Chronic Myeloid Leukemia

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Novel types of *bcr-abl* transcript with breakpoints in BCR exon 8 found in Philadelphia-positive patients with typical chronic myeloid leukemia retain the sequence encoding for the DBL- and CDC24-homology domains but not the pleckstrin homology one

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Background and Objectives. We previously described a novel type of the chimeric *bcr-abl* mRNA transcript in a patient with a Philadelphia chromosome positive chronic myeloid leukemia. A similar *bcr-abl* transcript has also been described by others.

Design and Methods. Sequence analysis of the fusion region showed a join between part of exon e8 of the *bcr* gene and an intronic sequence of *abl* intron 1b spliced on exon a2 of the *abl* gene, giving rise to an in-frame e8-int-a2 *bcr-abl* transcript, translated into a 197.5 kDa BCR-ABL protein of 1804 amino acid residues, which we named p200 BCR-ABL.

Results. In this work, employing protein comparison analysis (pFAM) we show that these novel *bcr-abl* transcripts retain the DBL homology (DH) domain and the recently recognized CDC24 homology domain, but not the pleckstrin homology (PH) domain of the *bcr* gene.

Interpretation and Conclusions. This observation, along with the myeloid immunophenotype of the tumor and, at least in one case, the patient's correspondingly good response to α-interferon therapy, suggests that p200 BCR-ABL is more similar to p210 BCR-ABL, in which the DH, CDC24 and PH domains are all maintained, than to p185, in which these domains are all lost.

Key words: chronic myeloid leukemia, *bcr-abl* gene, functional domains, Philadelphia chromosome translocation.

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Several forms of the *bcr-abl* oncogene responsible for the pathogenesis of Philadelphia chromosome positive (Ph^+) human leukemias[1] are generated by a specific reciprocal translocation t(9;22)(q34;q11).[2] The breakpoint locations on the *bcr* gene and the specific parts of bcr that are retained determine the forms of the *bcr-abl* oncogene. The main forms of BCR-ABL are P210, which is found in chronic myeloid leukemia (CML),[3, 4, 6] P185, which is found in acute lymphocytic leukemia (ALL)[5-7] and P230, found in chronic neutrophilic leukemia (CNL).[8-11]

We recently reported[12] a novel type of *bcr-abl* transcript detected by reverse transcription polymerase chain reaction (RT-PCR)[13] in a patient with Ph^+ CML.[12-14] Sequence analysis of the fusion region of the amplified cDNA fragment showed a join between part of exon e8 of the *bcr* gene and an intronic sequence of *abl* intron 1b spliced on exon a2 of the *abl* gene, giving rise to an in-frame *e8-int-a2 bcr-abl* transcript. The consequent *bcr-int-abl* transcript was translated into a BCR-ABL oncoprotein slightly larger than P185 BCR-ABL, but smaller than either P210 BCR-ABL or P230 BCR-ABL. This protein, which has 1804 amino acid residues and a molecular mass of 197.5 kDa, has been called P200 BCR-ABL.

P185 BCR-ABL contains only the first exon-encoded *bcr* sequence. On the other hand, P210 BCR-ABL contains the encoded sequences from exons 1 to 13 (or 14, depending on the breakpoint) of the *bcr* gene where there is a homology with the catalytic domains of GDP-GTP exchangers such as DBL, CDC24, and VAV.[15] Consideration of the primary structures of P185, P210 and P230 BCR-ABL reveals that P210 and P230 contain several potential functional motifs encoded by the *bcr* portion of the fusion gene, including the CDC24-DBL (DH) and plekstrin homology (PH) domains and a portion of

a RacGAP catalytic domain. These motifs could alter the oncogenic activity of these larger proteins with respect to P185. Indeed, characterization of the tyrosine kinase of P185 and P210 suggested that these two proteins possess distinct transforming properties. In an immune complex kinase assay, P185 had higher tyrosine kinase than P210.[16] Furthermore, P185 is more efficient than P210 in *in vitro* transformation of Rat-1 fibroblasts.[17] It is not yet known which parts of the BCR gene are responsible for these transformation differences.

