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Review of Clinical, Cytogenetic, and Molecular Aspects of Ph-Negative CML

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ABSTRACT: *Between 1985 and 1989, many cases of Philadelphia (Ph) chromosome negative chronic myelogenous leukemia (CML) were reported. For this review, the following selection criteria were used: the original articles on Ph-negative cases should provide clinical, hematologic, cytogenetic as well as molecular data. In addition, eight unpublished cases of Ph-negative CML are included that were studied in our institute during the last two years. Our purpose was to correlate presence or absence of the Ph rearrangement with the clinical features in an attempt to test whether the entity "Ph-negative CML" really exists and to identify the pathologic characteristics, frequency of occurrence, prognosis for survival, and underlying molecular mechanisms. Data on Ph-negative CML patients were compared with data on Ph-positive CML, atypical CML (aCML), and chronic myelomonocytic leukemia (CMMoL), reported in the same papers as the Ph negative patients. Essential for comparison of data from the different investigators appeared to be a clear description of criteria they used to establish the diagnosis CML, or alternatively a complete presentation of data for all patients reported in the articles. In most cases, Ph-negative CML was distinguishable from CMMoL and aCML, using simple criteria, e.g., differential count of peripheral blood and absence of dysplasia in the bone marrow. Cytogenetic analysis showed normal karyotype in most cases of Ph-negative CML. Interestingly, in cases with abnormal karyotype, chromosome 9 band q34 was relatively frequently involved in translocations with other chromosomes than chromosome 22, suggesting a variant Ph translocation not visible by cytogenetic techniques. This assumption was confirmed by molecular analysis, demonstrating bcr–abl rearrangement in 9 out of 10 of the latter cases. Results of cytogenetic and molecular investigations in 136 cases of Ph-negative CML reviewed in this article clearly indicated that molecular techniques are valuable tools for identification of bcr–abl rearrangements, indicative for the Ph translocation. The different mechanisms responsible for bcr–abl rearrangement in Ph-negative CML patients are discussed. The question remains whether all Ph-negative CML patients will have bcr–abl rearrangements, or whether alternative mechanisms will be identified that are responsible for this disease.*

INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematopoietic malignancy arising from neoplastic transformation of the pluripotent bone marrow stem cell. Standard findings at presentation are leukocytosis, increased granulopoiesis, sometimes increased thrombopoiesis, presence of immature granulocytic progenitors in peripheral blood, basophilia and/or eosinophilia, decreased leukocyte alkaline phosphatase (LAP), and hepatosplenomegaly. The course of the disease is biphasic. During the chronic phase, with a median duration of 1–4 years, the response to chemotherapy is usually good;

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Table 1 Phenotypes of 200 males in the registry of X-linked lymphoproliferative disease

Phenotype	n (%)
Fatal infectious mononucleosis	101 (51%)
Hypogammaglobulinemia	61 (31%)
Post-EBV	19 (19/29 = 66%)
Pre-EBV	10 (10/29 = 34%)
Malignant Lymphoma	52 (26%)
Hyperimmunoglobulinemia M	12 (6%)[a]
Marrow hypoplasia	10 (5%)
RFLP +, EBV negative	9 (5%)[b]
Lymphoid vasculitus	2 (1%)

Abbreviations: EBV, Epstein-Barr virus; RFLP, restriction fragment length polymorphism.

[a] Not directly associated with infectious mononucleosis.

[b] Six of these patients are hypogammaglobulinemic, but asymptomatic.

ciency, such as the early illness or death of males due to IM or ML are obtained from responses of families to medical- and family-history questionnaires. Files for each of the affected males and family members are maintained confidentially in the registry by kindred number. Each person is assigned a unique identification number. This information and laboratory data are stored in an IBM System/370 4381 computer (International Business Machines, Boca Raton, FL) which is accessed through several IBM and Apple (Apple Computers, Cupertineo, CA) personal computers. Data are stored and evaluated using the Statistical Analysis System (Cary, NC) [10].

Diagnosis of XLP

Each patient is assessed for the diagnostic criteria of XLP: One or more of the phenotypes (Table 1) has to occur in two or more maternally related males [2–4]. A morphological evaluation is made of slides of peripheral blood smears, surgical biopsy specimens, bone marrow, and tissues obtained at autopsy.To document the involvement of EBV, we perform a battery of tests depending on the availability of samples. Antibody titers of IgM, IgG, and IgA isotypes against viral capsid antigen (VCA) [11, 12], early antigen (EA) [13], and EBNA [14] are measured. We also have measured anti-EBNA titers by enzyme-linked immunosorbent assay (ELISA), using a synthetic EBNA peptide [15]. We stain available tissue imprints for EBNA [14] and probe for EBV genome using Southern blots hybridized with a cocktail of EBV DNA probes (cosmid clones 301–99 and 302–23, provided by Beverly Griffin) in DNA extracted from cryopreserved tissues obtained at autopsy or from surgical biopsy specimens [16]. We have also performed immunoblotting studies to search for EBV-encoded proteins in extracts of tissues from 15 male patients who died of IM [17]. Use of in situ hybridization techniques specific for EBV [18] permits us to identify EBV genome in archival tissues, and use of the polymerase chain reaction [19, 20] enables detection of low levels of EBV in blood [21].

Immunoglobulin levels are quantitated in plasma by radial immunodiffusion (RID) (Kallsted, Austin, TX) or in serum by nephelometry (Beckman ICS, Brea, CA). Serum IgG subclasses are measured by RID (ICN Immunobiologicals, Lysle, IL or The Binding Site, Birmingham, England). Reference ranges for immunoglobulin levels were established from measurements made on serum from healthy Nebraskans being evaluated for cholesterol levels (provided by our colleague, Bruce McManus, M.D., Ph.D.) and from laboratory controls.

