The impact of fluorescence in situ hybridization on the detection of genetic aberrations in haematological oncology

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Abstract

Interphase fluorescence in-situ hybridization study has become more or less routinely utilized for the rapid detection of a BCR/ABL fusion in chronic myeloid leukaemia. Its applications in cancer cytogenetics have vastly multiplied, and probes for many different genetic loci involved in chromosomal translocations have been developed. Commonly occurring translocations such as t(8;21)(q22;q22), t(15;17)(q24;q21.1), and inv(16)(p13q22) in acute myeloid leukaemia (AML), as well as t(12;21)(p13;q22) in acute lymphoblastic leukaemia (ALL), are associated with a favourable prognosis. In addition, t(9;22)(q34;q11.2) and MLL gene rearrangement at 11q23, which are associated with a poor prognosis, can also be readily identified with gene-specific probes. This is particularly helpful in cases in which these genes are suspected to be involved in complex translocation, especially when the chromosome morphology is poor. This review will summarize the current utilization of these translocation probes in the clinical cytogenetic laboratory.

Key words: FISH, cytogenetics, gene rearrangement, chromosome translocation, leukaemia

熒光原位雜交技術在檢測白血病遺傳異常中的應用

摘要

細胞分裂間期熒光原位雜交技術已經被常規應用於慢性骨髓白血病BCR/ABL融合的快速檢測。它在腫瘤細胞遺傳學上的應用也不斷增加，而且已經生產出針對不同染色體基因易位的探針。經常發生的易位，例如急性粒細胞性白血病的t(8;21)(q22;q22)、t(15;17)(q24;q21.1)與inv(16)(p13q22)易位；急性淋巴細胞性白血病的t(12;21)(p13;q22)易位都與良好的預後有關。另外，t(9;22)(q34;q11.2)易位及MLL基因在11q23的重組通常有很差的預後，應用基因特殊性探針很容易鑒別出這些基因改變。熒光原位雜交技術在基因多點易位，特別是在染色體形態很差的情況下特別有用。本文將概述這些易位探針目前在臨床細胞遺傳學研究室的應用。

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Introduction

Conventional cytogenetic study plays an indispensable role in the diagnosis and prognosis of haematological malignancies. Although banding techniques represent the central theme at every cytogenetic laboratory, it is sometimes difficult to karyotype the tumour cells from a patient owing to unfavourable factors such as low specimen yield, low mitotic index, poor quality metaphases and other technical difficulties. With the advent of fluorescence in situ hybridization (FISH) technique, it is possible to detect both numerical and structural cytogenetic changes. FISH exploits the use of DNA probes to hybridize to known chromosomal loci of interest. The technique of in situ hybridization was originally described by Pardue and Gall1, where isotypically labelled probes were used with subsequent autoradiographic detection by photographic emulsion overlaying the metaphase chromosomes, nuclei or whole cells.

FISH has provided a new armamentarium for cancer cytogeneticists. The provision of information for more accurate and specific diagnosis of malignant disorders has been a major contribution of molecular cytogenetics. FISH can be applied both at diagnosis (as guided by the morphology and immunophenotype) and to monitor the size of the leukaemic clone post chemotherapy.[2] Molecular cytogenetic techniques also serve to supplement conventional cytogenetics in answering many important questions and bridging the gap between convention and molecular genetics in oncology.[3-5] In clinical practice, FISH is quite an attractive and practical way to assess those chromosomal abnormalities in haematological malignancies at a shortened turnaround time. Requests for FISH analysis are obviously increasing in clinical cytogenetic laboratories.

Recently, a number of commercially available probes are directly conjugated to fluorochromes, which have the advantage of eliminating the secondary detection procedure. They also provide strong signal intensity with low background. The advantage of direct labelling for in situ hybridization is that more than one probe may be used simultaneously, each labelled with different fluorochromes. When cloned chromosomal breakpoints are used as dual colour dual fusion probes, there is a decrease in false positivity and hence an increase in sensitivity over single colour probes for the detection of genes rearrangement. This has been successfully illustrated using BCR/ABL, the fusion gene that results from the Ph translocation in chronic myeloid leukaemia (CML). Probe selection for clinical use is based on the intended use and available technology in the laboratory. The different types of DNA probes useful for clinical applications can be categorized according to the complexity of the target sequences. Here, we discuss and illustrate the utility of DNA FISH probes for the detection of chromosomal translocation in haematological malignancies.

Translocation t(9;22)(q34;q11.2) and BCR/ABL gene rearrangement

The Philadelphia chromosome (Ph) was the first consistent cytogenetic abnormality noted in human cancer, arising from a reciprocal translocation[6-7], t (9;22)(q34;q11.2), and at the molecular level characterized by the fusion of the proto-oncogene ABL, located on the long arm of chromosome 9, with the BCR gene of chromosome 22, known as the breakpoint cluster region (bcr) (Fig 1b). The translocation is identified in bone marrow or peripheral blood metaphases of 90-95% of CML patients. The Ph chromosome is not unique to CML since it may also be detected in 20-35% of adults with acute lymphoblastic leukaemia (ALL), 2% of those with acute myeloid leukaemia (AML), and 2 - 10% of childhood ALL cases.[8]

