

Evidence of clonality in chronic neutrophilic leukaemia

J Böhm, S Kock, H E Schaefer, P Fisch

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Background: Chronic neutrophilic leukaemia (CNL) is a rare myeloproliferative disorder of elderly patients characterised by sustained neutrophilia and splenomegaly. The diagnosis of CNL requires the exclusion of BCR/ABL positive chronic myelogenous leukaemia (CML) and of leukaemoid reactions (LRs). The differentiation between CNL and LR is problematic because both conditions share similar morphological features; it is also important because patients with CNL generally have a poor prognosis.

Aims: To determine whether CNL and LR could be distinguished on the basis of different clonality patterns.

Methods: Blood samples from 52 women were studied using the human androgen receptor gene assay (HUMARA).

Results: Monoclonality was found in the neutrophils in all 17 patients with different myeloproliferative syndromes (MPSs), including those with CNL. In four of the patients with CNL, autologous T cells were also monoclonal, suggesting that they belonged to the neoplastic clone. This finding was in contrast to other MPSs in which T cells were almost always polyclonal. Of nine patients with clinically suspected LR, the neutrophils of five were polyclonal, whereas three patients had monoclonal neutrophils, suggesting that they might be in the process of developing an MPS. Among 26 healthy blood donors, 20 had polyclonal neutrophils and five showed skewed clonality patterns. One case of LR and one normal blood donor were scored "not informative" at the HUMARA locus.

Conclusions: Clonality studies of blood neutrophils using HUMARA aid in distinguishing female patients with monoclonal CNL from those with LR. For the diagnosis of CNL, monoclonality of the neutrophils should be demonstrated whenever possible.

See end of article for authors' affiliations

Correspondence to:
Dr J Böhm, Department of Pathology, University of Freiburg, Postfach 214, D-79002 Freiburg, Germany;
jboehm@ukl.uni-freiburg.de

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Chronic neutrophilic leukaemia (CNL) is a rare myeloproliferative syndrome (MPS) of elderly patients showing sustained neutrophilia and splenomegaly.^{1–4} To date, only 143 cases of CNL have been reported in the literature, including 14 of our own cases reported recently.³ The diagnosis of CNL is based on the exclusion of chronic myelogenous leukaemia (CML) and of leukaemoid reactions (LRs).² In contrast to CML, which is characterised by a BCR/ABL translocation, no definite molecular marker is known in CNL.^{1,2} The differential diagnosis between CNL and LR may be difficult or even impossible because both conditions share identical morphological features, including a raised neutrophil alkaline phosphatase (NAP) score. The spectrum of disorders capable of causing LR is so wide that clinicians may not be able to exclude all possible causes of LR. It is important to differentiate CNL from LR because the prognosis of patients with CNL is poor, even worse than that of those with CML.⁶

"The diagnosis of chronic neutrophilic leukaemia is based on the exclusion of chronic myelogenous leukaemia and of leukaemoid reactions"

The human androgen receptor gene assay (HUMARA) for the analysis of clonality in tissues from female patients examines the inactivation patterns of the human androgen receptor gene on the X chromosome.⁷ This method relies on the length polymorphism of a human androgen receptor gene exon, which has restriction sites for methylase sensitive enzymes. Ideally, in polyclonal conditions such as LR, 50% of the cells show methylation of the maternal allele and 50% of the cells show methylation of the paternal allele. In contrast, neoplastic tissues such as leukaemic CML cells typically show complete methylation of one allele and demethylation of the other allele. In this study, we investigated blood samples of patients using HUMARA to determine whether CNL and LR

could be distinguished on the basis of X chromosomal inactivation patterns.

MATERIALS AND METHODS

Selection of patients

In the archives of our department, which receives more than 10 000 bone marrow biopsies annually, we found five living female patients fulfilling the morphological and clinical criteria of CNL. These five patients belonged to a group of 14 CNL cases that we have reported previously.³ Clinically, these patients showed chronic neutrophilia, a variable degree of splenomegaly, and no thrombocytosis. The bone marrow was strongly hypercellular with expansion of the neutrophilic granulopoiesis, which was not left shifted. In the blood, moderate leucocytosis was present, with an excess of mature neutrophils and bands. In four of the cases the blood also contained myelocytes, but there were no blasts (table 1). In all our CNL cases, the NAP score was increased and the BCR/ABL translocation was excluded by reverse transcription polymerase chain reaction (PCR) and, in addition, by fluorescence in situ hybridisation (FISH).⁸ At the time of diagnosis, four of the patients with CNL had normal cytogenetics, but patient 4 showed an abnormal clone with trisomy 9 (20 of 21 metaphases). We performed clonality studies in blood samples using HUMARA in our five cases of CNL and compared the results with 12 patients who had untreated, newly diagnosed MPS, nine patients with clinically suspected LR, and 26

