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Diagnostic utility of dual fusion *PML/RARα* translocation DNA probe (D-FISH) in acute promyelocytic leukemia

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Abstract. Translocation(15;17) leading to the formation of fusion gene *PML/RARα* is the diagnostic hallmark of acute promyelocytic leukemia (APL). Interphase fluorescence *in situ* hybridization (FISH) is one of the diagnostic tools employed for the detection of *PML/RARα* rearrangement. Using a dual color dual fusion (D-FISH) *PML/RARα* translocation DNA probe which hybridises both to *PML/RARα* and *RARα/PML* fusion genes, we characterised the FISH pattern of 52 APL patients at diagnosis and correlated the findings with conventional cytogenetics and RT-PCR analysis. The diagnostic sensitivity of the probe for *PML/RARα* was 100%. Seven patients had atypical D-FISH patterns; two had a masked *PML/RARα* fusion signal caused by the insertion of *PML* into *RARα* on 17q; 3 had an extra copy of *PML/RARα* in the form of isochromosome der(17)(q10)t(15;17) and one had duplication of the normal *RARα* gene with an ider(17q) masquerading as i(17)(q10). There was also one case of t(7;17;15) with a typical D-FISH pattern and in which metaphase FISH suggested an unusual 4-point break. In summary, *PML/RARα* D-FISH is a highly sensitive method for confirming diagnosis of APL. However D-FISH cannot be solely relied on for the diagnosis of APL owing to atypical patterns which are infrequently observed in cases with additional 17q structural abnormalities, gene insertion and gene duplication.

Introduction

Acute promyelocytic leukaemia is a distinct type of acute myeloid leukemia (AML) with accumulation of clonal myeloid cells at the promyelocytic stage of differentiation. Due to a high propensity for bleeding, patients with APL are associated with a very high morbidity and mortality rate. APL cells can harbour one of 4 types of fusion genes and patients which bear either *PML/RARα*, the most common subtype, or the rarer *NuMa-RARα* (1) carry the best prognosis owing to their sensitivity to all-trans retinoic acid (ATRA), arsenic trioxide (ATO) in combination with anthracycline-based chemotherapy. Thus, a correct diagnosis of APL based on careful morphological assessment and laboratory test confirmation for *PML/RARα* and *NuMa-RARα* is important for timely and optimal clinical management.

APL with *PML/RARα* has traditionally been diagnosed based on cytogenetic analysis which reveals t(15;17)(q22;q12) in which there is a reciprocal balanced translocation between the promyelocytic leukemia (*PML*) gene located on the long arm of chromosome 15 and the retinoic acid receptor *α* chain (*RARα*) gene leading to the formation of the pathogenic *PML/RARα* on 15q and *RARα/PML* on 17q. However many external factors such as marrow cellularity, variable culturing conditions and poor banding quality can negatively influence the success of chromosome culture. Since all APL patients with t(15;17) as well as a minor subset without any karyotypic abnormality show *PML/RARα* (2), FISH probes have been developed to detect *PML/RARα* in APL; firstly, a dual color single fusion *PML/RARα* translocation DNA probe (S-FISH) followed by a dual color dual fusion *PML/RARα* translocation DNA probe (D-FISH) with increased sensitivity and specificity. D-FISH signals are easy to interpret with a typical pattern diagnostic of *PML/RARα* in cases of APL with a simple karyotype with t(15;17)(q22;q12). However in cases with additional changes or complex karyotypes, atypical patterns are observed which can lead to uncertainties in interpretation. In this study, we studied 52 patients with APL using D-FISH and show that whilst the probe shows excellent specificity and sensitivity for diagnosis of *PML/RARα*, the interpretation of atypical patterns in some cases requires additional studies with S-FISH as well as metaphase FISH. Taken together, our studies suggest that D-FISH should not be solely relied on for the routine diagnosis of *PML/RARα* in APL.

