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Detection of BCR/ABL Gene Rearrangement and the Elimination of Rearranged Clone in Chronic Myelocytic Leukemia Patients

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Cytogenetic and molecular studies were performed on 20 interferon-α receiving Turkish chronic myelocytic leukemia patients. Four different restriction endonucleases and bcr-G probe were used for southern blot analysis to detect rearrangements of the bcr gene. The RT-PCR method was also applied to detect chimeric bcr/abl mRNA. Seventeen patients showed a chromosomal break within the 5.8 kb M-bcr region by southern blot analysis while three cases out of 20 have not shown any rearrangement. These three cases were further analysed by RT-PCR and they were also found to be carrying the Philadelphia translocation (Ph). However, in four years of follow-up this RT-PCR positivity has disappeared, which suggests an elimination of Ph clone with prolonged interferon-α treatment. Am. J. Hematol. 63:85–89, 2000. © 2000 Wiley-Liss, Inc.

Key words: chronic myelocytic leukemia; CML; Ph; bcr/abl; interferon-α; RT-PCR

INTRODUCTION

Chronic myelocytic leukemia (CML) is a myeloproliferative disorder characterised in 90% of patients by the presence of a Philadelphia chromosome (Ph) that results from a reciprocal translocation t(9:22) in 90–95% of patients [1–3]. This translocation locates the proto-oncogene c-abl, normally found on 9q34, to the breakpoint cluster region (bcr) on 22q11 [4,5]. Most of the chromosomal breaks occur in a 5.8 kb bcr region, which is called as M-bcr [6]. Formation of this rearrangement generates a bcr/abl hybrid gene that is expressed as an 8.5 kb chimeric mRNA and translated into a 210 kb protein product (p210) having a tyrosine kinase activity which is higher than that of the normal abl protein [7–9].

Previous studies suggested that there is a relation between these molecular events and the development of CML. Suppressing the Ph clones might change the natural course of the disease and its prognosis. Interferon-α therapy produces haematological responses in 60–80% and cytogenetic responses 35–55% of patients [10]. Major molecular responses also occur and defined by southern blotting. However, in almost all of the cells residual leukemic cells can only be detected by RT-PCR analysis, which amplifies Ph clone specific bcr/abl cDNA. This study has shown that extensive interferon-α treatment appears to be able to reduce the bcr/abl fusion transcript level so that it is not detectable even by RT-PCR analysis.

MATERIALS AND METHODS

Patients

Twenty patients with cytogenetically and clinically diagnosed as CML in Hacettepe University Institute of Oncology, Ankara, have been studied during their follow-up period.

The patients received only interferon-α therapy, and before the therapy was begun all patients had a complete medical and physical examination, complete blood counts, serum and coagulation studies, and bone marrow aspirate for the examination of morphologic and cytoge-

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TABLE I. Clinical Findings and Results of Molecular Studies in Patients With bcr/abl Translocation (Initial Data)*

Patients no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sex/age	F/48	M/32	M/38	M/23	M	F/37	M/40	M/44	M/28	M/48	M/40	M/2 8	F/37	F/34	F/23	F	F/39	M/28	M/48	M/20
No. of splenomegaly[a]	NT	+	+	+	+	+	+	+	+	+	++	+	++	+	++	+	+		+	+++
WBC × 10^9/1	620	570	820	870	650	590	350	680	280	380	320	350	400	594	948	480	118	400	370	264
PMN	45	53	76	50	64	75	68	73	63	33	53	45	65	78	60	67	75	65	54	30
Monocytes (%)	0	2	6	4	1	5	4	7	8	1	5	8	7	6	0	6	5	7	7	0
Hb (g/dl)	9.6	11	11.4	11.2	12.2	13.4	12.8	12.1	12.2	12.6	10.9	12.9	13.8	12.3	8.6	12.3	10.5	13.8	11.7	11.6
Plt × 10^9/dl	140	156	184	IS	128	34	104	102	106	100	101	134	158	154	NT	154	157	128	49	336
Blasts (bone marrow)	–	–	–	1	–	–	–	1	–	–	–	–	–	–	3	–	–	–	–	1
Chronic phase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Karyotype	Ph+	Ph+	Ph+	Ph+	Ph+	Ph–	Ph+	Ph+	Ph–	Ph–	Ph–	Ph–	Ph+	Ph+	Ph+	Ph+	Ph+	Ph–	Ph+	Ph–
bcr/abl rearrangement	+	+	+	+	+	–	–	+	–	–	+	+	+	+	+	+	+	+	+	–
RT-PCR (b2a2)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