Abbreviations: CML, chronic myelogenous leukaemia; CNL, chronic neutrophilic leukaemia; FISH, fluorescence in situ hybridisation; HPRT, hypoxanthine phosphoribosyl transferase; HUMARA, human androgen receptor gene assay; LR, leukaemoid reaction; MPS, myeloproliferative syndrome; NAP, neutrophil alkaline phosphatase; PCR, polymerase chain reaction; PHA, phytohaemagglutinin

Table 1 Haematological data of patients and results of the human androgen receptor gene assay (HUMARA) clonality studies

Patient	Diagnosis	Age	BCR/ ABL	Hb	WBC	Differential blood count	Plt	N-clon	L-clon
1	CNL	52	Absent	140	30.0	Seg 70, Bn 3, My 9, Bas 1, Eo 2, Ly 10, Mo 5	182	M	M
2	CNL	81	Absent	108	35.0	Seg 90, Bn 0, My 0, Bas 0, Eo 0, Ly 9, Mo 1	189	M	P
3	CNL	37	Absent	143	36.2	Seg 64, Bn 15, My 13, Bas 0, Eo 0, Ly 7, Mo 1	273	M	M
4	CNL	72	Absent	89	38.0	Seg 41, Bn 13, My 37, Bas 0, Eo 0, Ly 6, Mo 3	47	M	M
5	CNL	63	Absent	123	24.6	Seg 60, Bn 6, My 17, Bas 5, Ery 2, Ly 9, Mo 1	210	M	M
6	CML	60	b2a2	98	83.5	Seg 28, Bn 14, My 39, Bas 1, Eo 1, Ly 10, Mo 7	718	M	P
7	CML	53	b3a2	101	46.1	Seg 63, Bn 4, My 12, Bas 0, Eo 1, Ly 13, Mo 7	365	M	P
8	CML	41	b3a2	98	239.0	Seg 45, Bn 5, My 39, Bas 3, Eo 0, Ly 2, Mo 6	619	M	P
9	aCML	48	Absent	142	32.7	Seg 27, Bn 4, My 46, Bas 3, Eo 1, Ly 15, Mo 4	460	M	P
10	CML	64	b2a2	116	39.2	Seg 80, Bn 7, My 0, Bas 0, Eo 1, Ly 7, Mo 5	486	M	P
11	CML	58	b3a2	136	24.1	Seg 63, Bn 7, My 0, Bas 2, Eo 0, Ly 22, Mo 6	439	M	M
12	CML	65	b3a2	130	33.9	Seg 59, Bn 4, My 22, Ery 4, Bas 5, Eo 2, Ly 6, Mo 1	246	M	P
13	CMML	83	Absent	100	37.2	Seg 63, Bn 0, My 0, Bas 0, Eo 0, Ly 9, Mo 28	191	M	P
14	CMML	74	Absent	114	8.2	Seg 20, Bn 6, My 0, Bas 0, Eo 0, Ly 51, Mo 23	95	M	P
15	PCV	61	Absent	91	15.4	Seg 80, Bn 4, Ery 4, Bas 0, Eo 0, Ly 10, Mo 2	44	M	ND
16	CIMF	73	Absent	105	12.2	Seg 78, Bn 0, My 0, Bas 1, Eo 4, Ly 16, Mo 1	124	M	P
17	CIMF	83	Absent	116	30.4	Seg 38, Bn 4, My 19, Bas 5, Eo 7, Ly 20, Mo 7	403	M	P
18	LR (smoker)	39	Absent	Normal	14.0	Seg 63, Bn 2, My 2, Bas 1, Eo 4, Ly 25, Mo 3	Normal	M	P
19	LR (diabetes)	50	Absent	88	10.4	Seg 61, Bn 0, My 0, Bas 0, Eo 3, Ly 32, Mo 4	348	P	P
20	LR (fever)	63	ND	99	18.1	Seg 87, Bn 1, Eo 2, Ly 8, Mo 2	518	P	P
21	LR	67	ND	ND	21.5	ND	ND	P	P
22	LR (diabetes)	93	ND	ND	20.1	ND	ND	M	ND
23	LR (smoker)	37	ND	134	13.6	Seg 69, Bn 1, Ly 24, others 6	204	P	P
24	LR (Sharp sy.)	50	ND	90	7.0	Seg 80, Bn 1, Bas 1, Eo 5, Ly 7, Mo 6	307	M	P
25	LR	53	Absent	142	13.8	Seg 56, Bn 2, Ly 35, Mo 7	354	NI	NI
26	LR (smoker)	45	Absent	129	12.5	Seg 66, Bn 4, Eo 6, Ly 20, Mo 4	403	P	P
27–52	Blood donors	20–77	ND	ND	Normal	*	ND	See text	

