Breakpoints on Chromosomes 9 and 22 in Philadelphia Chromosome–positive
Chronic Myelogenous Leukemia (CML)
Amplification of Rearranged c-abl Oncogenes in CML Blast Crisis

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Abstract

We surveyed 20 Philadelphia chromosome (Ph') positive chronic myelogenous leukemia (CML) samples by Southern blot hybridization to determine the location of the breakpoints that occur on chromosomes 9 and 22 in the Ph' translocation. Only 3 of 20 samples exhibited breakpoints on chromosome 9 within 18 kilobases (kb) of the v-abl homologous sequences. Mapping of these three chromosome 9 breakpoints indicates that each is at a separate location within this 18-kb region, indicating that there are no breakpoint “hot spots” in this area. In contrast, all 20 CML samples exhibited breaks on chromosome 22 within a 5.0-kb Bgl II fragment that lies within the previously described breakpoint cluster region (bcr). Several patients with CML blast crisis exhibiting multiple Ph' chromosomes/metaphase exhibited amplified and rearranged c-abl-related fragments. These additional Ph' chromosomes in blast crisis cells do not arise from a second, independent 9:22 translocation but rather result from a duplication of the preexisting Ph' chromosome.

Introduction

Over 90% of cases of chronic myelogenous leukemia (CML) exhibit the Philadelphia chromosome (Ph') that results from a reciprocal translocation between chromosomes 9 and 22. In this reciprocal exchange a portion of the q arm of chromosome 22 is translocated to chromosome 9 to form 9q+, while a small portion of the distal end of the q arm of chromosome 9 harboring the c-abl oncogene is translocated to chromosome 22 (1, 2). Therefore, Ph' is a deleted form of chromosome 22 (22q-) which harbors the c-abl oncogene translocated from chromosome 9 (Fig. 1).

The c-abl oncogene is defined by virtue of its homology to v-abl, the transforming sequences of the Abelson murine leukemia virus (3). The normal physiologic function of c-abl is presently unknown although its homologue v-abl is known to have tyrosine kinase activity (4). An abnormally large 8-kb abl-related RNA transcript has been noted in CML cells (5, 6) and this aberrant transcript appears to be transcribed from the translocated c-abl oncogene on the Ph'. This abnormally large mRNA may code for P210, the abnormal-sized abl-related protein product that has been noted recently in CML cells (7, 8). Thus the c-abl oncogene appears to be intimately related to the pathogenesis of CML.

Initial reports have indicated that the breaks on chromosome 9 in CML occur at a variable distance from the 5' end of c-abl (9). In contrast, chromosome 22 breaks appear to occur in a more restricted region termed the breakpoint cluster region (bcr) (10). However, more recent studies indicate that some Ph'-positive leukemic cells harbor chromosome 22 breaks outside the bcr (11, 12). To more thoroughly characterize the breakpoint locations on chromosomes 9 and 22, which are involved in the formation of Ph', we studied 20 Ph'-positive CML cells and cell lines using Southern blot hybridization with c-abl and bcr probes. During this study we noted several patients in CML blast crisis with multiple Ph' chromosomes/metaphase who demonstrated amplified, rearranged c-abl-related fragments. We provide evidence that these additional Ph' chromosomes in blast crisis cells arose from a duplication of a preexisting Ph' chromosome rather than resulting from a second independent 9:22 translocation event.

Methods

Cells. The Ph'-positive CML cell line KCL-22 was cultured by means of standard techniques as referenced (13). The remaining cells were isolated from peripheral blood or bone marrow samples of CML patients being treated at the Seattle Veterans Administration Medical Center or the Fred Hutchinson Cancer Research Center. The presence of the Ph' was confirmed in clinical samples by standard cytogenetic analysis performed in the cytogenetic laboratories of the University of Washington Hospital and Fred Hutchinson Cancer Research Center. Leukocytes were isolated from patient samples using dextran sedimentation. The samples were diluted 1:1 in RPMI, and the red cells sedimented by incubation for 1 h in an equal volume of 3% dextran T-500 at room temperature. The supernate cells were pelleted by centrifugation at 1,500 rpm, and the contaminating red cells in this pellet were subjected to hypotonic lysis in 0.2% NaCl, then an additional equal volume of 1.6% NaCl. Before DNA extraction, the cells were washed three times in plain RPMI.