Recently, a CDC24-homology domain of BCR was recognized between the BCR residues DH and PH, and was found to bind to the xeroderma pigmentosum group B protein (XPB).[18] This domain is missing in P185 but retained in P210 BCR-ABL. Like BCR, CDC24 is a guanine-nucleotide exchange factor (GEF).[19] Whereas CDC24 is known to act on the CDC42 protein,[19, 20] the function of the 1271 residues of the BCR protein (including the CDC24-like domain) is currently unknown. This part of BCR is thought to act as a GEF for P21-RAC. Like some GEFs, it is similar to the CDC24 family (e.g. mammal p140-RAS GEF, Dbl oncogene, VAV oncogene). Indeed, BCR contains a DH domain located between residues 502 and 707, and a PH domain located between residues 590 and 777.[21] In several other proteins, such as Dbl gene, the interaction between the DH and PH domains may be critical for oncogenic activity. For instance, the Dbl becomes oncogenic when the conjunction between the DH and PH domains is disrupted.[22]

In order to investigate which of the potential functional motifs are retained in the novel P200 *BCR-ABL* (capable of modifing its oncogenic activity with respect to P185, P210 and P230) we performed protein comparison analysis with protein family alignment (pFAM) on the NBCI database. We show that P200 retains the DH domain and the recently recognized CDC24 homology domains, but not the PH domain of the *bcr* gene. To our knowledge, this is the first reported case of separation of the PH domain from the DH and CDC24 BCR domains. Study of this case may provide an *in vivo* model to help identify the functions of the three known BCR domains in the context of BCR-ABL.

Design and Methods

RT-PCR, DNA amplification, cloning and sequencing

After red blood cell lysis, RNA was extracted from total white blood cells by standard protocols. Conditions for RT and PCR of *bcr-abl* have been described elsewhere.[12, 13] *bcr-abl* PCR products were gel-separated, excised and, after the purification step, were sequenced in both strands as previously reported.[13] Sequence comparison analysis was performed using the FASTA3 software program.[20]

Immunophenotype analysis

The morphologic appearance of the blasts from the CML expressing P200 BCR-ABL was typically myeloid; immunophenotyping was performed as previously reported.[23]

Western blot and protein analysis

Western blotting was performed on lysates of 1×10^6 cells from the patient's peripheral blood leukocytes, from leukocytes of another Ph+ ALL patient, and from the K562 cell line. The lysates were run on 6.5% polyacrylamide gel, blotted, incubated with a 1:20,000 dilution of monoclonal anti-*abl* antibody *abl* (oncogene) for 2 hours, and developed by enhanced chemoluminescence (ECL) methods.[24]

Computer analysis

The modeling procedure was performed with SWISS-Model version 2.0, as reported elsewhere.[24] The prediction of molecular mass was performed according to Brendel *et al.*[24] Protein comparison analysis was obtained with Bic2 on the Swiss-prot database (swall database).[21] The isoelectric point analysis was performed as previously described.[25] Protein comparison analysis was performed with PSORT[26] on the EXPASY database. pFAM was performed on the NBCI protein database.[27]

Results

Sequence analysis showed the real bcr-abl nature of the fusion transcript

As described elsewhere,[12] the fusion transcript e8-int-a2 is a *bcr-abl* fusion. Briefly, sequence analysis of both strands of a PCR fragment revealed that the e8-int-a2 fusion transcript has high homology between the 5' part of exon 8 of the *bcr* gene (e8) and exon a2 of the *abl* gene.

The homology with the exon 8 of *bcr* gene ends at nucleotide (nt) 107,807 of the reported *bcr* sequence.[19] Thirty-one bp of the fragment, positioned between the homologs of the *bcr* and *abl* exons, do not match with either *bcr* exon 8 or the *abl* sequences (Figure 1, a).

