The prognostic value of FISH as an adjunct to conventional cytogenetics for the detection of cryptic gene rearrangements on chromosome 16. A retrospective investigation of 13 patients from Northern Ireland diagnosed with M4Eo acute myeloid leukaemia

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SUMMARY

M4Eo acute myeloid leukaemia (AML) patients with the typical chromosome 16 abnormalities have a favourable prognosis. These subtle 16q22 gene rearrangements can be difficult to detect by conventional cytogenetic methods and if missed could lead to the incorrect assignment of prognostic group and hence subsequent treatment strategies. We retrospectively studied 13 patients diagnosed with M4Eo AML for such chromosome 16 abnormalities comparing conventional cytogenetic (G-banding) and molecular (FISH) methods. G-banded analysis detected only 2 patients with definite chromosome 16 abnormalities whereas FISH detected 4 patients, one with the typical inversion and three with the typical chromosome 16 translocation. FISH analysis also confirmed a false +ve G-banded result in one patient and a false -ve G-banded result in another patient. Finally, FISH confirmed a deletion of one chromosome 16 homologue in another patient indicating a poor prognosis. The overall survival of patients with the typical 16q22 rearrangements (n=4) was also significantly better (P=0.007) than patients with normal chromosome 16 homologues or having other numerical and/or structural abnormalities (n=9). This set of data shows that FISH is a more accurate method for the detection of cryptic 16q22 gene rearrangements and because of the prognostic implications has become a mandatory test along with conventional cytogenetics for all newly diagnosed M4Eo AML patients in Northern Ireland.

INTRODUCTION

Reports have shown that karyotyping a leukaemic cell population using conventional cytogenetic methods is one of the most important prognostic determinants in acute myeloid leukaemia (AML).1,2,3 Both conventional cytogenetic and molecular methods have led to the definition of three prognostic groups (poor, intermediate & favourable) in AML. Results from the Medical Research Council (MRC) AML 10 trial and other international groups have clearly demonstrated that AML patients with chromosome abnormalities such as inv(16)/t(16;16), t(15;17) and t(8;21) have a favourable prognosis.4 Patients with an intermediate prognosis are those who have a cytogenetically normal karyotype or other chromosome abnormalities not associated with a good or poor prognosis. Patients with a poor prognosis are those with complex chromosome abnormalities (i.e. numerical and structural) or abnormalities such as abnormalities of chromosomes 5 or 7.

The typical chromosome 16 rearrangements consisting of either an inversion, inv(16)(p13q22), or a translocation, t(16;16)(p13;q22), are most closely associated with a distinct subtype of acute myelomonocytic leukaemia characterised by bone marrow eosinophilia. This subtype has been assigned the FAB-type M4Eo AML5 and although the eosinophilia can be variable it has been shown to be part of the leukaemia cell population.

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Molecular studies have shown that these chromosome 16 abnormalities generate a fusion protein (CBFβ-MYH11) between the core binding factor (CBFβ) gene at 16q22 and the smooth muscle myosin heavy chain (MYH11) gene at 16p13 which plays a vital role in myeloid cell transformation leading to leukaemia. Despite such a transformation, it is still highly desirable to detect those M4Eo AML patients who possess such favourable chromosome 16 abnormalities.

Conventional cytogenetic methods consisting of cell synchronisation and Giemsa banding (G-banding) are currently used to detect chromosome 16 abnormalities but visualisation can often be difficult especially if metaphase preparations are of a poor quality. It is therefore likely that the frequency of these abnormalities is higher than reported and individual patients unknowingly assigned to the wrong prognostic group and subsequent treatment strategy. The need for a more sensitive detection method as an adjunct to conventional cytogenetics at diagnosis is important for the correct stratification of such AML patients.

In this retrospective study we have assessed a molecular technique known as fluorescence in situ hybridisation (FISH) using a dual colour CBFβ DNA probe for the detection of chromosome 16 abnormalities and compared the results with our own conventional cytogenetic (G-banded) method and published data.

