresponding to known copy numbers, were run in triplicate to obtain the corresponding standard curves. These stan-

Figure 2. Chromatograms obtained by direct sequencing of PCR products, indicating the junction b3-a2 (two samples) and b2-a2 (two samples). Break-points (bkpt) are indicated.

dards were systematically run in every experiment. Test samples were analyzed by our duplex assay, amplicons b2-a2 and b3-a2 were identified by the melting peak and quantified using the standard curve for MBCR-ABL. The e1-a2 amplicon could be easily distinguished from the previous transcripts by the melting peak and quantified based on the corresponding standard curve for MBCR-ABL.

Results

Detection of Different BCR-ABL Transcripts by Real-Time Monochrome RT-PCR

To standardize an easy, simple, and cost-effective method to identify and quantify *BCR-ABL* transcripts, we designed a new duplex real-time RT-PCR for the Light-Cycler instrument using only Sybr Green I. We have previously detected *mBCR-ABL* transcripts using a single pair of primers that yield a product with a characteristic melting peak of $92.2 \pm 0.3^\circ\text{C}$.³² We have now added another BCR primer that allows amplification of the *MBCR-ABL* transcripts in a duplex reaction. This strategy takes advantage of the different melting profiles for each amplicon generated for proper identification.

As shown in Figure 1A, each amplicon, ie, b3-a2, b2-a2, and e1-a2, yielded a unique, characteristic, and reproducible melting profile detected only from amplification of the corresponding positive cDNA. The melting peak for e1-a2 was $91.85 \pm 0.25^\circ\text{C}$ while the peaks for b3-a2 was $85.6 \pm 0.3^\circ\text{C}$ and for b2-a2 was $86.55 \pm 0.25^\circ\text{C}$. Although these last two peaks were close to each other, we could unequivocally resolve them (see the confirmation below) in three independent runs, indicating that these two peaks can be distinguished when clinical samples are analyzed. Negative-control cDNA consistently demonstrated absence of any of these signals, indicating the absence of false positives.

These observations were confirmed by the size of the PCR products following electrophoresis in a 4% agarose gel (Figure 1B). The primers generate PCR products of 326 bp (b3-a2), 251 bp (b2-a2), and 418 bp (e1-a2). Final confirmation of the nature of the amplicons was obtained by direct sequencing of seven *MBCR-ABL* products (Figure 2).

Figure 3. Standard curves for quantification of number of transcripts using serial 10-fold dilutions of a plasmid containing either e1-a2 (left) or b3-a2 (right) junctions. Copy numbers are indicated for the highest and lowest concentrations.

We applied this assay to 260 samples from Indian childhood ALL, including 215 precursor B-cell and 45 T-ALL. We identified 19 samples positive for *BCR-ABL* (7.3%). While eight patients carried e1-a2 (indicative of p190), 11 patients, including one with T-ALL, carried *MBCR-ABL* fusions indicative of p210 (six b2-a2 and five b3-a2). One of these patients with precursor B-ALL demonstrated co-expression of b3-a2 and e1-a2.

Quantification of BCR-ABL Transcript Levels

The same assay can be used to specifically quantify relative transcript levels with respect to *GAPDH* expression as the endogenous RNA control. A real-time RT-PCR for *GAPDH* was previously established with a characteristic melting profile ($89.1 \pm 0.3^\circ\text{C}$).³² To generate a calibration curve for *GAPDH* transcripts, serial fivefold dilutions of leukemic cell line cDNA were run in triplicate. Similar data were obtained for different cell lines.

We cloned the *MBCR-ABL* amplicon obtained from K562 and the *mBCR-ABL* amplicon obtained from SupB15 in a TA vector (pCR2.1) and used known amounts of these plasmids to independently generate a standard curve for each fusion template, measured as copy number. Data from triplicates were plotted as curves with $r = -1.00$ and error < 0.103 (Figure 3). These standard curves indicate a linear detection of *BCR-ABL* transcripts over at least six logs. We could easily detect as little as 20 copies of plasmid. Standard deviation (SD) of less than 0.5 cycles was consistently observed, even in the reactions containing the least amount of copies (Table 1).

