Original Article

The Incidence and Patterns of *BCR/ABL* Rearrangements in Chronic Myeloid Leukaemia (CML) Using Fluorescence In situ Hybridisation (FISH)

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Abstract

Introduction: Chronic myeloid leukaemia (CML) is characterised by the formation of the *BCR/ABL* fusion gene, usually as a result of the Philadelphia (Ph) translocation between chromosomes 9 and 22. **Materials and Methods:** The incidence of both typical and atypical *BCR/ABL* gene rearrangements was determined in 110 patients suspected of CML using dual fusion fluorescence in situ hybridisation (DF-FISH) probes. **Results:** Eighty-seven per cent of CML patients showed Ph translocation while 13% were negative for the Ph chromosome. About 71.9% of Ph-positive patients displayed the typical DF-FISH signal pattern. Atypical patterns among the Ph-positive patients included the concurrent loss of residual proximal 9q and distal 22q (10.4%), complex translocation with additional partners (9.4%), supernumerary Ph (3.1%), loss of residual 9q sequences proximal to breakpoint (3.1%), and deletion of distal derivative 22q signal (2.1%). Cryptic genetic alterations with loss of proximal 9q sequences were found in 13.5% of CML Ph-positive patients, which is associated with poor prognosis. Fusion signals were detected in 57.1% of CML Ph-negative patients, indicating cryptic *BCR/ABL* rearrangements (i.e., masked Ph). **Conclusion:** FISH is able to detect *BCR/ABL* fusion in CML with masked or variant Ph not apparent with conventional karyotyping. Establishment of signal patterns with FISH is important as atypical patterns may have clinical prognostic implications.

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Key words: Chromosomes, Dual fusion probes, Philadelphia chromosome, Variant patterns

Introduction

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of the haematopoietic stem cells. About 90% to 95% are characterised cytogenetically by the presence of the Philadelphia (Ph) chromosome, due to a translocation between chromosomes 9 and 22. This rearrangement results in the formation of a chimeric *BCR/ABL* fusion gene on the derivative chromosome 22.[1] The fusion gene is transcribed and spliced into a 8.5 kb chimeric messenger RNA with b2a2 and b3a2 configurations.[2] The translation product is a 210 kD *BCR/ABL* protein that contributes to CML pathogenesis.

The Ph chromosome is not demonstrable by cytogenetic studies in about 5% to 10% of CML patients and these patients are classified as having Ph-negative CML. Molecular cytogenetic techniques are being increasingly used as a routine investigation tool both in the diagnosis and subsequent monitoring of CML. Fluorescence in situ hybridisation (FISH) analysis using dual colour *BCR/ABL* translocation probes allows the visualisation of *BCR/ABL* rearrangements in both interphase and metaphase cells,[3] and the presence of the *BCR/ABL* fusion gene on chromosome 22 has been reported in a substantial subset of these patients.[4-6] A smaller subset of Ph-negative variants shows the presence of the *BCR/ABL* fusion gene located on chromosome 9.[7,8]

The *BCR/ABL* dual colour dual fusion (DF) probe set consists of the *ABL* probe spanning 650 kb from an area centromeric of the *ASS* (arginosuccinate synthetase) gene to the telomeric end of the *ABL* gene. The *BCR* probe target spans about 1.5 Mb, beginning within the variable segments of the immunoglobulin lambda light chain locus (IGLV), and extends through the *BCR* gene to approximately 900 kb telomeric of *BCR*. The *ABL* probe is directly labelled with SpectrumOrange™ and the *BCR* probe with Spectrum Green™. Thus, a normal cell will exhibit 2 red and 2 green

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signals (2R2G) and a typical translocation pattern will exhibit 2 fusion, 1 red and 1 green signals (2F1R1G). The normal cut-off is below 1% for the DF probe.[3]

Variations in the FISH patterns include the gain of a Ph chromosome and deletions of sequences proximal to chromosome 9 breakpoint or distal to chromosome 22 breakpoint. In this study, the incidence and patterns of *BCR/ABL* gene rearrangements were determined in 110 suspected CML patients at diagnosis and/or during the course of the disease. The underlying genetic variations seen in atypical patterns were established using molecular and chromosome analysis.

Materials and Methods

A total of 110 patients suspected of CML due to clinical presentations of leukocytosis, anaemia, thrombocytosis, splenomegaly and fatigue were included in the study. The data collected for this study are part of the clinical service in which IRB consent has been sought and approved. Ninety-five patients were studied at the onset of the disease and 15 patients during the course of the disease. Heparin-anticoagulated bone marrow aspirates were obtained for cytogenetic and molecular analysis.