Breakpoints in the two genes show variation but do occur within defined regions. In ABL, almost all breaks are located within a 200 kb region 5' to exon 2 (exons 1b to 1a).[9] In BCR, there are two breakpoint regions. In CML, most occur within the Major Breakpoint Cluster Region (M-BCR) located between approximately exons 12 to 16, producing a 210 kD oncoprotein. On the other hand, in ALL most occur within the Minor Breakpoint Cluster Region (m-BCR) located between exons 1 and 2, produce a 190 kD oncoprotein (Fig 1a). The first generation of BCR/ABL dual colour single fusion FISH (S-FISH) probes hybridizes to chromosome 9q34 (red: R) and chromosome 22q11.2 (green: G) respectively. This gives, in a normal cell, two distinct signals for each colour (2G2R) (Fig 1c). In the interphase nuclei of Ph+ cells, the probe signals appear as one signal of each colour and one fusion signal (yellow) on the derivative chromosome 22 (1G1R1F) (Fig 1c). This system detects the fusion gene with high sensitivity. However, when the percentage of Ph+ cells falls to < 10%, residual disease cannot be distinguished from what we call juxtaposition artefact[10].

In order to tackle this problem, an extra signal (ES-FISH) translocation probe was subsequently developed, which is designed to reduce the rate of false-positive results by marking the argininosuccinate synthetase (ASS) gene (extended up to ~600 kb) and thus providing an extra signal (red) on chromosome 9[11] (Fig 1a). In the interphase nuclei of normal cells, the probe may appear as 2G2R pattern (Fig 1d). In the interphase nuclei of Ph+ cells, one fusion signal, two orange signals (native ABL and residual ABL) and one green signal (1G2R1F) should be present to indicate the occurrence of the translocation (Fig 1d). Recently, the advent of dual colour dual fusion (D-FISH) probe represents a significant technological advancement in the monitoring of minimal residual disease. Using the strict scoring criteria, and scoring at least 200 nuclei, it is now possible to further reduce the cut off level of false positive cells to 0. 25% (SD, 0.39%; range, 0-1.5%). When extended to the analysis of 6000 nuclei, the detection limit was improved to 0.079%.[12] The design of these probes is to make them span the breakpoints, leaving behind a residual signal on the derivative 9q proximally and extending the signal on the derivative 22q (extended up to ~500 kb) distal to the break (Fig 1a). In the interphase nuclei of normal cells, the probe system gives a 2G2R pattern (Fig 1e). In an interphase nucleus containing a simple reciprocal t(9;22), individual red and green signals from the normal chromosomes 9 and 22 and two fusion signals, one each from the derivative chromosomes 9 and 22, are detected (1G1R2F) (Fig 1e).

In clinical practice, the spectrum of hybridization patterns seen in Ph variants using different BCR/ABL probes and the underlying mechanisms are recently described.[13-17] Neoplastic cells with submicroscopic deletion of the reciprocal ABL/BCR fusion gene on the der(9) chromosome (with 1G1O1F pattern) are particularly problematic16 (Fig 1f and 1g), because normal cells with coincidental overlap may have the same pattern. More recently, a new method that incorporates an aqua-labeled probe for the ASS gene into the BCR/ABL D-FISH probe set has been introduced. This tricolour D-FISH (TD-FISH) method takes advantage of the ASS probe to distinguish between neoplastic and normal cells (Fig 1a).[18] Finally, three-way translocation of t(9;22) (with 2G2R1F pattern) are recurrently found in 5-10% of CML Ph+ cases (Fig 1h and 1i). Taken together, exact interpretation of each atypical interphase FISH pattern should be supported by FISH on metaphases and molecular determination of BCR breakpoint. Furthermore, without conventional cytogenetics or molecular genetics data, the simplest way would be to perform S-FISH to confirm the presence of BCR/

ABL gene fusion.

Translocation t(8;21)(q22;q22) and AML1/ETO gene rearrangement

The t(8;21)(q22;q22) is observed in roughly 15% of all AML patients with an abnormal karyotype and in 40% of AML-M2 patients (Fig 2b), and identifies a morphologically and clinically distinct subset.[19] The t(8;21) is associated with a high remission rate and a better prognosis for AML. However, there were very few long-term survivors before the introduction of modern intensive chemotherapy. The AML1/ETO dual colour, dual fusion (D-FISH) translocation probe is designed to detect the fusion of the AML1 gene locus on chromosome 21q22 with the ETO gene locus on chromosome 8q22. The ~1.3 Mb AML1 probe (green) hybridises to the 21q22 band containing the AML1 gene. The ETO is a 480 kb probe that hybridizes to the 8q22 band containing the ETO gene (Fig 2a). In a normal cell, the pattern of two green and two red signals (2G2R) will be observed (Fig 2c). In an abnormal cell containing the t(8;21), the one green, one red, and two fusion signal pattern (1G1R2F) is observed (Fig 2d).

Translocation t(15;17)(q22;q21.1) and PML/RAR(gene rearrangement

t(15;17)(q22;q21.1) is the cytogenetic marker of acute promyelocytic leukaemia (APL, FAB-M3) which results in the formation of fusion between the PML gene on chromosome 15 and the retinoic acid receptor alpha (RARα) gene on chromosome 17 (Fig 3b). Establishing the diagnosis of APL with the typical t(15;17) is important for disease management because this disease is responsive to therapy with all-trans retinoic acid (ATRA), whereas other cases of AML and other M3-like disorders associated with variant translocations (~2%) do not respond to this treatment.[20]

The PML/RARα single fusion (S-FISH) probe contains the 5' region of the PML gene (~180 kb), including exons 1 and 2, and is proximal to the 5' and 3' breakpoint regions. The probe for RARα (~400 kb) contains sequences from exons 2 to 9 including the APL breakpoint region in intron 2 (Fig 3a). In a normal cell, the expected pattern is two green and two red (2G2R) signals (Fig 3c). In an abnormal cell containing a PML/RARα fusion, the one green (RARα), one red (PML) and closely adjacent or fused signal pattern (1G1R1F) is observed (Fig 3d).

