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BCR-ABL mRNA Expression in Patients with Chronic Myeloproliferative Disorders — Absence of BCR-ABL Fused Clone except Chronic Myelocytic Leukemia —

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ABSTRACT

Bcr-abl mRNA expression was studied in patients with chronic myeloproliferative disorders (CMPD) by the reverse transcriptase-polymerase chain reaction (RT-PCR) method. A bcr-abl transcript was not found in any patient with polycythemia vera, essential thrombocythemia or primary myelofibrosis, suggesting that the bcr-abl rearranged clone is not present in CMPD other than chronic myelogenous leukemia (CML). In CML clinical and laboratory data were compared from three bcr-abl types: the bcr exon 2-abl exon 2 (B2-A2) type, bcr exon 3-abl exon 2 (B3-A2) type and the co-expression type. Age at diagnosis tended to be younger (p=0.08) in the co-expression type, and the platelet count tended to be lower (p=0.11) in the B2-A2 type. However, there was no difference in other data, including the duration of the chronic phase and the phenotype of blasts at blast crisis.

Key words: *Breakpoint cluster region, c-Abl, Chronic myeloproliferative disorders*

Chronic myeloproliferative disorders (CMPD) consist of four diseases: chronic myelogenous leukemia (CML), essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). In CML more than 95% of patients have Philadelphia chromosome (Ph^1), but Ph^1 negative CML is also recognized in 3-5%[7,8]. In ET some Ph^1 positive cases have been reported[1,9,12,22].

In Ph^1 positive CML the major breakpoint cluster region (M-bcr) on chromosome 22 is spliced to the cellular abl gene (c-abl) on chromosome 9, giving rise to a bcr-abl fused gene[21]. The bcr-abl gene product, $P^{210bcr-abl}$, has high tyrosine kinase activity, and by introducing the bcr-abl gene into cell line and transgenic mouse, the tumorgenic potential is proved[2,3,11]. In most patients with Ph^1, the break points locate either between bcr exon 2 and exon 3 or between bcr exon 3 and exon 4 within 5.8 kb length M-bcr. Each breakage of the bcr gene generates bcr-abl mRNA with bcr exon 2-abl exon 2 (B2-A2) and bcr exon 3-abl exon 2 (B3-A2). These two transcripts differ in size only by 75 bp. Both $P^{210bcr-abl}$ gene products have been demonstrated to have elevated tyrosine kinase activity *in vitro*, but the difference between their effects *in vivo* is not known. Using southern blot analysis some investigators[14,20] have reported a correlation between the location of the bcr breakpoint and clinical parameters observed during the course of CML. The average duration of the chronic phase was correlated with the site of breakpoint. On the other hand, several papers have shown that the chronic phase duration does not correlate to the site of the breakpoint[5,17,23].

In this paper we studied whether bcr-abl mRNA was expressed in cells from CMPD other than CML using the reverse transcriptase-polymerase chain reaction (RT-PCR) method. Some cases of this disease category, especially ET, can not be differentiated from CML clinically. As described above, Ph^1 positive CMPD other than CML have been reported. Therefore, it is possible that a bcr-abl mRNA positive clone exists in this disease, and that this clone occurs as a result of clonal evolution. We also studied bcr-abl mRNA type in CML and compared it with the clinical and laboratory data.

MATERIALS AND METHODS

Patients

There were thirteen patients with PV (10 males and 3 females) ranging in age from 27 to 70 years old (median 57, mean 55.7±11.4). Four atomic bomb (AB) survivors were included. Four patients showed normal karyotypes, and one had 46,XX / 46,XX, r (7) mosaic karyotype. They were under treatment with busulphan and/or phlebotomy. There were eight patients with ET (5 males and 3 females) including 4 AB survivors ranging from 47 to 77 years old (median 66, mean

64.5±10.7). None had Ph^1 chromosome. Busulphan had been prescribed for all of them. There were six PMF patients (3 males and 3 females) including one AB survivor ranging from 52 to 77 years old (median 65, mean 64.2±10.3). Four patients had normal karyotypes, while one had an abnormality of chromosome 13. They had been treated with 6-mercaptopurine, vitamin D3, oxymetholone and/or busulphan.