Diagnosis: aCML, atypical chronic myeloid leukaemia; CIMF, chronic idiopathic myelofibrosis; CML, chronic myelogenous leukaemia (chronic phase); CMML, chronic myelomonocytic leukaemia; CNL, chronic neutrophilic leukaemia; LR, leukaemoid reaction; PCV, polycythaemia vera; Sharp sy., Sharp syndrome (a mixed connective tissue disease). Age, age at time of diagnosis; BCR/ABL, status of BCR/ABL translocation; Hb, haemoglobin concentration (g/l); WBC, white blood cell count ($\times 10^9/l$); differential blood count: Seg, segmented neutrophils; Bn, bands; My, myelocytes; Bas(ophils), Eo(sinophils), Ly(mphocytes), Mo(nocytes), Ery(throblasts); Plt, platelet count ($\times 10^9/l$); N-clon/L-clon, clonal status of neutrophils and phytohaemagglutinin expanded lymphocytes using HUMARA with the patterns (M) monoclonal and (P) polyclonal; NI, not informative; ND, not determined.

healthy blood donors (table 1). All the investigations were done according to the guidelines of our institute.

Blood sample clonality assay

The neutrophils of all 52 patients were enriched on a buffy coat by centrifuging 5 ml of blood at $800 \times g$ rpm for 15 minutes, followed by the lysis of red blood cells. Lymphocytes were isolated on a Ficoll gradient from 5 ml of blood (Nycomed Pharma AS, Oslo, Norway) and T cells were expanded in vitro using phytohaemagglutinin (PHA) and interleukin 2. Thus, in each patient, at least 3 million cultured T cells were available for investigation. DNA samples from the neutrophils and the PHA expanded T cells were isolated for each patient (PNA Blood Minikit; Qiagen, Hilden, Germany). These DNA samples were digested overnight at 37°C with Hpa II (Roche Diagnostics, Mannheim, Germany) and Hha I (Promega Corporation, Madison, Wisconsin, USA). A third DNA sample remained undigested. All samples were heated to 95°C for 10 minutes to stop the digestion and to denature the DNA. HUMARA sequences were amplified from the undigested DNA, the Hpa II and Hha I digested DNA samples obtained from the neutrophils, and the T cells for each patient by PCR using AmpliTaq Gold (Perkin-Elmer, Rodgau, Germany) with the following primers: 5'-GCTGTGAAGGTTGCTGTTCCCTCAT-3' (sense) and 5'-TCCAGAATCTGTTCCAGAGCGTGC-3' (antisense).⁷ The sense primer was 5'-fluorescence labelled (6-FAM). The PCR conditions were 96°C for 10 minutes (first cycle), then 95°C for 30 seconds, 65°C for 45 seconds and 72°C for 90 seconds for 30 cycles, followed by 72°C for seven minutes. The PCR products were analysed by capillary electrophoresis on an ABI-310 sequencer (Perkin-Elmer, Weiterstadt, Germany).

RESULTS AND DISCUSSION

We studied the clonality patterns of leucocytes, which consisted mainly of neutrophils and PHA expanded T cells,

from patients with MPS and LR. The T cells were used as an internal control cell population because both neutrophils and T cells are derived from haemopoietic progenitor cells.^{9–11} The results of the HUMARA study are summarised in table 1, and representative findings in two patients with CNL and one normal blood donor are illustrated in fig 1. In all five cases of CNL, the leukaemic neutrophils displayed a monoclonal HUMARA pattern. Similarly, all seven patients with CML or atypical CML and all five patients with other MPS had monoclonal leucocytes. The PHA expanded T cells of four of five patients with CNL showed a monoclonal HUMARA pattern. This finding suggests that in CNL the T cells are frequently derived from the neoplastic clone. However, we cannot completely exclude the possibility that the finding of monoclonal T cells in four of the five CNL cases could represent an extreme form of "skewing", meaning an unbalanced, "skewed" pattern of X chromosomal inactivation in which cells with the inactivated maternal or paternal allele are predominant. Even though it is not always possible to differentiate clearly between true monoclonality and extreme skewing in the individual case, we think that such a concentration of cases showing extreme skewing in a small group of patients with CNL would be very unlikely. In contrast, in five of six patients with CML, in the patient with atypical CML, and in all five patients with other MPSs the T cells did not show a monoclonal HUMARA pattern, suggesting that they may be derived from residual normal progenitor cells. Thus, the different clonality patterns of the T cells may indicate that in CNL the neoplastic transformation occurs at an earlier stage of progenitor cell differentiation than in CML.

