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ORIGINAL ARTICLE

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Megakaryocytes carry the fused bcr-abl gene in chronic myeloid leukaemia: a fluorescence in situ hybridization analysis from bone marrow biopsies

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Abstract Histological examination of bone marrow biopsies shows that about one-third of chronic myeloid leukaemia (CML) patients exhibit an increase of megakaryocytes. The megakaryocytic predominance may be so striking that differentiation from other chronic myeloproliferative disorders (CMPD) may be difficult in some CML patients. Megakaryocytes in CML are clonal as demonstrated by loss of glucose-6-phosphate dehydrogenase isoenzymes. The Ph translocation, fusing the abl and bcr genes on chromosomes 9 and 22, however, obviously occurs as a second step in tumour development. So far, the Ph translocation has not been assigned explicitly to megakaryocytes. The question is whether the megakaryocytic cell lineage could harbour the bcr/abl fusion in those CML cases with striking proliferation of megakaryocytes but lack this genetic defect in cases with normal or decreased megakaryocyte counts. We therefore performed triple-colour fluorescence in situ hybridization (FISH) for portions of the bcr and abl genes flanking the breakpoint in CML in paraffin sections of CML cases with normal and with increased numbers of megakaryocytes. This method allows identification of the bcr/abl fusion in single, morphologically intact cells, whereas conventional cytogenetics requires lysis and thus destruction of the cell. Among the 21 CML patients examined by FISH, 10 were informative for bcr and abl genes and displayed distinct hybridization signals within nuclei of bone marrow cells. Besides the granulopoietic cells, megakaryocytes of all those patients (4 without and 6 with varying grades of megakaryocytic increase) displayed bcr/abl fusion signals indiciative of a Ph translocation. The lack of hybridization signals in the remaining 11 cases indicates that this technique is not of value diagnostically and should be reserved for scientific questions. Positive controls consisted of conventional chromosome preparations from bone marrow aspirates demonstrating the Ph chromosome in all patients examined, and negative controls of paraffin sections of bone marrow biopsies from non-CML patients. These showed no fusion signals in bone marrow cells, including megakaryocytes, using FISH. Our results demonstrate clearly that not only the transforming event but also the Ph translocation leading to the bcr/abl fusion happens prior to the differentiation of the pluripotent stem cell into different myeloid lineages. The megakaryocytic proliferation evident in some CML cases is probably a consequence of the disease progress.

Key words Chronic myelogenous leukaemia · Philadelphia chromosome · FISH · Megakaryocyte

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Introduction

The Ph-chromosome in chronic myeloid leukaemia (CML) results from a reciprocal translocation between chromosomes 9 and 22, fusing the bcr and abl genes [21, 23]. According to a model of CML as a multistep disease [7, 15], the Ph translocation happens secondary to an initial event in neoplastic transformation and there is thus a Ph-negative but clonal stage in at least some cases of CML [20]. The second step, leading to the Ph-positive phenotype, is believed to be responsible for the typical cytological and clinical picture associated with CML [20].

Progenitor cells of macrophages, granulocytes, erythrocytes and the megakaryocytic lineage are involved in the malignant clone, as shown by the loss of an isoenzyme of the glucose-6-phosphate-dehydrogenase (G6PD) in heterozygous female CML patients [6]. The question of whether these cells are not only clonal but also Ph-positive has not been answered definitely for the megakaryocyte (MK), although a study by Knuutila et al. [12] suggested megakaryocytic involvement. This question is of some interest, since histological examination of

the bone marrow reveals increased proliferation of megakaryocytes in about one third of CML cases [1, 8]. The latter can present as a predominant feature in diagnostic biopsies and is often accompanied by elevated platelet counts, making differentiation from Ph-negative groups of CMPD (essential thrombocythaemia or megakaryocytic myelosis) difficult. Moreover, megakaryocytic proliferation and the often developing myelofibrosis have been shown to herald a bad prognosis in CML [1–4, 14, 25].