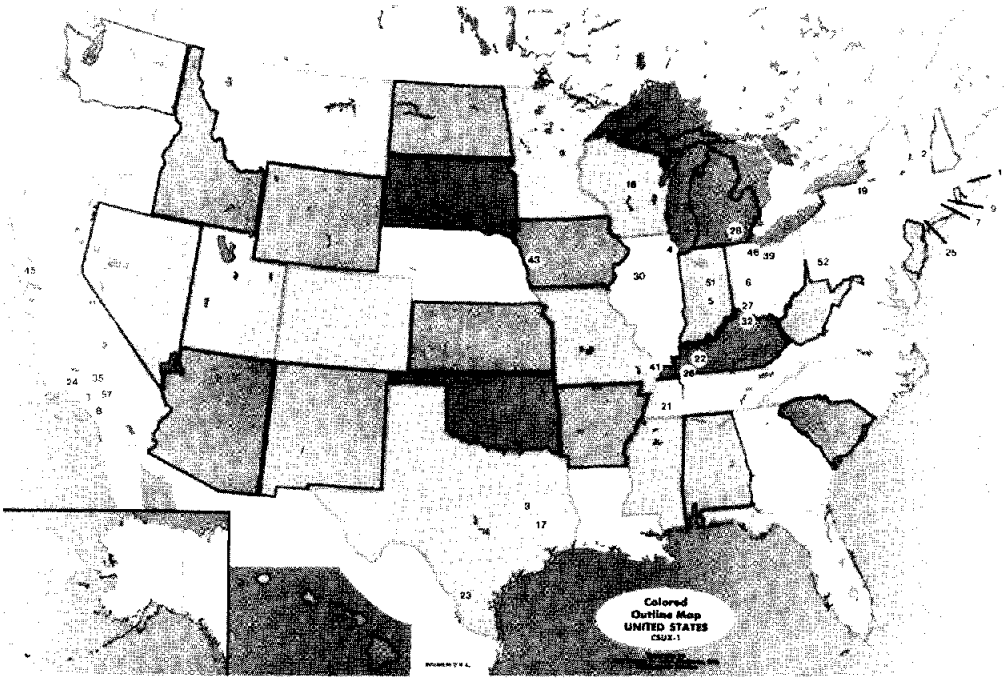

Figure 1 Map shows locations of 32 families with XLP in the United States. Numbers refer to kindred numbers.

After initial evaluation of the patient, we obtain blood from pertinent family members. We measure antibody titers to EBV to seek EBV-negative males at risk for XLP. In addition, we measure antibody responses to EBV in women at risk of being carriers because mothers of XLP patients often have elevated antibody titers to the virus [22]. We have also continued to pursue karyotyping of affected males and carriers in search of chromosomal abnormalities that might occur at the XLP locus [23]. Genetic analysis using DNA probes showing restriction fragment length polymorphisms to loci in the X chromosome (including DXS42, DXS37, and DXS12, which have been linked to the XLP locus) is performed as described previously [24–26]. When RFLP analysis is not informative, males at risk are challenged intravenously with bacteriophage ØX174 because males with XLP do not switch from IgM to IgG antibody production on secondary challenge with ØX174 [27, 28].

RESULTS

Two hundred forty males with XLP within 59 unrelated kindreds had been referred to the International XLP Registry by December 1989. Among the 222 males whose fate is known, 181 (82%) have died and 41 (18%) are living. Figures 1 and 2 show the geographical locations of the families. Noteworthy is the frequent recognition of XLP in the United States, Canada, the United Kingdom, Europe, and the Mideast, and the lack of cases referred from Central and South America, Africa, the nations of the Warsaw Pact, and the highly populated countries of Asia, including China, India, Indonesia, and Japan.

CML, atypical CML (aCML), and CMMoL. Not all hematologists are in agreement with these rather strict proposals, but they reflect on it and describe their own discriminating features for establishing the diagnosis of CML. The criteria for CML followed by the different investigators are summarized in Table 2.

Generally, there is consensus on the most essential features, i.e., leucocytosis, basophilia, hepatosplenomegaly, absence of absolute monocytosis, and absence of MDS or ANLL characteristics. As a consequence, the percentage of cases of Ph negative CML has been reduced from 10%–15% [2–4] to less than 5%, mainly by elimination of cases fulfilling the presently established criteria for CMMoL, jCML, MDS, and ANLL. Discrimination between Ph-positive and Ph-negative CML is not possible using clinical or hematologic characteristics only. The remaining group of patients with Ph-negative CML still appears heterogeneous and comprises cases that are clinically and hematologically indistinguishable from Ph-positive CML, including long survival and good therapeutic response. Other cases are atypical but resemble CML more than other well defined hematologic disorders. These are designed as aCML [30, 31].

Cytogenetic Findings in Ph Negative CML

In 127 patients, cytogenetic studies were performed at diagnosis or during the chronic phase of CML. In 9 other patients analysis was performed after blastic transformation. During chronic phase, the karyotype was found to be normal in 48 patients (Table 3); abnormal in 15 cases (Table 4); and Ph-negative, not specifying other chromosomal abnormalities, in 64 cases (Table 5). Among the 15 abnormal karyotypes, 10 showed a translocation involving chromosome 9 band q34, which is the chromosomal site involved in the Ph translocation (Table 4A). This is highly suggestive for a variant Ph translocation, in which the microscopic aspect of chromosome 22 is not visibly altered. Molecular studies confirmed this assumption, as discussed later. The rest of this group of chronic-phase CML patients with cytogenetically abnormal karyotype showed random clonal abnormalities (Table 4B). In a few patients, other translocations are detected, usually associated with subtypes of ANLL such as t(8;21), described by Wiedemann et al. [31], in a patient with atypical CML (Table 4D).