*NT, not tested; (–), not detected; (+), detected; b2, bcr exon 2; a2, abl exon 2. Aberrations: PMN, polymorphonuclear cells; Plt, platelets; IS, insufficient.
[a]+, 0–4 cm; ++, 5–9 cm; +++, >10 cm.

netic features of malignant cells. Patients received recombinant human interferon-α (5×10^6 U/m^2/day) alone up to 18 months. For each case, from the start of interferon-α treatment in no instance was the treatment interrupted or modified.

Probes

The bcr-G probe (0.6 kb) was used for Southern blot analysis.

Cytogenetic Analysis

Cytogenetic analysis was carried out on cultures of peripheral blood or bone marrow cells harvested after 24 hr in culture. Chromosomes were identified using G-banding technique. Well-banded 20 metaphases were analysed according to the International System for Human Cytogenetic Nomenclature (ISCN) [11].

Primers

The following primers were kindly provided by Dr. S.P. Guy (Regional Molecular Genetics Laboratory, St. Mary's Hospital, Manchester, U.K.) and used in RT-PCR analysis [12].

M-bcr b2 having sequence 5′ GAAGAAGTGTTTCAGAAGCTTCTCCC
c-abl a2 having sequence 5′ GGTACCGAATTCAGCGGCCAGTAGCATCTGACTT
c-abl a3 having sequence 5′ TGTGATTATAGCCTAAGACCCGGAG
β-actin (F) having sequence 5′ GTGGGGCGCCCCAGGCAGCAACGCACGAT
β-actin (R) having sequence 5′ CTCCTTAATGTC
G3PDH (F) having sequence 5′ GATGATCTTGAGGCTGTTGTC
G3PDH (R) having sequence 5′ TGGTCACCAGGGCTGCTTTTA

The Oncogene Science CML primer kit was used for nested PCR amplifications. The kit has the sensitivity for selecting one K562 cell in 10^6 cells.

Southern Blotting

DNA was isolated from peripheral blood samples, digested with restriction endonucleases according to the manufacturer's guidelines, electrophoresed on 0.8% agarose gels, and transferred to N^+Hybond membrane (Amersham International, Amersham, U.K.). Probes were isolated from pUC plasmids by digestion with restriction

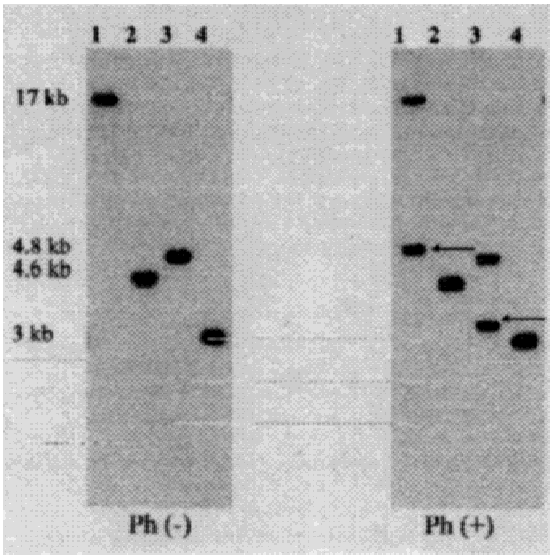

Fig. 1. Autoradiography results of CML patients. DNA samples were digested with *Eco*RI, *Hin*dIII, *Bgl*I and *Bam*HI restriction enzymes, respectively. The arrows indicate rearranged bands.