We calculated the inter- and intra-experiment variability using 10-fold dilutions of cell line cDNA (two independent reverse-transcription reactions) in three independent experiments in the LightCycler for *MBCR-ABL* and *mBCR-ABL* transcripts (Table 1). Normalized b3-a2 transcript levels in K562 ranged from 102 to 133 per ng of RNA, with an average of 119 and a SD of 16 (13%). Normalized e1-a2 transcript levels in SupB15 were lower,

Table 1. Quantification of BCR-ABL Transcripts in Control Samples

	MBCR-ABL (b3-a2)		mBCR-ABL (e1-a2)		BCR-ABL copies*	Relative level (copies/ng RNA) [†]
Plasmid copy number	Mean Cp	SD	Mean Cp	SD		
21,000,000	ND	ND	12.13	0.25		
2100,000	12.71	0.17	16.05	0.06		
210,000	16.35	0.13	20.42	0.26		
21,000	20.02	0.16	24.64	0.94		
2100	23.59	0.07	28.51	0.11		
210	26.94	0.27	32.23	0.40		
21	30.33	0.22	36.60	0.19		
K562 (25 ng)	21.08	0.15			6649	132.98 [‡]
K562 (2.5 ng)	24.80	0.01			595	123.96 [‡]
K562 (0.25 ng)	28.36	0.39			61	101.67 [‡]
SupB15 (25 ng)			26.23	2.11	2639	97.74 [§]
SupB15 (2.5 ng)			30.75	1.60	189	75.60 [§]
SupB15 (0.25 ng)			33.83	0.74	19	72.69 [§]

*Average copy number from three independent experiments.

[†]Average BCR-ABL level (measured as copies) after normalization with GAPDH level (measured as ng RNA).

[‡]Average from the three dilutions shown, 119.53; standard deviation, 16.12.

[§]Average from the three dilutions shown, 82.01; standard deviation, 13.70.

ranging from 73 to 98/ng RNA, with an average of 82 and a SD of 14 (16%). Inter-experiment variability was higher than intra-experiment variability; however, these variations were less than 1.4-fold. Further serial 10-fold dilutions of cell line cDNA were run in three independent experiments and fusion transcripts were easily detectable up to 10^{-4} for SupB15 and 10^{-5} for K562, indicating linearity of the assay over 4 and 5 logs, respectively, when cDNA is used as template.

Having established these quantification parameters, we estimated the expression level of BCR-ABL in the positive patients. The level of fusion transcripts in individual patients varied notably, from 0.108 to 340.5 copies/ng RNA, representing more than 3000-fold difference at the

time of presentation (Table 2). The average expression level was 64.3 copies/ng RNA. Patients with *mBCR-ABL* expressed higher levels (median 78.1 copies/ng RNA) than patients with *MBCR-ABL* (median 17.7 copies/ng RNA) ($P = 0.05$). Correlation of laboratory and clinical features including age, WBC counts, and gender is shown in Table 3.

We also tested 97 samples from normal healthy individuals. All these samples were first run for *GAPDH* expression to ensure quality and quantity of cDNA. However, under our conditions of amplification, none of these samples demonstrated expression of *BCR-ABL*. This result (0 of 97) is statistically different from patient data (19 of 260) ($P = 0.003$) suggesting that quantification of