Materials and methods

Patients, conventional cytogenetics. Between 1991 and 2004, 52 consecutive patients with APL diagnosed in the Queen Mary Hospital, Hong Kong, were identified. The patients comprised of 24 males and 28 females with a median age of 38 years at presentation (range, 8-73 years). Morphologic,

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Key words: acute promyelocytic leukaemia, cytogenetics, fluorescence *in situ* hybridization, PML-RARα, genetic marker

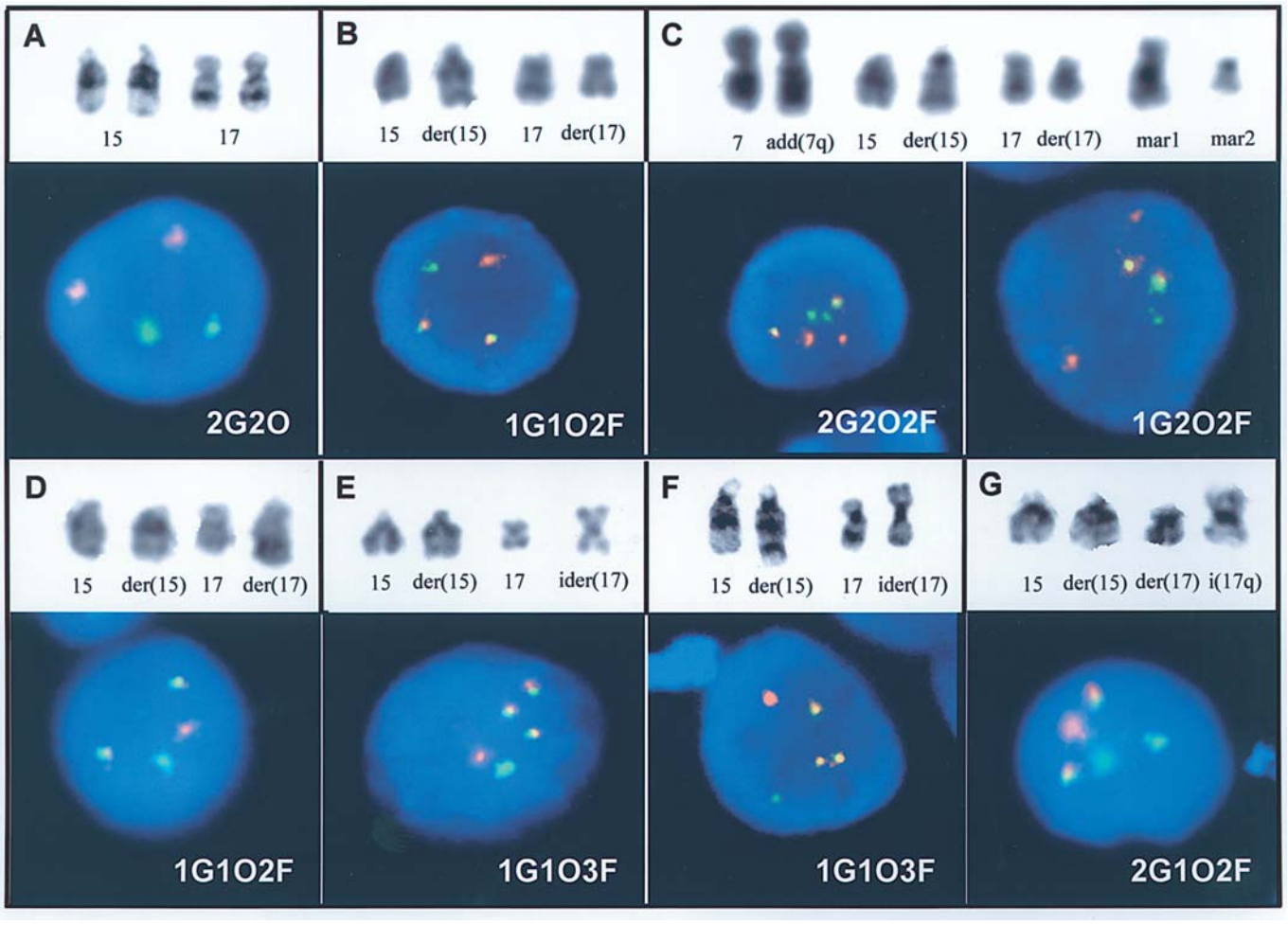

Figure 1. Representative partial karyotypes and interphase FISH patterns for the *PML/RARα* probe set. G designates a green *RARα* signal; O designates an orange *PML* signal; F designates a yellow signal, indicating fusion of *PML* and *RARα*. (A) Representative normal karyotype (upper) and normal nuclei (lower). (B) Representative partial karyotype (upper) and nuclei (lower) with t(15;17)(q22;q12). (C) Partial karyotype (upper) and nuclei (lower) with add(7)(q22), der(15)t(15;17), der(17)t(15;17), marker chromosome 1 and marker chromosome 2 of case 46. (D) Partial karyotype (upper) and nuclei (lower) with der(15)t(15;17) and der(17)add(17q)t(15;17) of case 47. (E) Partial karyotype (upper) and nuclei (lower) with der(15)t(15;17) and ider(17)t(15;17) of case 48. (F) Partial karyotype (upper) and nuclei (lower) with der(15)t(15;17) and ider(17)t(15;17) of case 51. (G) Partial karyotype (upper) and nuclei (lower) with der(15)t(15;17), der(17)t(15;17) and i(17)(q10) of case 50.