Conventional Karyotyping

Bone marrow aspirate samples were cultured and processed by conventional cytogenetic procedures with GTG banding (G-bands by trypsin and Giemsa). In each case, at least 20 metaphases were analysed and the karyotypes were described according to International System for Human Cytogenetic (ISCN) (1995).

FISH Analysis

FISH analysis was performed on both interphase and metaphase cells using the LSI *BCR/ABL* dual colour DF probe (Vysis Inc, Downers Grove, IL, USA). For samples suspected of an *ASS* gene deletion, the LSI *ASS* gene probe was used in conjunction with the LSI *BCR/ABL* probe to elucidate the molecular rearrangement.

FISH procedures were carried out on fixed bone marrow cells according to the manufacturer's instructions, with slight modifications. Briefly, the slide was incubated in 2XSSC/0.5% NP-40 for 30 minutes at 37^0C, followed by an alcohol series for dehydration. Co-denaturation was carried out for 3 minutes at 75^0C, followed by overnight hybridisation at 37^0C. Evaluation of the FISH signals was performed using a fluorescence microscope (Olympus BX60, Tokyo, Japan) under 1000x magnification. For each case, a minimum of 200 interphase cells was evaluated for the signal pattern.

A case was considered positive for *BCR/ABL* rearrangement when the percentage of *BCR/ABL* positive interphase nuclei was $\geq 0.8\%$ with the DF probe.[3] The FISH results were interpreted in the context of routine G-banded karyotypes, and metaphases were used to verify the interphase FISH (iFISH) pattern as well as to confirm the chromosomal position of the fusion gene. Reverse transcriptase-polymerase chain reaction (RT-PCR) of the *BCR/ABL* transcript was used to confirm the presence of the chimeric transcript in 75% (n = 82) of the cases.

Results

Of the 110 patients, 95 patients were studied at first diagnosis and the remaining 15 patients were monitored for minimal residual disease (MRD). There was no difference in the iFISH patterns between the 2 groups.

Eighty-seven per cent (n = 96) of the 110 suspected CML patients showed a translocation between chromosomes 9 and 22, resulting in a Philadelphia chromosome, by conventional cytogenetics. The remaining 13% (n = 14) were negative for the Ph chromosome. However, with adjunct FISH using DF-FISH probe for *BCR/ABL* gene, the pick-up rate was increased to 94.5% (n = 104).

The great majority of Ph-positive patients (71.9%, n = 69) displayed the typical iFISH DF-FISH signal patterns, i.e., 2F1R1G. Bone marrow morphological examination showed CML in chronic phase for 79.7% of the cases (n = 55), 8.7% (n = 6) showed CML in accelerated phase, 5.8% (n = 4) showed erythroid hyperplasia, 2.9% (n = 2) showed myeloid megakaryocytic hyperplasia and 2.9% (n = 2) indicated a myeloproliferative disorder. All the cases with erythroid hyperplasia, myeloid megakaryocytic hyperplasia and myeloproliferative disorder (n = 8) were positive for *BCR/ABL* transcript with RT-PCR analysis. Out of the 69 cases showing typical iFISH signal pattern, 51 cases had RT-PCR results carried out, in which the type of *BCR/ABL* transcript configuration was defined for 45 cases. Among these cases, 73.3% (n = 33) showed *BCR/ABL* transcript with a p210 b3a2 configuration,[9] 24.4% (n = 11) with p210 b2a2 transcript and 2.2% (n = 1) with an e8a2 configuration.[10]

In the remaining 27 Ph-positive cases, 5 different atypical iFISH patterns were observed. To elucidate the exact nature of the molecular rearrangements, FISH analyses using DF probe were performed on metaphases. Atypical patterns among Ph-positive patients include (1F1R1G) the concurrent loss of residual proximal 9q and distal 22q (10.4%), (1F2R2G) complex translocations with additional partners (9.4%), (1F2G1R) the loss of residual 9q sequences proximal to the breakpoint (3.1%), (3F1R1G) supernumerary Ph (3.1%), and (1F2R1G) deletions of distal der(22q) (2.1%). Table 1 shows the frequency of the various types of atypical iFISH patterns in Ph-positive patients. For cases that exhibit the 1F1R1G atypical pattern,

Table 1. Frequency of Various Types of Atypical iFISH Patterns in Ph-positive CML Patients