Three out of nine cases in blast crisis showed cytogenetic abnormalities (Table 4C). Remarkably, trisomy 8 and i(17q) were found in the latter cases [32]. These abnormalities are identical to the ones associated with blastic transformation of Ph-positive CML. The resemblance between Ph-negative and Ph-positive CML is also expressed in the clonal and multipotent stem cell origin of both Ph-positive and Ph-negative CML [33] and in the occurrence of lymphoid, myeloid, mixed and undifferentiated blast crisis of Ph-negative and Ph-positive CML [34, 35].

Molecular Investigations in Ph-Negative CML

The purpose of molecular investigations in Ph-negative CML is to identify the patients with *bcr–abl* rearrangement on DNA, RNA, or protein level. Comparison of cytogenetic, molecular, and clinical data between Ph-negative CML patients with or without *bcr* rearrangement and Ph-positive CML patients is important to determine the functional meaning of the Ph chromosome itself. Therefore, the strategy followed by all investigators was to screen Ph-negative CML cases for:

1. The presence of *BCR* breakpoint using Southern blot analysis.
2. Localization of c-*abl*, *bcr*, and c-*sis* oncogenes on the chromosomes applying in situ hybridization techniques.
3. Expression of *bcr–abl* mRNA using Northern blot, RNAse protection assay, or polymerase chain reaction (PCR) techniques. Both the RNAse protection assay and PCR technique give the opportunity to identify which *BCR* exon is fused to *abl*. In CML patients with t(9;22), usually *BCR* exon 2 (b2) or *BCR* exon 3 (b3) is fused to *abl* exon 2 (a2), resulting in b2a2 or b3a2 *bcr–abl* fusion region [36].

Table 2 Clinical criteria used to establish CML diagnosis and number of patients reviewed

Reference	Kurzrock et al. [44]	Bartram et al. [32, 47, 58]	Fitzgerald et al. [59]/ Morris et al. [46]	Dreazen et al. [41]/ Ganesan et al. [42]	Kantarjian et al. [60]	Ohyashiki et al. [61]	Shepherd et al. [30]/ Wiedemann et al. [31]	Others[a] [34, 37, 42, 43, 45, 55, 62–67]
Splenomegaly		+		+				
WBC (10^9/L)	>20	>30[b]		>100	>20	>20		
Absolute basophilia in PB		+	+	+			+	
Lack of absolute monocytosis (i.e., <1000/mm^3)	+		+			−		
Decreased LAP		−						
BM hyperplasia without dysplasia	+	+	+		+	+	+	
Absence of ANLL features, <20% myeloblasts + promyclocytes in BM at presentation	+	+				+		
Absence of MDS features	+	+			+	+		
Other criteria		[c]		[d,e]			[f]	
No. of patients with CML	4	41	2	4	23	1	25[g]	19
No. of patients with CML BC		3				1		5
No. of patients with aCML				5		3	10	7

[a]No detailed criteria for CML diagnosis were presented in these case reports and papers, but often clinical and hematologic data of the individual patients were mentioned.

[b]WBC > 100 × 10^9 L in Ref. [58].

[c]All stages of neutrophilic series present in differential count and good response to hydroxyurea or busulphan.

[d]Platelets more than 300 × 10^9 L.

[e]Survival more than 1 year from diagnosis.

[f]Peaks of neutrophils and myelocytes plus metamyelocytes in differential count, more than 15% immature granulocytes in peripheral blood.

[g]CML patients in which the diagnosis was not verified are included [31].

Table 3 Molecular data on CML patients with normal karyotype of leukemic cells

No. of cases	BCR breakpoint	bcr-abl mRNA	P210 bcr-abl	In situ hybridization	Reference
A. Patients with CML ($n = 48$)					
1	ND			c-*sis* on 22	Bartram et al. (1984) [62]
1	+	−		c-*abl* on 9q34	Bartram et al. (1985) [47]
1	−		−		Kurzrock et al. (1986) [44]
2	−		ND		Kurzrock et al. (1986) [44]
1	+				Bartram et al. (1986) [58]
6	−				Bartram et al. (1986) [58]
1	−			c-*abl* on 9q/22q c-*sis*, 3′*bcr* on 22	Morris et al. (1986) [46] and Fitzgerald et al. (1987) [59]
1	+			ND	Morris et al. (1986) [46] and Fitzgerald et al. (1987) [59]
1	+[a]	ND		ND	Ganesan et al. (1986) [42] and Dreazen et al. (1987) [41]
1	+	+, (b_2a_2 + b_3a_2)		c-*abl* on 9/22 *bcr* on 22q11 c-*sis* on 22	Ganesan et al. (1986) [42] and Dreazen et al. (1987) [41]
1	+	+, (b_3a_2)		c-*abl*, *bcr* on 22q11	Dreazen et al. (1987) [41]
1	+	ND		c-*abl*, *bcr* on 22q11	Dreazen et al. (1987) [41]
1	+				Ohyashiki et al. (1988) [61]
2	+				Weinstein et al. (1988) [63]
1	+				Eisenberg et al. (1988) [64]
7[c]	+		+(4/4)[b]		Wiedemann et al. (1988) [31]
4[c]	−		−		Wiedemann et al. (1988) [31]
4	+				Bartram et al. (1988) [32]
1	+				LoCoco et al. (1989) [65]
1	+	+, (b_2a_2)		5′*bcr*, c-*abl* on 1p 3′*bcr* on 9 c-*sis* on 22	Van der Plas et al. (1989) [45]
1	+	+, (b_3a_2)		5′*bcr*, c-*abl* on 1p 3′*bcr*, c-*sis* on 22	Van der Plas et al. (1989) [45]
1	+	+, (b_2a_2)			Van der Plas et al. (this report)

Table 3 (*Continued*)

No. of cases	BCR breakpoint	bcr-abl mRNA	P210 bcr-abl	In situ hybridization	Reference
7	–	- (5/5)[b]			Van der Plas et al. (this report)
B. Patients with CML BC (n = 6)					
1					Bartram et al. (1986) [58]
4			–		Maxwell et al. (1987) [34]
1	+				Ohyashiki et al. (1988) [61]
C. Patients with atypical CML (n = 16)					
1	+[a]				Ganesan et al. (1986) [42]
1	+				Ganesan et al. (1986) [42]
2	+	+ (b_2a_2)			Dreazen et al. (1987) [41]
2					Ohyashiki et al. (1988) [61]
4	–		–, (2/4)[b]		Wiedemann et al. (1988) [31]
6	–				Cogswell et al. (1989) [55]

Abbreviation: ND, not done.

