Communication

Influence of BCR/ABL fusion proteins on the course of Ph leukemias

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The hallmark of chronic myeloid leukemia (CML) and a subset of acute lymphoblastic leukemia (ALL) is the presence of the Philadelphia chromosome as a result of the t(9;22) translocation. This gene rearrangement results in the production of a novel oncoprotein, BCR/ABL, a constitutively active tyrosine kinase. There is compelling evidence that the malignant transformation by BCR/ABL is critically dependent on its Abl tyrosine kinase activity. Also the bcr part of the hybrid gene takes part in realization of the malignant phenotype. We supposed that additional mutations accumulate in this region of the BCR/ABL oncogene during the development of the malignant blast crisis in CML patients. In ALL patients having p210 fusion protein the mutations were supposed to be preexisting.

Sequencing of PCR product of the BCR/ABL gene (Dbl, PH region) showed that along with single-nucleotide substitutions other mutations, mostly deletions, had occurred. In an ALL patient a deletion of the 5th exon was detected. The size of the deletions varied from 36 to 220 amino acids. For one case of blast crisis of CML changes in the character of actin organization were observed. Taking into account the functional role of these domains in the cell an etiological role of such mutations on the disease phenotype and leukemia progression is plausible.

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Abbreviations: ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CNL, chonic neutrophilic leukemia; DH, Dbl homology; GEF, guanine nucleotide exchange factor; PTK, protein tyrosine kinase.
More than 90% of cases of chronic myeloid leukemia (CML) and 10–25% of cases of acute lymphoblastic leukemia (ALL) are characterized by a reciprocal translocation between chromosomes 9 and 22 (Clark et al., 1988; Cortes et al., 1995). As a result, a BCR/ABL hybrid gene is formed on the derivative Philadelphia chromosome (Ph). Depending on the location of the breakpoint in BCR, three types of fusion protein can be formed, all of which exhibit deregulated protein tyrosine kinase (PTK) activity compared to normal ABL (Konopka et al., 1984; Quackenbush et al., 1997). As a result, there is excessive tyrosine phosphorylation of many intracellular proteins including the BCR/ABL protein itself (Laneuwille, 1995; Liu et al., 1993).

Several forms of the BCR/ABL oncogene responsible for the pathogenesis of Philadelphia chromosomes positive (Ph+) human leukemias are generated by this translocation. The breakpoint locations in the BCR gene and the specific parts of BCR that are left intact determine the forms of the BCR/ABL oncogene. The main forms of BCR/ABL are as follows: P210, which is found in most cases of CML and in 50% of cases of ALL, P185, which is found in ALL and P230, found in chronic neutrophilic leukemia (CNL) (Pane et al., 2000; Martinelli et al., 2002).

The differences between the p210 and p185 proteins are associated with additional Dbl homology (DH) and PH domains (exons 3–12 of the BCR/ABL gene) which are present only in p210. The Dbl family proteins catalyse guanine nucleotide exchange on the Rho family of small GTPases. Members of this family control cell progression, transcription and actin cytoskeletal arrangement. DH functions together with the PH domain (membrane targeting module).

CML is a biphasic disease with an initial chronic phase during which the disorder is easily controlled. However, chronic-phase CML is followed by a terminal blastic phase that resembles acute leukemia and is usually refractory to treatment. Transformation of the chronic phase to blast crisis is accompanied by secondary cytogenetic changes in approximately 85% of cases (Gribble et al., 1999). However, the genetic events responsible for the transformation of CML are poorly understood. We supposed that additional mutations accumulate in the Dbl, PH region of BCR/ABL oncogene during the development of the malignant blast crisis in CML patients. In ALL patients, having the p210 fusion protein, we suppose that these mutations were already pre-existent.

**MATERIALS AND METHODS**

*Patient samples and cell culture.* Peripheral blood cells from patients with a referring diagnosis of CML were obtained for molecular studies as part of diagnosis workup. The Ph-positive cell line K562 and the Ph-negative cell line U937 were cultured in the presence of 10% fetal calf serum in RPMI 1640 medium in a humidified atmosphere with 5% CO2 at 37°C.

*RT-PCR, DNA amplification, cloning and sequencing.* Total RNA was extracted from white blood cells as well as from the control cell lines by the method of Chomczynski and Sacchi (1987). The Ph-chromosome was detected as described (Kawasaki et al., 1988). For amplification of the Dbl homology region bp 1955–2810 of BCR/ABL cDNA were: 30 s of denaturation at 94°C, 30 s annealing at 56°C and 90 s of extension at 72°C (30 cycles). The following primers were used: ext1 dbl (5’-GGCTGCCCTACATTGATGACTCGC-3’) and extr1 dbl (5’-GATGTTGGGCACTGCTCCAGTTC-3’) for the first round and ext dbl (5’-AAGCTTGCCCTGGAGTCCACTAAAG-3’) and extr dbl (5’-GAATTCTGCCTCCAGTTCA-TCCAC-3’), for the second round. PCR products were gel separated, excised and after purification were cloned in pUC19 and sequenced using the T7-sequencing TM Kit (Amersham).
**Fluorescence microscopy.** Cells were plated onto cover slips and grown overnight before preparing them for immunofluorescence. Cells were fixed in paraformaldehyde, washed with phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100. FITC-labeled phalloidin (Sigma-Aldrich, U.S.A.) was applied to the cells as previously described (Wulf et al., 1997.)