Molecular probes. A molecular probe from the 5' end of the c-abl oncogene was cloned from a genomic library constructed from the K-562 CML blast crisis cell line (14). DNA from this cell line was partially digested with Sst I, fractionated by sucrose density gradient centrifugation, and the 15-23-kb DNA fragments were isolated. This DNA was ligated with Bam-digested EMBL 3 phage vector DNA, and the ligation mixture was packaged in vitro. Approximately 500,000 recombinant clones were screened with a nick-translated 550-bp Hinc II-Sma I fragment derived from the pAb1 sub9 plasmid, which contains a v-abl insert (15). This 550-bp fragment represents the most 5' sequences of v-abl. It was isolated from a polyacrylamide gel following endonuclease digestion of pAb1 sub9 (16). After screening, 10 positive clones were isolated and purified. One of these clones contained a 17-kb genomic insert that exhibited a restriction map similar to that of the previously published 5' end of c-abl (9). From this clone a 0.3-kb Hind III–Eco R1 fragment, free of human repetitive sequences, was isolated and used as a molecular probe.

The bcr probe consisted of a 1.2-kb Hind III–Bgl II fragment lying within the previously described bcr region on chromosome 22 (10) and was purchased from Oncogene Science, Inc., Mineola, NY.
DNA extraction, Southern blot hybridization. DNA was extracted from the isolated leukocytes by digesting nuclei with protease K, then phenol/chloroform extracting and ethanol precipitating, as previously described (17). Standard techniques were used to perform restriction endonuclease digestion and Southern blotting. The blots were hybridized to nick translated probes and then washed under exactly the same conditions described previously (17).

Results

Localization of the breakpoints on chromosome 22. The Philadelphia chromosome results from a reciprocal translocation between chromosomes 9 and 22 (Fig. 1). Groffen et al. originally reported that 17 of 17 Ph1-positive CML cells exhibited a breakpoint on chromosome 22 within a limited 5.8-kb bcr (10). In contrast, more recent reports indicate that some Ph1-positive patients may exhibit chromosome 22 breakpoints outside this bcr (11, 12). To examine this question, we analyzed 20 Ph1-positive CML samples by Southern blot hybridization to determine the chromosome 22 breakpoint. We used a 1.2-kb Hind III-Bgl II bcr fragment which lies at the 3' end of a 5.0-kb Bgl II fragment within the bcr as a molecular probe (Fig. 2). If chromosome breaks occur within this 5.0-kb Bgl II fragment, then this probe, when hybridized to Southern blots of Bgl II digests of the CML DNA, should detect an extra fragment or fragments in addition to the germline 5.0-kb Bgl II fragment. As shown in Fig. 3, all 20 of the CML samples exhibited an additional Bgl II fragment as well as the germline 5.0-kb Bgl II fragment. Thus, the breakpoint on chromosome 22 lies within this 5.0-kb Bgl II fragment in all 20 of these CML samples.

Localization of the breakpoint on chromosome 9. The c-abl oncogene is translocated from chromosome 9 to the Philadelphia chromosome in cases of CML. In contrast to the breakpoint on chromosome 22, the location of the breakpoint on chromosome 9, while invariably occurring at the 5' end of the c-abl gene, has been reported to be variable (9, 10). To access the breakpoint location on chromosome 9 in our 20 CML samples, we used a 0.3-kb Eco R1-Hind III fragment that we cloned from a K-562 genomic EMBL 3 library. This library was constructed and screened as described in the Methods section. This subcloned 0.3-kb Eco R1-Hind III fragment is free of repetitive sequences and lies within a 12-kb Bgl II as well as within a 15-kb Bam fragment which are both 5' to the v-abl homologous sequences on chromosome 9 (Fig. 4). This probe therefore can detect breaks as far as 18 kb from the v-abl homologous sequences (Fig. 4).

Figure 1. Genesis of Ph' in chronic myelogenous leukemia. The c-abl oncogene is translocated from chromosome 9 to bcr on chromosome 22 q- to form Ph'. The reciprocal translocation involves a portion of the q arm of chromosome 22 moving to chromosome 9 to form 9q".

Figure 2. Restriction map of bcr on chromosome 22. The indicated bcr probe is a 1.2-kb Hind III-Bgl II fragment that hybridizes to a 5.0-kb Bgl II germline fragment. The solid arrow depicts the approximate bcr breakpoint of CML patient K. This restriction map is derived from reference 10.

Figure 3. Chromosome 22 breakpoints in CML cells. DNA from the indicated samples were digested with Bgl II, electrophoresed on a 1.2% agarose gel, transferred to nitrocellulose, and hybridized to a nick-translated 1.2-kb Hind III-Bgl II bcr probe (see Fig. 2). Lane F contains the Ph' positive CML blast crisis cell line KCL-22 DNA. Lane C contains Ph'-negative HL-60 promyelocytic leukemia cell line DNA. All other samples represent fresh patient Ph'-positive CML cells. The germline 5.0-kb Bgl II fragment detected by the bcr probe is indicated. Arrows indicate additional bcr fragments detected by this probe.

“Smudges” seen in the lower molecular weight region of lanes G and Q were not present in other Southern blots of the same samples and most likely represent blot artifacts.