In our experience, this method accurately detected PML/RARα fusion, but the analytical false positive rate was estimated at 4.8% (unpublished data). The analytical sensitivity of this probe is not adequate to detect low level of disease, especially for the minimal residual disease patients. Recently, a dual fusion PML/RARα (D-FISH) probe has been developed, which is designed to reduce the rate of false-positive results and thus providing an extra fusion signal on derivative chromosome 17q. The probe mixture contained a 700 kb DNA probe (green) that spanned the RARα locus at 17q21.1 and a 515 kb DNA probe (red) that spanned the PML locus at 15q22 (Fig 3a). In normal cell, two green and two red signals (2G2R) will be observed. In an abnormal cell containing the t(15;17), one green, one red, and two fusion signal pattern (1G1R2F) is observed (Fig 3e). Interestingly, a failure to detect small insertion of PML gene on 17q using this very large D-FISH probe was described in a case of atypical APL associated with lack of PML/RARα expression.[21]

inv(16)(p13q22) or t(16;16)(p13;q22) and CBF / MYH11 gene rearrangement

A well characterized AML subtype, AML-M4Eo (M4 with abnormal marrow eosinophils), is strongly associated with inv(16)(p13q22) (Fig 4b) and the rarer t(16;16)(p13q22). This abnormality has been associated with a relatively good prognosis,