Table 2. Relative Level of BCR-ABL Transcripts in Patient's Samples

	BCR-ABL Cp	GAPDH Cp	Copies BCR-ABL/ngRNA*	Age	Sex	WBC ($\times 10^9/L$)
e1-a2 ([†])						
Pt 2	30.62	24.46	79.29	8	F	38.50
Pt 3	27.70	24.49	176.03	17	M	21.60
Pt 4	34.16	24.30	4.15	3	F	3.50
Pt 5	26.17	22.10	120.47	14	M	4.87
Pt 6	26.60	22.57	120.26	6	M	384.00
Pt 7	33.87	24.52	41.58	8	F	3.75
Pt 8	28.47	21.53	24.22	17	F	10.10
b2-a2						
Pt 10	22.80	20.06	69.81	7	M	546.00
Pt 11	37.23	19.09	0.36	14	F	4.62
Pt 12	32.62	29.69	17.71	11	M	76.60
Pt 13	29.87	22.52	2.51	4	M	9.40
Pt 14	21.35	21.28	340.54	8	F	54.50
Pt 15 ([‡])	29.37	24.22	8.44	17	M	2.30
b3-a2						
Pt 16	33.07	31.08	27.14	18	F	10.70
Pt 17 ([§])	23.61	20.77	59.80	16	M	5.00
Pt 18	33.51	25.76	1.27	3	F	30.90
Pt 19	30.85	29.54	51.70	10	M	6.60
Pt 20	34.01	21.32	0.11	5	M	2.10

*BCR-ABL level (measured as copies) after normalization with GAPDH level (measured as ng RNA).

[†]Patient 1 carries p190 but was not quantified.

[‡]T-ALL.

[§]Patient expressing both p210 and p190. The relative level of p190 was quantified in an independent reaction as 0.55 copies/ng RNA.

Table 3. Clinical and Laboratory Features of BCR-ABL-Associated ALL Patients

		p190	p210
Sex	Male	4	7
	Female	4	4
Age (years)	<10	4	5
	≥10	4	6
	Median	8	10
	Range	3–17	3–17
WBC ($\times 10^9/L$)	< 10	3	7
	10–49.9	3	1
	50–99.9	1	2
	>100	1	1
	Median	15.85	6.64
BM blasts (%)	Range	3.5–384	2.1–546
	Median	73	82
Phenotype	Range	35–98	42–94
	CALLA	8	10
Status*	T-ALL	0	1
	Complete Tx	2	4
	Relapse	4	2
	Early death	1	2
	LFU	1	3

*Tx, treatment. LFU, lost follow-up

BCR-ABL levels among patients at diagnosis, even when low, is unlikely (power of 0.95) to result from background non-leukemic cells expressing BCR-ABL.

Discussion

It is well-established that monitoring disease during IFN- α or ST1571 treatments for CML are relevant for patient management.^{5,15,17,34} Similarly, identification of a BCR-ABL fusion defines the type of treatment in ALL.^{1,3,7,8,16,35} However, very little information is available on the distribution of the three most common *BCR-ABL* fusions, ie, e1-a2, b2-a2, and b3-a2 and their clinical relevance.

Although many methodologies have been developed to detect and/or quantify the transcript levels, none is easy, simple, and time- and cost-effective.^{18,20–31} Therefore, we have now developed a new strategy, based on real-time RT-PCR that allows correct quantification simultaneously with correct identification of the type of transcript, using only Sybr Green I.

We have previously validated a monochrome multiplex real-time RT-PCR for the four most common translocations in childhood acute lymphoblastic leukemia (ALL) using the LightCycler instrument (Roche).^{32,33} These rearrangements included the *TEL-AML1*, *E2A-PBX1*, *MLL-AF4*, and *mBCR-ABL* fusions. This approach takes advantage of the melting program that allows proper identification of products detected with Sybr Green I. Each amplicon was designed to efficiently amplify virtually all of the variant splice products yielding a characteristic and reproducible melting profile. The *mBCR-ABL* reaction was independent with a melting peak at $92.2 \pm 0.3^\circ\text{C}$.

In this report, we expand this approach to incorporate detection of other *BCR-ABL* fusions, namely b2-a2 and b3-a2, which are translated into a p210 protein. This new assay is faster and more convenient than other methods

previously reported. It would also facilitate, especially in terms of time and cost, studies on CML.