FISH pattern with DF-FISH probes	Chromosome localisation of signals			% (n)	Interpretation
	Fusion	Red	Green		
1F1R1G	1F (Ph)	1R (9)	1G (22)	10.4% (n = 10)	t(9;22), loss of residual proximal 9q and distal 22q
1F2R2G	1F (Ph)	1R (9) 1R (third translocation partner)	1G (22) 1G (der 9)	9.4% (n = 9)	Complex translocation with additional partners
3F1R1G	3F (Ph,Ph,der 9)	1R (9)	1G (22)	3.1 % (n = 3)	t(9;22), +Ph
1F2G1R	1F (Ph)	1R (9)	1G (22) 1G (der 9)	3.1% (n = 3)	t(9;22) with loss of residual 9q red signal
1F2R1G	1F (Ph)	1R (9) 1R (der 9)	1G (22)	2.1% (n = 2)	t(9;22) with loss of distal der (22) signal

der: derivative; F: fusion; G: green; Ph: Philadelphia; R: red

the LSI *ASS* gene probe was used in conjunction with the LSI *BCR/ABL* probe set to confirm *ASS* gene deletion. All 1F1R1G cases showed loss of the *ASS* gene and this probe combination was subsequently used for residual disease monitoring.

Bone marrow aspirate examination showed CML in chronic phase for all the cases with atypical iFISH pattern except for the 3 cases with supernumerary Ph, which revealed CML in blast crisis, accelerated phase and CML in transformation to blast crisis, respectively. Out of the 27 cases with atypical iFISH pattern, RT-PCR results were available for 18 cases. However, the type of *BCR/ABL* transcript configuration was only defined for 15 cases. Transcript with p210 b3a2 configuration was detected in 53.3% (n = 8) cases, p210 b2a2 in 40% (n = 6) and p190 e1a2 in 6.7% (n = 1) of the cases.

Eight patients suspected of CML had normal karyotypes but with FISH were found to harbour a cryptic *BCR/ABL* rearrangement (Table 2). Three of these 8 patients had the

BCR/ABL fusion gene on chromosome 9. The iFISH pattern of 2 of these cases were similar to those with Ph-positive cases with concurrent loss of 9q (i.e., 1F1R1G and 1F2G1R) and 1 of these showed the presence of the *BCR/ABL* fusion on both chromosomes 9. The remaining 5 patients had the fusion gene on chromosome 22. Bone marrow morphological examination on these 8 cases showed CML in chronic phase for all but one case with a 1F1R1G signal pattern, which showed CML in blast crisis. RT-PCR results were available for 7 cases and the type of configuration was only defined for 5 cases. *BCR/ABL* transcript with p210 b3a2 configuration was detected in 80% (n = 4) of the cases while p210 b2a2 was found in 20% (n = 1) of patients with a masked Ph.

Six patients with normal karyotypes were diagnosed with suspected CML because of elevated leukocyte count with increased eosinophils or basophils and/or clinical presentations of splenomegaly, anaemia and thrombocytosis. All 6 patients showed normal karyotypes. Neither

Table 2. Frequency of Various Types of Atypical iFISH Patterns in Ph-negative CML Patients

FISH pattern with DF-FISH probes	Chromosome localisation of signals			% (n)	Interpretation
	Fusion	Red	Green		
IF2R1G	1F (22)	1R (9) 1R (9)	1G (22)	50% (n = 4)	Cryptic insertion of *ABL* into *BCR* gene on chromosome 22
1F1R1G	1F (9)	1R (9)	1G (22)	12.5% (n = 1)	Fusion gene on chromosome 9
1F1R1G	1F (Ph)	1R (9)	1G (22)	12.5% (n = 1)	Fusion gene on chromosome 22 with loss of residual proximal 9q and distal 22q
2F2G	1F (9) 1F (9)		1G (22) 1G (22)	12.5% (n = 1)	Localisation of fusion gene on both chromosome 9
1F2G1R	1F (9)	1R (9)	1G (22) 1G (22)	12.5% (n = 1)	Localisation of fusion gene on chromosome 9

F: fusion; G: green; R: red

fusion signals with FISH nor a *BCR/ABL* transcript by RT-PCR was observed in these patients. Bone marrow morphological examination revealed trilineage dysplasia (n = 1), myelofibrosis (n = 1), hypereosinophilia (n = 2), myeloproliferative disorder (n = 1) and acute lymphoblastic leukaemia (n = 1). These patients were therefore diagnosed as not having CML and were excluded from this study.