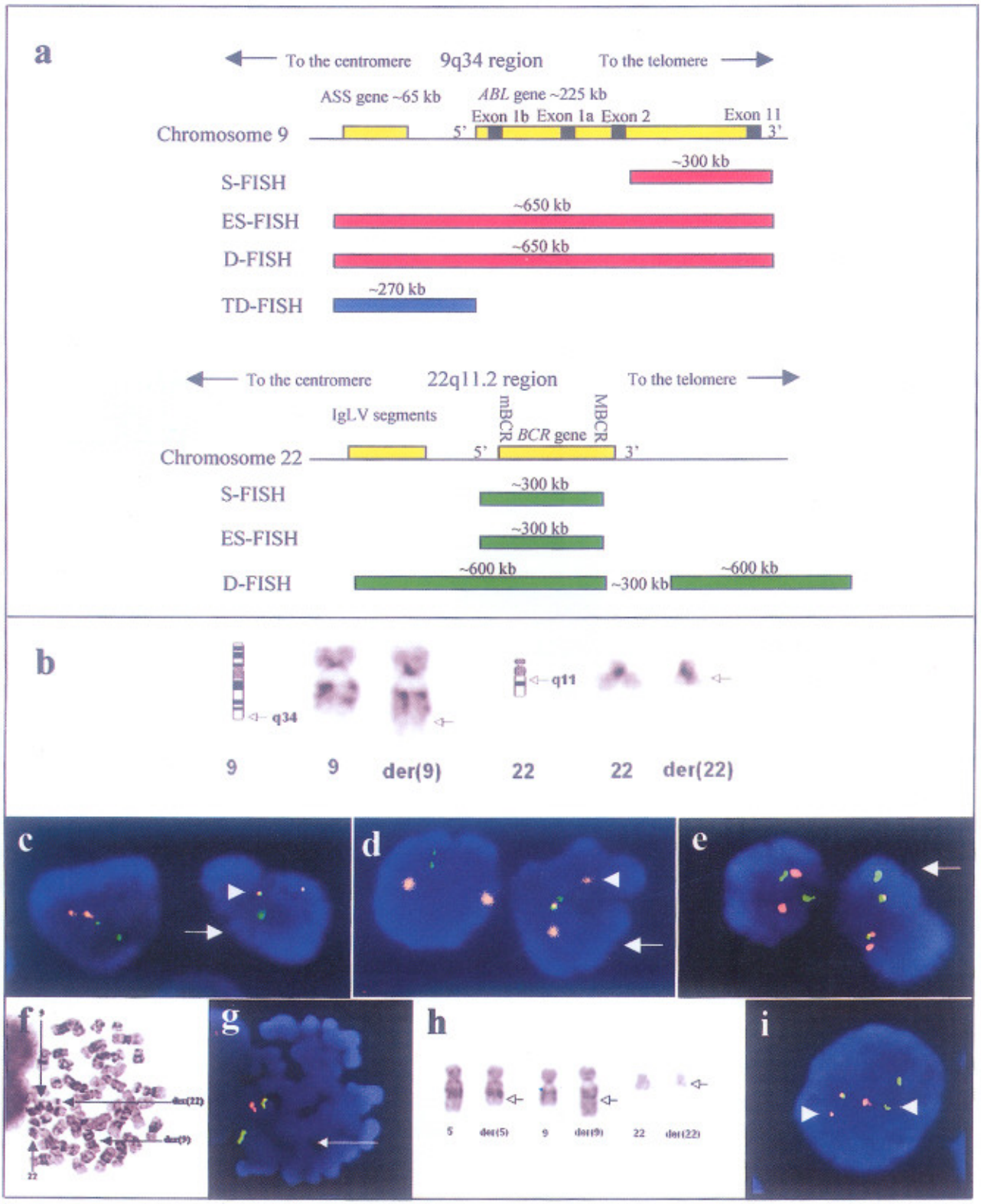

Figure 1. (a) Schematic representation of four different BCR/ABL dual color gene probes: signal fusion (S-FISH) probe, extra signal (ES-FISH) probe, dual fusion (D-FISH) probe and tricolour dual fusion (TD-FISH) probe. (b) Partial karyotype showing t (9;22)(q34;q11.2). G-banding with trypsin/Giemsa. (c) Interphase FISH using BCR/ABL S-FISH translocation probe, showing 2G2R pattern in a normal cell, and 1G1R1F pattern (fusion signal with arrowhead) in a Ph+ cell (arrow). (d) Interphase ES-FISH translocation probe, showing 2G2R pattern in a normal cell and 1G2R1F pattern (residual signal pointed by arrowhead) in a Ph+ cell (arrow). (e) Interphase D-FISH translocation probe, showing 2G2R pattern in a normal cell and 1G1R2F in a Ph+ cell (white arrow). (f, g) Metaphase FISH with the D-FISH probe in a case of CML with reciprocal ABL/BCR deletion on derivative chromosome 9. No ABL/BCR fusion signal is detected on the derivative chromosome 9 (white arrow). (h) Partial karyotype showing three-way translocation, t(9;22;5)(q34;q11.2,q31) in a CML patient. G-banding with trypsin/Giemsa. (i) Interphase D-FISH translocation probe, showing 2G2R1F pattern with ABL/BCR split signals (arrowhead) in a three-way Ph+ cell.

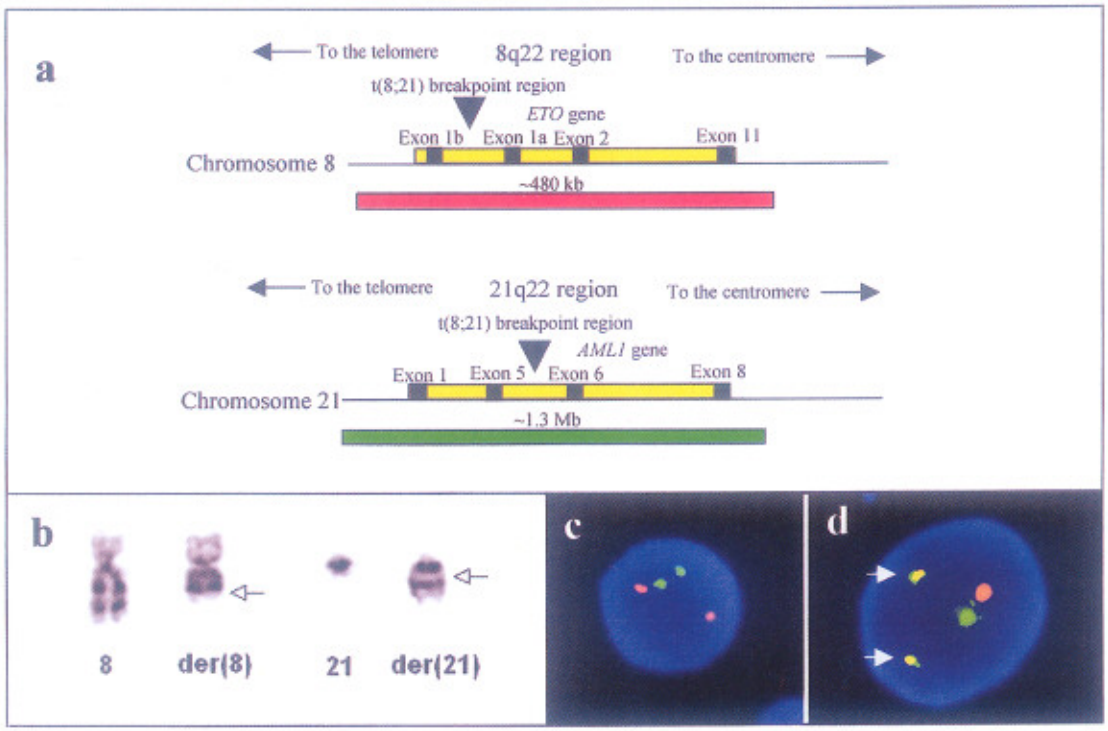

Figure 2. (a) Schematic representation of AML1/ETO dual color dual fusion gene probe. (b) Partial karyotype showing t(8;21)(q22;q22). G-banding with trypsin/Giemsa. (c) Interphase FISH using AML1/ETO D-FISH translocation probe, showing 2G2R pattern in a normal cell, and (d) 1G1R2F pattern (fusion signal pointed by arrowhead) in a t(8;21) positive cell.