Thirty four CML patients were also studied. Since bcr-abl transcript was not detected in 8 patients, the analysis was performed on 26 patients. Three patients were AB survivors. The age of the patients ranged from 22 to 75 years old (median 51, mean 51.0±14.5). Twelve patients were studied in the chronic phase and the other 14 patients were in blastic crisis. Ph^1 was positive for all the patients. Among the 12 patients in the chronic phase, 5 were examined for their bcr-abl type before treatment and 7 were under treatment by either busulphan, interferon alpha, hydroxyurea or ranimustine. A diagnosis of blastic crisis was made essentially on the basis of peripheral white blood cells or bone marrow cells with more than 30% blasts indicating unresponsiveness to therapy. Three of the 14 patients were identified as lymphoid crisis on the morphology of blasts with negative per-oxidase as well as lymphoid surface markers (CD 10, CD 25, CD 19, CD 20, CD 21). One of the 3 was induced to the complete remission state, but relapse with myeloid blasts occurred. Eleven were identified as myeloid crisis on the basis of morphology of blasts with positive per-oxidase or myeloid surface markers (CD 13, CD 14, CD 33, CD 41b).

Detection of bcr-abl mRNA

Peripheral blood or marrow aspirate was drawn from patients after informed consent. For peripheral blood, white blood cells were collected by the dextran method, and for marrow aspirate, light density mononuclear cells were obtained by the Ficoll-Hypaque density gradient. For RNA extraction the cells were mixed with guanidinium isothiocyanate, layered over a CsCl cushion and centrifuged at 130,000 g for 10 hours at 18°C. The deposit was suspended with 10mM Tris-1mM EDTA, extracted by phenol and precipitated with 70% ethanol. The bcr-abl mRNA detection by RT-PCR was performed essentially according to Kawasaki et al[6]. RNA (1 μg) was incubated with 100 ng 3'primer in annealing buffer for 3 min at 81°C and 45 min at 31°C. The mixture was added to 1mM dNTPs (each of four), reverse transcriptase and RNase inhibitor, and then incubated at 43°C for 1 hour. The cDNA was then mixed with 25μl of amplifying buffer containing 0.2 mM dNTPs, 1 μM 5'primer, 1 μM 3'primer and 1 unit of Taq-polymerase. PCR was carried out with 40 cycles of 94°C 1 min, 55°C 1 min and 72°C 2 min. The amplified product was run on agarose gel containing 1% Seaken-3% Nusieve agarose. The gel was stained with ethidium bromide, photographed and transferred onto nylon-membrane by the alkaline transfer procedure. The membrane was hybridized at 65°C for 10 hours with a detecting probe endlabeled with ^{32}P-ATP, washed and autoradiographed. The following primers and detecting probes were used according to Kawasaki et al[6].

major bcr exon 2 primer,	5-GGAGCTGCAGATGCTGACCAAC-3,
abl exon 2 primer,	5-TCAGACCCTGAGGCTCAAAGTC-3,
B2-A2 detecting probe,	5-GCTGAAGGGCTTCTTCCTTATTGATG-3,
B3-A2 detecting probe,	5-GCTGAAGGGCTTTTGAACTCTGCTTA-3,

Statistical analysis

Median values at diagnosis for age, log (WBC), Hgb, log (Plt) and NAP score were analyzed by both the t-test and multiple range test. Sex and the presence or absence of anemia, splenomegaly and marrow fibrosis were compared among the three bcr-abl mRNA types by χ^2-test. The chronic phase duration was compared with the bcr-abl type by both the t-test and multiple range test.

