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Rearrangement of the *bcr* Gene in Philadelphia-Chromosome-Negative Chronic Myeloid Leukemia

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A. Introduction

The majority of patients with chronic myeloid leukaemia (CML) have a characteristic deletion of a portion of the long arm of one chromosome 22, the Philadelphia (Ph^1) chromosome, in their myeloid cells. The missing material is reciprocally translocated to chromosome 9 such that the usual karyotype is described as $t(9;22)(q34;q11)$. In Ph^1-positive CML, the oncogene (c-*abl*) normally present on chromosome 9 is translocated to chromosome 22 [1, 2] where it comes into juxtaposition with a region named the "breakpoint cluster region" (*bcr*) [3]. A chimeric *abl*-related mRNA has been identified in cells from patients with CML [4] and is associated with the presence of a fusion protein that has tyrosine kinase activity [5]. Thus, patients with Ph^1-positive CML show evidence of rearrangement of DNA within the *bcr* region.

About 10% of patients regarded on clinical and haematological grounds as having CML lack the Ph^1 chromosome. Some of these have a leukaemia that is totally indistinguishable from Ph^1-positive disease in all other respects, while in other cases clinical and haematological features suggest that the disease is different. We report the results of studying the clinical, haematological, cytogenetic and molecular biological features in seven patients with Ph^1-negative CML. Two further patients who were classified as having chronic myelomonocytic leukaemia (CMML) are also included.

B. Materials and Methods

I. Patients

We selected for study nine patients who had attended the Hammersmith Hospital within the previous 2 years with a diagnosis of Ph^1-negative CML. Their clinical and haematological features at diagnosis are shown in Table 1.

II. Cytogenetic Analysis

Cytogenetic studies were carried out on fresh bone marrow cells or on fresh or cryopreserved buffy-coat cells collected from the patients at the time of diagnosis. Mononuclear cells were prepared from the blood samples by centrifugation on a Lymphoprep (Nyegaard, Oslo, Norway) density gradient. Cells from marrow or blood were then incubated at 37 °C at a final concentration of 1×10^6/ml in RPMI 1640 medium supplemented with 20% foetal calf serum, L-glutamine and antibiotics for a period of 24–72 h. Colcemid was then added, and the cells were harvested 1 h later. Chromosomes were prepared according to standard methods and analysed after Giemsa banding [6]. In a number of the cultures, mitoses were synchronized by addition of fluorodeoxyuridine (FDU) in accordance with the method of Webber and Garson [7]. For this purpose,

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Table 1. Clinical and haematological details of nine patients with Ph^1-negative CML

Patient	Age/ sex	Spleen cm	Peripheral blood at diagnosis											NAP	Treatment and response
			Hb g/dl	WBC × 10.9/1	Platelets × 10,9/1	Differential (% of 300 cells)									
						B1	Pm	My +Met	Neut	Eos	Baso	Mono	Lymph	NRBCs	
1.	M/24	25+	8.3	174	941	6	6	21	41	6	14	0	4	0	0	HU; PR
2.	M/58	0	11.2	170	53	1	3	28	61	0	0.5	0.5	6	0	0–12	HU; PR
3.	M/31	0	12.1	46	375	0	0	10	56	17	2	1	14	0	NA	BU, Hu; CR. BMT
4[a]	F/33	0	10.5	77	492	0	0	17	68	4	2	3	6	0	0	BU, HU; CR. BMT
5.[a]	M/62	3	10.6	105	87	0	4	31	58	0	0	1	2	2	NA	SRT; CR
6.	F/43	12	10.3	506	319	2	4	28	59	2	3	1	1	0.3	0	BU; CR
7.	F/54	3	7.0	163	545	2	4	22	58	2	9	1	1	0.3	0	BU, HU; CR
8.[b]	M/63	0	9.4	15	116	9	–	22	28	6	1	12	27	0	94	NYT
9.[b]	M/61	15	14.2	42	74	0	0	6	73	4	1	13	4	4	NA	NYT

HU, hydroxyurea; BU, busulphan; SRT, splenic irradiation; NYT, not yet treated; CR, complete haematological ressponse; PR, partial haematological response; BMT, Bone marrow transplantation; NA, not available; NAP, neutrophil alkaline phosphatase (normal range 30–100 units).