[a]Extra bands in one restriction enzyme digest.

[b]Number of cases observed/number of cases investigated.

[c]CML diagnosis could not be verified.

4. Detection of 210 kD *bcr–abl* protein (P210), e.g., by means of autophosphorylation assays.

The results of these molecular investigations are presented in detail in Tables 3–6 together with the corresponding cytogenetic data. (An overview of these data for CML patients is provided in Table 7.)

In summary, we can make the following points:

1) Fifty-eight out of 136 Ph-negative CML patients (including the cases in which CML diagnosis could not be verified [31]) showed evidence of *BCR* rearrangement, *bcr–abl* mRNA expression, or the presence of a 210 kD *bcr–abl* protein.

2) Southern blot analysis detected a BCR breakpoint in 9 out of 10 Ph-negative CML patients with cytogenetic abnormalities involving chromosome 9 band q34, indicative for variant Ph translocation. Only one patient showed involvement of chromosome 9 band q34 without *BCR* rearrangement, although clinical and hematologic data were in favor of CML diagnosis [37]. However, it should be noticed that molecular data are scarce in this article: No details are mentioned about number of restriction enzyme digestions or probes used. Therefore, it cannot be ruled out that this patient also has a *BCR* rearrangement that was not detected in this study. When really no *BCR* breakpoint can be found using Southern blot analysis, it is worthwhile to search for a breakpoint more 5′ in the BCR gene, e.g., using PCR technique on cDNA or pulse field gel electrophoresis (PFGE) on DNA. This case is possibly comparable with Ph-positive, *BCR*-negative cases that are described by Selleri et al. [38, 39] and had a breakpoint in the first intron of the *BCR* gene or with the Ph-positive *BCR*-

Table 4 Cytogenetic and molecular data on patients with abnormal karyotype of leukemic cells

No. of cases	Karyotype	*BCR* breakpoint	*bcr–abl* mRNA	P210 *bcr–abl*	Reference
A. CML patients with translocations involving 9q34 (n = 10)					
1	t(9;12)(q34;q21)[a]	+			Bartram et al. (1985) [43]
1	t(9;11)(q34;q13)	+	+	+	Kurzrock et al. (1986) [44]
1	t(8;9)(?;q34)	+			Bartram et al. (1988) [32]
1	t(9;18)(q34;?)	+			Bartram et al. (1988) [32]
1	t(9;12)(q34;q13)	+			Weinstein et al. (1988) [63] and Eisenberg et al. (1988) [64]
1	t(9;11)(q34;q11)	+			Weinstein et al. (1988) [63] and Eisenberg et al. (1988) [64]
1	t(8;9)(q22;q34)	+			Weinstein et al. (1988) [63]
1	t(2;9)(?;q34)	+			Wiedemann et al. (1988) [31]
1	t(3;7)(q21;q32), t(4;9)(q21;q34),del(8)(q22)	–[b]			Wang et al. (1988) [37]
1	t(9;9)(p13;q34)	–			Sessarego et al. (1989) [67]
B. CML patients with translocations not involving 9q34 (n = 5)					
1	t(20;21)(q11;q22)	–			Weinstein et al. (1988) [63]
1d	N/t(5;6)	+		ND	Wiedemann et al. (1988) [31]
1d	t(3;5)	–		ND	Wiedemann et al. (1988) [31]
1d	t(9;15)(q22;q22),t(11;20)	–		–	Wiedemann et al. (1988) [31]
1	t(11;22)(q23;q13),del 7q, del(13)	–		–	Wiedemann et al. (1988) [31]
C. CML patients in BC with abnormal karyotype, 9q34 not involved (n = 3)					
1	46,XY/47,XY, + 8	–			Bartram et al. (1986) [58]
1	46,XY/46,XY,i(17q)	–			Bartram et al. (1986) [58]
1	46,XX,t(7p q),13q + ,13q –).	ND	+		Andrews et al. (1987) [66]
D. Atypical CML patients with abnormal karyotype (n = 4)					
1	del(16)(q22)	+[c]			Ganesan et al. (1986) [42] and Dreazen et al. (1987) [41]
1	7q –	–			Ohyashiki et al. (1988) [61]
1	t(8;21)	–			Wiedemann et al. (1988) [31]
1	47,XY, + 8	–			Cogswell et al. (1989) [55]

Abbreviation: ND, not done.

[a]Results in situ hybridization studies: c-*abl* on 12q –, 5′-*bcr* on 12q –, 3′-*bcr* on 9q +, c-*sis* on 22.

[b]No detailed molecular data presented in this article.

[c]Extra bands in one restriction enzyme digest only.

[d]CML patient in which diagnosis could not be verified.

? Localization of breakpoint not mentioned.