A homology search demonstrated that this stretch of nucleotides matches with an intronic sequence of ABL intron 1b[19] spanning from nt 53,340 to 53,370 (Figure 1, a). Homology of our *bcr-abl* fragment with the second exon of the *abl* gene is complete starting from nt 49,888 of the

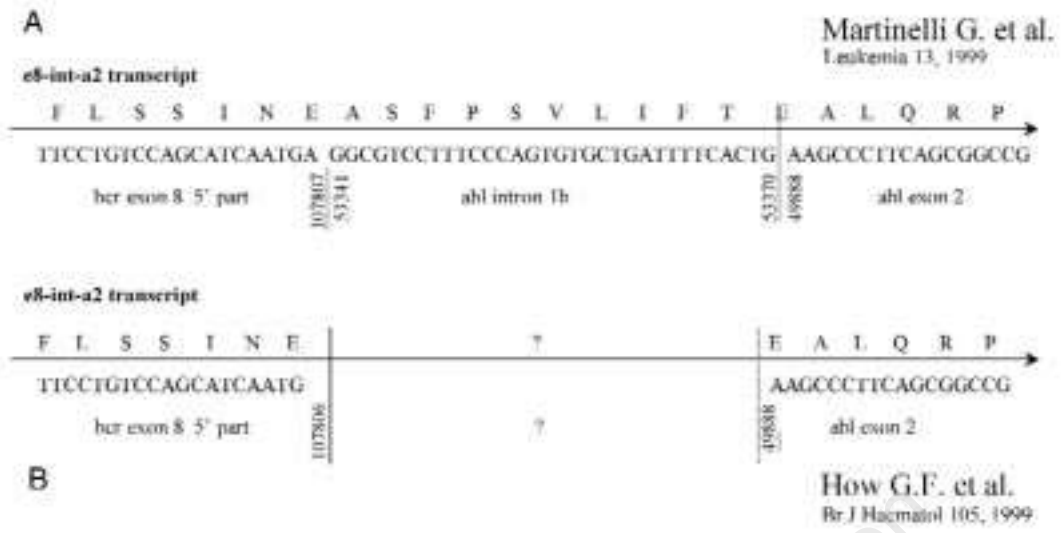

Figure 1. Schematic representations of e8-int-a2 transcript by Martinelli *et al.* (A)[14, 12] and How *et al.* (B)[1] showing amino acids (upon the arrow) and nucleotides (below the arrow) around the breaks. Numbers represent the nucleotides according to ref. no. 19. Question marks represent possible intervening nucleotides to maintain *the frame* for the BCR-ABL protein.

ABL2 clone,[19] corresponding to ABL exon 2 (Figure 1, a). If BCR exon 8 (e8) and ABL exon 2 are spliced they are not in-frame. Therefore, the insert of 31 nt from the ABL intron 1b sequences may be important to maintain the new type of *bcr-abl* transcript in-frame. A similar breakpoint was described by How *et al.*[1] (Figure 1, b).

Western blot and protein comparison analysis showed that P200 is a BCR-ABL protein

A strong BCR-ABL protein signal was detected on Western blots of our patient's leukocytes (*data not shown*). The patient's protein was slightly larger than the p185 BCR-ABL protein. The translated BCR-ABL protein of 1804 amino acid residues has a predicted molecular mass of 197.5 kDa, with an isoelectric point of 8.01. We have called this protein P200 BCR-ABL.[2,14] At sequence analysis, we found perfect protein alignment between P200 BCR-ABL and the 1-690 portion of the BCR protein plus the 700-1804 segment of the ABL protein. P200 BCR-ABL showed 50.2% similarity and 44.9% identity with the entire BCR protein, and 70.5% similarity and 70.3% identity with the entire ABL protein. Due to insertion of sequences from intron ABL 1b, P200 was predicted to contain a further stretch of 10 amino acids ASFPSVLIFT, located between the BCR and ABL portions (Figure 1), not present in any reported BCR-ABL fusion protein. Protein comparison analysis of this stretch showed alignment with the HRPC2 protein (identified as HRC2 XANCV) (*Xanthomonas campestris* protein) (Accession Number P80150) with 100% similarity and 70% identity. Prediction of the P200 BCR-ABL localization site, as obtained with PSORT version 6.4, showed that this protein could be 84% nuclear.

Our BCR-ABL is similar to other P200 BCR-ABL proteins.