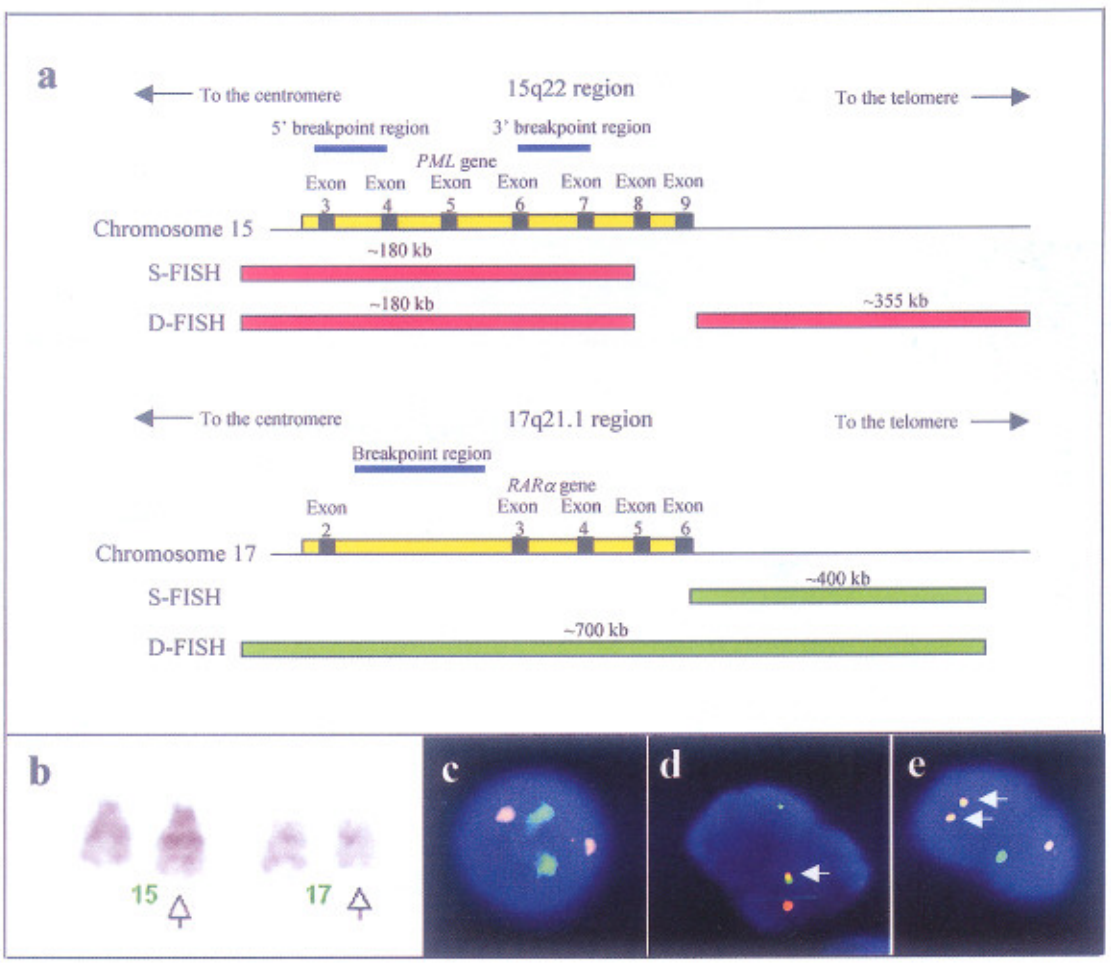

Figure 3. (a) Schematic representation of two different PML/RAR(dual color gene probes: signal fusion (S-FISH) probe and dual fusion (D-FISH) probe. (b) Partial karyotype showing t(15;17)(q22;q21). G-banding with trypsin/Giemsa. (c) Interphase FISH using PML/RARA S-FISH translocation probe, showing 2G2R pattern in a normal cell, and (d) 1G1R1F pattern (fusion signal pointed by arrowhead) in a t(15;17) positive cell. (e) Interphase FISH using PML/RARA D-FISH translocation, showing 1G1R2F pattern (two fusion signals pointed by white arrow) in a t(15;17) positive cell.

although with tendency to central nervous system relapse.[22] In this rearrangement the core binding factor beta (CBFß) gene on 16q22 is fused to the smooth muscle heavy chain gene (MYH11) on 16p13 resulting in the transcribed fusion gene CBFß-MYH11.

There are two kinds of commercially available dual colour break-apart DNA probe for detecting this chromosomal rearrangement (Fig 4a). (1) The CBFß break-apart rearrangement probe is a mixture of a 5' CBFß (red) and 3' CBFß (green) probes labelled with different fluorochromes. The 5' CBFß probe is ~150 kb and is positioned centromeric to the inv (16) breakpoint region. The 3' CBFß probe is ~170 kb and is positioned telomeric to the inv(16) breakpoint and does not extend over the breakpoint. (2) The MYH11 break-apart rearrangement probe contains dual labelled sequences proximal (green) and distal (red) to the 16p13.1. The two probe sequences are determined to be approximately 100-150 kb apart and both are of ~100 kb in size. The expected number of spots in a normal interphase nucleus is two fusion signals (2F) (Fig 4c). In inv (16) rearrangements the observed pattern will be one fusion signal and one green and one red signal (1G1R1F) (Fig 4d).

Translocation t(12;21)(p13;q22) and TEL/AML1 gene rearrangement

The t(12;21)(p13;q22) is one of the most common translocations in childhood acute leukaemia, accounting for 25% of childhood precursor-B ALL, and defines a distinct group of patients characterized by age between 1 and 10 years, B-lineage immunophenotype ($CD10^+$, $CD19^+$), and favourable outcome.[23] The reciprocal translocation t (12;21) fuses the helix-loop-helix domain of TEL gene to the DNA binding and trans-activation domains of the AML1 gene.[24] As the translocation is cryptic and usually escapes diagnosis on conventional cytogenetic study, molecular techniques such as FISH have to be employed for its detection.

