Detection of BCR/ABL Gene in Chronic Myeloid Leukaemia: Comparison of Fluorescence *in situ* Hybridisation (FISH), Conventional Cytogenetics and Polymerase Chain Reaction (PCR) Techniques

[1]Reena RMZ, [1]Julia Munchar MJ, [1]Salwati S, [2]Zubaidah Z, [1]Hamidah NH, [1]Sharifah NA, [1]Cheong SK.

[1] Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, Bandar Tun Razak, Cheras, Kuala Lumpur.
[2] Division of Haematology, Institute of Medical Research, Jalan Pahang, Kuala Lumpur

ABSTRAK

Translokasi salingan t(9; 22)(q34; q11) yang menghasilkan kromosom Philadelphia (Ph[1]) dan juga gen lakuran BCR/ABL memainkan peranan utama dalam diagnosis dan patogenesis penyakit leukemia myeloid kronik (CML). Didalam kajian ini, kami mengkaji peranan teknik pendaflor *in situ* hibridasasi (FISH) dalam mengesan gen lakuran BCR/ABL pada 18 sampel aspirasi sumsum tulang daripada pesakit CML dan pesakit yang disyaki menghidap CML. Kami juga membuat analisa perbandingan mengenai kesensitifan, spesifisiti dan kadar pengesanan gen lakuran BCR/ABL di antara teknik penghibridan pendaflor (FISH), kariotip sitogenetik dan tindak balas berantai polimerase (PCR). Sensitiviti, spesifisiti dan kadar pengesanan untuk FISH adalah 100% untuk kesemuanya. Untuk kariotip sitogenetik pula, sensitiviti, spesifisiti dan kadar pengesanan adalah 85%, 100% dan 100% masing- masing. Kariotip sitogenetik juga telah mengesan sejenis aberasi kromosom di samping Ph[1]. Kajian ini menunjukkan kaedah FISH adalah sangat sensitif dalam mengesan gen lakuran BCR/ABL. Kariotip sitogenetik tetap menjadi salah satu kaedah utama dalam penyiasatan pesakit CML kerana terdapat kebarangkalian mengesan aberasi kromosom disamping Ph[1]. Oleh itu, kariotip sitogenetik dan FISH adalah dua teknik (komplementari) yang saling melengkapi dalam pengesanan gen lakuran BCR/ABL pada pesakit CML.

Kata kunci: BCR/ABL, kromosom Philadelphia, FISH, sitogenetik, RT-PCR

ABSTRACT

The reciprocal translocation t(9;22)(q34;q11) which gives rise to the Philadelphia (Ph[1]) chromosome and BCR/ABL fusion gene, plays a pivotal role in the diagnosis and pathogenesis of chronic myeloid leukemia (CML). In this study, we evaluated the role of fluorescence *in situ* hybridisation (FISH) in detecting the BCR/ABL rearrangement in CML patients. The sensitivity, specificity and detection rate of BCR/ABL gene using FISH, PCR and conventional cytogenetics (karyotyping) methods were also compared. 18 bone marrow samples of patients with clinically diagnosed CML and suspected of CML were collected. The sensitivity, specificity and positive predictive values of FISH were altogether

Address for correspondence and reprint requests: *Dr Reena Rahayu Md Zin, Cytogenetics Unit, Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, Bandar Tun Razak, Cheras, 56000 Kuala Lumpur. Email: ayureena@hotmail.com*

100% while the sensitivity, specificity and positive predictive values for conventional cytogenetics (karyotyping) were 85%, 100% and 100% respectively. Convetional cytogenetics (karyotyping) detected an additional chromosomal aberration in addition to the Ph^1 chromosome. In conclusion, FISH is a highly sensitive method in detecting the BCR/ABL gene. Conventional cytogenetics (karyotyping) remains an important investigation in the work up of suspected CML patients since there is a possibility of detecting chromosomal aberrations in addition to the Ph^1 translocation. Therefore, conventional cytogenetics (karyotyping) and FISH are complementary techniques and their results should be interpreted together with clinical information.

Key words: BCR/ABL, Philadelphia chromosome, FISH, cytogenetics, RT-PCR

INTRODUCTION

Chronic myeloid leukaemia (CML) is a malignant clonal myeloproliferative disorder affecting the pluripotent haematopoietic stem cell that can give rise to an increase of myeloid, erythroid cells and platelet in the peripheral blood, and myeloid hyperplasia of the bone marrow. The natural history of CML involves a triphasic phase from the chronic phase to an accelerated phase and then finally the blast phase.