Other P200 BCR-ABL fusion genes have been reported to cause a CML-like disease

Reichert *et al.* studied a similar breakpoint in a different *bcr-abl* fusion gene found in a patient with *atypical* CML.[28] Sequence analysis of the fusion region of their amplified cDNA fragment revealed juxtaposition of the entire exon 8 of the *bcr* gene with exon a2 of the *abl* gene. A 91-bp-insertion coding for 32 unusual amino acids of a sequence independent from *bcr* and *abl* was found to give rise to an e8-ins-a2 *bcr-abl* transcript and a BCR-ABL protein of about 202 kDa. Remarkably, a search of the Genbank database revealed that the inserted sequence was derived from KIAA0376, a gene that is known to map close to *bcr* on chromosome 22q. As in our CML case, their novel P202 BCR-ABL protein was sufficient to cause typical CML.

How *et al.* reported another CML case with *bcr* exon 8-*abl* exon 2 type of junction.[1] No insertion sequences (and consequently extra amino acids) were reported in this case. A single nucleotide insertion (or double nucleotide deletion) must be necessary to maintain in frame *bcr* exon 8 and *abl* exon 2 sequences at RNA level. Both these genom-

ic features are missed in the reported paper.[1] Also in this case the predicted BCR-ABL translated protein of 1,794 aminoacids residues has a molecular mass of 197.5 kDa. We conclude that these two rare types of BCR-ABL protein could be considered as versions of P200 BCR-ABL.

The new P200 BCR-ABL protein retains the DH and CDC24 homology domains of the BCR gene but not the PH homology domain

pFAM revealed that the CDC24 homology domain of BCR, which is known to bind to the xeroderma pigmentosum group B protein XPB,[18] is missing in P185 BCR-ABL but is retained in P200, P210 and P230 BCR-ABL. Furthermore, the DH homology domain, located between residues 502-585 of BCR, is also maintained in P200, P210 and P230 BCR-ABL. The PH domain, which is located between residues 590-777 and is maintained in the P210 and P230, is lost in P200 BCR-ABL (Figure 2).

Discussion

In this work, we used pFAM comparison analysis to assess the novel P200 BCR-ABL protein. We found that P200 BCR-ABL retains the DH homology domain of BCR, together with the recently recognized CDC24 domain, but not the PH domain.

The presence of the P200 BCR-ABL coding transcript is a rare but not random occurrence

Reichert *et al.* reported a similar breakpoint in a different *bcr-abl* fusion gene found in a patient with *atypical* CML.[28] Sequence analysis of the fusion region of their amplified cDNA fragment revealed juxtaposition of the entire exon e8 of the *bcr* gene with exon a2 of the *abl* gene. A 91-bp-insertion coding for 32 unusual amino acids of a sequence derived from KIAA0376, a gene that is known to map close to *bcr* on chromosome 22q and independent from *bcr* and *abl*, was found to give rise to an e8-ins-a2 *bcr-abl* transcript and a BCR-ABL protein of about 202 kDa. As in our CML case, their novel P202 BCR-ABL protein was sufficient to cause typical CML. A third case of typical CML with *bcr* exon 8 and *abl* exon 2 junction at RNA level was reported by How *et al*.[1] Both these novel BCR-ABL proteins retained the DH and CDC24 homology domains of BCR, but not the PH one.

The BCR-ABL P200 transcript retains the CDC24 domain and probably also its related activity

The role of the CDC24 homology domain in BCR has recently been elucidated.[18] It was found to bind the BCR-ABL protein to the *Xeroderma pigmentosum* group B protein, which in turn is tyrosine-phosphorylated by BCR-ABL. The interaction not only reduces both the ATPase and the helicase activities of XPB, but also impairs XPB-mediated cross- complementation of the repair deficiency in rodent UV-sensitive mutants of group 3. It was also reported that P210 BCR-ABL potentially inactivates XPB, leading to reduced DNA repair ability and a higher probability (or perhaps faster development) of blast crisis evolution, which is more evident in cells expressing P185 than in those expressing P210 BCR-ABL. It is reasonable to hypothesize that cells from the cases of CML expressing P200 or P202 BCR-ABL probably also have similar XPB binding activity and DNA-repair property and reduced blast crisis evolution with respect to cases expressing P185.