cytochemical and immunophenotypic diagnoses of APL (AML-M3) were performed according to standard French-American-British (FAB) criteria. Cytogenetic studies were performed on Giemsa-banded metaphases obtained through short-term synchronized and unsynchronized cultures of bone marrow cells supplemented by direct harvest, according to standard protocols previously published (3). Details of the karyotypes were reported in accordance with the International System for Human Cytogenetic Nomenclature (ISCN2005) (4).

FISH analyses and detection of PML/RARα fusion by reverse transcriptase polymerase chain reaction. For each case, 300 Carnoy's fixative-fixed interphase nuclei on cytospin smears were analyzed for the presence of *PML/RARα* D-FISH probes (Vysis, Downers Grove, IL, USA), according to the manufacturer's instructions. *PML/RARα* S-FISH probes (Vysis) were used for further confirmation of the presence of the *PML/RARα* fusion gene in 2 cases with complex karyotypes, metaphase FISH was performed on G-banded t(15;17)(q22;q12)-positive metaphases relocated using microscope coordinates, as described previously (5). This allowed direct morphological correlation of fusion signals and abnormal chromosomes.

RNA from marrow cells was extracted according to standard protocols and all cases were screened for the presence of *PML/RARα* mRNAs by fully nested RT-PCR, according to routine procedures (6,7).

Results

There were 12 cases of no growth, 5 cases of an apparent normal karyotype and 19 samples with t(15;17)(q22;q12) only. 16 samples showed t(15;17)(q22;q12) together with additional numerical and/or structural chromosomal abnormalities. All 52 cases of APL were confirmed by RT-PCR to contain *PML/RARα*.

Forty-five cases showed a typical D-FISH pattern diagnostic of the presence of *PML/RARα* and *RARα/PML* as well as the presence of normal cells not carrying the translocation; these