Most patients with the clinical diagnosis of LR showed polyclonal patterns for neutrophils and T cells, as expected. Three patients with clinically suspected LR had monoclonal neutrophils and polyclonal T cells, displaying a clonality pattern similar to the patients with MPS. Thus, these patients with

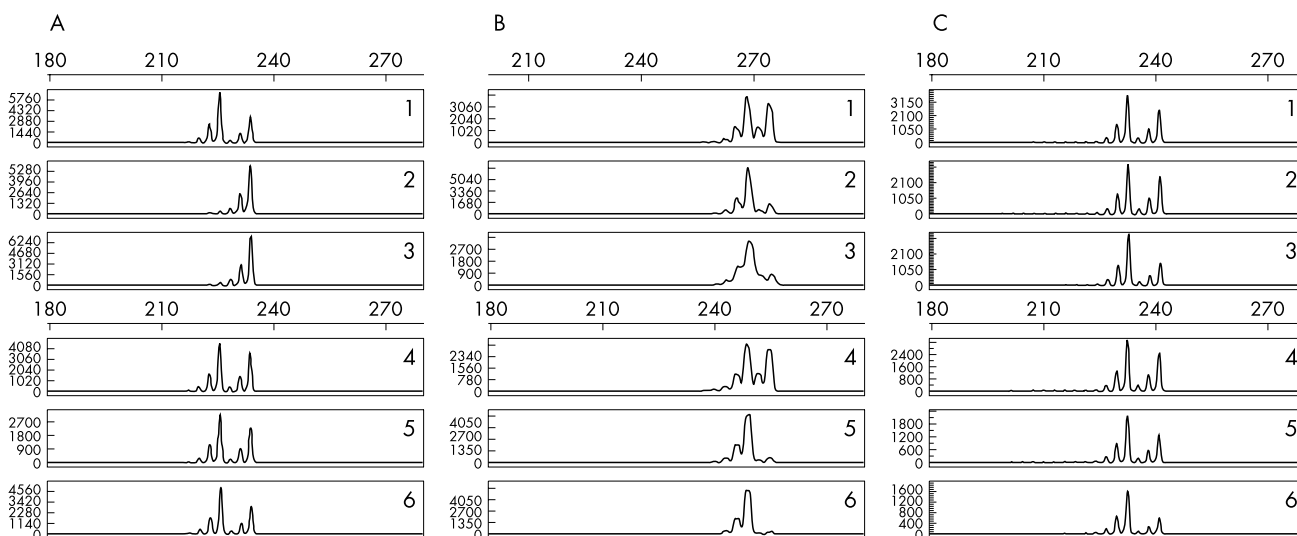


Figure 1 Clonality analysis using human androgen receptor gene assay. (A) Chronic neutrophilic leukaemia (CNL) case 2: after predigestion of the DNA from the leukaemic neutrophils with the methylase sensitive enzymes only one of the human androgen receptor gene microsatellites can be amplified by PCR, whereas both X chromosomal alleles are amplified without predigestion (monoallelic pattern; rows 1–3). In contrast, PCR amplification of the human androgen receptor gene microsatellites from the DNA of this patient's T cells is not affected by predigestion (polyallelic pattern; rows 4–6). A monoallelic pattern in the gene scan analysis is suggested if one allele peak completely disappears after enzymatic digestion, whereas a polyallelic profile is characterised by the persistence of two distinct allele peaks. The additional smaller peaks are caused by slippage of the Taq polymerase.¹² (B) CNL case 1: monoallelic patterns in both neutrophils and T cells. (C) Normal blood donor: polyallelic patterns in both neutrophils and T cells. Rows 1 and 4, PCR from undigested DNA; rows 2 and 5, PCR from DNA predigested with HpaII; rows 3 and 6, PCR from DNA predigested with HhaI.