RESULTS

Detection of bcr-abl mRNA type by RT-PCR

Amplified DNA derived from bcr-abl mRNA with bcr exon 2-abl exon 2 (B2-A2 type) and that with bcr exon 3-abl exon 2 (B3-A2 type) were detected as 125 bp and 200 bp band, respectively (Fig. 1). Sometimes both bands were found simultaneously in some patients (co-expression type).

Detection of bcr-abl mRNA in PV, ET and PMF

We first examined whether cells from PV, ET and PMF were expressing B2-A2 type or B3-A2 type. None of the 13 PV, 7 ET and 6 PMF cases showed the expression (data not shown).

Bcr-abl type and clinical data in the chronic phase of CML

Twenty-six CML patients including 12 at chronic phase and 14 at blastic crisis were examined for the major bcr-abl mRNA type. Clinical and laboratory data at diagnosis according to bcr-abl type are shown in Table 1. For the patients examined at blastic crisis, the data were analysed assuming that their bcr-abl type had not changed during the chronic phase. Patient numbers for B3-A2 type, B2-A2 type and co-expression type were 13, 7 and 6, respectively. In the

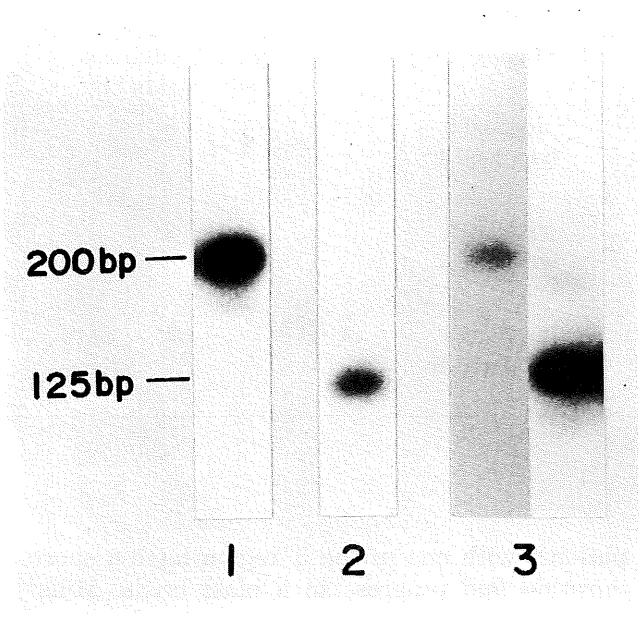

Fig. 1. Detection of bcr-abl mRNA with RT-PCR
Three types of bcr-abl mRNA, B3-A2 type (1), B2-A2 type (2) and co-expression type (3), are shown from CML patients at chronic phase

co-expression type, the B3-A2 type transcript was more intensely expressed than the B2-A2 type in all the patients, judging from our method. Age at diagnosis was compared among the three bcr-abl types. The co-expression type was diagnosed at a younger age (median 42.5, mean 41.7±10.4) than the other two types, B3-A2 (54, 55.0±14.1) and B2-A2 (58.5, 54.7±17.9). The difference tended to be significant (p=0.08). The platelet count in the patients was generally higher than in normal subjects. The platelet count of the B2-A2 type (median $371\times10^3/\mu l$, mean $455\pm248\times10^3/\mu l$) was lower than that of the B3-A2 type (675×10^3, $814\pm553\times10^3$) or the co-expression type (702×10^3, $928\pm809\times10^3$). The difference tended to be significant (p=0.11). There was no difference in the white blood cell count, hemoglobin, NAP score nor presence of splenomegaly among the three types. A bone marrow biopsy was performed for the 14 patients at diagnosis and the presence of marrow fibrosis was evaluated. Fibrosis was found in the 3 of the 8 patients with the B3-A2 type, 2 of the 3 with B2-A2 type and 2 of the 3 with the co-expression type, and there was no difference among them.