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This probe was hybridized to Southern blots of Bam and Bgl II digests of genomic DNA from our 20 CML samples. 3 of the 20 samples revealed an extra Bgl II fragment in addition to the germline 12-kb Bgl II fragment, indicating a break within this fragment in these three CML cells (Fig. 5). This was confirmed by hybridizing the same probe to Southern blots of Bam digests of the CML samples. Only the three samples with an extra Bgl II fragment exhibited an extra Bam fragment (Fig. 6).

To further localize the chromosome 9 breakpoint region in these 3 CML patients, we localized the breakpoint within the chromosome 22 bcr by restriction analysis and then determined the chromosome 9 breakpoint location from the size of the aberrant bcr Bgl II fragment, which is a fusion fragment containing sequences from both chromosomes 22 and 9. In patient K, for example, the bcr probe detected not only a germline 4.5-kb Hind III fragment, but also an extra 7.5-kb Bam fragment in addition to the germline 3.5-kb Bam fragment (Fig. 7, lane 3). Therefore the breakpoint on chromosome 22 in patient K must lie in the 1.3-kb Bam--Hind III fragment within the bcr (Fig. 2, arrow) and is 1.3–2.6 kb from the 3' Bgl II site (Fig. 2). The aberrant Bgl II fragment detected by the bcr probe in patient K is 6 kb (Fig. 3) and represents a junction fragment with chromosome 9 sequences at its 5' end and chromosome 22 sequences at its 3' end (Fig. 8). Since this 9q' junction fragment is 6 kb, the distance from the 5' end of this junction fragment to the chromosome 9 breakpoint must be 4.7–3.4 kb (Fig. 8). By similar reasoning we mapped the approximate breakpoint on chromosome 9 in patients E and A. Unlike patient K, these patients exhibited germ-line fragments in both Hind III and Bam digests, induced by the 1.2-kb Hind III--Bgl II bcr probe (Fig. 7). Therefore the chromosome 22 breakpoints in patients A and E must lie in the 2.4-kb Bgl II--Bam fragment at the 5' end of the bcr (Fig. 2) and therefore are located 2.6 to 5.0 kb from the 3' Bgl II site. The 9q' Bgl II junction fragments detected by the bcr probe in patients A and E (Fig. 3) were 10.8 and 14.8 kb, respectively. Therefore the distance from the chromosome 9--5' Bgl II end of this junction fragment to the breakpoint on chromosome 9 must be somewhere from 5.8 to 8.2 kb for patient A and 9.8–12.2 kb for patient E. These approximate chromosome 9 breakpoint locations are noted by the solid arrows in Fig. 4.

Multiple Ph1 chromosomes in CML blast crisis: amplification of rearranged c-abl fragments. The evolution of CML from chronic phase to blast crisis is commonly associated with karyotypic changes, the most frequent of which is the development of two or more Ph1 chromosomes (18). It is uncertain whether these new Ph1 chromosomes arise from a new 9;22 translocation event or whether they represent duplication of the existing Ph1 chromosome. Karyotypic analysis of cells from patients K and A, both of whom were in blast crisis, revealed the presence of two and three Ph1 chromosomes, respectively, per metaphase. In both these cells, the aberrant Bgl II fragment detected by the Hind III--Eco R, 5' abl probe appears to be amplified, since this probe hybridizes more intensely to the rearranged Bgl II fragment than to the germline fragment (Fig. 5, lanes A and K). This amplification most likely results from a duplication (or tripling) of the original Ph1 chromosome rather than from a new 9;22 translocation event in Ph1-positive cells. This interpretation is confirmed by the Southern blot analysis of patients A and K. The bcr probe reveals only a single rearranged Bgl II fragment in each patient's DNA (Fig. 3, lanes A and K).
Discussion

We undertook this survey of 20 Ph' positive CML cells and cell lines in an attempt to localize the breakpoints on chromosomes 9 and 22 that are involved in the generation of Ph'. We were particularly interested in determining the breakpoint locations on chromosome 9, since initial reports indicated that these breaks occurred at a variable distance from the 5' end of the c-abl oncopGene (9, 10). Using a probe at the 5' end of c-abl, which can determine breakpoints within 18 kb of the v-abl homologous sequences when hybridized to the appropriate Southern blots, we found breaks in this region in only 3 of the 20 CML samples analyzed. Using similar molecular probes, Heisterkamp et al. (9) initially described breaks in this region in one of three Ph' positive CML samples while Leibowitz et al. (11) more recently described breaks in this location in only 2 of 15 CML samples. Together with our own findings, these data indicate that only ~15% (6 of 38) of CML samples exhibit breaks on chromosome 9 within 18 kb of the v-abl homologous sequences. Most chromosome 9 breaks presumably occur at a distance more 5' to this region.