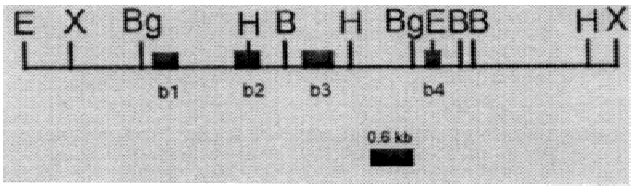

Fig. 2. Restriction map of M-bcr on chromosome 22, translocation regions, and position of the bcr-G probe.

enzymes and electrophoresed on low-melting point agarose (Sigma). For hybridisation, probes were labelled with ^{32}P-dCTP (Amersham International) and hybridised to filters overnight at 65°C in hybridisation oven. Final post-hybridisation washes were carried out in SSC (3×, 2×, 1×, and 0.1×, for 40 min) solution containing 0.1% SDS at 65°C. The autorads of filters were prepared by Kodak X-AR film (exposed two days at −80°C).

RNA Extraction

Total RNA was extracted from blood cells using the method of Chomcznsky and Sacci [13].

cDNA Synthesis

Total RNA (1 μg) obtained from nucleated blood cells was dissolved in 15 μl dH_2O, 100 ng of random primers was added, and the mixture was heated to 70°C for 10 min. cDNA synthesis was carried out at 42°C for 30 min in a reaction mixture containing 2 mM dNTPs, 10 mM DTT, and 10 U of AMV reverse transcriptase (Promega), and reaction buffer.

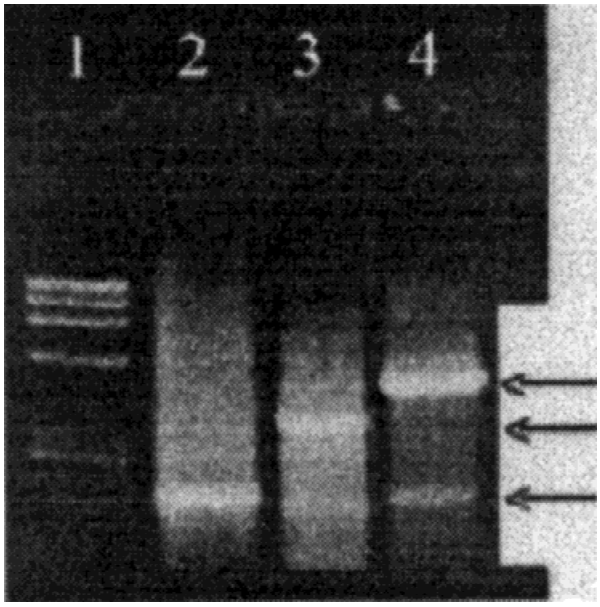

Fig. 3. RT-PCR products on agarose gel. Well no. 1: ϕ X174/*Hae*III size marker DNA. Well no. 2: amplification product of normal cDNA by using a2a3 primer (218 bp). Well no. 3: amplification product of patient cDNA by using b2a2 primer (360 bp). Well no. 4: amplification product of β-actin gene (548 bp).

PCR Amplification Procedures

The PCR was carried out using a Thermocycler (COY Model 50/60). Amplification was performed by using 5 μl cDNA, 1.5 mM $MgCl_2$, 2 U of Taq DNA polymerase (Promega), 10 mM Tris-HCl, and 50 μmol of each primer. PCR amplification was performed by one cycle at 94°C for 3 min, 35 cycles at 94°C for 1 min, 65°C for 1 min, 72°C for 1 min, two extenuation cycles at 72°C for 10 min, and 10°C for 10 min.

β-Actin and G3PDH genes were amplified separately in the same reaction mixture as a control.

Detection of Amplified DNA

Aliquots (5 μl) of the reaction product were run on a 2% agarose gel containing ethidium bromide.