Chronic myeloid leukaemia is distinguished from other chronic myeloproliferative disorders by the presence of a distinctive chromosomal abnormality, termed the Philadelphia or Ph^1 chromosome. This was the first consistent chromosomal abnormality identified in cancer (Nowell 1960). The Philadelphia (Ph^1) chromosome results from a reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11] and is present in at least 95 % of patients with CML (Nowell 1960). The Ph^1 chromosome is also present in 5% of children and 20% of adults with acute lymphoblastic leukaemia (ALL) (Hagemeijer, 1987). In CML, there is a translocation of part of the long arm of chromosome 22 to the long arm of chromosome 9 and reciprocal translocation of part of the long arms of chromosome 9 to chromosome 22 (Ph^1 chromosome). In the BCR gene, the breakpoint occurs within a region of 5.8 kb, known as the major breakpoint cluster region (M-BCR) (Heisterkamp 1985). The majority of breakpoints occur between exons b2 and b3 or b3 and b4 of the M-BCR giving rise to b2a2 or b3a2 chimeric messenger RNA (mRNA) (Heisterkamp 1985). Both transcripts encode a p210 protein with prominent tyrosine kinase activity and transforming ability. This elevated tyrosine kinase activity plays an important role in the pathogenesis of the disease (Daley 1990). In the ABL gene, the breakpoint may occur anywhere within a region larger than 300kb upstream of the exon 1b, between exons 1b and 1a, or downstream from exon 1a.

Due to this distinctive chromosomal and molecular abnormality, the detection of the t(9;22)(q34;q11) or the BCR/ABL rearrangement is necessary for the diagnosis of CML and for monitoring of treatment response. The techniques of detection include conventional karyotyping and molecular analysis. In about 5% of cases diagnosed clinically as CML, no Ph^1 chromosome is observed (De Klein 1982). These cases have either more complex translocations involving other chromosomes besides chromosome 9 and 22 or presence of cryptic BCR/ABL rearrangement (Kurzrock 1990). However, molecular analyses have shown that BCR/ABL is present in about half of these patients (Kurzrock 1990). Hence molecular analysis complement karyotyping by identifying abnormal clones undetected by karyotyping.

Conventional cytogenetics or karyotyping, is the standard method to study proliferating cells in CML. Generally, they are based on analysis of up to 25 metaphases and can be used as a screening method to detect chromosome anomalies. The major advantage of chromosome analysis in CML is the possibility of detecting chromosome aberrations such as trisomy 8 and isochromosome 17, in addition to the Ph^1 chromosome (Nanjangud 1994). On the other hand, chromosome analyses are cumbersome, time consuming, labour intensive and dependent on high numbers of viable dividing cells.

Fluorescence *in situ* hybridisation or FISH is rapidly becoming part of clinical practice in the workup of patients with haematological malignancies. FISH permits analysis of proliferating (metaphase cells) and non-proliferating (interphase nuclei) cells by detecting the location of specific nucleic acid sequences, using chromosome specific DNA probes. These DNA probes contain a label (fluorescence) that allows their detection after hybridisation to the target of interest (Cox 1998). FISH has been used to reliably identify the BCR/ABL gene at the time of diagnosis (Cox 1998). Increasing usage of FISH probes have also successfully detected a variety of molecular-cytogenetics variants. Using FISH analysis, several studies have identified cases with additional phenomena to the translocation, such as large deletions of the derivative chromosome 9, which has been reported to have a worse prognosis (Sinclair 2000).

The advantages of FISH are significant. It can be performed overnight and so it is particularly useful for situations which require diagnostic urgency. It is also easy to analyse large numbers of cells and the results are reproducible. It can be performed on various types of specimens, which include fixed cell pellets left over from cytogenetic studies on bone marrow or blood (Dewald 1998). They also work well with peripheral blood smears, touch preparations and paraffin embedded tissues (Paternoster, In press).