[a] Blood film morphology not typical of Ph^1-positive CML.

[b] Morphological diagnosis: CMML.

cultures were incubated for an initial period of either 24 or 48 h, after which FDU was added at a concentration of 0.1 μM. Incubation was continued for a further 18 h, after which thymidine was added to release cells from the FDU-induced block of division. Incubation was continued for a further 6 h, after which colcemid was added and the cells were harvested 30 min later. Chromosomes were prepared as described above.

III. Southern Analysis

Leukaemic cells were isolated from the peripheral blood or marrow samples, and high molecular weight DNA was prepared by standard procedures. All the samples of DNA were digested with restriction enzymes (*Bam*HI, *Bg*III, *Eco*RI, and *Hin*dIII), separated on a 0.8% agarose gel by electrophoresis and transferred to Hybond-N (Amersham) by the Southern technique [8]. The filters were prehybridized and then hybridized [9] at 43 °C with radiolabelled (^{32}P) probe. They were washed twice for 60 min in 0.1 × SSC/0.1% sodium dodecyl sulphate at 43 °C and autoradiographed for 3–5 days at −70 °C.

The 0.6-kb intron fragment (*bcr*-G) was subcloned from a commercially available *bcr* probe (Oncogene Sciences, Mineola, New York) and labelled by the oligonucleotide priming method to a specific activity of 1–3 × 10^9 cpm/μg [10].

C. Results

I. Haematology

The haematological findings for the nine patients are shown in Table 1. Their leucocyte counts ranged from 46 to 506 × 10^9/l at diagnosis. Minor dysplastic changes were present in the granulocyte series in three cases (patients 2, 5, and 7). The neutrophil alkaline phosphatase score was low in the five patients studied at diagnosis. Two of the patients were thrombocytopenic. In each case the bone marrow in aspirate smears, trephine sections or in both was hypercellular and showed gross granulocytic hyperplasia with maturation and no excess of blast cells. The morphology in five cases (1, 2, 3, 6, and 7) was indistinguishable from that characteristic of Ph^1-positive chronic granulocytic leukaemia (CGL) [11]; in two cases (4 and 5) it was atypical, and in two others (8 and 9) was characteristic of CMML. In five cases, treatment usually effective in Ph^1-positive CGL was effective in restoring the patient's blood counts to normal, but in two cases only partial responses were observed. All seven patients were alive at the time of writing. Two patients (3 and 4) had been treated

Table 2. Cytogenetic findings in the nine patients

Patient	Source of cells	Number of metaphases	Karyotype
1.	PB	38	46, *XY* (*n*=38)
		2	46, *XY, del*(22) (*pter*—*q*11:)
2.	BM	30	46, *XY del* (16) (*pter*→*q*22:)
3.	BM[a]	30	46, *XY*
4.	PB	20	46, *XX*
5.	BM[a]	20	46, *XY*
6.	PB	25	46, *XX*
7.	BM	30	46, *XX, t*(4;9;22) (9*pter*→9*q*34::22*q*11→*qter*; 22*pter*→22*q*11::4*p*11→4*pter*)
8.	BM	20	46,*XY*
9.	BM	20	46,*XY*

[a] Except in two cases, blood (PB) or bone marrow (BM) for these studies was collected before any treatment was administered. Marrow cells from patients 3 and 5 were studied after the patients had been treated with chemotherapy and splenic irradiation, respectively.