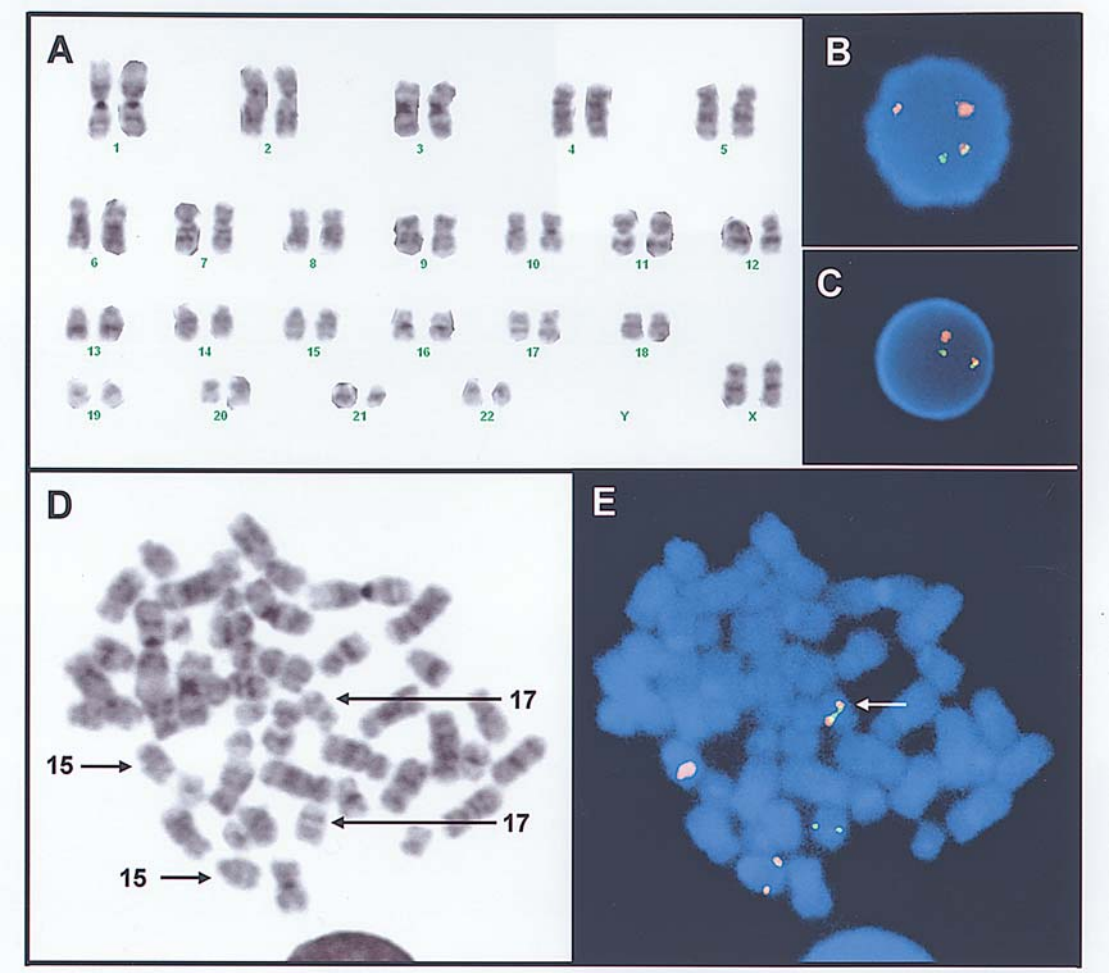

Figure 2. Conventional and molecular cytogenetic analysis of case 52. (A) Complete karyotype showing normal female karyotype. G-banding with trypsin/Giemsa. (B) Interphase FISH with *PML/RARα* D-FISH translocation probe, showing one green signal, two orange signals and one fusion (yellow) signal (ie 1G2O1F pattern). (C) Interphase FISH with *PML/RARα* S-FISH translocation probe, showing the presence of *PML/RARα* gene fusion (yellow). (D and E) Metaphase FISH analysis on a relocated G-banded chromosome using the D-FISH probe, showing the presence of fusion signal on chromosome 17 and the missing green signal (i.e. 3'portion of the *RARα* gene) on chromosome 15.

are indicated by two orange (*PML*) and two green signals (*RARα*), 2G2O (Fig. 1A), in contrast to APL cells which in addition showed two fusion signals (1G1O2F) representing *PML/RARα* and *RARα/PML* on derivative chromosome 15 and 17 respectively (Fig. 1B).