Table 1. Clinical and laboratory data of CML patients at diagnosis in relation to bcr-abl mRNA type

Type	Sex[1]	Age[2] (year)	WBC[2] ($\times10^3/\mu l$)	Hb[2] (g/dl)	Plt[2] ($\times10^3/\mu l$)	NAP score[2]	Spleno-megaly[3]	Bone marrow fibrosis[4]
B3-A2	M7	54	28.2	13.0	675	55	4	3 (8)
	F6	55.0±14.1	68.4±67.0	13.0±2.3	815±553	67.3±50.5		
		30~70	16.3~196.4	8.4~16.3	153~1800	11~183		
B2-A2	M4	58.5	49.6	12.7	371	70.1	2	2 (3)
	F3	54.7±17.9	71.3±65.4	13.6±1.5	454±228	71.5±27.4		
		31~75	12.2~174.8	12.3~16.1	252~903	41~104		
Co-expression	M1	42.5	35.1	13.2	70.2	65	1	2 (3)
	F5	41.7±10.4	67.5±87.0	12.7±1.6	927±808	103.6±62.2		
		22~53	18.6~226.8	9.5~13.8	237~2415	60~206		

1) M: male, F: female
2) Values indicate median, mean±SD and range from the top
3) Number of patients with splenomegaly
4) Parentheses indicate number of patients with bone marrow biopsy performed

Bcr-abl type and clinical data in CML blast crisis

The correlation between bcr-abl type and clinical data was investigated for the 14 patients at the time of the blast crisis (Table 2). The chronic phase duration was compared, assuming that the type of bcr-abl mRNA had not changed during the chronic phase. The median and mean (±SD) duration of the B3-A2 type, B2-A2 type and co-expression type were 36 and 40.1±43.5 months (7 patients), 17 and 29.7±31.0 months (3 patients), 53.5 and 60.8±47.6 months (4 patients), respectively. The phenotype of the blasts was then compared in relation to the bcr-abl type. Three of the 14 patients were lymphoid while the other 11 were myeloid type. No bcr-abl type had any specific phenotype of the blasts. The karyotype of the blasts was also compared to the bcr-abl type for the 14 patients. Standard Ph^1 alone was observed in 5 patients, while additional karyotype changes were found in the other 9 patients. Again, no specific bcr-abl type was associated with the frequent occurrence of additional karyotype change.

Table 2. Clinical and laboratory data of CML blast crisis in relation to bcr-abl mRNA type

Type	Chronic phase duration (month)[1)]	Phenotype of blasts	Karyotype change additioal to Ph^1
B3-A2	36 40.1±43.5 0~132	myeloid 6 lymphoid 1	5
B2-A2	17 29.7±31.0 7~65	myeloid 2 lymphoid 1	1
Co-expression	53.5 60.8±47.6 11~125	myeloid 3 lymphoid 1	2

1) Values are median, mean±SD and range from the top

DISCUSSION

We confirmed the detection by RT-PCR of the three bcr-abl mRNA types: B2-A2, B3-A2 and the co-expression type. Since this RT-PCR method was able to detect a small cell population with a bcr-abl transcript, this experiment was performed for CMPD patients. In ET the existence of Ph^1 is excluded from the criterion by the Polycythemia Vera Study Group (1986)[16)]. However, Ph^1 positive ET cases have been reported[1,9,12,22)]. Ph^1 positive cases have been described even in myelodysplastic syndrome[19)]. Therefore, we sought the possibility that the bcr-abl rearranged clone exists in CMPD patients other than CML. We tried to detect the bcr-abl transcript in PV, ET and PMF. However, the transcript was not detected in any patient examined. The bcr-abl rearranged clone is thought to be rarely existent in CMPD other than CML.