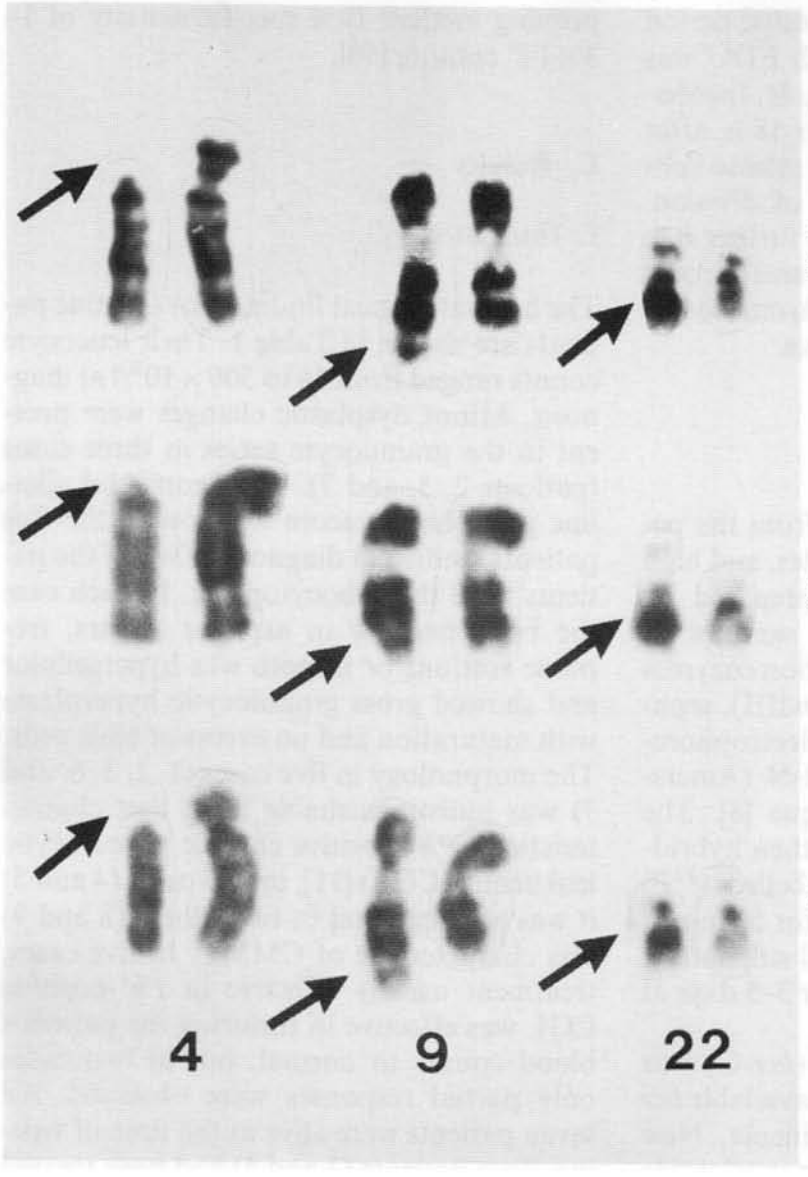

Fig. 1. Partial karyotype of three G-banded metaphases from myeloid cells of patient 1, showing *t*(4;9;22)(*p*11;*q*34;*q*11). Chromosomal material has been reciprocally translocated between chromosomes 9 and 22, resulting in 9*q*+. Moreover, material from the short arm of chromosome 4 has been translocated to the long arm of chromosome 22. These changes are indicated by *arrows*

by allogeneic bone marrow transplantation and were well 6 and 12 months after transplant, respectively.

II. Cytogenetics

The results of cytogenetic studies are summarized in Table 2. Patient 7 had a complex translocation and her karyotype may be regarded as showing a "masked" Ph^1 chromosome (Fig. 1). Patient 2 had a deletion involving chromosome 16, but the karyotype was otherwise normal. A third patient (1) had a minority population of myeloid metaphases that showed 22*q*- (but no 9*q*+), but the majority of metaphases were normal.