We wondered whether the striking variances in megakaryocytic proliferation in CML might be associated with differences in the molecular genetic aetiology, involving the megakaryocytic cell line by the Ph translocation in some, but not all, cases of CML. This question was addressed by triple-colour FISH for bcr and abl genes as a technique that allows the simultaneous analysis of genotype and morphology on a single-cell base [26]. The advantage of FISH compared with conventional cytogenetic analysis is that the cell analysed need not be destroyed, thus making it possible to assess cell morphology and even immunophenotype simultaneously [19, 27].

Patients and methods

Yamshidi bone marrow biopsies were obtained from 21 patients with CML. The biopsies were fixed in Hannover solution (1 part 40% formalin, 2 parts 80% methanol), then one part was decalcified with EDTA and paraffin embedded. The other was embedded in methyl-metacrylate [9]. CML was subtyped according to the distribution of megakaryocytes, presence and degree of myelofibrosis and the number of blasts [1, 8], which were graded into 4 categories semiquantitatively (Table 1). Bone marrow specimens of three healthy patients with inconspicuous marrows served as negative controls.

For the molecular cytogenetic analysis, 4-μm paraffin sections were cut onto Poly-L-lysine-coated slides. After dewaxing and rehydrating through graded ethanols, sections were pretreated by irradiation in citrate-monohydrate buffer (pH 6) in a household microwave oven (900 W) for 30 min. FISH was performed with a commercially available kit (Oncor, Gainsborough, USA) using a mixture of probes specific for the bcr/abl translocation major breakpoint. Briefly, after preincubation in 2× SSC at 37°C, sections were dehydrated with graded ethanols and the target DNA was denatured in a waterbath at 70°C for 12 min. A mixture of digoxigenin-labelled cosmid DNA probes specific for the major breakpoint cluster region of the bcr gene and a single biotin-labelled cosmid specific for the abl gene [30] were applied to the sections and allowed to hybridize overnight at 37°C. After stringency washes (50% formamide, 2× SSC), the bound probes were detected with Texas-red coupled anti-digoxigenin and FITC-labelled anti-biotin antibodies. Nuclei were counterstained with DAPI. A morphologically identified megakaryocytic cell was regarded as bcr/abl-positive when at least one green, one red and one red/green double or one yellow fusion signal, respectively, were present in the nucleus.

Table 1 Staging of CML [8, 9] regarding the distribution of megakaryocytes and fibres in bone marrow biopsies

Acronym	Semi-quantitative definitions
CML.MI0	No megakaryocitic increase
CML.MI1	Slight megakaryocytic increase
CML.MI2	Conspicuous megakaryocytic increase
CML.MI3	Predominance of megakaryocytes
CML.MF0	No myelofibrosis
CML.MF1	Reticulin sclerosis
CML.MF2	Collagen fibrosis
CML.MF3	Advanced myelofibrosis

In addition to the Yamshidi biopsies, in 10 of the cases bone marrow aspirates were availabe for cytogenetic analysis. The bone marrow cells isolated from the buffy coats were cultured for 22 and 44 h, respectively, in RPMI medium (Gibco) supplemented with 15% fetal calf serum, the last hour in the presence of Colcemid (Serva). Chromosome preparation and banding analysis were done as described earlier [28].

Briefly, cells were incubated after 22 and 44 h of incubation, the last hour in the presence of Colcemid (Serva, 0.4 mg/ml), lysed by hypotonic treatment with 0.075 M potassium chloride for 20 min at 37°C, pelleted and then fixed in methanol/acetic acid. Nuclei were dropped onto cold, wet slides, G-banded and at least 10 cells were karyotyped in detail.

Results

Histological classification regarding the megakaryocytic distribution from methylmetacrylate-embedded biopsies was as follows: 7/21 cases with normal or even decreased megakaryocyte numbers (Fig. 1) (CML.MI 0), 5/21 cases with slight (CML.MI 1) and 7/21 with moderate increase (Fig. 2) (CML.MI 2), and 2 cases in which a striking predominance of megakaryocytes was noted (CML.MI 3). Fibrosis was seen in 5/21 cases, all of them with grade 2 or 3 of megakaryocytic increase (3 CML.MI 2 and 2 CML.MI 3).