The TEL/AML1 ES-FISH probe hybridizes to chromosome 12p13 (green) and to chromosome 21q22 (red) (Fig 5a). The TEL probe starts between exons 3 and 5 and extends approximately 350 kb towards the telomere on chromosome 12. The AML1 probe, approximately 500 kb in size, spans the entire gene and therefore gives an extra split signal when TEL/AML1 gene fusion is present, the absence of which helps to prevent co-localization of signals from chromosome 12 and 21 to be falsely interpreted as TEL/AML1 gene fusion. In the interphase nuclei of normal cells, the probe signals appear as two distinct signals of each colour (2G2R) (Fig 5b). In the interphase nuclei of t(12; 21) cells, one fusion signal, two red signals (native AML1 and residual AML1), and one green signal (native TEL) should all be present to indicate the occurrence of the translocation (1G2R1F) (Fig 5c). Additional or secondary genetic changes including TEL deletion (Fig 5d) and AML1 amplification (Fig 5e) are commonly encountered in childhood ALL with TEL/AML1 gene fusion, which are envisaged to play significant roles in disease progression.[25-26]

t(11q22) and MLL gene rearrangements

A variety of recurrent translocations involving the MLL gene at 11q23 are of great interest in acute leukaemia. More than 30 other chromosome bands have been implicated as fusion partner sites with 11q23.[27] It appears to be clinically important to determine whether or not this gene is involved, where cases in which an abnormality of MLL generally have a worse prognosis than those without. If there is any suspicion that this abnormality may be present, a FISH study using a break-apart DNA probe for the MLL gene is strongly advised. Recently, we showed that del(11q) is heterogeneous at the molecular level and may be a pointer for cryptic rearrangements involving the

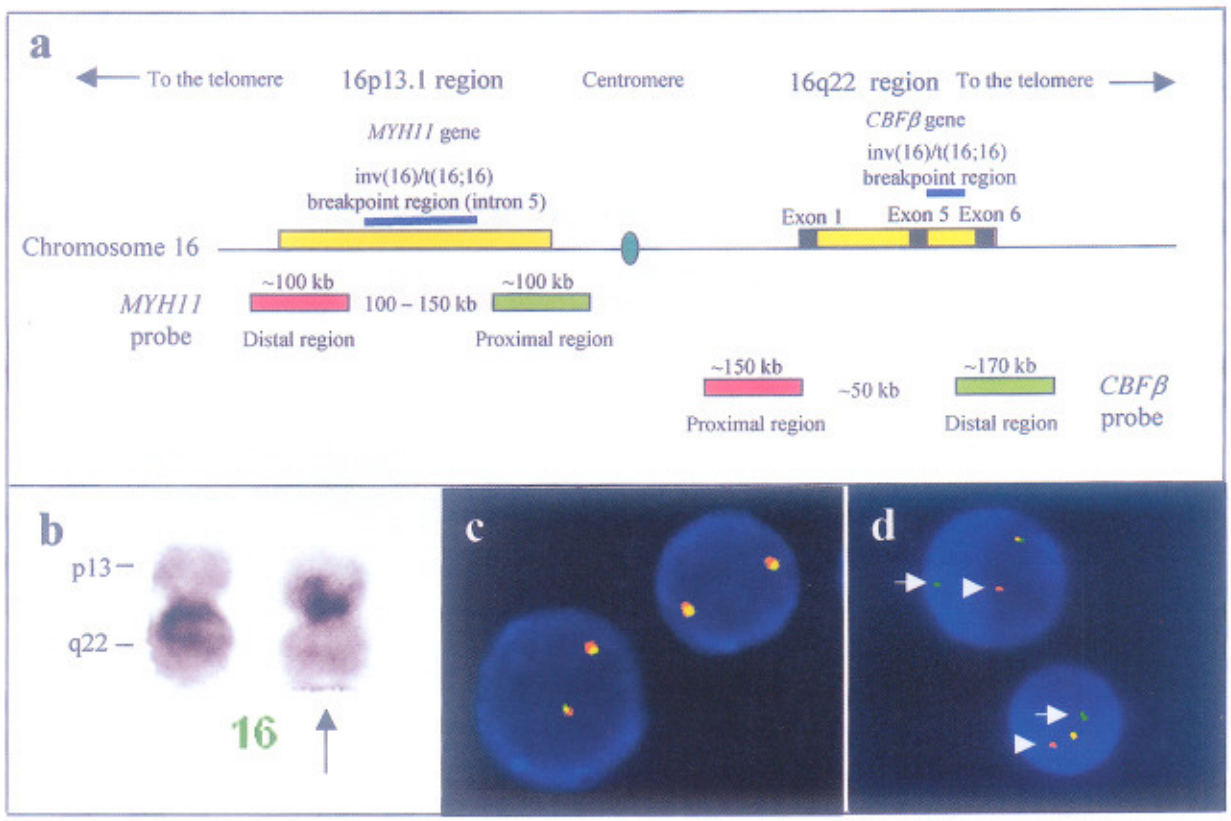

Figure 4. (a) Schematic representation of two different CBFß / MYH11 dual color gene probes: MYH11 break-apart probe and CBFß break-apart probe. (b) Partial karyotype showing inv(16)(p13q22). G-banding with trypsin/Giemsa. (c) Interphase FISH using CBFß break-apart rearrangement probe, showing 2F pattern in normal cells. (d) Interphase FISH with CBFß split signal (distal region, arrow; proximal region, arrowhead) in two cells, indicating CBFß gene rearrangement.