**RESULTS AND DISCUSSION**

The medical diagnoses “Ph-positive CML” and “Ph-positive ALL” were confirmed by nested RT-PCR. Polymorphonuclear cells of peripheric blood from patients C. and K. (CML, blast crisis), patients F. and Y. (ALL), normal donors A. and B., cell lines K562 (CML, erythrocyte blast crisis), U937 (Ph-negative promonocytic leukemia) were used for fluorescence microscopy. Comparative analysis of actin distribution in polymorphonuclear leukocytes of the different donors made it possible to distinguish the following types of cell staining: 1) diffuse distribution (normal donors) (Fig. 1, D); 2) paramembrane actin distribution in the cells of patients C., F., Y., cell line K562 and U937 (Fig. 1, AB); 3) formation of amorphous cytoplasm accumulation, “dot-like structures” in one case of patient K. (CML, blast crisis) (Fig. 1, C). The BCR/ABL

![Figure 1. Comparative analysis of actin distribution in cells of patients with Ph-positive leukemias, cells lines K562 (Ph-positive) and U 937 (Ph-negative).](image)

A, B, paracortical actin distribution which is peculiar to cells from lines K562, U937 and to cells of patients C. (CML, blast crisis), Y. (ALL), F. (ALL); C, “dot-like” structures, which were detected in patient K. (CML, blast crisis); D, diffuse actin distribution in cells of normal donors.
protein is a polyfunctional protein composed of several domains with diverse properties (Gishizky et al., 1996; Butturini et al., 1996). This functional diversity of the BCR/ABL protein enables its participation in different signalling pathways. The DH domain of is presented in p210 BCR/ABL but not in p185 BCR/ABL. This domain encodes a guanine nucleotide exchange factor activity specific for Rho GTPases which modulate the cell actin structure (Chuang et al., 1995). The modulatory effect of the Dbl domain on actin structure may underlay the different transforming properties of the two types of BCR/ABL fusion proteins. We supposed here that the observed dot-like distribution of actin correlated with some mutation, which could arise in the Dbl-homology part of the BCR/ABL gene. Indeed, sequencing of the BCR/ABL amplification product from patient K. confirmed the presence of mutational changes in the Dbl homology region in positions 2127 (replacement T→C) and 2449 (replacement C→A) of BCR/ABL cDNA that corresponded to substitution in position 547 (Phe→Leu) and 654 (Thr→Lys) of the protein molecule. Therefore the mutations were likely to influence GEF function of the Dbl domain and, as a consequence, change the BCR/ABL transforming potential leading to progression in CML. The data obtained may shed light on the nature of CML blast crisis development and can be used for early detection of CML tumor progression as well as for elaboration of more effective treatment protocols.

During electrophoretic analysis in some samples together with the full-length amplification products shorter fragments were detected. Alteration of PCR conditions (i.e. increase in temperature of primer annealing, decrease of elongation time) did not significantly affect the pattern of the PCR products. For further analysis the PCR products were cloned in pUC19 vector and sequenced. Analysis of those clones revealed that the changes in length are caused by deletions in the Dbl domain of BCR/ABL gene.

<table>
<thead>
<tr>
<th>The form of disease</th>
<th>Clone number</th>
<th>The deleted domain, amino-acid residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1 (ALL)</td>
<td>CL2</td>
<td>514–733 (220)</td>
</tr>
<tr>
<td></td>
<td>CL6</td>
<td>585–620 (36)</td>
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<tr>
<td></td>
<td>115</td>
<td>556–683 (129)</td>
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<tr>
<td>Case 2 (CML)</td>
<td>2K10</td>
<td>558–712 (155)</td>
</tr>
<tr>
<td></td>
<td>2K19-1</td>
<td>517–573 (57) + 581–734 (154)</td>
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<tr>
<td></td>
<td>2K19-2</td>
<td>493–660 (168)</td>
</tr>
<tr>
<td></td>
<td>2K23</td>
<td>493–660 (168)</td>
</tr>
<tr>
<td>Case 3 (CML)</td>
<td>2L1</td>
<td>582–729 (148)</td>
</tr>
</tbody>
</table>

Table 1. Analysis of Dbl domain of BCR/ABL protein from clones obtained from blood samples of patients with CML and ALL.

Figure 2. Localization of deletions in Dbl domain of BCR/ABL gene.

Numbers of clones are on the left. Numbering of nucleotides is according to human bcr protein mRNA 5′ end (Genbank, HUMBCRD, Accession: M24603).
domain. These deletions did not affect the reading frame. Localization of the deletions in comparison with the structure of full-length Dbl domain of the BCR/ABL gene is presented in the Fig. 2. The respective deletion-dependent alterations in the p210 BCR/ABL protein are shown in the Table 1.

It is well known that in 50–60% of CML patients simultaneous expression of p210 BCR/ABL and p185 BCR/ABL genes is observed (Saglio et al., 1996; Lichty et al., 1998). Our study found novel mutations in the Dbl region of p210 encoding transcripts of CML patients. It may be suggested that the different functional forms of the fusion proteins studied affect the factors determining cell morphology and this may influence the course of the disease. The results obtained show that a mutational change occurred in the Dbl region of the BCR/ABL gene giving rise to a p210 protein whose properties could be similar to those of p185. Long term monitoring of patients with such mutations as well as increasing the number of such cases may contribute to our understanding of progression in this disease and aid in patients management.

**REFERENCES**