In the assay we describe here, the use of only one dye, Sybr Green I, and a melting program in the LightCycler yields reproducible and characteristic peaks for each amplicon for identification of the majority of *BCR-ABL* transcripts. It is noteworthy that in this multiplex system, we obtained a temperature profile of $91.85 \pm 0.25^\circ\text{C}$ for e1-a2 transcripts, which was very similar to our previous approach on a single assay. Transcripts b2-a2 and b3-a2 can be easily identified even with a one degree difference (Figure 1A). We confirmed these results by conventional RT-PCR (Figure 1B) and sequence analyses (Figure 2). A housekeeping gene, *GAPDH*, is initially and independently amplified for controlling the quality of the cDNA and for normalization of the levels of the target transcript.

We have used cDNA from K562 and SupB15 cell lines, known to express b3-a2 and e1-a2 transcripts, respectively. We have also cloned the amplified products from a standard RT-PCR reaction and these plasmids were used to generate a standard curve for quantification purposes (Figure 3). Independent experiments ran in triplicates using these controls as templates indicate that the inter- and intra-assay variability is low (<0.5 cycles) which is represented in normalized amounts of *BCR-ABL* transcripts less than 1.4-fold (Table 1). We demonstrate that we can detect a wide range of expression with a sensitivity of at least 10^{-4} , making this assay very convenient for monitoring minimal residual disease.

Since *BCR-ABL* transcripts can be detected in peripheral blood from up to 30% of healthy individuals when a large number of cells (usually one million) are screened,¹⁹ it is critical in MRD studies that no false-positive results are obtained. We demonstrate here that in none of 97 normal samples did any potential background BCR-ABL transcripts interfere with quantification under the conditions used in this duplex assay ($P = 0.003$).

The distribution of the relative contribution of each of these types of BCR-ABL may vary in childhood ALL from different geographic regions. Furthermore, limited data are available for the characterization of the type of fusion transcript. Thus we applied this monochrome multiplex real-time RT-PCR assay in clinical samples from patients with childhood ALL from Mumbai, India. Two hundred sixty samples were analyzed. More than 7% of patients carry BCR-ABL (including one patient with T-ALL), a frequency higher than previously reported for the West.^{1,4} More interestingly, we observed that 60% of the cases were *MBCR-ABL* (11 of 19, 4.2% of all ALLs). This incidence of p210 in Indian childhood ALL is notably higher than previously reported from German (1.1%) and Taiwanese (0.34%) patients.^{35,36} Considering the prognostic value of the presence of BCR-ABL in childhood ALL,³⁷ this observation is clinically relevant and should be taken into consideration. This highlights the importance of screening all BCR-ABL variants in childhood ALL in the Indian population.

With respect to the quantification of the relative level of *BCR-ABL* transcripts, we showed that the assay is specific within a very wide range (at least 4 to 5 logs) and the

sensitivity is adequate for MRD studies with a minimal error. Since there appeared to be a significant difference in the expression levels between the control cell lines, we compared the level of expression among patients with p190 and p210 (Table 2). The normalized level in SupB15 cell line (p190) is lower than that in K562 (p210), as previously observed.³¹ Interestingly enough, the opposite was observed in clinical samples: patients with p190 expressed an average of 80.4 copies/ng RNA while patients with p210 expressed an average of 52.7 ($P = 0.05$). Even among p210-positive cases, b2-a2 transcripts were 10-fold higher than b3-a2, although the numbers are too small to achieve statistical significance. Since adult ALL patients expressing p210 have higher levels, up to 10-fold, than patients with p190,³⁸ the clinical consequence of these observations needs further study. Although two samples express very low levels of *BCR-ABL* (Table 2), it is likely that these represent leukemic cells, since no *BCR-ABL* transcripts are detected in 97 independent samples from healthy individuals. A wide range of expression level of *BCR-ABL* has been repeatedly noted in acute and chronic patients at diagnosis.^{24,25,31,37}

We correlated this molecular assessment with known clinical prognostic factors in childhood ALL, including age, WBC counts, and gender (Table 3). Although these numbers are too small to achieve statistical significance, they indicated a positive trend for boys and the presence of p210. In contrast, p190-positive patients were equally distributed between males and females. Considering that the median age of all patients was 7 years old, it appears that p210 may occur in association with older age (median 10 years) in childhood ALL. WBC counts varied similarly in both subgroups.