Seven cases had atypical patterns with additional signals. These were of one of three patterns: a) 2 cases (case 16 and 17) with normal karyotypes (Fig. 2A) showed an atypical two orange, one green and one fusion (1G2O1F) pattern in over 90% of cells analyzed. Strikingly, diminished fluorescence intensity of one orange signal was observed (Fig. 2B and Table I). The presence of *PML/RARα* gene fusion was subsequently confirmed on interphase nuclei using the *PML/RARα* S-FISH probe (Fig. 2C). Metaphase FISH on G-banded metaphases showed the only fusion signal on chromosome 17q, no green fluorescence signal was detected on chromosome 15 (Figs. 2D and E). Taken together, these results indicated a cryptic insertion of *PML* at 17q12 leading to *PML/RARα* fusion on chromosome 17q. This is in contrast to the typical balanced translocation where the pathogenic *PML/RARα* fusion gene is located on der(15). b) Two cases showed duplication of *PML* and/or *RARα* genes. Case 46 which has complex chromosomal abnormalities with marker chromosomes showed abnormal signal patterns of 2G2O2F (85%) and 1G2O2F (10%) (Fig. 1C). This implies duplication of *PML* and *RARα* in the former clone and duplication of *PML* in the latter clone. However, hidden trisomy or triploid clone cannot be excluded, especially if these cells were not mitotically active and hence missed by conventional cytogenetic analysis. Case 50 harbored i(17)(q10) (Fig. 1G) but not ider(17)(q10)t(15;17). This case had a signal pattern of 2G1O2F (Fig. 1G) which indicated duplication of a normal *RARα* gene. c) Three cases showed extra fusion signals (1G1O3F); case 48 (Fig. 1E), 49 and 51 (Fig. 1F) (60%, 39% and 98% respectively). This signal pattern was consistent with an extra copy of *RARα/PML* in the form of isochromosome der(17)(q10)t(15;17).

Finally, the karyotype of case 52 was an apparent three-way translocation involving chromosomes 7, 15 and 17 (Fig. 3A) but with typical D-FISH patterns, i.e. 1G1O2F and 2G2O patterns (Fig. 3B). Further studies by metaphase FISH showed that the two fusion genes were located on der(15) and der(7) (Fig. 3C and D). This suggests a 4-break mechanism to account for the FISH results and a more definitive karyotype would

Table I. Fifty-two APL patients with either atypical *PML/RARα* D-FISH patterns or t(15;17)(q22;q12) with additional chromosomal abnormalities.

Case	Karyotype	D-FISH patterns: % cells positive
No growth (n=12)		
1-12	no growth	typical: 70%-100%
Normal karyotype (n=5)		
13-15	normal karyotype	typical: 95%-98%
16	46,XX[11]	1G2O1F: 93%
17	46,XY[15]	1G2O1F: 91%
t(15;17) only (n=19)		
18-36	t(15;17)(q22;q12) only	typical: 90%-100%
t(15;17) with additional numerical changes (n=4)		
37	47,XX,+8,t(15;17)(q22;q12)[5]/46,XX[1]	typical: 92%
38	47,XX,+8,t(15;17)(q22;q12)[7]/46,XX[1]	typical: 92%
39	47,XY,t(15;17)(q22;q12),+21[9]	typical: 100%
40	47,X,-Y,+8,t(15;17)(q22;q12),+21[6]	typical: 100%
t(15;17) with additional structural abnormalities not involving 17q (n=6)		
41	46,XX,t(2;7)(p13;q22),t(15;17)(q22;q12)[12]	typical: 99%
42	46,XY,del(10)(q22),t(15;17)(q22;q12)[2]/46,XY[5]	typical: 99%
43	46,XY,inv(9)(p11q13),t(15;17)(q22;q12)[9]	typical: 100%
44	46,XX,inv(9)(p11q13),t(15;17)(q22;q12)[5]	typical: 97%
45	46,XY,t(15;17)(q22;q12),add(20)(q)[10]	typical: 99%
46	44~47,XX,add(2)(q37)[3],add(7)(q22)[15] der(15)add(15)(p11)t(15;17)(q22;q12)[16],-16[18], der(17)t(15;17)(q22;q12)[18],+mar1[12],+mar2[4][cp18]	1G2O2F: 10%, 2G2O2F: 85%
t(15;17) with additional structural abnormalities of 17q (n=6)		
47	46,XY,der(15)t(15;17) (q22;q12), der(17)add(17q)t(15;17)(q22;q12)[5]/46,XY[4]	typical: 92%
48	46,XY,t(15;17)(q22;q12),i(17)(q10)[4][a]	typical: 30%, 1G1O3F: 60%
49	46,XX,t(15;17)(q22;q12)[6]/46,XX,der(15)t(15;17) (q22;q12),ider(17)(q10)t(15;17)(q22;q12)[5]	typical: 58%, 1G1O3F: 39%
50	46,XY,t(15;17)(q22;q12),i(17)(q10)[5]	2G1O2F: 85%
51	46,XY,der(15)t(15;17)(q22;q12), ider(17)(q10)t(15;17)(q22;q12)[8]	1G1O3F: 98%
52	47,XY,+8,t(7;17;15)(q22;q12;q22)[15]/46,XY[1][b]	typical: 76%