MATERIALS AND METHODS

Patients

Thirteen patients were diagnosed with M4Eo AML in Northern Ireland at the Belfast City and Royal Victoria Hospitals during a five year period (January 1995 to December 1999). The diagnosis and classification of patients were based on standard morphologic, cytochemical and immunophenotypic studies of leukaemic cells according to criteria proposed by the FAB and the MIC cooperative study groups.

Conventional cytogenetics (G-banding)

Chromosomes were prepared and analysed from bone marrow aspirates using conventional techniques. Routine cytogenetic analysis was performed on all patients using a trypsin-giemsa banding technique. Metaphase cells were examined from short-term (24 hour) unstimulated bone marrow cultures and chromosome abnormalities were described according to ISCN (1995). The number of metaphase cells analysed fully varied from 10 to 20 depending upon the quality of individual cell preparations.

Fluorescence in situ hybridisation (FISH)

FISH was carried out using a commercially available dual colour LSI CBFβ 16q22 probe mixture (Vysis, UK) containing a red coloured 5’CBFβ probe (R) positioned centromeric to the 16q22 breakpoint and a green coloured 3’CBFβ probe (G) as well as a mixture containing a 5’CBFβ probe (G) and a red coloured 3’CBFβ probe (R).

Fig 1. Representative fluorescence in situ hybridisation (FISH) analysis on metaphase spreads using the LSI dual colour CBFβ (16q22) probe mixture (Vysis, UK). (A) Characteristic inversion 16 split signal pattern consisting of a Red signal (R) at 16p13 and a Green signal (G) at 16q22 on the inverted chromosome 16. (B) Characteristic chromosome 16 translocation split signal pattern consisting of a fused Red/Green signal (R/G) at 16q22 and a Green signal (G) at 16p13 on one chromosome 16 with a Red signal (R) at 16q22 on the other chromosome 16. (Published by permission of Vysis UK).
probe (G) positioned telomeric to the 16q22 breakpoint. Hybridisation to a normal chromosome 16 homologue should therefore show a fused red/green signal (R/G) at the 16q22 region (Fig 1a). Likewise, hybridisation to a chromosome 16 homologue containing an inv(16)(p13q22) will cause this fused R/G signal to split with the individual red (R) and green (G) signals appearing on opposite arms of the inv(16) chromosome homologue (Fig 1a). Hybridisation to chromosome 16 homologues involved in a t(16;16) translocation preparation will result in a fused red/green signal (R/G) on the q arm at 16q22 on one chromosome 16 homologue and a green signal (G) on the p arm at 16p13 while the second chromosome 16 homologue will only contain the red signal (R) on the q arm at 16q22 (Fig 1b).

**Statistical analysis**

The outcome and survival of individual patients from the time of diagnosis was assessed in August 2001. Survival curves were estimated by the Kaplan-Meier method and compared M4Eo AML patients that were +ve by FISH for the typical 16q22 gene rearrangements (n=4) and the remaining M4Eo AML patients that were –ve by FISH for the typical 16q22 gene rearrangements (n=9) using the log-rank test. A P value <0.05 was considered statistically significant. All statistical computations were performed using SPSS for Windows (version 10).

**RESULTS**

Patient and cytogenetic/FISH data for the thirteen M4Eo AMI, patients are presented in Table 1.