Conventional chromosome analysis from bone marrow aspirates was successful in the 10 CML cases examined and revealed the Ph chromosome in haematopoietic cells of all those patients (Table 2). In 8 cases, the Ph chromosome was the sole aberration; 1 patient had an additional Ph chromosome with 47,XY,t(9;22),+Ph (patient 6, CML.MI 2), 1 a translocation t(X;5) in addition to the Ph translocation (patient 5, CML.MI.2). Because of the practically inevitable cell lysis that occurs during chromosome preparation, abnormal metaphases could not be assigned to distinct cell lines by karyotyping.

FISH performed in paraffin sections of decalicified bone marrow biopsies of the 21 CML cases was informative in 10 of the cases showing distinct hybridization spots within nuclei (Fig. 3, Table 2). In the other 11 biopsies, no signals were obtained even after repetition of the experiments and replacement of the microwave pretreatment by enzymatic digestion with pronase (in 1% solution for 30 min). The signal distribution in granulopoietic nuclei of Ph-positive CML cases was one red signal for the unaffected chromosome 22, one green hybridization spot highlighting the normal chromosome 9, and one red/green double spot or a yellow fusion signal, respectively, for the bcr/abl fusion. However, as described earlier [19], owing to sectioning of nuclei not all cells showed all the signals expected. For this reason, exact quantification of the percentage of Philadelphia-positive cells in each section was not possible.

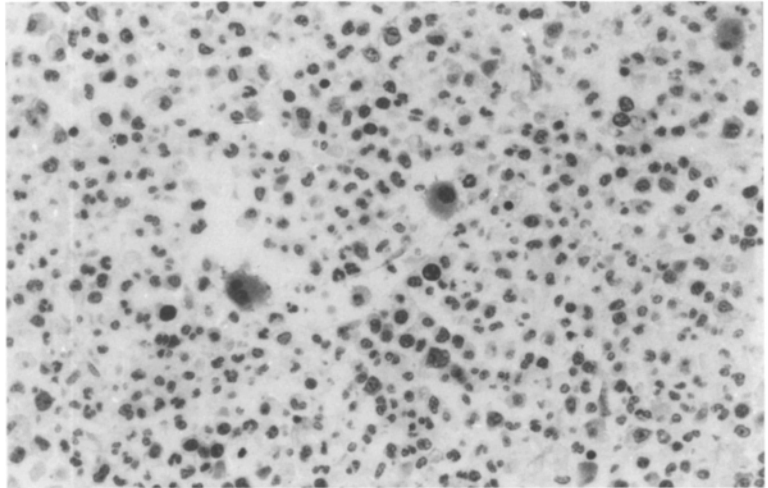

Fig. 1 Scattered small megakaryocytes in the bone marrow histopathology of patient 2 with a normal number of megakaryocytes (CML.MI.0). Methylmetacrylate embedding, Giemsa staining, magnification ×200

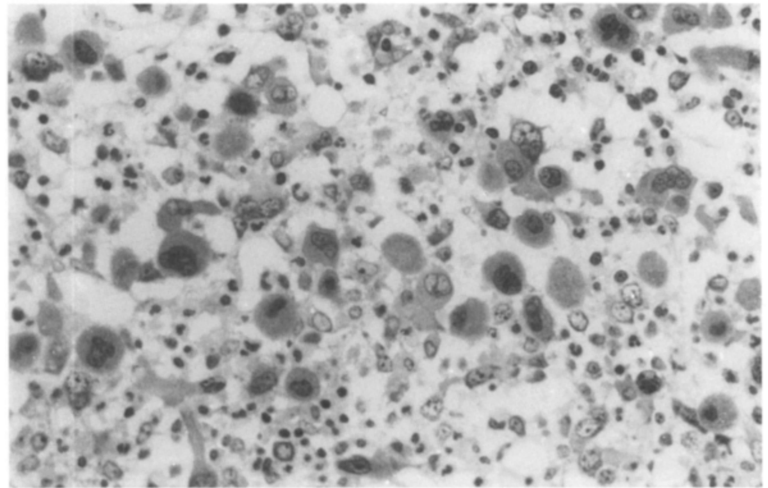

Fig. 2 Striking proliferation of megakaryocytes in patient 9 with a conspicuous increase of megakaryocytes (CML.MI2). Methylmetacrylate embedding, Giemsa staining, magnification ×200