[a]The definitive karyotype as resolved by FISH was as follows: 46,XY,der(15)t(15;17)(q22;q12), ider(17)(q10)t(15;17)(q22;q12)[4]. [b]The definitive karyotype as resolved by FISH was as follows: 47,XY,+8,der(15)t(15;17)(q22;q12),t(7;17)(q22;q11)t(15;17)(q22;q12).ish, der(15)(PML+,RARα+),t(7;17)(RARα+,PML+;RARα-). G, green signal of LSI probe for *RARα* gene; O, orange signal of LSI probe for *PML* gene; F, fusion signal of LSI probe for *PML/RARα* or *RARα/PML* gene arrangement.

therefore be represented as 47,XY,+8,der(15)t(15;17)(q22;q12), t(7;17)(q22;q11)t(15;17)(q22;q12).ish der(15)(PML+,RARα+), t(7;17)(RARα+,PML+; RARα-). A summary of cytogenetics and D-FISH patterns is shown in Table I.

Discussion

A correct diagnosis of APL with *PML/RARα* fusion is important since a high remission rate and improved survival can be achieved using ATRA or ATO-containing regimens. Conventional cytogenetic analysis alone cannot provide definitive evidence of the underlying molecular event in a significant proportion of cases, owing to poor growth of APL cells in culture and the cryptic translocation in some cases, both negatively influencing the detection rate. It is shown in the present study that 17 out of 52 cases (32.7%) failed to demonstrate t(15;17) but were indeed harboring *PML/RARα* fusions as demonstrated by D-FISH analysis. The detection