| Patient | Age (yrs) | WCCa (x10^9/l) | Conventional Cytogenetics | FISH | Outcome* (months) |
|---------|-----------|----------------|---------------------------|------|------------------|
| 1       | 21        | 179            | 46,XX,inv(16)(p13q22)[18a]/46,XX[2] | +i   | Alive 32         |
| 2       | 43        | ND             | 46,XY,inv(16)(p13q22)[71/46,XX[13] | +i   | Alive 80         |
| 3       | 81        | 144            | 46,XX,?inv(16)(p13q22)[4]/46,XX[16] | –    | Dead; <1         |
| 4       | 22        | 20             | 46,XX,?add(16)(?q12)[151/46,XX[5] | +i   | Alive 28         |
|         |           |                | 46,XY,del(16)(q22)[16a]/46,XY[4] |      |                  |
| 5       | 50        | ND             | 46,XY,t(16;16)(p13;q22)[16]/46,XY[4] | +d   | Dead; <1         |
| 6       | 40        | 3              | 46,XX[10]a                | –    | Alive 50         |
| 7       | 52        | 170            | 46,XX[12]a                | –    | Dead; <1         |
| 8       | 59        | 120            | 46,XX[10]a                | –    | Dead; 9          |
| 9       | 47        | 145            | 46,XY[20]                 | –    | Dead; 4          |
| 10      | 72        | 31             | 48,XY,+8,+22[cpl4]        | +i   | Alive 38         |
| 11      | 42        | 15             | 47,XY,+8[3]/46,XY[27]     | –    | Dead; 7          |
| 12      | 77        | 100            | 47,XY,+8,t(12;21)(p13.3;q11)18]/46, XY[2] | –    | Dead; <1         |
| 13      | 71        | 100            | 46,XY,del(18)(q21)[12]    | –    | Dead; 7          |

aWhite cell count; aqualified result (i.e. <20 cells analysed); iinv(16)(p13q22), t(16;16)(p13;q22); del(16)(q22); *Outcome in months from date of diagnosis; ND not determined.
Their median age at diagnosis was 50 years (range 21-81 years) with a median white cell count (WCC) of 100 x 10^9/l (range 3 to 179 x 10^9/l).

Conventional cytogenetic and FISH studies
Conventional cytogenetic and FISH data for all thirteen M4Eo AML patients are presented in Table 1. Patients were grouped into 4 categories according to conventional cytogenetic findings.

‘Definite’ 16q22 rearrangement
Conventional cytogenetics revealed ‘definite’ 16q22 rearrangements in two patients (patients 1 & 2) both of which were confirmed by FISH. Furthermore, FISH was also able to further characterise these abnormalities as the typical chromosome 16 inversion in patient 1 and the typical chromosome 16 translocation in patient 2.

‘Suspected’ 16q22 rearrangement
Conventional cytogenetics indicated ‘suspected’ 16q22 rearrangements in three patients (patients 3-5). However, FISH analysis excluded a 16q22 rearrangement in patient 3 (i.e. a false +ve G-banded result) and confirmed the typical chromosome 16 translocation in patient 4. FISH also confirmed a deletion of one chromosome homologue at 16q22 in patient 5 due to the loss of the green telomeric CBFβ probe signal, the prognostic significance of which will be discussed later.

‘Apparently normal’ cytogenetics
Conventional cytogenetics was apparently normal for all autosomal chromosomes in 4 patients (patients 6-9). However, a ‘qualified’ normal result (i.e. <20 cells analysed) could only be obtained in 3 of these patients (patients 6, 7, 8) due to the overall yield and poor quality of metaphase preparations. FISH, however, was able to exclude any 16q22 gene rearrangement in all four patients.

‘Other numerical and/or structural’ abnormalities
Conventional cytogenetics detected other numerical and/or structural chromosome abnormalities in four patients with ‘apparently normal’ chromosome 16s (patients 10-13). FISH excluded 16q22 rearrangements in three of these patients (patients 11-13). FISH detected the typical chromosome 16 translocation in patient 10 (i.e. a false -ve G-banded result) who was reported to have only a trisomy for chromosomes 8 and 22 by conventional cytogenetics, the significance of which will be discussed later.

Survival of M4Eo AML patients
We compared the survival of M4Eo AML patients with the typical chromosome 16 abnormalities (n=4) and those patients with either normal chromosome 16s or other chromosome abnormalities (n=9) as illustrated in Figure 2. This Kaplan-Meier survival curve clearly demonstrated a significant longer survival and hence better prognosis for M4Eo AML patients with the typical chromosome 16 abnormalities (log rank test $\chi^2 = 7.27; P=0.007$).