Table 5 Molecular data on patients with no abnormalities of chromosome 22 in leukemic cells (karyotype not further specified)

No. of cases	*BCR* breakpoint	*bcr–abl* mRNA	P210 *bcr–abl*	Reference
A. CML patients (n = 64)				
6	+			Shepherd et al. (1987) [30]
2	–			Shepherd et al. (1987) [30]
4	–			Eisenberg et al. (1988) [64]
11	+	5/5 –		Kantarjian et al. (1988) [60]
12	–			Kantarjian et al. (1988) [60]
1[a]	–		–	Wiedemann et al. (1988) [31]
27	–			Bartram et al. (1988) [32]
1	+			LoCoco et al. (1989) [65]
B. Atypical CML patients (n – 5)				
4				Shepherd et al. (1987) [30]
1	–			Wiedemann et al. (1988) [31]

[a]CML patient in which diagnosis could not be verified [31].

negative CML patient described by Bartram et al. [40], which had a breakpoint in the *bcr* gene located 5′ of the *BCR* region but 3′ of the region described by Selleri et al. [38, 39]

3) In 20 out of 25 cases of aCML, no *BCR* breakpoint was detected. The five exceptions with a *BCR* breakpoint were all reported by the same research group [41, 42]. It would be interesting to reexamine the differential count and other clinical data to check if these patients really belong to the group of aCML or resemble more CML. To the best of our knowledge, no CMMoL or juvenile CML cases are published in which a *BCR* rearrangement was identified. In conclusion, *bcr–abl* rearrangement is strongly associated with the morphologic features of CML, although few exceptions still exist.

4) The percentage of Ph-negative CML patients with *BCR* rearrangement versus no *BCR* rearrangement varied between the different authors, e.g., Bartram et al. [32, 58] reported 3 out of 12 cases *BCR*-positive; Ganesan et al. [42] and Dreazen et al. [41], 5 out of 5; Fitzgerald and Morris [46, 59], 2 out of 2; Wiedemann et al. [31], 8 out of 8 (5 out of 9 among the cases that were not morphologically reexamined); Kantarjian et al. [60], 11 out of 23; and our group, 5 out of 12 [45, this report]. In our opinion, there are two main reasons responsible for these differences. First, the different authors used clinical, hematologic and morphologic criteria that are not exactly the same, resulting in differences in diagnosis. Second, some authors [41, 42] diagnosed *BCR* breakpoints on extra bands in only one out of several different restriction enzyme

Table 6 Molecular data on CMMoL patients

No. of cases	*BCR* breakpoint	*bcr abl* mRNA	P210 *bcr–abl*	In situ hybridization	Reference
A. Normal karyotype (n = 3)					
2	–				Morris et al. (1986) [46]
1	ND			c-*abl* on 9q	Fitzgerald et al. (1987) [59]
B. Ph negative, karyotype not further specified (n = 18)					
1	–				Shepherd et al. (1987) [30]
17	–				Kantarjian et al. (1988) [60]

Abbreviation: ND, not done.

digests. In such cases, the occurrence of a restriction enzyme polymorphism is a more likely cause for the aberrant fragment than the presence of a *BCR* breakpoint. In such cases, additional analysis, e.g., at the protein or RNA level, is required to prove *bcr–abl* rearrangement.

5) Molecular data presented in Tables 3–5 indicate that several mechanisms can play a role in Ph-negative CML. A summary follows.

Bcr–abl recombination takes place in the same way as in Ph-positive CML but is cytogenetically not visible. Examples of complex Ph translocations in Ph-negative CML are provided by Bartram et al. [43], Kurzrock et al. [44] and our data [45]. In situ hybridization studies of Ph-negative CML patients reported by Bartram et al. [43] and our own group [45] provided evidence that 5′-*bcr* and c-*abl* were localized on the same chromosomal segment. However, in these special cases, the hybrid *bcr–abl* gene was present on a third chromosome instead of on the Ph chromosome. In these cases, the localization of the hybrid *bcr–abl* gene indicated that complex Ph translocations had occurred, although the aspect of chromosome 22 was visibly unaltered.

Insertion of part of the *abl* gene in the *bcr* gene without reciprocal translocation to chromosome 9 has been described by Morris et al. [46] and Dreazen et al. [41].

Based on investigations in a Ph-negative CML patient in which BCR was rearranged without juxtaposition of c-*abl*, Bartram [47] proposed the hypothesis that *bcr* or *abl* can work in combination with yet another oncogene. Thus far, there is no further evidence for this hypothesis.

Several other possibilities remain open for discussion in Ph-negative, BCR-negative cases indistinguishable from Ph-positive CML on clinical and hematologic as well as morphologic criteria. Three hypothetic mechanisms could explain these phenomena:

1) The breakpoint might be located outside the BCR, but within the BCR gene as described by Selleri et al. [38, 39] and Bartram et al. [40] in Ph-positive CML cases. Both authors reported breakpoint localizations more 5′ in the *BCR* gene.

2) Abl possibly cooperates with an as yet unknown oncogene.

3) Neither *bcr* nor *abl* are responsible for the disease in exceptional cases, but other oncogenes might be. Thus far, the few data available on this subject do not identify candidate genes for this latter hypothesis [48–54]. Recently, Cogswell et al. [55] reported that using the polymerase chain reaction very few *ras* mutations were detectable in CML, i.e., in 1 out of 18 Ph-positive CML patients in blast crisis and in 0 out of 39 Ph-positive CML cases in chronic phase. However, in Ph-negative, BCR-negative atypical CML (aCML), they [55] demonstrated the presence of *ras* mutations in 54% (i.e., 7/13) of the cases. This high frequency of *ras* mutations is comparable with results obtained by Padua et al. [56] in CMMoL patients. CMMoL and aCML also share several clinical and hematologic features. The authors therefore conclude that aCML is a subgroup of CMMoL and that both diseases belong to MDS rather than CML.

