FISH Detection of PML-RARA Fusion in ins(15;17) Acute Promyelocytic Leukaemia Depends on Probe Size

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The diagnosis of acute promyelocytic leukaemia (APL) is usually confirmed by cytogenetics showing the characteristic t(15;17), but a minority of patients have a masked PML/RARA fusion. We report ten patients with APL and no evidence of the t(15;17), in whom the insertion of RARA into PML could not be demonstrated by initial FISH studies using a standard dual fusion probe but was readily identified using smaller probes. Given the need for rapid diagnosis of APL, it is important to be aware of the false negative rate for large PML/RARA FISH probes in the setting of masked rearrangements.

1. Introduction

Acute promyelocytic leukaemia (APL) is characterised by the reciprocal 15;17 translocation involving the PML gene on 15q24, and RARA gene on 17q21 in more than 90% of cases. This translocation creates a PML/RARA fusion gene on the derivative chromosome 15 [1]. Occasional cases of complex translocations involve 15, 17 and other partner chromosomes, or insertions of 15 into 17 and vice versa, all resulting in a PML/RARA fusion [2]. There are also rare variant translocations involving RARA and other partner genes: PLZF, NPM, NuMA, STAT5b, PRKARIA, FIP1L1, and BCOR [3–6]. In a series of APL cases without the standard t(15;17), most contained the PML/RARA fusion caused by an insertion and the fusions were usually demonstrated by both RT-PCR and fluorescence in situ hybridization (FISH) [7].

We present ten APL cases without cytogenetic evidence of t(15;17) in whom RT-PCR identified the PML/RARA fusion transcript, but initial FISH with standard probes showed no abnormality. Subsequent FISH revealed a small PML/RARA fusion signal in all cases on an apparently normal chromosome 15. Thus, all cases appeared to represent insertions of RARA into 15q24. Using the dual fusion probe from Abbott Molecular Inc., the PML signal swamped the tiny RARA signal. Careful examination showed a fusion signal in 3 cases, but, even in retrospect, there was no evidence of a fusion signal in 7 cases. Depending on which probe is used, a negative FISH result in APL does not, therefore, exclude the diagnosis.

2. Patients and Materials and Methods

Seven cases were identified from the records of 135 APL patients analysed by the Victorian Cancer Cytogenetics Service (VCCS) (cases 1, 3, 4, 5, 8, 9, and 10) over an 11-year period...
from 2002, two cases were identified from 25 APLs analysed by the Cytogenetics Department of LabPLUS, Auckland City Hospital (cases 2, 7) over a 5-year period from 2002 and one case was analysed by the Cytogenetics Department of Sullivan and Nicolaides Pathology, Brisbane (case 6) in 2005.

Cytogenetic studies were performed using standard protocols and FISH was performed according to the manufacturers’ instructions. Five FISH probes were used: LSI PML/RARA dual colour translocation probe (Abbott Molecular Inc., Des Plaines, IL, USA), LSI PML/RARA dual colour dual fusion translocation probe (Abbott), LSI RARA dual colour break apart rearrangement probe (Abbott), PML/RARA translocation probe (extra signal) and PML/RARA translocation dual fusion probe (Cytocell Technologies, Cambridge, UK).

FISH was performed on cytogenetic preparations fixed in 3:1 v:v methanol/glacial acetic acid, derived preferentially from short-term (usually less than 24 hour) cultures. Slide preparations were hybridized with the various locus-specific probes using codenaturation and overnight hybridization. Analysis was performed using a Zeiss Axioplan 2 Epifluorescence microscope and analysed by ISIS software (MetaSystems, Altlufheim, Germany). A minimum of 200 cells were scored for each probe by two scorers. Cut-off values for false positive results (below which the result was regarded as normal) were <1% for the dual fusion probes, 3% for the RARA break apart probe, 10% for the Cytocell PML/RARA extra signal probe, and 10% for the PML/RARA single fusion probe. Karyotypes were described according to ISCN (2009) [8].