The p200 BCR-ABL transcript may give rise to a leukemic myeloid phenotype whose transforming activity is more similar to p210 and p230 than to p185 BCR-ABL

The clinical presentation of our patient with P200 BCR-ABL was typical of CML. Her laboratory findings at diagnosis and response to α-interferon therapy were very similar to those of CML patients expressing P210. In an experimental model, Shaoguang Li *et al.* recently reported[29] that whereas there was no difference between the three main BCR-ABL oncogenes in the induction of a CML-like disease, animals receiving P185-transduced marrow had significantly reduced survival as compared with P210- and P230-transduced marrow recipients.[29] Under these conditions, rather than succumbing to CML-like disease, most of the animals that received P185-transduced marrow went on to develop B-lymphoid leukemia. Although several recipients of P210- and P230-transduced marrow also developed B-lymphoid leukemia, in these cases the disease developed significantly later. These findings indicated that P185 BCR-ABL induces lymphoid leukemia *in vivo* with a shorter latency than P210 or P230. This is in line with previous studies which found P185 to be a more potent transformer of B-lymphoid cells *in vitro* than P210.[30] Alternative forms of BCR-ABL have quantitatively different potencies for stimulation of immature lymphoid cells[30] and induction of lymphoid leukemia after marrow transduction.[31] The mechanism of the increased lymphoid leukemogenic activity of P185 with respect to P210 and P230 is not known. One possibility is that the increased intrinsic tyrosine kinase activity of P185 allows elevated tyrosine phosphorylation of a substrate critical to proliferation or transformation of lymphoid cells. One can-

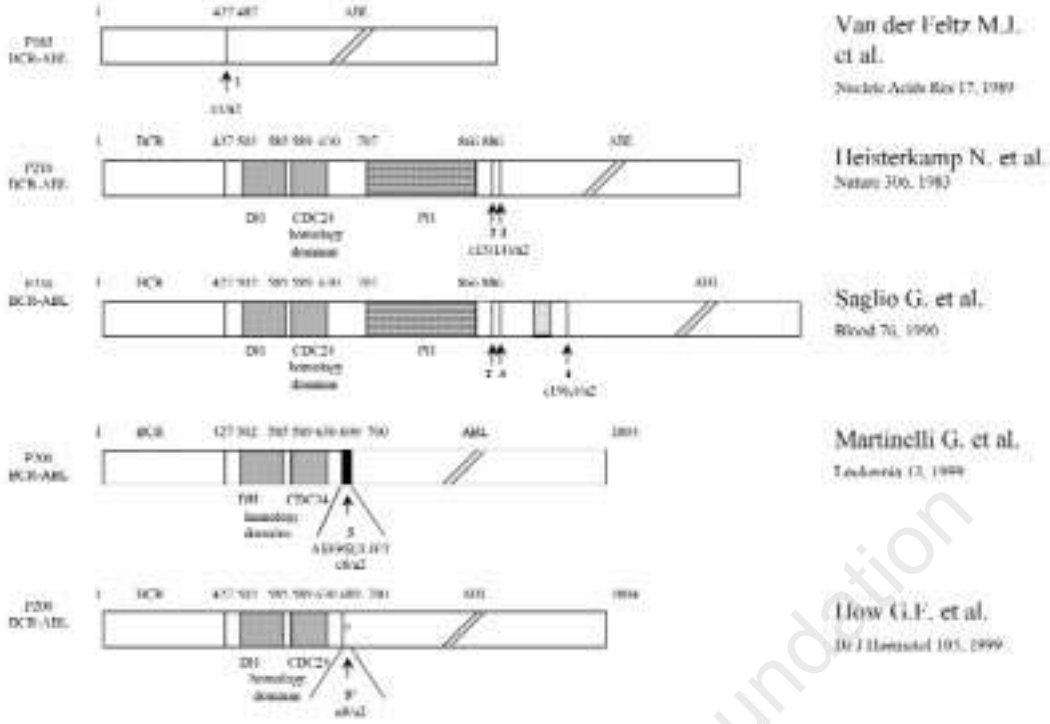

Figure 2. Schematic representation of most common types of BCR-ABL proteins (P185; P210; P230) and rare BCR-ABL proteins (P200). Arrows with numbers (1 to 5') represent different BCR-ABL fusion points, and *bcr* (e) and *abl* (a) exons are signed under the number. DH, CDC24, and PH domains are depicted with different filled boxes. BCR amino acids are signed over each domain.