Although further clinical correlations are beyond the scope of this report, it is noteworthy that 4 of 8 *mBCR-ABL* leukemias relapsed while only 2 of 11 *MBCR-ABL* did so. We conclude that this assay is sensitive and reliable for identifying and quantifying frequent *BCR-ABL* fusion transcripts in ALL and CML.

References

1. Pui C-H: Treatment of acute leukemias. New Directions for Clinical Research. Edited by Pui C-H. Totowa, NJ, Humana Press, 2003, pp 87-157
2. Melo JV, Hughes TP, Apperley JF: Chronic myeloid leukemia. Hematology. Edited by Broudy VC, Prehal JT, Berliner N, Tricot GJ. Washington, DC, American Society of Hematology, 2003, pp 132-152
3. Cortes JE, Kantarjian H: Acute lymphoblastic leukemia. Medical Oncology: A Comprehensive Review, ed 2. Edited by Pazdur R. PRR 1997, pp 1-45; www.cancernetwork.com/textbook/mores01.htm
4. Greaves M: Childhood leukaemias. Br Med J 2002, 324:283-287
5. Kantarjian H, Melo JV, Tura S, Giralt S, Talpaz M: Chronic myelogenous leukemia: disease biology and current and future therapeutic strategies. Hematology. Edited by Schechter GP, Berliner N, Telen MJ. Washington, DC, American Society of Hematology, 2000, pp 90-109
6. Kurzrock R, Gutterman JU, Talpaz M: The molecular genetics of Philadelphia chromosome-positive leukemias. N Engl J Med 1988, 319:990-998
7. Gleissner B, Gokbuget N, Bartram CR, Janssen B, Rieder H, Janssen JW, Fonatsch C, Heyll A, Voliotis D, Beck J, Lipp T, Munzert G, Maurer J, Hoelzer D, Thiel E, German Multicenter Trials of Adult Acute Lymphoblastic Leukemia Study Group: Leading prognostic relevance of the *BCR-ABL* translocation in adult acute B-lineage lymphoblastic leukemia: a prospective study of the German Multicenter Trial Group and confirmed polymerase chain reaction analysis. Blood 2002, 99: 1536-1543
8. Arico M, Valsecchi MG, Camitta B, Schrappe M, Chessells J, Baruchel A, Gaynon P, Silverman L, Janka-Schaub G, Kamps W, Pui CH, Masera G: Outcome of treatment in children with Philadelphia chromosome-positive acute lymphoblastic leukemia. N Engl J Med 2000, 342:998-1006
9. Melo JV: *BCR-ABL* gene variants. Baillieres Clin Haematol 1997, 10:203-222
10. Saglio G, Guerrasio A, Rosso C, Zaccaria A, Tassinari A, Serra A, Rege-Cambrin G, Mazza U, Gavosto F: New type of *BCR/ABL* junction in Philadelphia chromosome-positive chronic myelogenous leukemia. Blood 1990, 76:1819-1824
11. van Rhee F, Hochhaus A, Lin F, Melo JV, Goldman JM, Cross NC: p190 *BCR-ABL* m-RNA is expressed at low levels in p210-positive chronic myeloid and acute lymphoblastic leukemias. Blood 1996, 87:5213-5217