For quantitative t(15;17) PML-RARA gene analysis, RNA was purified using Trizol as per the manufacturer’s instructions (Invitrogen), 1st-strand cDNA transcribed using SuperScript II (Invitrogen) and absolute quantitative PCR performed using Taqman assay on a Fast Real-Time ABI7500 PCR instrument (Applied Biosystems) using absolute quantitation standards.

3. Results and Discussion

APL is usually diagnosed on the bone marrow morphology and confirmed by the presence of the t(15;17) and detection of the PML/RARA fusion transcript via RT-PCR [3]. The t(15;17) is reported in 92% of APL cases, with 2% having simple or more complex variants, another 4% with insertions of RARA into PML or PML into RARA, and the rest with RARA fused to partner genes other than PML or, in 1%, with no RARA rearrangement [3]. We have identified ten cases of APL without a t(15;17) that appear to have produced a PML/RARA fusion gene by inserting a small segment of RARA into the PML gene on one cytogenetically normal chromosome 15. Seven of these cases were studied at the VCCS between 2002 and 2012, during which time 135 new cases of APL were diagnosed. Thus, the incidence of these cryptic insertions was 7/135 (5%), comparable to previously published series of cryptic abnormalities in APL [3, 9]. No specific morphological features distinguished this group as there were both classic hypergranular (n = 8) and variant hypogranular cases (n = 2). The RT-PCR results showed that there was no uniformity with regard to the PML breakpoint (Table 1), and immunophenotyping did not show any striking differences compared with the majority of APL cases (results not shown).

Clinical details of all patients are summarized in Table 1. The median age at diagnosis was 43 years (range 22–78 years) and there was an equal sex distribution. Survival data is available for 9/10 patients and 7/9 remain in complete remission 2–119 months after diagnosis. Two patients received modified Pethema protocols [11] and six were treated according to the Australian Leukaemia and Lymphoma Group studies APML3 [10] or APML4 [12]. All but two patients developed DIC and there were two early deaths—one of a presumed cerebral haemorrhage prior to the commencement of therapy (case 3) and one attributed to infection at 1 month post diagnosis (case 4).

The karyotypic, molecular, and FISH data are presented in Table 1. Seven of the ten patients had a normal karyotype; three contained additional abnormalities unrelated to the PML-RARA fusion. FISH in all cases using either the LSI PML/RARA t(15;17) dual colour dual fusion translocation probe (Abbott) in 9/10 cases or a single fusion translocation probe (Abbott) in one case failed to reveal a PML-RARA fusion signal despite RT-PCR identifying a PML/RARA transcript in all cases.

The Abbott dual fusion probe contains fluorescently labelled DNA that covers approximately 180 kb and 335 kb either side of the PML loci on chromosome 15 including all of the known BCR regions of PML and approximately 700 kb of chromosome 17 spanning all the breakpoint region of RARA (http://www.abbottmolecular.com/products/oncology/fish/hematology-probes.html). It was, therefore, puzzling that these probes were unable to detect a fusion signal in patients producing the PML/RARA transcript, whereas the Cytocell probes clearly revealed a fusion signal in all cases (Figure 1(a)).

The major difference between the probes lies in the size of the respective probes. Both the PML and RARA segments of the extra signal probe from Cytocell are only approximately 40 kb in size, 100x smaller than their Abbott counterparts. The Cytocell PML/RARA translocation dual fusion probe is larger than the extra signal probe but the fluorescently labelled segments that span either side of the PML locus are only 151 kb and 174 kb and those spanning RARA are 167 kb and 164 kb in size. In three cases, a review of the Abbott dual fusion probe result revealed a tiny green (RARA) signal underlying one PML signal, only visualized using the single-colour Spectrum Green filter (Figure 1(b)). In the remaining 7 cases, despite careful examination, the extra RARA signal could not be seen. Apparently, the discrepancy between the size of the Abbott PML signal and very small RARA segment inserted into 15q24 allowed the PML signal intensity to quench the RARA signal, whereas the less disparate intensities of the two signals using the Cytocell probes allowed the fusion to be visualized.

