Philadelphia-Positive Chronic Myeloid Leukemia With a Chromosome 22 Breakpoint Outside the Breakpoint Cluster Region

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The hallmark of chronic myeloid leukemia (CML) is the presence of a shortened chromosome 22, the Philadelphia (Ph') chromosome, in more than 90% of cases.1 The Ph' chromosome, which can be considered the hallmark of CML, contains a reciprocal translocation t(9;22)(q34;q11). The breakpoint of chromosome 22 is within a 100-kilobase (kb) region. The breakpoint of chromosome 22 is found in the middle portion of a transcribed gene of unknown function.7

The breakpoint of chromosome 9 has been found to occur at variable sites in a region of up to 100 kilobases (kb) upstream from the first v-abl-related human c-abl sequence.5 On the other hand, the breakpoint of chromosome 22 has been shown to be clustered within a limited region of about 5.8 kb, the breakpoint cluster region (bcr), which is the middle portion of a transcribed gene of unknown function.7

The reciprocal translocation therefore brings the 5' end of the c-abl oncogene into head-to-tail juxtaposition with the truncated 3' portion of bcr on chromosome 22. This mechanism leads to the formation of a new chimeric transcriptional unit containing genetic information from both c-abl and bcr genes7 whose resulting transcript is a new fused mRNA of about 8.5 kb.8

The translocation product of this hybrid mRNA is a 210-kD phosphoprotein with tyrosine kinase activity. This protein is larger than the normal p145 kD c-abl protein because of a replacement of aminoterminal c-abl sequences by bcr sequences.9 Interestingly, the aminoterminal alteration is in fact responsible for the transactivation activity of v-abl in Abelson murine leukemia virus.10 This seems to raise the question whether the altered abl protein may play a role as a transforming protein in the pathogenesis of CML.

Therefore the most important genomic event in CML seems to be the juxtaposition of c-abl with the broken bcr gene. Although the breakpoint on chromosome 9 is quite variable and therefore the length of the hybrid bcr/abl gene also varies, the fused mRNA is consistent from patient to patient as a result of primary transcript splicing.8

It is now well established that in Ph'-positive acute lymphocytic leukemia (ALL) the breakpoint of chromosome 22 can take place outside the bcr region.11 In such cases a novel 6.5-kb chimeric mRNA12 and a novel 190-kD chimeric protein13 were observed. On the other hand, some observations seemed to suggest that perhaps in CML too the breakpoints on chromosome 22 are more heterogeneous than previously recognized.14 This focused our attention on the importance of analyzing as many as possible Ph'-positive CML patients to examine, by Southern blot analysis with a bcr probe, how large a variation there could be in the location of chromosome 22 breakpoints that still allows the formation of the novel chimeric mRNA.

Patients

Twenty adult patients affected by typical Ph'-positive CML were studied. In all cases leukapheresis procedures as well as bone marrow aspirations were performed after informed and explicit consent of the patient was obtained, as required by our institutional guidelines. Ten patients were in chronic phase, three were in accelerated phase, and 7 were in blast crisis (BC). Leukemic cells were obtained by leukapheresis.

Cytogenetic studies. Cytogenetic studies were performed on bone marrow and/or peripheral blood cells after a 24-hour incubation in tissue culture media without mitogen, and standard trypsin Giemsa chromosome preparations were made. The karyotype was expressed by using the recommendations of the International System for Human Cytogenetic Nomenclature.16

Southern and Northern blots. Leukocyte-enriched plasma was diluted in RPMI and centrifuged to obtain cell pellets from which nucleic acids were extracted. DNA was isolated according to the technique of Gross-Bellard et al with minor modifications.15 Control DNA was extracted from leukocytes of two normal blood donors.