Reverse transcriptase polymerase chain reaction (RT-PCR) is another method of molecular analysis in detecting the BCR/ABL translocation. It is a highly sensitive technique for the detection of BCR/ABL mRNA. Using PCR, one can detect leukaemia specific mRNA sequences extracted from as few as one cell. The technique consists of two parts. The first part involves the synthesis of cDNA (complementary DNA) from RNA by reverse transcription. The second part involves the amplification of a specific cDNA by PCR. In our study, we have considered PCR as the gold standard.

Several studies have addressed the issue of which is the clinically most useful approach for the detection of BCR/ABL rearrangement in Ph^1 positive patients. Cox et al in 1998 reported that the sensitivity of PCR and FISH related to Ph^1 positive cases were 97% and 100% respectively with 5% false positive results by PCR. In another study on 40 samples of Ph^1 positive related disorders, Tbakhi et al (1998) found that FISH and PCR were positive in 100% of CML samples. In these two studies, both of them had utilised conventional cytogenetics (karyotyping) as their gold standard. Both of these studies had concluded that either FISH or PCR procedure offers a specific, cost effective and faster alternative to karyotyping. Both of these studies had analysed all the Ph^1 related disorders which include CML, acute lymphoblastic leukaemia and acute myeloid leukaemia.

The present study aims to evaluate the role of FISH in detecting the BCR/ABL rearrangement in clinically diagnosed CML patients. A comparative analysis between FISH (molecular cytogenetics) and conventional karyotyping were carried out utilising PCR as our gold standard. The signal patterns obtained using the BCR/ABL ES (Extra Signal) FISH probes were also studied.

METHODOLOGY AND MATERIALS

This is a cross sectional study on all cases diagnosed with CML at various stages of therapy and those suspected of CML from Hospital Universiti Kebangsaan Malaysia (HUKM) recruited between January 2003 and December 2004. Cytogenetics, PCR and double colour ES FISH analyses were performed on the samples for the presence of BCR/ABL gene rearrangement. Routine diagnostic procedures for these patients consisted of morphology and differential white blood cell counts on bone marrow and peripheral blood. Diagnosis of CML was made by clinical findings, bone marrow aspirate and neutrophil alkaline phospahatase score. Patients with other forms of myeloproliferative disease including Acute Lymphocytic Leukemia (ALL) and Acute Myeloid Leukemia (AML) were not included.

Conventional Cytogenetics (Karyotyping)

Samples of bone marrow were cultured and harvested following standard protocol (Barch 1997). Details of the karyotype were reported according to ISCN 1995.

Fluorescence in situ Hybridisation (FISH)

ES (extrasignal) FISH analysis was performed on the same bone marrow samples harvested for cytogenetics examination and following manufacturer's protocol (Vysis Inc. Downers Grove, Illinois, USA). Hybridisation signals were evaluated using BX60 fluorescence microscope equipped with appropriate filters for simultaneous visualization of fluorescein isothiocynate (FITC) and tetra-methylrhodamine isothiocynate (TRITC) fluorescence.

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

In this study, we considered PCR as the gold standard in the diagnosis of CML. We adopted strict precautions and conditions to avoid contamination.

Total RNA isolation and cDNA synthesis

Mononuclear cells from fresh bone marrow aspirates were isolated by Histopaque density gradient centrifugation. The mononuclear layer was aspirated into a 5 mL tube and stored at –80^{o}C. The RNA extraction was performed using the acid guanidinium thiocyanate - phenol - chloro-form extraction (AGPC) method.

Complementary DNA (cDNA) synthesis was done using 5X RT buffer, $MgCl_2$, PCR nucleotide mix, Rnasin, random hexamers and Moloney murine leukemia virus reverse transcriptase (M-MLVRT, Promega Corp). RNA sample was initially transferred into a PCR tube and heated at 70^{o}C for 10 minutes using a thermocycler. After a quick chill in ice, cDNA mix was then added to the RNA sample. PCR was performed on the mixed cDNA sample consisting of 1 cycle at 37^{o}C for 90 minutes and 65^{o}C for 10 minutes. The sample was then stored at –20^{o}C.

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

Master mix for PCR (distilled water, 10x PCR buffer, primers, 10mM of PCR nucleotide mix and 25 mM of $MgCl_2$, Promega Corp) was added to the PCR tube along with cDNA sample. After a hotstart of 1 min at 94^{o}C, 0.5 uL of Taq DNA polymerase was added to each sample. PCR was performed on an automated thermocycler which consisted of 35 cycles at 94^{o}C for 1 minute, 65^{o}C for 50 seconds, 72^{o}C for 1 minute, and final extension at 72^{o}C for 10 minutes. Reaction products were electrophoresed on a 1.5% agarose gel with ethidium bromide staining for 45 minutes at 80V. The products were then visualised under ultraviolet light.