Occasional cases of APL with normal cytogenetics and normal FISH studies have been reported previously [14, 15]. Indeed, Brockman et al., when reporting on the efficacy of the original dual fusion PML/RARA probe, noted the difficulty in identifying masked PML/RARA fusions. In their series
Table 1

| Case no. | Age/sex | WBC × 10^9/L | Dx | DIC | Karyotype | FISH probes | Treatment protocol | Survival (months) |
|----------|---------|--------------|----|-----|-----------|-------------|-------------------|------------------|
| 1        | 22/F    | 0.6          | M3 | No  | 46,XX     | NT – NT + +  | ALLGAPML3        | 2+               |
| 2        | 25/F    | 6.5          | M3 | Yes | 46,XX     | – –/+ NT + NT | Modified         | 103+             |
| 3        | 30/M    | 161          | M3V| Yes | 46,XX,add(4)(q34),add(5)(q12)[4]/46,XY[17] | NT – NT + + 1 | Nil               | 0                |
| 4        | 30/F    | 1.9          | M3 | Yes | 46,XX,add(7)(q22)[10]/46,XX[20] | NT – – + 1/2 | ALLGAPML4        | 1                |
| 5        | 43/M    | 2.0          | M3 | Yes | 46,XY     | NT – /+ – + + 3 | ALLGAPML3       | 119+             |
| 6        | 43/F    | 42           | M3V| Yes | 46,XX     | NT – – + NT 3 | NA                | NA               |
| 7        | 50/F    | 11.5         | M3 | Yes | 46,XX     | – –/+ NT + NT | Modified         | 105+             |
| 8        | 57/M    | 1.1          | M3 | No  | 46,XY,t(2;13)(p25;q22)[18]/45,X,-Y[3]/46,XY[26] | NT – – + + 1 | ALLGAPML4        | 17+              |
| 9        | 59/M    | 6            | M3 | Yes | 46,XY     | NT – NT + + 1 | ALLGAPML4        | 41+              |
| 10       | 78/F    | 7.5          | M3 | Yes | 46,XX     | NT – NT + + 3 | ALLGAPML4        | 55+              |

*FISH probes abbreviations: SF(A): single fusion probe—LSI PML/RARA dual colour translocation probe (Abbott Molecular Inc.); DF(A): dual fusion probe—LSI PML/RARA dual colour dual fusion translocation probe (Abbott); BA(A): break apart probe—LSI RARA dual colour break apart rearrangement probe (Abbott); ES(C): extra signal probe—PML/RARA translocation probe (Cytocell); DF(C): dual fusion probe—PML/RARA translocation dual colour probe (Cytocell); NT: not tested; NA: not available; –: not visible; –/+: only visible via single band-pass filters; +: observed; #PML/RARART-PCR identification of the variant transcripts: 1 refers to the PML bcr1 within intron 6 and 2 to the PML bcr2 with variable breakpoints within exon 6 and 3 to PML bcr3 within intron 3 [13].

Figure 1: (a) Metaphase spread with the Cytocell PML/RARA extra signal probe showing a fusion signal on one chromosome 15 and a diminished RARA (green) signal on one chromosome 17. (b) Interphase cells with the Vysis dual colour dual fusion probe showing two red and two green signals in the top panel, two red signals in the middle panel, and two green signals plus a tiny third green signal (arrowed) in the bottom panel.
of 38 APL cases, two did not initially show a fusion signal and it was only by observing a tiny additional RARA signal, via the single-pass SpectrumGreen filter, located in the same position as one PML signal that the PML/RARA fusion was revealed [15]. This is the first report to consistently identify these cryptic rearrangements using alternate FISH probes.

Given the importance of a rapid and reliable test to confirm the diagnosis of APL, it is critical that the possibility of a false negative result using standard FISH probes is considered and that alternate probes are available for rare cases of insertions resulting in the PML/RARA fusion.

Conflict of Interests
The authors declare that they have no conflict of interests.

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