Table 2 Histopathological diagnoses, results from conventional cytogenetics and FISH for bcr/abl fusion of the ten cases amenable to FISH analysis (*GRAN* granulocytic cells, *MEG* megakaryocytes)

Patient no.	Histological classification	Conventional cytogenetics	bcr/abl in	
			GRAN	MEG
1	CML.MI0	46,XY,t(9;22)	Positive	Positive
2	CML.MI0	46,XX,t(9;22)	Positive	Positive
3	CML.MI0	46,XY,t(9;22)	Positive	Positive
4	CML.MI0	46,XX,t(9;22)	Positive	Positive
5	CML.MI2	46,XY,t(9;22),t(X;5)	Positive	Positive
6	CML.MI2	46,XX/46,XX,t(9;22)	Positive	Positive
7	CML.MI2	46,XY,t(9;22)	Positive	Positive
8	CML.MI2	46,XX,t(9;22),+Ph	Positive	Positive
9	CML.MI2.MF2	46,XY,t(9;22)	Positive	Positive
10	CML.MI3.MF2	46,XY,t(9;22)	Positive	Positive

The small megakaryocytes typical for CML were easily identified by their characteristic morphology in the paraffin sections. As a rule the nuclei of megakaryocytes had more fusion signals, viz two or three red or green and more than one for bcr/abl (Fig. 3). In all histological subgroups, including those with and without increase in the number of megakaryocytes, Ph-positive megakaryocytes were found (Table 2). Despite the occasionally observed signal losses caused by sectioning of nuclei, in each section enough megakaryocytic cells showed all signals expected to determine the presence or absence of a bcr/abl fusion.

In the three control specimens, only red and green hybridization signals, clearly separated from each other, occurred. Megakaryocytes were consistently Ph-negative (i.e., they did not show bcr/abl fusion signals).

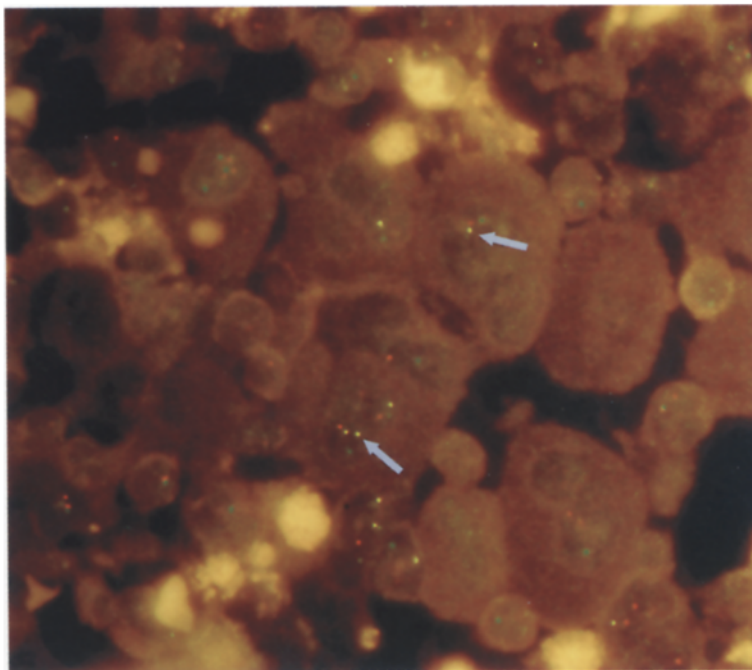

Fig. 3 Fusion of parts from the *bcr* and *abl* genes indicated by yellow fusion signals (*arrows*) in megakaryocytes from a CML case with megakaryocytic predominance (CML.MI.3, patient 10). Note that the megakaryocytes have more than the three signals (in a Ph-positive) or four (in a Ph-negative) expected in a mononuclear cell. In some cells, signals are lost due to sectioning of nuclei or because they are not in focus. Original magnification ×1000. Nuclear counterstain with DAPI