Discussion

Of the 110 patients in whom CML was suspected, 104 patients showed either cytogenetic or molecular evidence of the *BCR/ABL* fusion gene. The presence of a variable number of different iFISH patterns was unveiled with the use of the *BCR/ABL* FISH probes. The most frequently encountered FISH pattern involved the 2F1R1G comparable to the 75.6% figure that was recently reported.[11] Bone marrow morphological examination findings in this group of patients were similar to those of patients with atypical iFISH pattern and cryptic *BCR/ABL* rearrangement. Patients whose bone marrow morphology showed CML in accelerated phase or blast crisis can display either a typical iFISH pattern or an atypical pattern with gain of an extra fusion signal. However, all patients with FISH assays showing extra fusion signals (3F1R1G) signified additional chromosomal aberrations (i.e., gain of Ph) with clonal evolution and this often occurs in blast crisis. RT-PCR in this study showed that most of the CML cases have either b2a2 or b3a2 transcripts. Higher proportions of patients with atypical iFISH patterns showed b2a2 transcript (40%) compared to those with typical iFISH pattern (24.4%) or masked Ph (20%). However, no distinctive clinical features were found in the patients with b2a2 or b3a2 transcripts.

Additional karyotypic changes such as a supernumerary Ph or deletions of 9q and/or 22q can result in atypical iFISH patterns. This was observed in 28.1% of Ph-positive patients. But as cases with additional cytogenetic changes may present as a typical pattern, there is a possibility of a misdiagnosis. Hence, the exact interpretation of iFISH should be made in conjunction with metaphase analyses.

In our study, the most common atypical iFISH pattern among Ph-positive patients was the 1F1R1G rearrangement, in which large portions of the *BCR* and *ABL* regions are deleted. This may be a result of a translocation event, causing portions of the 9 and 22 to be lost. Such events would require 4 chromosome breaks. Lawce et al[11] and Primo et al[12] demonstrated the presence of such a pattern in about 12.5% and 9% respectively, and these were comparable to the 10.4% found in our study.

For this subgroup of patients, detection of residual disease is difficult since the pattern is indistinguishable from juxtaposition artifacts as a result of random overlapping between chromosomes 9 and 22. The use of the *ASS* gene (labelled with SpectrumAqua™), located proximally to the *ABL* gene, circumvents this problem. A normal nucleus with a juxtaposition artifact would display 1 fusion, 1 red, 1 green, and 2 aqua signals connected to the 2 *ABL* red signals. In a translocation, the *ASS* gene would remain on the 9q while the distal portion of the *ABL* gene would be located on the der(22). Thus, the *ASS* would be separated and no longer coincidental with the *ABL* or fusion signals. On the other hand, loss of the *ASS* gene would result in a signal pattern with 1 fusion, 1 red signal in tandem with 1 aqua signal, and 1 green signal. Therefore, the use of the *ASS* gene probe in conjunction with the *BCR/ABL* probe would be more useful in monitoring residual disease of cases that have a 1F1R1G baseline.

Deletion of the 9q region proximal to the rearranged *ABL* gene signal on the derivative chromosome 9 was demonstrated in 13 out of 96 (13.5%) Ph-positive patients. This frequency is comparable to the 15% reported by Cohen et al[13] and Reid et al.[14] Deletion of the 9q sequences proximal to the breakpoint and t(9;22) might represent a single one-step event occurring at the time of the Ph translocation.[15] In addition, it has been shown that the deletion is not acquired during disease progression as the deletion tends to be consistent throughout the course of the disease.[13] The high rates of deletions in the *BCR* and *ABL* regions may be associated with high-density alu repeats regions.[16] Similar observations are seen in MLL and CBFB/MYH11 rearrangements. In addition, patients with CBFB/MYH11 rearrangements seem to indicate a poorer prognosis with deletions. Indeed, Sinclair et al[17] found that deletions of the proximal sequences on the derivative chromosome 9q were associated with a poor prognosis on standard drug therapy. The study by Cohen et al[13] also indicated that patients with a major deletion of 9q encompassing the *ASS* gene showed a relatively poor prognosis and this subgroup had different cell properties. Subsequent studies by Kolomietz et al[16] and Huntly et al[15] led to the same conclusion. Molecular studies have shown that the poor prognosis is not related to loss of *BCR/ABL* expression, increased *BCR/ABL* transcripts, or genetic instability.[18] It was shown recently that there was no difference in the survival rates between patients with and without deletions when imatinib treatment was given.[19] Nevertheless, the time to disease progression was significantly shorter for patients on imatinib treatment regardless of chronic or advanced phases.