Table 3. Schematic representation of *bcr* gene rearrangements in the nine patients

Patient	Rearrangement detected with restriction			
	*Bam*HI	*Bg*III	*Eco*RI	*Hind*III
1.	+	+	+	−
2.	−	−	−	+
3.	−	+	−	−
4.	+	−	+	+
5.	−	−	+	−
6.	−	+	+	−
7.	+	+	+	−
8.	−	−	−	−
9.	−	−	−	−

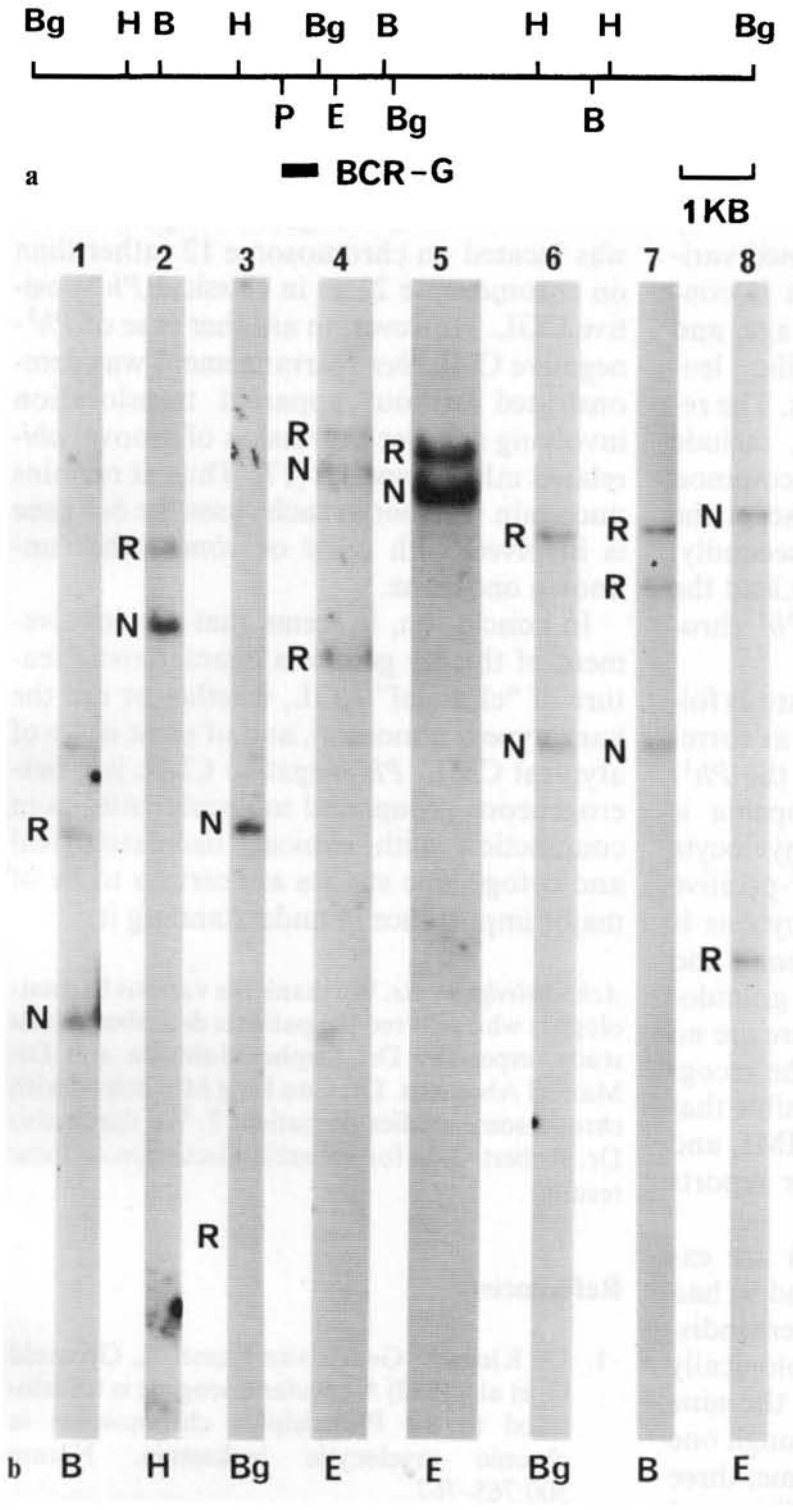

Fig. 2. a Schematic representation of the *bcr* region (3), showing the position of the probe (*bcr*-G) used. For the sake of clarity, not all PstI sites are indicated. *E, Eco*RI; *B, Bam*HI; *Bg, Bg*III; *H, Hind*III; *P,* PstI. **b** Composite of Southern blots from the seven patients, showing patterns of rearrangement for each patient. *Lanes* 1–7 show blots from each patient using DNA digested with one of the restriction endonucleases that displayed a rearrangement. The numbers correspond to the numbers given to each patient in Table 1. *Lane* 8 shows DNA from a patient with Ph^1-positive CML, digested with EcoRI and hybridized with the *bcr*-G probe. The enzyme used in each case is indicated below the corresponding lane. *N,* normal band; *R,* rearranged band(s)

The other six patients had entirely normal karyotypes.