RESULTS

bcr Rearrangement and Breakpoints Within M-bcr

bcr/abl translocation in 20 CML patients was first studied by using 0.6 kb bcr-G probe (Table I). The breakpoints were analysed by using four different restriction enzymes (*Eco*RI, *Hin*dIII, *Bgl*II, and *Bam*HI, respectively). The bands obtained from Ph (−) normal blood samples and patients blood samples were compared according to the autoradiography results (Fig. 1). The exact localisation of the breakpoints within M-bcr region was

TABLE II. Elimination Results of Ph Clone

Patients no.	Treatment	No. of positive PCR after onset of IFN-α (months)	Time of PCR negativation after onset of IFN-α (months)	No. of negative PCR after onset of IFN-α (months)	Comment
1	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
2	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
3	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
4	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
5	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
6	IFN-α	0, 3, 6, 12, 18, 24, 32	43	5(43, 46, 49, 52, 55)	Transiently positive at 44 months
7	IFN-α	0, 3, 6, 12, 18, 24, 32	40	5(40, 43, 46, 49, 52)	
8	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
9	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
10	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
11	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
12	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
13	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
14	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
15	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
16	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
17	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
18	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
19	IFN-α	0, 3, 6, 12, 18, 24, 32, 43, 52, 55	–	–	
20	IFN-α	0, 3, 6, 12, 18	20	5(20, 21, 25, 28, 32)	

TABLE III. Clinical Data of the Patients Showing RT-PCR Negativity

Patient no.	Months	WBC ($\times 10^9$/l)	Hg (g/dl)	Platelet ($\times 10^9$/dl)	Blasts (%) (bone marrow)	No. of splenomegaly[a]
6	43	620	10.8	40	–	+
	44	560	12.0	43	–	+
	46	560	12.6	42	–	+
	49	550	14.8	38	–	+
	52	580	10.0	54	–	+
	55	580	11.3	88	–	+
7	40	300	11.4	144	–	+
	43	320	11.8	183	–	+
	46	350	11.0	190	–	+
	49	380	11.3	224	–	+
	52	350	11.0	150	–	+
20	20	270	11.7	402	1	+
	21	294	12.0	458	1	+
	25	320	12.2	393	–	+
	28	300	11.9	398	–	+
	32	340	11.7	443	–	+

[a]+, 0–4 cm.

determined by looking at the restriction map of M-bcr on chromosome 22 (Fig. 2).

Polymerase Chain Reaction

The total RNA was extracted from CML patients, and the RNA samples were subjected to RT-PCR. The amplification of cDNA with a2a3 primer pair gave a 218 bp product; however, a2b2 primer pair gave a 360 bp product, indicating bcr/abl translocation (Fig. 3). Amplification product of β-actin gene gave a 548 bp and G3PDH gave a 423 bp long band. The disappearance of bcr/abl mRNA was also checked by nested PCR analysis. For this analysis, the primers of Oncogene Science CML Kit were used. Table II shows the elimination results of Ph clone.

DISCUSSION

In the present study, localisation of breakpoints within M-bcr region of Turkish CML patients treated with interferon-α extensively (5×10^6 U/m^2/day) was studied. Results of hybridisation with bcr-G probe have shown that the rearrangements are mainly localised in the exons 3 and 4 on bcr gene. There were no detectable bands in results of three patients (#6, #7, and #20). However, they have shown bcr/abl transcript that was not detectable by southern blot but was detected by RT-PCR analysis which is the most sensitive technique [14–16]. In addition, it has been shown that during the follow-up period RT-PCR results become negative after 20, 43, and 44 months of interferon treatment. One has been shown a transient positive result (patient #6, at 44th month), and the other two remained so during 43–55 and 40–52 months of follow-up. Detailed clinical data of these three patients are given in Table III.

Although in most of the cases bcr/abl transcript has shown persistence, there are some publications about RT-PCR negativity of chimeric mRNA in the patients that are receiving long-term treatment of recombinant interferon-α [17–19]. In conclusion, our data together with other previously published studies [14–16] suggest that interferon-α has reduced the fusion transcript product of Ph clone to the levels which is not detectable even RT-PCR analysis. Whether this corresponds to true eradication of Ph clone or rather to its persistence at low level is at present unknown.

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