CONCLUSION

Correct diagnosis of CML is essential when efforts are made to correlate clinical features with molecular changes in Ph-negative CML. The data reviewed in this article do not identify any clinical or hematologic characteristic that is unique for Ph-negative CML. We expected that in nearly all Ph-negative CML patients, indistinguishable from Ph-positive CML on clinical and hematologic grounds, *bcr–abl* rearrangement will be detected using molecular analysis. The data on Ph-negative CML reviewed in this article show the presence of *bcr–abl* rearrangement in 43% of the cases (Table 7). Although no evidence was found for *bcr–abl* rearrangement in the remaining 57%, in many cases no definitive proof was provided to rule out this possibility. On the other hand, it can not be denied that several CML patients are reported with classical CML disease without the presence of *bcr–abl* rearrangement. Very recently, this was confirmed by Kurzrock et al. [57], who reported on 11 Ph-negative, BCR-negative CML

Table 7 Distribution of Ph-negative CML patients in chronic phase or blast crisis according to results of cytogenetic and molecular studies

No. of cases	Karyotype	Molecular evidence for BCR rearrangement	
		Yes	No
54	Normal	28	26
10	Abnormal, 9q34 involved	9	1
8	Abnormal, q34 not involved	3	5
64	Ph-negative, karyotype not specified	18	46

cases investigated in the MD Anderson Cancer Center using Southern and Northern blot analysis. They represented about 3% of the CML cases studied in the same period in that institute. In addition to our findings, Kurzrock et al. reported that, although the early stage of BCR-negative and BCR-positive CML shows striking resemblance, disease progression manifests distinctly.

In the Ph-negative patients (the aCML patients) that do not fulfill all criteria for CML, a more heterogeneous picture can be expected, showing activation of other oncogenes than *bcr* and *abl* e.g., *ras*, in some cases.

A controlled multicenter study of Ph-negative CML patients who are clinically, hematologically, and cytogenetically well characterized should form the basis for future molecular investigations necessary to elucidate the mechanisms responsible for Ph negative CML and to apply this knowledge to determine choice of therapy and prognosis.

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REFERENCES

1. Nowell PC and Hungerford DA (1960): A minute chromosome in human chronic granulocytic leukemia. Science 132:1497.
2. Kantarjian HM, Keating MJ, Walters RS, McCredie KB, Smith TL, Talpaz M, Beran M, Cork A, Trujillo JM, Freireich EJ (1986): Clinical and prognostic features of Philadelphia chromosome negative chronic myelogenous leukemia. Cancer 58:2023–2030.
3. Sandberg AA (1980): The cytogenetics of chronic myelocytic leukemia (CML): chronic phase and blastic crisis. Cancer Genet Cytogenet 1:217–228.
4. Canellos GP, Whang-Peng J, DeVita VT (1975): Chronic granulocytic leukemia without Philadelphia chromosome. Am J Clin Pathol 65:467–470.
5. Bennett JM, Catovski D, Daniel MT, Flandrin C, Galton DAG, Gralnick HR, Sultan C (1982): Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189–199.
6. Laszlo J (1975): Myeloproliferative disorders (MPD): Myelofibrosis, myelosclerosis, extra medullary hematopoiesis, undifferentiated MPD, and hemorrhagic thrombocythemia. Semin Hematol 12:409–432.
7. Greenberg PL, Bagby GC (1982): The preleukemic syndrome (hematopoietic dysplasia). In:

Hematologic Malignancies in the Adult. SR Newcom, ME Kadin, eds., Addison-Wesley, Reading, MA, pp. 1–7.

8. Smith KL, Johnson W (1974): Classification of chronic myelocytic leukemia in children. Cancer 34:670–679.
9. Bennett JM, Catovski D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C (1976): Proposals for the classification of the acute leukemias. Br J Haematol 33:451–458.
10. Rowley JD (1973): A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature 243:290–293.
11. Sandberg AA (1980): Chromosomes and causation of human cancer and leukemia: XL. The Ph and other translocations in CML. Cancer 46:2221–2226.
12. Heim S, Billstrom R, Kristoffersson U, Mandahl N, Strömbeck B, Mitelman F (1985): Variant Ph translocations in chronic myeloid leukemia. Cancer Genet Cytogenet 18:215–227.
13. De Braekeleer M (1987): Variant Philadelphia translocations in chronic myeloid leukemia. Cytogenet Cell Genet 44:215–222.
14. Hagemeijer A, de Klein A, Gödde-Salz E, Turc-Carel C, Smit EME, van Agthoven AJ, Grosveld GC (1985): Translocation of c-abl to "masked" Ph in chronic myeloid leukemia. Cancer Genet Cytogenet 18:95–104.
15. Alimena G, Hagemeijer A, Bakhuis J, De Cuia MR, Diverio D, Montefusco E (1987): Cytogenetic and molecular characterization of a masked Philadelphia chromosome in chronic myelocytic leukemia. Cancer Genet Cytogenet 27:21–26.
16. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G (1984): Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 36:93–99.
17. Shtivelman E, Gale RP, Dreazen O, Berrebi A, Zaizov R, Kubonish I, Miyoshi I, Canaani E (1987): bcr–abl RNA in patients with chronic myelogenous leukemia. Blood 69:971–973.
18. Kurzrock R, Kloetzer WS, Talpaz M, Blick M, Walters R, Arlinghaus RB, Gutterman JU (1987): Identification of molecular variants of P210 bcr–abl in chronic myelogenous leukemia. Blood 70:233–236.
19. Konopka JB, Witte ON (1985): Detection of c-abl tyrosine kinase activity in vitro permits direct comparison of normal and altered c-abl gene products. Mol Cell Biol 5:3116–3123.
20. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartram CR, Grosveld G (1983): Localization of the c-abl oncogene adjacent to a translocation breakpoint in chronic myelocytic leukemia. Nature 306:239–242.
21. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, Geurts van Kessel A, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, Heisterkamp N, Stephenson JR, Groffen J (1983): Translocation of c-abl oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature 306:277–280.
22. Hagemeijer A, Bartram CR, Smit EME, van Agthoven AJ, Bootsma D (1984): Is chromosomal region 9q34 always involved in variants of the Ph translocation? Cancer Genet Cytogenet 13:1–16.
23. Bartram CR, Anger B, Carbonell F, Kleihauer E (1985): Involvement of chromosome 9 in variant Ph translocation. Leuk Res 9:1133–1137.
24. Morris CM, Rosman I, Archer SA, Cochrane JM, Fitzgerald PH (1988): A cytogenetic and molecular analysis of five variant Philadelphia translocations in chronic myeloid leukemia. Cancer Genet Cytogenet 35:179–197.
25. Ezdinly EZ, Sokal JE, Crosswhite L, Sandberg AA (1970): Philadelphia chromosome positive and negative chronic myelocytic leukemia. Ann Intern Med 72:175–182.
26. Pugh WC, Pearson M, Vardiman JW, Rowley JD (1985): Philadelphia chromosome-negative chronic myelogenous leukaemia: a morphologic reassessment. Brit J Haematol 60:457–467.
27. Travis LB, Pierre RV, DeWald GW (1986): Ph-negative chronic granulocytic leukemia: A nonentity. Am J Clin Pathol 85:186–193.
28. Spiers ASD, Bain BJ, Turner JE (1977): The peripheral blood in chronic granulocytic leukaemia. Scand J Haematol 18:25–38.
29. Galton DAG (1982): The chronic myeloid leukaemias. In: Blood and Its Disorders, RM Hardisty, DJ Weatherall, eds. Blackwell, Oxford, pp. 877–917.
30. Shepherd PCA, Ganesan TS, Galton DAG (1987): Haematological classification of the chronic