clinically suspected LR might be in the initial phase of an MPS. At present, one year after the investigations were done, these patients are free of disease. The clonality analysis of 26 blood donors demonstrated 20 cases with polyallelic patterns of neutrophils and T cells. In five blood donors we found a skewed pattern of X chromosomal inactivation. This was seen in both the neutrophils and the T cells of these blood donors. The skewing has been interpreted as a natural phenomenon, detectable in some women upon aging.^{9 11 13–15} By definition, a cell fraction is considered as monoallelic or “skewed” if the expression of the dominant allele exceeds 75%.¹⁴ Thus, the results of HUMARA clonality studies should only be interpreted together with the clinical, blood, and bone marrow findings. Among the patients with LR and blood donors there were two “non-informative” cases, where the analysis of clonality was impossible because the two different PCR amplified X chromosomal microsatellites were of approximately equal size.

“The different clonality patterns of the T cells may indicate that in chronic neutrophilic leukaemia the neoplastic transformation occurs at an earlier stage of progenitor cell differentiation than in chronic myelogenous leukaemia”

To our knowledge this is the first report of HUMARA clonality studies in CNL. This technique is naturally restricted to female patients and gives meaningful results in 80% to 90% of cases, owing to the high rate of heterozygosity at the HUMARA locus.^{7 16–19} However, the results may be blurred by the excessive skewing that is seen in some normal blood donors.^{9 11 13–15} Thus far, the clonal nature of blood neutrophils in CNL has been documented in two cases. Froberg *et al* reported monosomy for a 11q23 probe using FISH in a 67 year old woman with CNL evolving from a myelodysplastic syndrome.²⁰ In a 60 year old female patient, Kwong and Cheng²¹ found a monoallelic methylation pattern of the X linked hypoxanthine phosphoribosyl transferase (HPRT) gene. However, these authors did not provide data about the T cell clonality pattern, so that extreme skewing in that case cannot be excluded. Some of the cases that were reported in

the literature as being “CNL” occurred in association with plasma cell dyscrasias like myeloma.² However, when studied, a polyallelic pattern of the neutrophils at the HPRT gene of these suspected CNL cases was found,^{22 23} indicating that neutrophilia in these patients might have represented LR triggered by cytokines.² In our five patients with CNL, plasma cell dyscrasias were absent. Significant dysplasia of the haemopoietic cells in the bone marrow has been described in some cases of “CNL”,^{4 20 24 25} but is not a diagnostic feature of CNL,^{1 2} and was not seen in our five patients with CNL. Thus, on the basis of the CNL cases published up to 2001, in his review, Reilly²⁶ concluded that only 33 cases sufficiently fulfilled the criteria of “true” CNL, including the case of Kwong and Cheng,²¹ but excluding the case of Froberg *et al*.²⁰ As was seen in four of our five patients, nearly 90% of patients with CNL have normal cytogenetics, but a minority show diverse aberrations,^{1 2 26} probably suggesting monoallelicity. Among the karyotypic abnormalities reported in CNL, 20q deletions were the most frequent.²⁶ The finding of trisomy 9, as in our patient 4, has been described once by Di Donato *et al* in a patient with CNL after beginning radiotherapy and chemotherapy.²⁷ The heterogeneity of the cytogenetic aberrations found in some of the CNL cases indicates that these aberrations probably represent secondary phenomena in the course of the disease and are not primarily involved in the pathogenesis of CNL.¹

In conclusion, our HUMARA clonality studies prove the neoplastic nature of the leukaemic neutrophils in CNL, and provide evidence that CNL is a distinct myeloproliferative disorder. In female patients, HUMARA studies may help to distinguish CNL from polyallelic LR. We suggest that the monoallelicity of blood neutrophils should be demonstrated for the diagnosis of “true” CNL whenever possible. The importance of skewed or monoallelic patterns of haemopoiesis in individual blood donors and in patients with clinically suspected LR should be investigated in prospective studies.

Authors' affiliations

J Böhm, S Kock, H E Schaefer, P Fisch, Department of Pathology, University of Freiburg, Medical School, D-79002 Freiburg, Germany

Take home messages

- Clonality studies of blood neutrophils using the human androgen receptor gene assay can help to distinguish female patients with monoclonal chronic neutrophilic leukaemia (CNL) from those who have a polyclonal, non-malignant leukaemoid reaction
- This distinction is difficult using morphology alone but is important because those with CNL have a very poor prognosis
- For the diagnosis of CNL, monoclonality of the neutrophils should be demonstrated whenever possible

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