didate for such a key substrate is STAT6, whose DNA-binding activity is activated via tyrosine phosphorylation by P190 but not P210 but an alternative possibility is that the presence of distinct functional motifs in the BCR portion of P210 and P230 impairs their potential for lymphoid transformation. The only known domains common to P200, P210 and P230 but lacking in P185 are the CDC24 and DH homology domains, which belong to a class of guanine nucleotide exchange factors for small G proteins of the Rac/Cdc42 family. Study of our case of P200 BCR-ABL strongly suggests that the retention of two of these three domains (i.e. DH and CDC24 but not PH) may be critical to maintain the CML phenotype and the relatively low transforming activity associated with P210 and P230.

The P200 BCR-ABL protein loses the PH domain

The PH domain may play a role in the dimerization of various proteins[32-34] and/or in enzyme activation upon binding to lipids.[34-36] The most important function of PH domains, however, seems to be the transient targeting of proteins to membrane, either by binding phospholipids or by binding $\beta\gamma$ sub-units to heterotrimeric G proteins.[37] The fact that the PH domains of different proteins generally cannot be functionally exchanged between each other[39] suggests that they provide distinct targeting signals or that they function in concert with adjacent sequences or other domains. In several other proteins, such as Btk,[38] Akt[39] and Tec,[40] the PH domain is extended with approximately 30 amino acids, and in Akt this extension is essential for the formation of protein complexes.[39] In TIAM 1, which possesses two PH domains, only one of the two (the N-terminal one) can function co-operatively in Ras GRF activation.[41] Virtually all other GEFs, such as Dbl, Ost, Vav, Dbs and Lfc, contain only one PH domain, which is C-terminally adjacent to the DH domain,[41] as in BCR. These DH-flanking PH domains have also been shown to be essential to the activities of their GEFs, but it is not known whether lost sequences or domains also play a role.[42,43] Interestingly, deletions in the PH domains of Dbl, Ost, Dbs and Lfc abolished the transforming activity of these proteins. Similarly, disruption of the complex domain DH-CDC24-PH of BCR might lead to the transforming activity typical of BCR-ABL proteins

while leaving intact any functions that the DH-CDC24-XPB binding may have. In this regard, the transforming capability of P200, which contains the DH but not the PH domain, may be similar to that of P210. In this case, no difference in the transforming activity or blastic transformation of P200 should be expected with respect to P210 BCR-ABL. The clinical outcome of our patient supports this hypothesis, but further experiments with BCR-ABL constructs resembling either our P200 BCR-ABL or other described BCR-ABL protein variants,[44-51] will improve our knowledge on the transforming role of different BCR domains.

Contributions and Acknowledgments

GM was the principal investigator: he designed the study; MA analyzed the samples; BG set up PCR procedures; CT, EO, SS, GS and GR were responsible for clinical management of the patient; MB gave the final approval for submission. The order of authorship reflects the contribution given to the study.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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Peer Review Outcomes

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What is already known on this topic

Although Philadelphia chromosome translocation invariably involves BCR and ABL genes, the breakpoint locations on the BCR gene can vary, thus leading to the formation of a few fusion genes and oncogenic proteins. Very different diseases are associated with different fusion genes/proteins.

What this study adds

The authors have analyzed the sequence of a protein (P200) encoded by a recently identified fusion gene. P200 maintains the DH and CDC24 homology domains, but the PH one is lost.

Potential implications for clinical practice

These results suggest that patients with this type of molecular abnormality may behave clinically similarly as those with the more frequent BCR-ABL lesion (p210). Therefore, these findings may have an impact on the clinical management of these specific patients.

Francesco Dazzi, Associate Editor