Canaani has recently mapped several exons at the 5' end of c-abl which are not homologous to v-abl (Fig. 4, hatched boxes, reference 19). Conceivably there may be other more 5' c-abl exons that have not yet been mapped. The three chromosome 9 breakpoints we have mapped in this work, as well as the three others reported to date, all lie within a large intron between the first two of these recently described exons (Fig. 4). As is evident from Fig. 4, the reported chromosome 9 breakpoints are dispersed widely throughout this large intron, and there is no particular "hot spot" for chromosome breakage in this region.

In contrast to the breaks on chromosome 9, the breaks on chromosome 22 have been reported to be confined to a relatively small locus, the bcr (10). This ~6-kb region appears to lie within a much larger coding region of at least 45 kb (20). The function of the gene product of this region is unknown, but Canaani has recently demonstrated by cDNA cloning and DNA sequencing that the aberrantly large 8-kb RNA transcript observed in CML cells is a fusion transcript between bcr coding sequences on chromosome 22 and the translocated c-abl sequences from chromosome 9 (19). This aberrantly large RNA transcript may code for P210, an abnormally large abl-related protein product that has been observed in CML cells (7, 8). Groffen et al. originally noted 17 of 17 Ph' positive CML patients with chromosome 22 breaks in the bcr (10). However, several recent reports have described Ph' positive samples where the chromosome 22 breakpoint apparently lies outside the bcr (11, 12). In our survey, we found that all 20 of our Ph' positive CML samples exhibited breaks in a 5.0-kb Bgl II fragment within the bcr region. These findings confirm the original observations of Groffen and emphasize the usefulness of bcr probes in identifying by restriction analysis CML cells harboring Ph'. Indeed, we are currently using this probe to identify residual or recurrent disease among Ph' positive CML patients who have undergone bone marrow transplantation.

The bcr probe should be particularly useful in these bone marrow transplant studies because it provides a unique marker for individual Ph' chromosomes. Since the breakpoints on chromosomes 9 and 22 are variable for individual Ph' chromosomes, the aberrant restriction fragment detected by the bcr probe will vary in size in different Ph' positive CML samples. (Note that all 20 of the aberrant bcr fragments displayed by the Ph' positive CML cells in Fig. 3 are different sizes.) This probe, when used to analyze CML transplant patients who have relapsed, can distinguish patients who relapse with a Ph' chromosome identical to the one present before transplant from those who relapse with a Ph' arising de novo from a new translocation event.

The development of multiple Ph' chromosomes is the most frequent karyotypic abnormality that occurs as CML evolves from chronic to blast crisis phase. It is unclear whether these additional Ph' chromosomes are a consequence of duplication of a preexisting Ph' or whether they arise as a result of a new 9;22 translocation event. We were able to approach this question...
in our survey, since two of the three CML samples exhibiting breaks relatively close to the v-abl homologous sequences were blast crisis cells harboring two and three Ph1 chromosomes/mosaic, respectively. Both these samples exhibit amplification of the rearranged 5' c-abl fragment when compared with the germine fragment (Fig. 5, lanes A and K). Since this rearranged fragment is present on the Ph1, the presence of multiple Ph1 chromosomes harboring identically rearranged fragments is the most likely explanation for the c-abl fragment amplification. These extra Ph1 chromosomes most likely arose from a duplication of preexisting Ph1 chromosomes rather than from a new 9:22 translocation event. Southern blots of DNA digests using the bcr probe confirm this interpretation. If the new Ph1 chromosome arose from a second independent 9:22 translocation event, then a second rearranged bcr fragment should be noted. However, only a single rearranged bcr fragment is noted in the Southern blots from these samples (Fig. 3, lanes A and K), thus confirming that the new Ph1 chromosomes arose from a duplication (or triplication) of the initial Ph1.

The presence of these multiple Ph1 chromosomes in CML blast crisis vs. chronic phase cells and the consequent amplification of the translocated c-abl oncogene emphasize the important role this oncogene may play in the inevitable progression of CML from chronic phase to blast crisis. The data presented here provide the first direct evidence for amplification of the c-abl oncogene in fresh CML blast crisis cells. We have previously noted a four- to eight-fold amplification of the c-abl oncogene in the cultured CML blast crisis cell line K-562 (17) (Interestingly, unlike these fresh CML cells, the K-562 cell line lacks multiple Ph1 chromosomes, but the c-abl oncogene appears to be tandemly amplified on an aberrant marker chromosome [21].) We have also noted increased RNA expression of the c-abl oncogene in CML blast crisis vs. chronic phase cells (6). Oncogene amplification and enhanced expression may play an important role in the progression of certain human malignancies. For instance, amplification and enhanced expression of the N-myc oncogene has recently been related to progression of childhood neuroblastoma (22, 23). Whether the enhanced expression of c-abl is the major event in the evolution of CML from chronic phase to blast crisis or whether other important genetic events are involved in the progression of CML is presently unknown.

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References