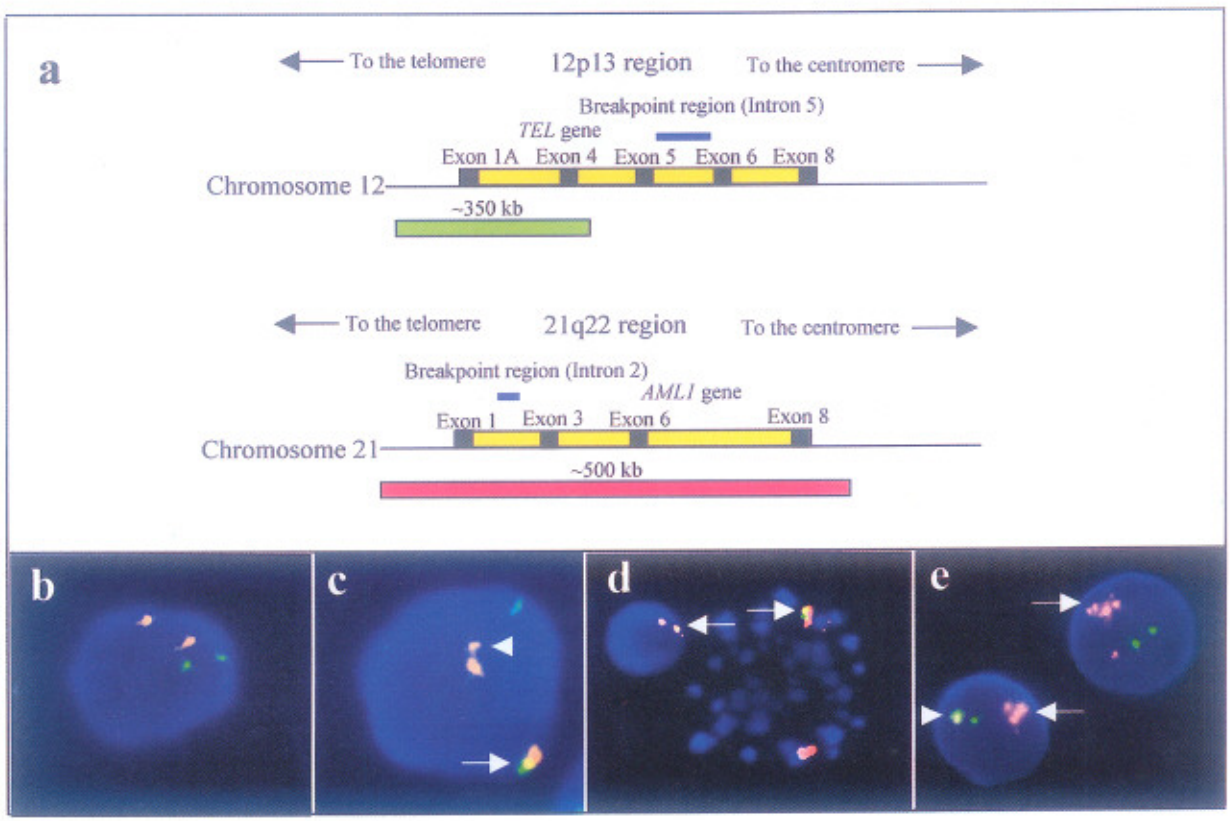

Figure 5. (a) Schematic representation of TEL/AML1 dual color extra signal gene probe. (b) Interphase FISH using TEL/AML1 ES-FISH probe, showing 2G2R pattern in a normal cell. (c) Interphase FISH showing fusion signal (arrow), AML1 split signal (arrowhead), normal TEL (green) and AML1 (red) in a t(12;21) positive cell. (d) t(12;21) positive cell associated with TEL deletion (fusion signal pointed by arrow). (e) Interphase FISH showing AML1 amplification (arrow), fusion signal (arrowhead), normal TEL and split signal (left cell), and interphase showing AML1 amplification (arrow) without TEL/AML1 fusion signal (right cell).