myeloid leukaemias. In: Baillière's Clinical Haematology (1:4), Chronic Myeloid Leukaemia, JM Goldman, ed. Baillière Tindall, London, pp. 887–906.

31. Wiedemann LM, Karhi KK, Shivji MKK, Rayter SI, Pegram SM, Dowden G, Bevan D, Will A, Galton DAG, Chan LC (1988): The correlation of breakpoint cluster region rearrangement and p210 phl/abl expression with morphological analysis of Ph-negative chronic myeloid leukemia and other myeloproliferative diseases. Blood 71:349–355.
32. Bartram CR (1988): Rearrangement of c-abl and bcr genes in Ph-negative CML and Ph-positive acute leukemias. Leukemia 2:63–64.
33. Fialkow PJ, Jacobson RJ, Singer JW, Sacher RA, McGuffin RW, Neefe JR (1980): Philadelphia chromosome (Ph)-negative chronic myelogenous leukemia (CML): A clonal disease with origin in a multipotent stem cell. Blood 56:70–73.
34. Maxwell SA, Kurzrock R, Parsons SJ, Talpaz M, Gallick GE, Kloetzer WS, Arlinghaus RB, Kouttab NM, Keating MJ, Gutterman JU (1987): Analysis of p210 bcr–abl tyrosine protein kinase activity in various subtypes of Philadelphia chromosome-positive cells from chronic myelogenous leukemia patients. Cancer Res 47:1731–1739.
35. Hughes A, McVerry BA, Walker H, Bradstock KF, Hoffbrand AV, Janossy G (1981): Heterogeneous blast cell crisis in Philadelphia negative chronic granulocytic leukaemia. Br J Haematol 47:563–569.
36. Hermans A, Gow J, Selleri L, von Lindern M, Hagemeijer A, Wiedemann LM, Grosveld G (1988): bcr–abl oncogene activation in Philadelphia chromosome-positive acute lymphoblastic leukemia. Leukemia 2:628–633.
37. Wang TY, Raza A, Fan YS, Sait SNJ, Kirschner J, Sandberg AA (1988): Complex cytogenetic changes in Ph-negative chronic myelogenous leukemia. Cancer Genet Cytogenet 31:241–245.
38. Selleri L, Narni F, Emilia G, Colò A, Zucchini P, Venturelli D, Donelli A, Torelli U, Torelli G (1987): Philadelphia-positive chronic myeloid leukemia with a chromosome 22 breakpoint outside the breakpoint cluster region. Blood 70:1659–1664.
39. Selleri L, von Lindern M, Hermans A, Meijer D, Torelli G, Groveld G (1990): Chronic myeloid leukemia may be associated with several BCR-ABL transcripts including the "ALL type" 7 kb transcript. Blood, 75:1146–1153.
40. Bartram CR, Bross-Bach U, Schmidt H, Waller HD (1987): Philadelphia-positive chronic myelogenous leukemia with breakpoint 5' of the breakpoint cluster region but within the bcr gene. Blut 55:505–511.
41. Dreazan O, Rassool F, Sparkes RS, Klisak I, Goldman JM, Gale RP (1987): Do oncogenes determine clinical features in chronic myeloid leukaemia? Lancet i:1402–1405.
42. Ganesan TS, Rassool F, Guo AP, Th'ng KH, Dowding C, Hibbin JA, Young BD, White H, Kumaran TO, Dalton DAG, Goldman JM (1986): Rearrangement of the bcr gene in Philadelphia chromosome-negative chronic myeloid leukemia. Blood 68:957–960.
43. Bartram CR, Kleihauer E, de Klein A, Grosveld G, Teyssier JR, Heisterkamp N, Groffen J (1985): c-abl and bcr are rearranged in a Ph-negative CML patient. EMBO J 4:683–686.
44. Kurzrock R, Blick MB, Talpaz M, Velasquez WS, Trujillo JM, Kouttab NM, Kloetzer WS, Arlinghaus RB, Gutterman JU (1986): Rearrangement in the breakpoint cluster region and the clinical course in Philadelphia-negative chronic myelogenous leukemia. Ann Intern Med 105:673–679.
45. Van der Plas DC, Hermans ABC, Soekarman D, Smit EME, de Klein A, Smadja N, Alimena G, Goudsmit R, Grosveld G, Hagemeijer A (1989): Cytogenetic and molecular analysis in Philadelphia negative CML. Blood 73:1038–1044.
46. Morris CM, Reeve AE, Fitzgerald PH, Hollings PE, Beard ME, Heaton DC (1986): Genomic diversity correlates with clinical variation in Ph-negative chronic myeloid leukaemia. Nature 320:281–283.
47. Bartram CR (1985): bcr rearrangement without juxtaposition of c-abl in chronic myelocytic leukemia. J Exp Med 162:2175–2179.
48. Collins SJ, Howard M, Andrews DF, Agura E, Radich J (1989): Rare occurrence of N-ras point mutations in Philadelphia chromosome positive chronic myeloid leukemia. Blood 73:1028–1032.
49. Slamon DJ, deKernion JB, Verma IM, Cline MJ (1984): Expression of cellular oncogenes in human malignancies. Science 224:256–262.