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Ten micrograms of each DNA was digested with the restriction endonucleases BamHI, BglII, BamHI together with BglII, EcoRI and HindIII; run in 0.7% agarose gel electrophoresis; and then transferred to a Gene Screen (NEN) membrane with a standard Southern blot technique.14

RNA was extracted by following the technique described by Torelli et al.15 Total RNA was then enriched for polyadenylate-containing RNA by one cycle of purification on oligo-dT-cellulose affinity chromatography.16 Ten micrograms of poly (A)+ RNA were size fractionated by electrophoresis on 0.8% agarose gel containing 7% formaldehyde and then transferred to nitrocellulose filters as described.20

Preparation of DNA probes. To study the rearrangement of bcr we used a subgenomic probe represented by a HindIII-BglIII genomic restriction fragment of 1.2-kb (Pr-1, Oncogene Sciences, Inc, Mineola, NY) that corresponded to an intron region of the gene located in the middle of the bcr region21 and two contiguous bcr cDNA fragments of 0.6 kb (6.13-0.6) and 0.3 kb (6.13-0.3) representing a coding portion of the gene 5' to the bcr region. The cDNA fragments used to study the expression of the chimeric mRNA were represented by a 0.5-kb human bcr cDNA restriction fragment (Pr-2, Oncogene Sciences) isolated from cDNA VI-322 and by bcr cDNA probes (6.13 and K31) corresponding together to a coding segment of about 1.4 kb 5' to the sequences represented by the bcr probe Pr-2.22 Furthermore, a human c-abl cDNA fragment of 1.7 kb (K38) corresponding to the 3' end of the c-abl cDNA was used.

The probes 6.13, K31, and K38 were developed and generously provided by Dr G. Grosveld, Erasmus University, Rotterdam, The Netherlands. A c-sis probe corresponding to a 1.7-kb BamHI subgenomic fragment cloned into pBR 32222 was used to examine whether a possible breakpoint had occurred in the subbands q12.3 to q13.1. A Cs probe corresponding to a 0.8-kb EcoRI subgenomic fragment containing the Cs light-chain gene23 was used to study the presence of a possible breakpoint in the subband q11.12.

To define as precisely as possible that some of the cases pertained to the myeloid lineage, a probe for Cs was used that corresponded to a 1.2 EcoRI restriction fragment containing a part of the first and all of the second and the third exon of the Ig heavy-chain μ gene.24 A probe for T cell receptor (TCR) β was further used and corresponded to a 0.77-kb insert containing J and C regions cloned in the PstI site of pBR 322.25

All the probes were nick-translated with α32P deoxyctydine triphosphate (dCTP) to a specific activity of about 2 × 108 cpm/μg DNA.

Hybridization and autoradiography. Southern and Northern blot hybridizations were performed according to procedures already described in detail.26 Washing of the Southern blot filters was carried out in 0.3× SSC at 65°C. Washing of the Northern blot filters was performed in 0.1× SSC at 65°C. Autoradiography was performed at −80°C with intensifier screens for two to seven days.

RESULTS

All 20 adult patients studied were affected by CML diagnosed according to French-American-British (FAB) morphological and cytochemical criteria.25 Ten patients were in fact in chronic phase, three in accelerated phase, and seven in BC. Cytogenetic analysis of all patients showed the typical t(9;22) translocations, except in one case in which a simple variant translocation of the type t(22;22) was present. Most patients in BC showed numerical chromosomal abnormalities (hyperdiploidy) and/or Ph1 duplication as expected.27 No c-abl rearrangement was detected in any of the cases studied,29 which did not seem surprising because the chromo-

some 9 breakpoints can occur in a region very far upstream to the first c-abl exons. Concerning chromosome 22 breakpoints, restriction data of 17 of 20 patients together with cytogenetic data have already been discussed in detail elsewhere29 and are consistent with the results already reported in the literature.6,7

In three cases rather unexpectedly (T.V., R.G., and Q.S., all in chronic phase), no rearranged bands were detectable after digestion of the DNA with the restriction endonucleases BamHI, BglII, HindIII, EcoRI (Fig 1), and BamHI together with BglII (not shown). In fact, all the aforementioned restriction enzymes in all three cases, the hybridization with the 1.2-kb HindIII-BglII bcr genomic fragment showed only the normal germ line band as in the control DNA from normal leukocytes; they measured, respectively, 3.3 kb in the BamHI-digested DNAs, 5.0 kb in the BglII-digested DNAs, 4.5 kb in the HindIII-digested DNAs, 23 kb in the EcoRI-digested DNAs (Fig 1), and 2.1 kb in the same DNAs digested with BglII together with BamHI. These experiments were performed twice for all three patients with different blood samples collected within an interval of few months. In all three cases both Ig heavy-chain and TCR β chain genes were in a germ line configuration (data not shown), which confirmed, in addition to the
FAB morphological and cytochemical data, that patients' leukemic cells were of the myeloid lineage.