Discussion

In the present study, FISH for detection of a bcr/abl fusion was applied to bone marrow paraffin sections of different CML subtypes to verify the presence or absence of the Ph translocation in megakaryocytes. Compared with conventional cytogenetics or other methods for determination of bcr/abl status, such as Southern blot or PCR techniques, FISH has the advantage of allowing genotypic analysis on a single-cell basis in a morphologically intact cell, which can be simultaneously immunophenotyped [19, 27, 29].

Whereas some authors were able to assign the Ph-positive clones to erythroid, myelomonocytic and also lymphoid precursor cells [5, 17, 22] besides granulopoietic cells, we found only two reports mentioning the analysis of megakaryocytic precursors or megakaryocytes, respectively [11, 12] but these lacked definite proof of the Ph chromosome in megakaryocytes. Knuutila et al. [12] separated cells from CML patients by fluorescence-activated cell sorting and then analysed the different cell lineages by conventional cytogenetics or FISH. They found trisomies which they had already noticed as additional aberrations in Ph-positive metaphases [trisomy 1 and 8) of the respective patients in some of the megakaryocytic cells analysed. However, this is only an indirect hint as to whether these cells really were representing the same clone. Moreover, the detection of polysomy in megakaryocytes is not per se proof for the presence of a malignant clone, as especially these cells are known to have a polyploid chromosome complement [13]. This was confirmed in our study by the finding that megakaryocytes usually had more than the three (in a Ph-positive cell) or 4 (in Ph-negative cells of the control specimens) signals expected, that is more than two chromosomes 9 and 22 per cell. Other indirect hints to involvement of megakaryocytes in the Ph-positive clone are reports from blast crises of CML patients presenting with different phenotypes, including – besides myeloblasts and even T- or B-lymphoblasts – megakaryocytic cells [10, 15, 18]. In general, these and other studies have suggested that in individual CML cases the transforming event and the occurrence of the Ph translocation obviously happen at different stages of differentiation. For these reasons, we wondered whether in different histological types of CML megakaryocyte numbers can be elevated or these cells may even predominate due to involvement of megakaryocytes in the Ph-positive clone in this type of leukaemia, but not in the "common" type with normal or decreased numbers of megakaryocytes.

The detection of unambiguous fusion signals in megakaryocytes of all histological subtypes of CML (with and without increase of megakaryocytes) makes this possibility less likely however. Thiele et al. [24] have interpreted the megakaryocytic increase in CML as a "concomitant phenomenon". The present results suggest that the changes in number and morphology of megakaryocytes are consequences of the neoplastic event.

The fact that 11 of the 21 formalin-fixed and paraffin-embedded bone marrow biopsies investigated here failed to give any hybridization signals indicates that the method has its limits. One reason for the negative results in more than 50% of the cases may be that DNA can also be "masked" by formalin fixation [16]. However, we used microwave preheating of the sections, which has been shown to improve not only immunostaining but also chromosomal in situ hybridization in formalin-fixed tissues [19]. Another disadvantage of using tissue sections is the loss of signals caused by sectioning of nuclei, making quantification of the cells involved difficult. This

technique should therefore be reserved for scientific questions and not used for routine analysis of specimens, since it lacks diagnostic significance. For diagnostic purposes, including grading of remission or relapse after chemotherapy or bone marrow transplantation in Ph-positive CML patients, optimal results can be obtained by using chromosome or nuclei preparations from bone marrow aspirates [29].

In conclusion, we have shown by FISH that megakaryocytes bear the Ph translocation in all histological subtypes of CML, which has not been shown for the megakaryocyte with a single-cell approach before. This further sustains the assumption that this translocation in CML happens in the majority of cases, if not in all, at a stage of the multipotent stem cell capable of differentiating into all lineages of the myeloid series, including megakaryocytes. The different histological types of CML, with and without megakaryocytic proliferation probably reflect different stages of the disease rather than true differences in aetiology.

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