12. Kantarjian HM, Talpaz M, Dhingra K, Estey E, Keating MJ, Ku S, Trujillo J, Huh Y, Stass S, Kurzrock R: Significance of the P210 versus P190 molecular abnormalities in adults with Philadelphia chromosome-positive acute leukemia. Blood 1991, 78:2411-2418
13. Voncken JW, Kaartinen V, Pattengale PK, Germersaad WT, Groffen J, Heisterkamp N: *BCR/ABL* P210 and P190 cause distinct leukemia in transgenic mice. Blood 1995, 86:4603-4611
14. Prejzner W: Relationship of the *BCR* gene breakpoint and the type of *BCR/ABL* transcript to clinical course, prognostic indexes, and survival in patients with chronic myeloid leukemia. Med Sci Monit 2002, 8:191-197
15. Kantarjian HM, O'Brien S, Cortes JE, Shan J, Giles FJ, Rios MB, Faderl SH, Wierda WG, Ferrajoli A, Verstovsek S, Keating MJ, Freireich EJ, Talpaz M: Complete cytogenetic and molecular responses to interferon- α -based therapy for chronic myelogenous leukemia are associated with excellent long-term prognosis. Cancer 2003, 97: 1033-1041
16. Cazzaniga G, Lanciotti M, Rossi V, Di Martino D, Arico M, Valsecchi MG, Basso G, Masera G, Micalizzi C, Biondi A: Prospective molecular monitoring of *BCR-ABL* transcript in children with Ph+ acute lymphoblastic leukemia unravels differences in treatment response. Br J Haematol 2002, 119:445-453
17. Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, Gathmann I, Bolton AE, van Hoomissen IC, Goldman JM, Radich JP, International Randomised Study of Interferon versus STI571 (IRIS) Study Group: Frequency of major molecular responses to imatinib or interferon α plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med 2003, 349:1423-1432
18. van Dongen JJ, Macintyre EA, Gabert JA, Delabesse E, Rossi V, Saglio G, Gottardi E, Rambaldi A, Dotti G, Griesinger F, Parreira A, Gameiro P, Diaz MG, Malec M, Langerak AW, San Miguel JF, Biondi A: Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease: report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. Leukemia 1999, 13:1901-1928
19. Janz S, Potter M, Rabkin CS: Lymphoma- and leukemia-associated chromosomal translocations in healthy individuals. Genes Chromosomes Cancer 2003, 36:211-223
20. Schoch C, Schnittger S, Bursch S, Gerstner D, Hochhaus A, Berger U, Hehlmann R, Hiddemann W, Haeflrich T: Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. Leukemia 2002, 16:53-59
21. Nashed A, Rao KW, Gulley ML: Clinical applications of *BCR-ABL* molecular testing in acute leukemia. J Mol Diagn 2003, 5:63-72
22. Kreuzer KA, Lass U, Bohn A, Landt O, Schmidt CA: LightCycler technology for the quantification of *bcr/abl* fusion transcripts. Cancer Res 1999, 59:3171-3174
23. Emig M, Saussele S, Wittor H, Weissner A, Reiter A, Willer A, Berger U, Hehlmann R, Cross NC, Hochhaus A: Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia 1999, 13:1825-1832