50. Liu E, Hjelle B, Bishop JM (1988): Transforming genes in chronic myelogenous leukemia. Proc Natl Acad Sci USA 85:1952–1956.

51. Eva A, Tronick SR, Gol RA, Pierce JH, Aaronson SA (1983): Transforming genes of human hematopoietic tumors: Frequent detection of ras-related oncogenes whose activation appears to be independent of tumor phenotype. Proc Natl Acad Sci USA 80:4926–4930.

52. Blick M, Westin E, Gutterman J, Wong-Stahl F, Gallo R, McCredie K, Keating M, Murphy E (1984): Oncogene expression in human leukemia. Blood 64:1234–1239.

53. Bartram CR (1985): Activation of proto-oncogenes in human leukemias. Blut 51:63–71.

54. Mars WM, Florine DL, Talpaz M, Saunders GF (1985): Preferentially expressed genes in chronic myelogenous leukemia. Blood 65:1218–1225.

55. Cogswell PC, Morgan R, Dunn M, Neubauer A, Nelson P, Poland-Johnston NK, Sandberg AA, Liu E (1989): Mutations of the ras protooncogenes in chronic myelogenous leukemia: A high frequency of ras mutations in bcr/abl rearrangement-negative chronic myelogenous leukemia. Blood 74:2629–2633.

56. Padua RA, Carter G, Hughes D, Gow J, Farr C, Oscier D, McCormick F, Jacobs A (1988): RAS mutations in myelodysplasia detected by amplification, oligonucleotide hybridization, and transformation. Leukemia 2:503–510.

57. Kurzrock R, Kantarjian HM, Shtalrid M, Gutterman JU, Talpaz M (1990): Philadelphia chromosome-negative chronic myelogenous leukemia without breakpoint cluster region rearrangement: A chronic myeloid leukemia with a distinct clinical course. Blood 75:445–452.

58. Bartram CR, Carbonell F (1986): bcr rearrangement in Ph-negative CML. Cancer Genet Cytogenet 21:183–184.

59. Fitzgerald PH, Beard MEJ, Morris CM, Heaton DC, Reeve AE (1987): Ph-negative chronic myeloid leukaemia. Br J Haematol 66:311–314.

60. Kantarjian HM, Shtalrid M, Kurzrock R, Blick M, Dalton WT, LeMaistre A, Stass SA, McCredie KB, Gutterman J, Freireich EJ, Talpaz M (1988): Significance and correlations of molecular-analysis results in patients with Philadelphia chromosome-negative myelogenous leukemia and chronic myelomonocytic leukemia. Am J Med 85:639–644.

61. Ohyashiki JH, Ohyashiki K, Ito H, Toyama K (1988): Molecular and clinical investigations in Philadelphia chromosome-negative chronic myelogenous leukemia. Cancer Genet Cytogenet 33:119–126.

62. Bartram CR, de Klein A, Hagemeijer A, Grosveld G, Heisterkamp N, Groffen G (1984): Localization of the human c-sis oncogene in Ph positive and Ph-negative chronic myelocytic leukemia by in situ hybridization. Blood 63:223–225.

63. Weinstein ME, Grossman A, Perle MA, Wilmot PL, Verma RS, Silver RT, Arlin Z, Allen SL, Amorosi E, Waintraub SE, Shapiro LP, Benn PA (1988): The karyotype of Philadelphia chromosome-negative, bcr rearrangement-positive chronic myeloid leukemia. Cancer Genet Cytogenet 35:223–229.

64. Eisenberg A, Silver R, Soper L, Arlin Z, Coleman M, Bernhardt B, Benn P (1988): The location of breakpoints within the breakpoint cluster region (bcr) of chromosome 22 in chronic myeloid leukemia. Leukemia 2:642–647.

65. Lo Coco F, Saglio G, De Fabritiis P, Diverio D, Guerrasio A, Rosso C, Meloni G, Mancini M, Mandelli F (1989): Molecular evidence of transient complete remission after autographting in Ph⁻/bcr rearranged chronic myelogenous leukemia. Br J Haematol 72:285–290.

66. Andrews III DF, Collins SJ (1987): Heterogeneity in expression of the bcr–abl fusion transcript in CML blast crisis. Leukemia 1:718–724.

67. Sessarego M, Mareni C, Vimercati R, Defferrari R, Origone P, Damasio E, Ajmar F (1989): Translocation t(9;9)(p13;q34) in Philadelphia-negative chronic myeloid leukemia with breakpoint cluster region rearrangement. Cancer Genet Cytogenet 43:51–56.