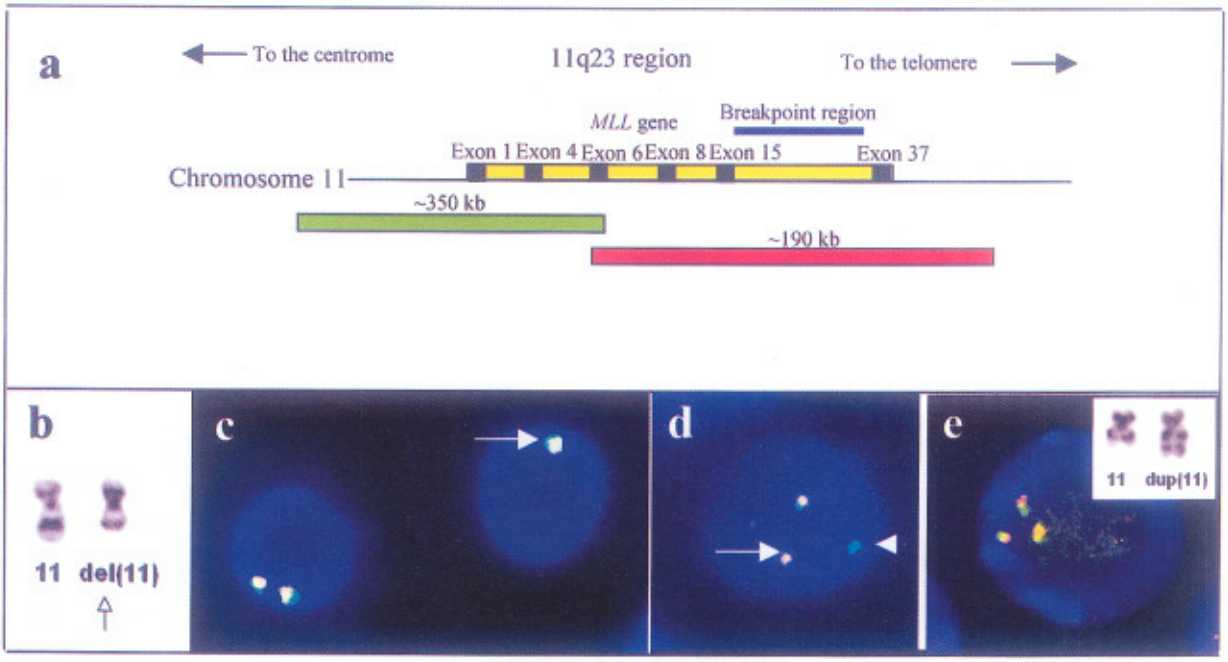

Figure 6. (a) Schematic representation of MLL dual color break-apart gene rearrangement probe. (b) Partial karyotype showing del(11)(q23). G-banding with trypsin/Giemsa. (c) Interphase FISH with MLL break-apart rearrangement probe, showing deletion of one MLL allele in a cell (arrow) and preservation of two MLL alleles in another cell. (d) Interphase FISH with MLL probe, showing MLL split signal (distal MLL region, arrow; proximal MLL region, arrowhead), indicating MLL gene rearrangement. (e) Interphase FISH with MLL probe, showing MLL duplication (3F pattern). Inset: Partial karyotype showing dup(11)(q13q23). G-banding with trypsin/Giemsa

chromosome 11q or the MLL gene.[28] It follows therefore that the presence of del(11q) in the myeloid malignancies necessitates further characterization by FISH techniques, in particular to detect cryptic MLL translocation that are of prognostic significance.

The MLL dual colour break-apart rearrangement probes consists of a 350 kb portion centromeric fragment of MLL gene breakpoint cluster region (green) and a 190 kb portion largely telomeric fragment of MLL gene breakpoint cluster region (red) (Fig 6a). The expected number of spots in a normal interphase nucleus is two fusion signals (2F) (Fig 6c). In rearrangements involving the MLL gene region the observed pattern will be one fusion signal and one green and one red signal (1G1R1F) (Fig 6d). The probe will also identify MLL deletions as a signal fusion with the loss of the other fusion signal (1F) (Fig 6b and 6c), consistent with preservation of one allele and deletion of the other. Duplication or amplification of chromosome band 11q23, including the wild type MLL gene, is a recurrent abnormality in therapy-related MDS and AML, and is closely related to mutation of the TP53 gene and to previous therapy with alkylating agent.[29] The copy number of the fusion signal will be increased (>2) in these cases (Fig 6e).

Conclusions and Perspectives

The cytogenetic and FISH study of haematological malignancies can provide important information regarding clonality, prognosis and pathogenic mechanism. It has a sensitivity clearly higher than conventional karyotyping and it may prove also superior to that reverse transcription polymerase chain reaction (RT-PCR) in cases with unusual gene arrangement breakpoints. Where specific chromosome abnormalities characterize particular types or subtypes of leukaemia, cytogenetics can confirm diagnosis or aid in the classification of the disease which, in certain instances, dictates the choice of therapy. Thus, detection of specific translocations would be a particularly useful investigational tool in the diagnostic sample of leukaemia, especially when dry tap or low mitotic marrow blood is obtained. Recently, using cryptic insertion of BCR at 9q34 in CML as an example, we have illustrated that, in clinical practice, atypical FISH results should not be interpreted in isolation, and should be integrated with information gathered through conventional cytogenetics, metaphase FISH, and if necessary molecular genetic studies.[17] Therefore, cytogeneticists and haematologists should be working closely together in performing and interpreting these tests.

FISH technology has evolved in the past few years to become as routine a test as a special stain in the clinical laboratory. In practice, getting a reliable result from a FISH study requires experience, as well as efforts to determine the precise local conditions needed for optimum hybridization, to assess and score positive and negative controls, and to determine local baseline levels. Therefore, the main obstacle to FISH testing in clinical laboratory is training experienced technologists to read slides on the fluorescence microscope. An automated spot reader is a solution once test volume exceeds a certain level for counting the fluorescent signal. Automated readers replace the currently cumbersome manual method to scan all cells and to archive images at the time of scanning. However, it is realistic to expect that automated detection of fusion signal still await elucidation, especially issues of sensitivity and accuracy.

With these technological advancements, the future continues to remain bright for molecular cytogenetics. FISH technologies are continually being developed, tested and incorporated within the clinical laboratories, which ultimately must

determine how best to utilize this diagnostic tool for betterment of patient care.

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