Our findings suggested that in these three patients the breakpoint was located either 5' or 3' to the bcr, but certainly not within it despite the fact that in all three cases a t(9;22) translocation was present (Fig 2) in two successive cytogenetic analyses carried out within an interval of several months and despite the typical CML phenotype. It must be added that the peripheral cell populations studied were formed by a vast majority (>80%) of largely immature myeloid elements and that the Ph1 translocation was observed in more than 90% of the metaphases analyzed in each case.

Because it was suggested that in Ph1-positive ALL cases the breakpoints of chromosome 22 could be located 5' to the bcr region, we explored the possibility that a similar genomic event could have taken place in our CML patients. Therefore in Southern blot experiments we used the probes 6.13-0.6 (0.6 kb) and 6.13-0.3 (0.3 kb), which represent together 0.9 kb corresponding to the middle portion of the bcr cDNA sequences 5' upstream the bcr region (G. Grosveld, personal communication). Again the pattern obtained after digestion with BamHI, BglII, and EcoRI in all three cases was identical to that of normal leukocyte DNA, both with the 0.6 kb probe (data not shown) and with the 5', 0.3-kb probe (Fig 3).

The DNAs of the three patients were then hybridized to the c-sis probe after digestion with EcoRI and HindIII and to the C1 probe after digestion with EcoRI, BamHI, and HindIII. No rearranged bands were found in any case, either with c-sis or with C1, with all the restriction enzymes used (data not shown). With the c-sis probe, however, two of the three patients (T.V. and R.G.) exhibited a polymorphic pattern already described after digestion with HindIII that gave bands of about 21.7 kb and 14.5 kb (not shown). Furthermore, in one of the three patients (R.G.) a polymorphic restriction pattern, already described, was also observed in the EcoRI-digested DNA hybridized to the C1 probe that gave an additional band of about 22 kb (not shown).

As far as the expression of bcr is concerned, the poly (A) + RNAs of the three described patients together with the poly (A) + RNAs derived from two other classic Ph1-positive CML cases were hybridized on the same filters with the bcr cDNA probe (Pr-2) representing exons 1 and 2 of the bcr region and at least two further bcr exons 5' upstream to them. In all cases two faint bands representing normal bcr mRNA species were detected (Fig 4). A major 8.5-kb mRNA species was detectable only in the RNAs analyzed as controls and was apparently absent in the RNAs of the three patients already mentioned (Fig 4) despite the fact that the chimeric mRNA, when present, is usually more abundant than the normal bcr mRNA transcripts.

We then submitted the same RNAs to a further Northern blot analysis using a c-abl cDNA probe (K38). This experiment revealed, both in poly (A) + and total RNA of one case (T.V.), the presence, in addition to the normal expected bands, of a faint but still detectable band of about 8.5 kb while confirming the absence of a chimeric mRNA species in the remaining two cases (Fig 5). The results obtained in patient T.V. led us to investigate whether the chimeric mRNA might be detected by bcr cDNA probes representing coding sequences located more 5' to those represented by Pr-2. In fact Northern blot analysis of the poly (A) + RNA of patient T.V., which was carried out with the bcr cDNA probes 6.13 and K31 and represented together about 1.4-kb sequences 5' to the Pr-2 sequences, revealed extremely faint bands, one of which apparently corresponds to the chimeric mRNA present in a classic CML case (Fig 6). The same experiment did not show any detectable level of chimeric mRNA in patient Q.S. (Fig 6) or patient R.G. (not shown).