24. Bolufer P, Sanz GF, Barragan E, Sanz MA, Cervera J, Lerma E, Senent L, Moreno I, Planelles MD: Rapid quantitative detection of BCR-ABL transcripts in chronic myeloid leukemia patients by real-time reverse transcriptase polymerase-chain reaction using fluorescently labeled probes. *Haematologica* 2000, 85:1248–1254
25. Amabile M, Giannini B, Testoni N, Montefusco V, Rosti G, Zardini C, Terragna C, Buonamici S, Ottaviani E, Soverini S, Fiacchini M, Bassi S, de Vivo A, Trabacchi E, Saglio G, Pane F, Baccarani M, Tura S, Martinelli G: Real-time quantification of different types of bcr-abl transcript in chronic myeloid leukemia. *Haematologica* 2001, 86:252–259
26. Lee WI, Kantarjian H, Glassman A, Talpaz M, Lee MS: Quantitative measurement of BCR/abl transcripts using real-time polymerase chain reaction. *Ann Oncol* 2002, 13:781–788
27. Yokota H, Tsuno NH, Tanaka Y, Fukui T, Kitamura K, Hirai H, Osumi K, Itou N, Satoh H, Okabe M, Nakahara K: Quantification of minimal residual disease in patients with e1a2 BCR-ABL-positive acute lymphoblastic leukemia using a real-time RT-PCR assay. *Leukemia* 2002, 16:1167–1175
28. Neumann F, Herold C, Hildebrandt B, Kobbe G, Aivado M, Rong A, Free M, Rossig R, Fenk R, Schneider P, Gattermann N, Royer-Pokora B, Haas R, Kronenwett R: Quantitative real-time reverse-transcription polymerase chain reaction for diagnosis of BCR-ABL-positive leukemias and molecular monitoring following allogeneic stem cell transplantation. *Eur J Haematol* 2003, 70:1–10
29. Jones CD, Yeung C, Zehnder JL: Comprehensive validation of a real time quantitative bcr-abl assay for clinical laboratory use. *Am J Clin Pathol* 2003, 120:42–48
30. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, Barbany G, Cazzaniga G, Cayuela JM, Cave H, Pane F, Aerts JL, De Micheli D, Thirion X, Pradel V, Gonzalez M, Viehmann S, Malec M, Saglio G, van Dongen JJ: Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia: a Europe Against Cancer Program. *Leukemia* 2003, 17:2318–2357
31. Luthra R, Sanchez-Vega B, Medeiros LJ: TaqMan RT-PCR assay coupled with capillary electrophoresis for quantification and identification of bcr-abl transcript type. *Mod Pathol* 2004, 17:96–103
32. Siraj AK, Ozbek U, Sazawal S, Sirma S, Timson G, Al-Nasser A, Bhargava M, El Solh H, Bhatia K, Gutierrez MI: Pre-clinical validation of a monochrome real-time multiplex assay for translocations in childhood acute lymphoblastic leukemia. *Clin Cancer Res* 2002, 8:3832–3840
33. Siraj AK, Kamat S, Gutierrez MI, Banavali S, Timpson G, Sazawal S, Bhargava M, Advani S, Adde M, Magrath I, Bhatia K: The frequencies of the major subgroups of precursor B-cell acute lymphoblastic leukemia in Indian children differ from the West. *Leukemia* 2003, 17:1192–1193
34. Oehler VG, Radich JP: Monitoring bcr-abl by polymerase chain reaction in the treatment of chronic myeloid leukemia. *Curr Oncol Rep* 2003, 5:426–435
35. Schlieben S, Borkhardt A, Reinisch I, Ritterbach J, Janssen JW, Ratei R, Schrappe M, Repp R, Zimmermann M, Kabisch H, Janka-Schaub G, Bartram CR, Ludwig WD, Riehm H, Lampert F, Harbott J: Incidence and clinical outcome of children with BCR/ABL-positive acute lymphoblastic leukemia (ALL): a prospective RT-PCR study based on 673 patients enrolled in the German pediatric multicenter therapy trials ALL-BFM-90 and CoALL-05–92. *Leukemia* 1996, 10:957–963
36. Ko BS, Tang JL, Lee FY, Liu MC, Tsai W, Chen YC, Wang CH, Sheng MC, Lin DT, Lin KH, Tien H: Additional chromosomal abnormalities and variability of BCR breakpoints in Philadelphia chromosome/BCR-ABL-positive acute lymphoblastic leukemia in Taiwan. *Am J Hematol* 2002, 71:291–299
37. Desai SA, Barbhaya SA, Parikh PM, Banavali S, Pai SK, Manjrekar N, Nair CN, Advani SH: Prognostic significance of BCR-ABL translocation in patients with acute lymphoblastic leukemia from India. *Indian J Hematol Blood Transfus* 2002, 21:59–60
38. Scheuring UJ, Pfeifer H, Wassmann B, Bruck P, Hoelzer D, Ottmann OG: Methodologic and biological variability of quantitative real-time polymerase chain reaction analysis of Bcr-Abl expression in Philadelphia chromosome-positive acute lymphoblastic leukemia. *Haematologica* 2003, 88:1074–1076