RESULTS AND DISCUSSION

Eighteen cases were studied by conventional karyotyping, FISH and RT-PCR. The results are presented in Table 1. BCR/ABL mRNA was detected in 13 of

TABLE 1. Patient's characteristics and results of cytogenetics, FISH and RT-PCR.

Patient No	Age	Sex	Hb (g/dL)	Leucocyte x10/mm3	Karyotyping result	FISH result	RT- PCR result
1	33	M	5.7	120	46XY, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
2	61	M	11	195	46XY, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL*
3	38	M	14.7	75.4	46XY,-22, t(9;22)(q34;q11.1)der (3), +mar	BCR/ABL	BCR/ABL
4	50	F	11.1	21.6	No metaphase	BCR/ABL	BCR/ABL
5	25	M	13.4	96.6	46XY, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
6	31	F	12.3	6.5	46XX, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
7	29	M	11.4	154.6	46XY t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
8	26	M	11.8	23.7	Very few cells	Not performed	BCR/ABL
9	39	F	8.4	17.6	47XX,+8,i(17q),t(9;22)(q34;q11.1)X2	BCR/ABL	BCR/ABL
10	36	M	14.6	22.1	46XY, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
11	40	M	8.8	158	46XY, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
12	32	F	8.3	182	46XX, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
13	29	F	10.5	84.2	46XX, t(9;22)(q34;q11.1)	BCR/ABL	BCR/ABL
14	77	M	9.4	9.0	46XY,No abnormal karyotype	No fusion gene	No fusion gene mRNA
15	45	F	9.8	7.3	46XX,No abnormal karyotype	No fusion gene	No fusion gene mRNA
16	53	F	7.8	12.2	46XX,No abnormal karyotype	No fusion gene	No fusion gene mRNA
17	34	M	7.5	3.1	46XY,No abnormal karyotype	No fusion gene	No fusion gene mRNA
18	43	M	7.6	3.5	46XY,No abnormal karyotype	No fusion gene	No fusion gene mRNA

* RT-PCR performed on initial sample showed insufficient mRNA. However, a repeat RT-PCR performed on follow up sample was BCR/ABL positive