**DISCUSSION**
This small retrospective investigation of 13 patients with M4Eo AML has highlighted the prognostic importance of FISH not only to detect but also to confirm and/or exclude 16q22 rearrangements that have previously been analysed using conventional cytogenetic techniques.

Conventional G-banded analysis detected ‘definite’ chromosome 16 abnormalities in only two (i.e. 15.4 %) of the 13 patients, both of which were confirmed by FISH. Because these 16q22 rearrangements can be difficult to detect, their frequencies are likely to be higher than reported so it is of little surprise that FISH was able to detect a further two patients with the typical chromosome 16 abnormalities in our group of patients. FISH not only highlighted a false +ve G-banded result in one patient who only survived one month but also highlighted a false -ve G-
banded result in another patient who was thought to be carrying only trisomies for chromosomes 8 and 22 (Fig. 3a). FISH clearly unmasked the typical chromosome 16 translocation abnormality in this patient who is still alive after 38 months. Although trisomy 22 itself is rare in AML, it is a common secondary chromosome abnormality in patients with 16q22 rearrangements, as are deletions of the long arm of chromosome 7 and trisomy 8, 5, 8. Interestingly, other groups have also unmasked cryptic chromosome 16 abnormalities upon reexamination of several AMLs that initially had been classified as having trisomy 8 or trisomy 22 as the only cytogenetic abnormality. The detection of false G-banded results, both +ve and -ve, using the FISH technique clearly demonstrates that, without FISH, patients can be assigned to the wrong prognostic groups and hence treatment stratification.

FISH confirmed a deletion with a breakpoint at 16q22 in a patient ‘suspected’ of having a 16q22 rearrangement by conventional cytogenetics. It has been shown that patients with such a deletion have a poorer prognostic outcome than patients with the typical 16q22 rearrangements. At the molecular level, these patients are also more than likely to have different consequences, since without the involvement of the smooth muscle myosin heavy chain gene located at 16p13, the generation of the typical chimeric CBFβ-MYH11 fusion gene is not expected. It has also been suggested that patients with the deleted chromosome 16 abnormality should be excluded from the current treatment recommendations reserved for patients with the typical 16q22 rearrangement. The survival of our patient with the 16q22 deletion (i.e. < 1 month) is concordant with the survival and poorer outcome reported for similar patients in other studies.

Overall, the four patients with typical 16q22 rearrangements have had a better prognostic outcome compared to the other nine patients. Only one of these nine patients was still alive at the time of assessment, the other eight having already died with a median survival of 2.5 months (range 1-9 months).

Two newly diagnosed M4Eo AML patients have been referred for cytogenetic investigations since the introduction of mandatory FISH screening for 16q22 rearrangements. The first patient, diagnosed in December 2001, showed the typical inversion by conventional cytogenetics and which was confirmed by FISH. The second patient, diagnosed in May 2002 also showed the typical inversion by conventional cytogenetics. The bone marrow sample for this second patient yielded metaphase spreads of extremely good quality and therefore the inversion was easily confirmed by conventional cytogenetics (Fig. 3b). FISH analysis, however, was still carried out and confirmed the conventional cytogenetic findings.
Although this series of patients is small it was notable that 50% of the chromosome 16 abnormalities detected showed the chromosome 16 translocation. This is in contrast to some previous studies that showed a higher incidence of chromosome 16 inversions.\textsuperscript{12} Whether this is a mere chance finding, a feature of the local population or reflects failure of other laboratories to characterise the chromosome 16 translocations is unclear.

In conclusion, FISH has proved to be a more sensitive technique compared to conventional cytogenetics for the detection of 16q22 rearrangements. Because of the simplicity of this technique and the availability of the commercially available CBFβ probe, FISH has now become a mandatory diagnostic test along side conventional cytogenetics for detection of 16q22 rearrangements in newly diagnosed M4Eo AML patients in Northern Ireland.

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