III. Southern Analysis

Southern blots prepared from leukaemic cells and probed with the *bcr*-G probe (Fig. 2A) showed abnormal restriction bands in seven of the nine cases (Table 3). A representative Southern blot for each patient exhibiting rearrangement with one of the restriction enzymes is shown in Fig. 2B. Patients 8 and 9 did not show any rearrangements of the *bcr* region with multiple enzymes and different probes of that region (data not shown).

D. Discussion

The common form of CML is CGL (90% of cases): it has a highly characteristic clinical

and haematological profile and is associated with the presence of the Ph^1 chromosome. The remaining 10% of Ph^1-negative cases are heterogeneous and include a number of possibly unrelated conditions that have little in common, except high leucocyte counts involving the granulocytic lineage and usually enlargement of the spleen. The named variants include juvenile CML, which is confined to children under 4 years of age, and CMML and chronic neutrophilic leukaemia, confined to elderly patients. The remaining cases, found at all ages, include firstly, examples identical with the common Ph^1-positive CGL in all respects except the lack of the Ph^1 chromosome, and secondly, others with atypical features which lead the haematologist to predict that the Ph^1 chromosome will not be found.

The atypical features referred to are as follows: (a) the spleen is often smaller at corresponding leucocyte counts than in the Ph^1-positive disease; (b) thrombocytopenia is common at presentation; (c) the myelocyte "peak" characteristic of the Ph^1-positive disease is often absent; (d) monocytosis is common; (e) basophilia is exceptional; and (f) dysplastic morphology in the granulocytic series is common. Because there are no generally accepted guidelines for the recognition of these latter cases, it is possible that some at least have been called CMML and myelodysplastic syndrome in other reports on Ph^1-negative CML [12, 13, 15].

Two of our patients (4 and 5) are examples of atypical CML, two (8 and 9) had CMML and five (1, 2, 3, 6, and 7) were indistinguishable clinically and haematologically from Ph^1-positive CGL. None of the nine patients had the classical $t(9;22)$, though one (7) had a "masked" Ph^1 chromosome; three had various chromosome abnormalities and six had normal karyotypes. This work suggests that among cases of Ph^1-negative CML, patients whose disease is clinically and haematologically indistinguishable from classical Ph^1-positive CGL are likely to show evidence of *bcr* rearrangement; the only two "atypical" cases studied also showed rearrangement, but the two cases of CMML did not. We do not know whether c-*abl* is the oncogene involved in the seven patients with rearranged *bcr*. *abl*-related genetic material was demonstrated on chromosome 22 in one of the cases reported by Morris et al. [14]. Moreover, Bartram et al. [16] described a patient with clinically typical CGL who had a complex chromosomal translocation $t(9;12;22)$; cytogenetic analysis and molecular biological studies suggested that a *bcr-abl* hybrid gene was present but was located on chromosome 12 rather than on chromosome 22 as in classical Ph^1-positive CGL. However, in another case of Ph^1-negative CGL, *bcr* rearrangement was demonstrated without apparent translocation involving c-*abl* or expression of a novel *abl*-related mRNA species [17]. Thus, it remains uncertain whether in such cases the *bcr* gene is involved with c-*abl* or some other unknown oncogene.

In conclusion, it seems that the involvement of the *bcr* gene is a characteristic feature of "classical" CGL, whether or not the karyotype is abnormal, and of some cases of atypical CML. Ph^1-negative CML is a heterogeneous group, and molecular studies in conjunction with clinical, haematological and cytogenetic studies are certain to be of major importance in understanding it.

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