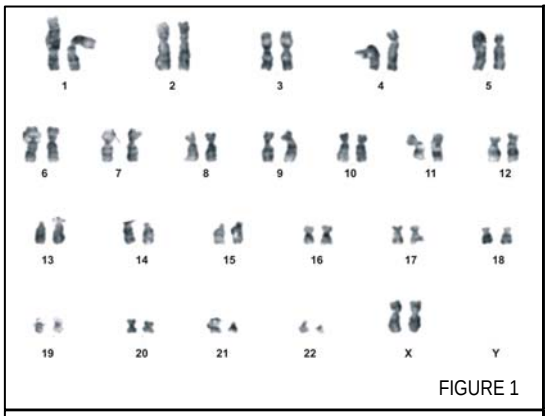

FIGURE 1

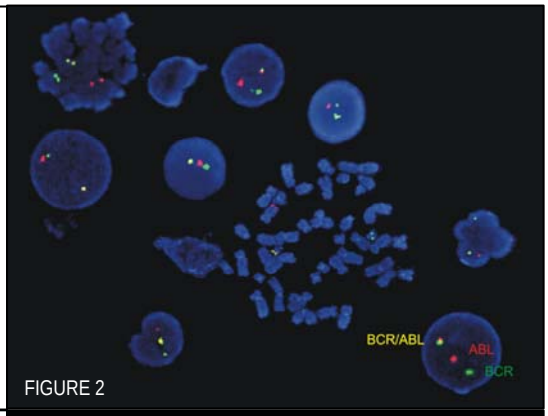

FIGURE 2

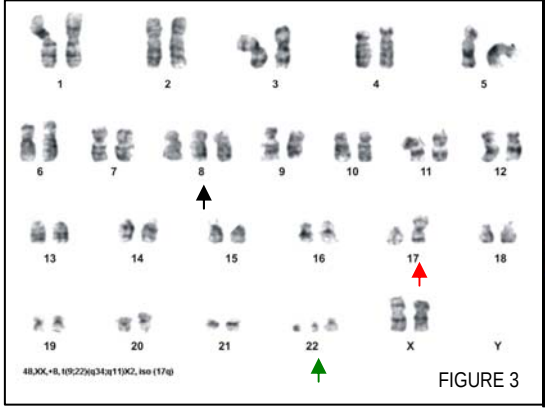

FIGURE 3

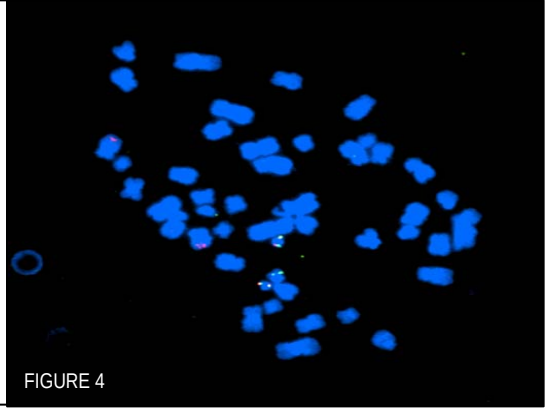

FIGURE 4

FIGURE 1. Presence of Ph[1] chromosome t(9;22)(q34;q11.1) by conventionl karyotyping.

FIGURE 2. Interphase FISH showing presence of BCR/ABL gene fusion. One orange, one green and one fused orange and green signal is seen in all the nucleus possessing t(9;22).

FIGURE 3. Presence of extra copy of chromosome 8 (trisomy 8)(↑) and isochromosome 17q (↑) in addition to Philadelphia chromosome (↑) in patient 9.

FIGURE 4. FISH analysis confirmed presence of two BCR/ABL fusion gene in patient 9. Cytogenetic analysis showed presence of two Ph[1] chromosomes, an extra copy of chromosome 8 (trisomy 8) and an isochromosome 17q (Figure 3)

TABLE 2. Correlation between Conventional Cytogenetics and RT-PCR.

Methods	RT-PCR	
	mRNA+	mRNA-
Cytogenetics Ph^1+	11	0
Cytogenetics Ph^1-	2	5
Total	13	5

TABLE 3. Correlation between FISH and RT-PCR.

Methods	RT-PCR	
	mRNA+	mRNA-
FISH BCR/ABL +	12	0
FISH BCR/ABL -	0	5
Total	12	5

TABLE 4. Correlation between Conventional Cytogenetics and FISH.

Methods	FISH	
	BCR/ABL +	BCR/ABL -
Cytogenetics (Ph^1 +)	11	0
Cytogenetics (Ph^1 -)	0	5
Total	12	5

these samples (72%). The remaining five samples did not show presence of BCR/ABL mRNA. Eleven patients (61%) were Ph^1 positive (Table 1 and Figure 1) and all of them showed presence of BCR/ABL mRNA by PCR. Two out of the remaining seven samples did not have any metaphase and showed very few cells respectively. However, these two samples were proven mRNA positive by PCR. The other five samples showed normal karyotype with no abnormalities seen. For conventional karyotyping, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 85%, 100%, 100% and 71% respectively (Table 2). The accuracy for conventional karyotyping method was 88%. The lower sensitivity was obviously due to a smaller number of samples utilised in our study. A previous larger study has quoted sensitivity of at least 95% (De Klein 1982). In patient 9, there was presence of an extra copy of chromosome 8 (trisomy 8) and iso-chromosome 17q (Figure 3). Seventeen samples were evaluable for FISH analysis. FISH was not performed on one sample due to insufficient material. Twelve samples (71%) showed presence of BCR/ABL fusion gene by FISH analysis (Table 1 and Figure 2) and all of these twelve samples showed presence of BCR/ABL mRNA by PCR. The remaining five samples did not show presence of the fusion gene by FISH. The sensitivity, specificity, PPV and NPV for FISH were all 100% (Table 3). The accuracy for FISH analysis was 100%. Previous studies have quoted sensitivities of FISH between 80% and 100% (Cox 1998, Tkachuk 1990). FISH had also confirmed presence of two BCR/ABL fusion genes in one patient. The cytogenetic analysis of this patients' sample also showed presence of two Ph^1 chromosomes (Figure 3 and Figure 4). From our study, we detected the BCR/ABL fusion gene in 12 of 17 samples analysed by FISH method (Table 4). The possibility of a false positive result by FISH was ruled out because the PCR finding on this sample was BCR/ABL mRNA positive.