In the study of CML, the clinical data at diagnosis of the chronic phase were compared among the three bcr-abl types: B2-A2, B3-A2 and the co-expression type. The patient group with the co-expression type was a little younger than that of the other two types. The reason for this was unknown. Recently, cases with the co-expression have been reported without analysis of age[10,15)]. There was no apparent difference in the white blood cell count, hemoglobin or NAP score among the three types. However, the platelet count of the B2-A2 type was lower than that of the other two types. This result is consistent with one recent report[4)], but not with another report[18)]. From our results, it may be thought that the CML clone in the B2-A2 type might differentiate less strongly into a megakaryocytic lineage than that of the other two types. There have been some reports showing that the site of the breakpoint within M-bcr, analysed by Southern blot, has a correlation to the duration of the chronic phase[13)]. Schaefer-Rego et al[20)] reported that patients with a breakpoint in the 5' region of M-bcr have a longer chronic phase duration, whereas patients with one in the 3' region have a shorter duration and progress to a blast crisis. Mills[14)] also reported that the chronic phase duration of patients with 5' side breakpoint is four times longer than that of patients with 3' side breakpoint. On the other hand, several papers have shown that the chronic phase duration does not correlate to the site of the breakpoint[5,17,23)]. In our study, no particular difference was found between the bcr-abl mRNA type and the duration of the chronic phase. The analysis in the above reports was performed on the basis of the location of the bcr breakpoint, whereas our analysis was based on the bcr-abl mRNA type. Therefore, it is impossible to compare these results. However, a very recent report employing the same method of analysis as ours was not inconsistent with our results. In addition, the phenotype of blasts at the blastic crisis and the bcr-abl type were not correlated. The co-expression of B2-A2 and B3-A2 mRNA, which may occur due either to alternative splicing or the presence of two populations of Ph^1-positive clone, was found not to be associated with the progression of CML into the blastic crisis[15)]. Karyotypic change occurs in many cases at the blastic crisis, but no correlation to specific bcr-abl mRNA type was recognized. These results suggest that the bcr-abl mRNA type does not determine the clinical features of CML progression from chronic phase to blastic crisis. However, since the number of patients studied was small, it will be necessary to examine more cases in future.