The densitometric analysis did not reveal relevant differences in the amount of RNA present in each lane in all experiments.

DISCUSSION

The analysis of our data led us to the conclusion that in these three patients affected by classic Ph1-positive CML, the breakpoint of chromosome 22 was certainly outside the bcr region. This finding seemed rather unusual, even though in the literature some evidence has already been reported of Ph1-positive CML in which a breakpoint inside bcr was not found, and it has been suggested that in CML the chromosome 22 breakpoints must be more heterogeneous than previously recognized. Southern blot hybridizations with the bcr (Pr-1) probe suggested that in these three cases the breakpoint should be located either 5' or 3' to the bcr region.

 Meanwhile, the absence of rearranged bands in the DNAs digested with different enzymes and hybridized with the probes Pr-1, 6.13-0.6, and 6.13-0.3 prevented us from localizing the breakpoint in the coding segments 5' to the bcr in a region of about 13 kb flanking the 5' site of the bcr.

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**Fig 2.** Partial karyotypes of the three patients showing a t(9;22) translocation in all cases. 1, the patient T.V.; 2, R.G.; and 3, Q.S.
Consequently, we reasoned that in these three cases the breakpoint should be located either in the far 3' extremity of the bcr gene or outside the gene.

The absence of rearranged bands with both C\(_a\) and c-sis probes made it clear that the breakpoint had occurred neither in the C\(_a\) locus located in the q11.12 subband nor in the c-sis locus harbored in the q12.3-13.1 subbands. At present we cannot explain the apparently high frequency of polymorphism of both these loci in these three cases.

With respect to the Northern blot hybridization data, we did not find a chimeric mRNA of 8.5 kb in two of these three Ph\(^{+}\)-positive CML patients. We are well aware of the uncertainties related to a quantitative evaluation of mRNA expressed at low level; nevertheless, our results indicate that in these two cases the level of transcription of the 8.5-kb mRNA, if present, must be at least much lower than in common Ph\(^{+}\)-positive CML. These findings seemed rather unusual because data reported in literature show the presence of this chimeric mRNA in practically all cases of Ph\(^{+}\)-positive CML studied thus far with rare exceptions.\(^{32,33}\)

So the possibility is raised that a breakpoint of chromosome 22 outside the bcr region might result in a novel genomic structure that does not allow the transcription of a 8.5-kb chimeric mRNA, at least at a detectable level, while leaving unaffected the formation of the mRNA species presumed to be normally transcribed by the bcr and abl genes.\(^{21}\)

A similar genomic event has been described in several patients affected by ALL with a t(9;22) translocation in which the bcr region was not involved in the chromosomal rearrangement.\(^{11,14,39}\) By analogy with the reports in some Ph\(^{+}\)-positive ALL cases,\(^{12,14}\) it would be worthwhile in these two cases to look for the presence of abl-derived kinases different from the chimeric p210 and from the normal p145 while taking in mind that the cells of these two cases are certainly not lymphoid.
The third case (T.V.), in which the c-abl probe reveals the chimeric 8.5-kb mRNA, seems to suggest that unusual breakpoints sometimes may still allow the formation of a chimeric mRNA. The same mRNA species shows an extremely low level of hybridization with 5' bcr cDNA sequences and apparently no hybridization at all with the 0.5-kb bcr probe representing only the sequences immediately flanking the breakpoint. These findings seem to suggest that the bcr portion of the chimera is in some way different from the classic one, possibly as a result of molecular events like deletions or alternative splicing.44

Because different molecular patterns underlie the same clinical features, it can be only suggested that the relationship between the genetic alteration of the Ph' event and the resulting neoplastic phenotype remains to be elucidated. While this manuscript was under preparation, other authors have reported identical molecular abnormalities, ie, bcr rearrangement as in Ph'-positive CML, in Ph'-negative CML cases with and without the classic clinical features of CML. They suggest that factors other than the bcr-abl rearrangement might underlie the heterogeneous clinical features of CML.39

In view of all these results it will be necessary to carefully define the genomic structure, the mRNA transcripts, and the presence of the related proteins in a larger number of CML patients.

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