Based on results of conventional cytogenetics (karyotyping) in our study, we acknowledge that conventional cyto-genetics (karyotyping) has its limitations. We could not obtain any metaphase spread in one of our samples and in another sample, only very few cells were present. The proportions of metaphases identified in cytogenetics depend on several factors, such as the number of metaphases analysed and the method used for obtaining metaphases. These metaphases only represent a small proportion of the total cells of a sample. However, conventional cytogenetics (karyotyping) has its strength because it is able to detect additional nonrandom chromosomal changes.

In conclusion, the ES fluorescence *in situ* hybridisation (FISH) technique is a highly sensitive and accurate method for detection of the BCR/ABL fusion gene. Despite several limitations, conventional karyotyping, however, will remain an important investigation in the work up of suspected CML patients since there is a possibility in detecting chromosomal aberrations in addition to the Ph^1 translocation. Therefore, conventional cyto-genetics (karyotyping) and FISH are complementary techniques and their results should be interpreted together with clinical information and other molecular studies.

REFERENCES

Barch M. 1997. The ACT Cytogenetics Laboratory Manual. 3rd Edition. Lippincott- Raven Publishers.

Cox MC, Maffei L, Buffolino S, et al. A comparative analysis of FISH, RT-PCR, and cytogenetics for the diagnosis of bcr-abl positive leukemias. Am J Cl Pathol 1998; 109:24-31.

Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the p210 bcr/abl gene of the Philadelphia chromo-some. Science 1990; 247:824-830.

De Klein A, Van Kessel AG, Grosveld G, et al. A cellular oncogene is translocated to the Philadel-phia chromosome in chronic myelocytic leukemia. Nature 1982;300:755-67.

Dewald GW, Wyatt WA, Juneau AL, et al. Highly sensitive fluorescence *in situ* hybridization method to detect double BCR/ABL fusion and monitor response to therapy in chronic myeloid leukaemia. Blood 1998; 91:3357-3365.

Hagemeijer A. Chromosomal abnormalities in CML. In Balliere's Clinical Haematology 1987; vol 1 no 4 963-981. London: Balliere Tindall.

Heisterkamp N, Stam K, Goffren J, de Klein a, Grosveld G. Structural organization of the BCR gene and its role in the Ph1 translocation. Nature 1985; 315:758-61.

Karger S. 1995. ISCN 1995. In: Mitelman F (ed.). Guidelines for Cancer Cytogenetics, Supplement to: An International System for Human Cytogenetic Nomenclature. Basel.

Kurzrock R Kantarjian HM, Shtalrid M, Gutterman JU, Talpaz M. Philadephia chromosome negative chronic myelogenous leukemia without breakpoint cluster region rearrangement: a chronic myeloid leukemia with a distinct clinical course. Blood 1990; 75: 445-52

Melo JV. The diversity of BCR/ABL fusion proteins and their relationship to leukemia phenotype. Blood 1996; 88: 2375-84.

Nanjangud G Kadam PR, Saikia T, Bhisey AN, Kumar A, Gopal R Chopr H, Hair CN, Advani SH. Karyotypic findings as an independent prognostic marker in chronic myeloid leukaemia blast crisis. Leuk Res. 1994; 18:385-392

Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science 1960; 132:1497.

Paternoster S, et al. A new method to extract nuclei from paraffin embedded tissue to study lymphomas using interphase FISH. Am J Patholo, In Press

Sinclair P et al. Large deletions at the t(9;22) breakpoint are common and may identify a poor prognosis subgroup of patients with CML. Blood 2000; 95:738-44.

Tbakhi A, Petty J, Sreenan JJ, et al. Comparative analysis of interphase FISH, RT-PCR to detect bcr-abl translocation in chronic myelogenous leukemia and related disorders. *Am J Clin Pathol* 1998; 109:16-23.

Tkachuk DC, Westbrook CA, Andreeff M, et al. Detection of BCR/ABL fusion in chronic myelogenous leukemia by *in situ* hybridization. Science 1990; 250:559-562.