Primo et al[12] showed that the presence of additional Ph chromosomes was 1 of the 2 most common underlying genetic abnormalities when using FISH to identify *BCR/ABL* rearrangements in CML and ALL. These gains were more frequently observed in ALL than in CML. In our present study, 3.1% of Ph-positive patients showed a gain

of a Ph chromosome. The additional Ph is usually associated with disease progression or secondary genetic changes, and is one of the major pathways of clonal evolution seen during blast crisis. Indeed, 2 of our patients had relapsed and were in blast crisis when FISH detected the additional Ph chromosome. Bone marrow morphological examination in these patients with supernumerary Ph showed CML in blast crisis, accelerated phase or CML in transformation to blast. Such findings emphasize the importance of performing baseline FISH studies and that these patterns are verified using metaphases since the signal patterns may change during the course of the disease. For example, a 1F1R1G pattern at diagnosis may change to a 2F1R1G pattern as a result of a gain of a Ph chromosome, and this may inadvertently be misinterpreted as a typical iFISH abnormal pattern while in fact it has the clinical implication of secondary clonal changes.

Although the loss of a red signal in *BCR/ABL* positive nuclei is often associated with an extensive loss of proximal 9q sequences, such atypical iFISH patterns (1F1R1G or 1F2G1R) can also be found in Ph-negative cases but may not be associated with a concomitant del9q. Three Ph-negative cases in our study had cryptic insertions of the *BCR* gene into the derivative 9. The iFISH patterns of 2 of these cases were similar to those found in Ph-positive cases with loss of 9q (i.e., 1F1R1G and 1F2G1R). Hagemeijer et al[20] and Nacheva et al[21] postulated a two-step rearrangement for the localisation of *BCR/ABL* gene on chromosome 9. A *BCR/ABL* translocation occurs initially, followed by a translocation between the derivatives 9 and 22. This results in the masking of the exchange, with the *BCR/ABL* transposition to chromosome 9. Alternatively, it may involve an insertion of the *BCR* gene from chromosome 22 into the *ABL* gene on chromosome 9.[22]

Of the 3 Ph-negative cases, one showed the presence of the *BCR/ABL* fusion on both chromosomes 9. Consequently, the iFISH had an atypical 2F2G pattern. Localisation of the fusion gene on both chromosomes 9 has been previously reported.[20,23] A possible mechanism for the origin of the 2 *BCR/ABL* fusion signals may be a result of a somatic crossover between the rearranged and normal chromosomes 9, or a duplication of the der(9) with a concurrent loss of the normal 9. The presence of *BCR* and *ABL* fusion genes on chromosome 9 appears to confer a rapid clinical course or unresponsiveness to treatment.[20,24] The appearance of 2 such fusion genes may easily be misinterpreted as 2 Ph chromosomes in interphase cells. The majority of reports of double fusion genes on both chromosomes 9 are cases of CML in transformation and/or blast crisis.[23,25] However, the case in our study was a newly diagnosed CML in the chronic phase. The patient had received bone marrow transplantation 3 months after diagnosis. Five years after

the initial diagnosis, the patient is in remission and the *BCR/ABL* transcript has not been detected even with RT-PCR. This contrasts sharply with the poor prognosis reported in earlier publications. Indeed, Macera et al[7] reported the case of a Ph-negative patient with a chimeric *BCR/ABL* gene on chromosome 9 and the clinical course of the disease seemed unaffected by the chromosomal localisation of the gene. They concluded that expression of the aberrant chimeric gene may not be effected by positional regulation. In addition, there are some conflicting data pertaining to the influence of the position of the breakpoint within the *BCR* gene on the duration of the chronic phase.[26] Some evidence exists indicating that patients with a b3a2 splice site generally have a shorter chronic phase[27] than patients with a b2a2 breakpoint who tend to show better response to α-interferon.[28] In our case, molecular studies with RT-PCR revealed a b3a2 configuration. This would imply that the patient is expected to have a short chronic phase, but this does not seem to be the case here.

This study demonstrates the efficiency of FISH in detecting *BCR/ABL* fusion in CML with masked or variant Ph, which is often not apparent with conventional karyotyping. Hence, in Ph-negative cases, a routine cytogenetic approach would be non-informative. Although iFISH can identify and diagnose the majority of *BCR/ABL* positive patients, verification of iFISH pattern using metaphases is essential since it allows a precise interpretation of the rearrangement. Even though the FISH pattern has been previously determined, patterns can evolve such as the gain of a Ph chromosome, loss of der(9) and der(22), or further translocation events involving the normal and rearranged chromosomes 9 and 22.[11] As such, analysis of both interphase and metaphase cells will provide a more accurate assessment of the aberration. Patients having a cryptic genetic alteration with loss of proximal 9q sequences may be associated with poor prognosis and the time to disease progression on Glivec treatment is shorter. Hence, establishment of signal patterns with FISH is important as atypical patterns may have clinical diagnostic and prognostic implications.

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