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REFERENCES

1. **Bornstein, R.** 1990. Does the Philadelphia chromosome exclude the diagnosis of essential thrombocythaemia? Am. J. Hemat. **35:** 70-71.
2. **Daley, G.Q., Van Etten, R.A. and Baltimore, D.** 1990. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. Science **247:** 824-829.
3. **Elefanty, A.G., Hariharan, I.K. and Cory, S.** 1990. Bcr-abl, the hallmark of chronic myeloid leukemia in man, induces multiple haemopoietic neoplasms in mice. EMBO J. **9:** 1069-1078.
4. **Inokuchi, K., Inoue, T., Tojo, A., Futaki, M., Miyake, K., Yamada, T., Tanabe, Y., Ohki, I., Dam, K., Ozawa, K., Asano, S. and Nomura, T.** 1991. A possible correlation between the type of bcr-abl hybrid messenger RNA and platelet count in Philadelphia-positive chronic myelogenous leukemia. Blood **78:** 3125-3127.
5. **Jaubert, J., Martiat, P., Dowding, C., Ifrah, N. and Goldman, J.** 1990. The position of the M-bcr breakpoint does not predict the duration of chronic phase or survival in chronic myeloid leukaemia. Brit. J. Haemat. **74:** 30-35.
6. **Kawasaki, E.S., Clark, S.S., Coyne, M.Y., Smith, S.D., Champlin, R., Witte, O.N. and McCormic, F.** 1988. Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-spesific mRNA sequences amplified in vitro. Proc. Natl. Acad. Sci. **85:** 5698-5702.
7. **Kurzrock, R., Gutterman, J.U. and Talpaz, M.** 1988. The molecular genetics of Philadelphia chromosome-positive leukemias. New Eng. J. Med. **319:** 990-997.
8. **Kurzrock, R. and Talpaz, M.** 1991. The molecular pathology of chronic myelogenous leukemia. Brit. J. Haemat. **79:** 34-37.
9. **LeBrun, D.P., Pinkerton, P.H., Sheridan, B.L., Chen-Lai, J., Dube, I.D. and Poldre, P.A.** 1991. Essential thrombocythemia with the Philadelphia chromosome and bcr-abl gene rearrangement. Cancer. Gen. Cyto. **54:** 21-25.
10. **Lee, M-S., LeMaistre, A., Kantarjian, H.M., Talpaz, M., Freireich, E.J., Trujilo, J.M. and Stass, S.A.** 1989. Detection of two alternative bcr/abl mRNA junctions and minimal residual disease in Philadelphia chromosome positive chronic myelogenous leukemia by polymerase chain reaction. Blood **73:** 2165-2170.
11. **Lugo, T.G., Pendergast, A., Muller, A.J. and Witte, O.N.** 1990. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. Science **247:** 1079-1082.
12. **Martiat, P., Ifrah, N., Rassool, F., Morgan, G., Giles, F., Gow, J. and Goldman, J.M.** 1989. Molecular analysis of Philadelphia positive essential thrombocythemia. Leukemia **3:** 563-565.
13. **Mills, K.I., Benn, P. and Birnie, G.D.** 1991. Does the breakpoint within the major breakpoint cluster region (M-bcr) influence the duration of the chronic phase in chronic myeloid leukemia? An analytical comparison of current literature. Blood **78:** 1155-1161.
14. **Mills, K.I., Hynds, S.A., Burnett, A.K., MacKenzie, E.D. and Birnie, G.D.** 1989. Further evidence that the site of the breakpoint in the major breakpoint cluster region (M-bcr) may be a prognostic indicator. Leukemia **3:** 837-840.
15. **Morgan, G.J., Hernandez, A., Chan, L.C., Hughee, T., Martiat, P. and Wiedemann, L.M.** 1990. The role of alternative splicing patterns of bcr/abl transcripts in the generation of the blast crisis of chronic myeloid leukemia. Brit. J. Haemat. **76:** 33-38.
16. **Murphy, S., Iland, H., Rosenthal, D. and Laszlo, J.** 1986. Essential thrombocythemia : an interim report from the Polycythemia Vera Study Group. Sem. Hemat. **23:** 177-182.
17. **Opalka, B., Wandl, U., Beer, U., Roggenbuck, U., Kloke, O. and Niederle, N.** 1991. Breakpoint location within the M-bcr and clinical course do not correlate in patients with chronic myelogenous leukemia undergoing alfa interferon therapy. Leukemia **5:** 452-456.
18. **Opalka, B., Wandl, U., Stutenkemper, R., Kloke, O., Seeber, S. and Niederle, N.** 1992. No correlation between the type of bcr-abl hybrid messenger RNA and platelet counts in chronic myelogenous leukemia. Blood **80:** 1854-1855.
19. **Roth, D., Richman, C.M. and Rowley, J.D.** 1980. Chronic myelodysplastic syndrome (Preleukemia) with the Philadelphia chromosome. Blood **56:** 262-264.
20. **Schaefer-Rego, K., Dudek, H., Popenoe, D., Arlin, Z., Mears, J.G., Bank, A. and Leibowitz, D.** 1987. CML patients in blast crisis have breakpoints localized to a specific region of the BCR. Blood **70:** 448-455.
21. **Shtivelman, E., Lifshitz, B., Gale, R.P. and Canaani, E.** 1985. Fused transcript of abl and bcr genes in chronic myelogenous leukemia. Nature **315:** 550-554.
22. **Tesch, H., Zankovich, R., Fonatsch, C. and Diel, V.** 1990. Analysis of bcr rearrangements in primary thrombocythemia. Leuk. Res. **14:** 241-246.
23. **Tien, H.F., Wang, C.H., Chen, Y.C., Shen, M.C., Wu, H.S., Lee, F.Y., Chuang, S.M. and Liu, C.H.** 1990. Chromosome and bcr rearrangement in chronic myelogenous leukemia and their correlation with clinical states and prognosis of the disease. Brit. J. Haemat. **75:** 469-475.