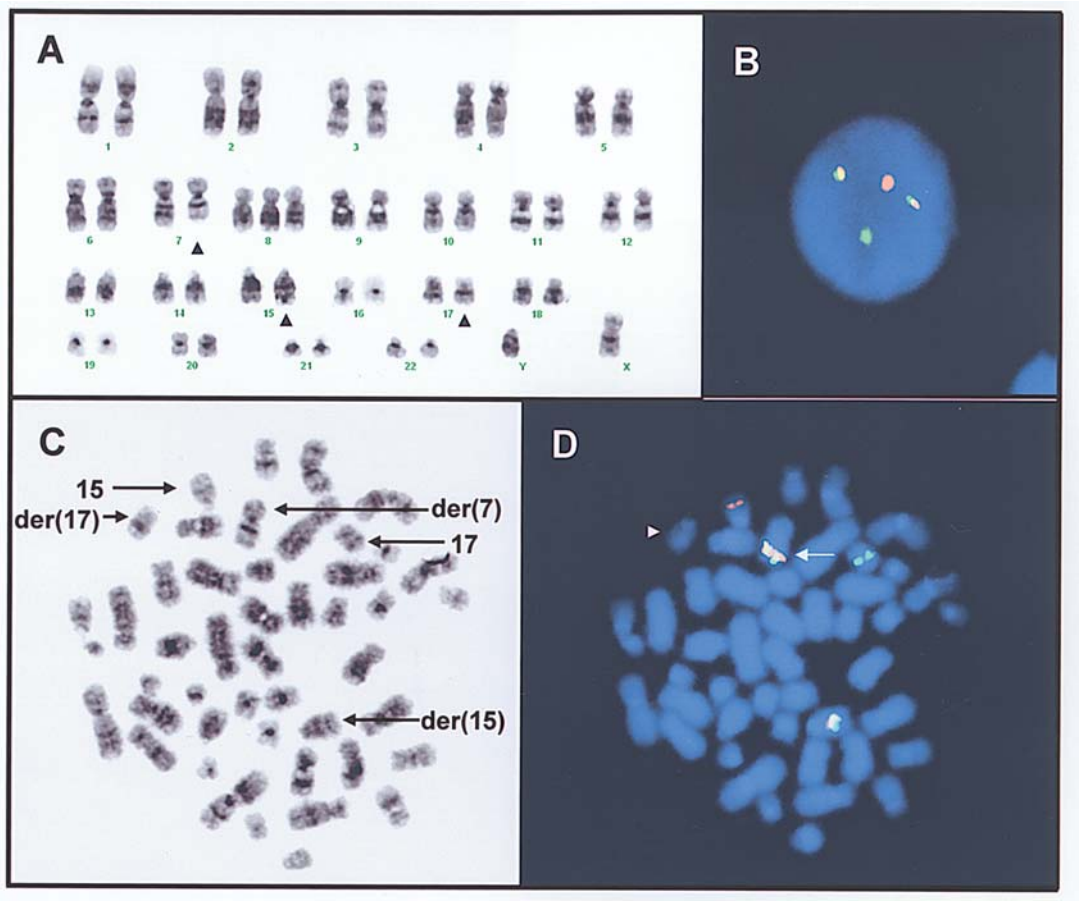

Figure 3. Conventional and molecular cytogenetic analysis of case 52. (A) Complete karyotype showing 47,XY,+8,t(7;17;15)(q22;q12;q22). G-banding with trypsin/Giemsa. (B) Interphase FISH with *PML/RARα* D-FISH translocation probe, showing one green signal, one orange signal and two fusion (yellow) signals (ie 1G1O2F pattern). (C and D) Metaphase FISH analysis on relocated G-banded chromosome using the D-FISH probe, showing the presence of fusion signal on der(7) (white arrow) and missing fusion signal on der(17) (white arrowhead).

sensitivity of the technique was 100%. The present study is the largest series reported which assessed the use of D-FISH in the diagnosis of APL cases.

Compared with the APL series reported by Brockman *et al* (8), our patients had a higher percentage of additional or complex cytogenetic abnormalities. This provided good opportunities to evaluate the variation of D-FISH patterns and its contribution to the understanding of complex karyotypic changes in APL. Complex chromosomal aberrations often lead to poorly characterized karyotypes and potentially important genetic changes may be masked. Seven patients showed atypical D-FISH patterns. Cases 16 and 17 showed cryptic insertion of *PML* into *RARα* on 17q which contrasted with the case with insertion of *RARα* into *PML* at 15q22 described by Brockman *et al* (8). Case 50 showed duplication of the *RARα* gene whereas case 46 showed duplication of *PML* and *RARα* genes. The duplicated *PML* and *RARα* genes in case 46 could either be located on the marker chromosomes or in the additional material on the short arm of der(15). Unfortunately, metaphase FISH could not be performed in this case to confirm this due to limitation of retrievable materials. Gene duplication which may have pathogenic significance would not have been suspected had FISH not been performed. Three cases (48, 49, and 51) showed that an extra fusion signal of the *PML/RARα* gene was only possible in cases with better chromosome morphology (case 49 and 51).

Recently, further APL cases with three-way translocation have been described. All of them represented a 3-point break as confirmed by FISH (9,10). By cytogenetics, case 52 showed an apparent 3 way translocation t(7;17;15)(q22;q12;q22) and one would expect 2G2O1F, i.e. the only fusion signal would be expected on der(15) where 3'*RARα* on chromosome 17 was translocated to and fused with 5'*PML* on chromosome 15. The other signals would be one orange signal on normal chromosome 15 and one orange signal from 3'*PML* on der(7), one green signal on normal chromosome 15 and one green signal from the residue 5'*RARα* on der(17). Instead D-FISH studies showed the pattern of 1G1O2F which was not consistent with a simple three-way translocation with a 3-point break. Metaphase FISH was performed to further characterize the location of the rearranged genes. Results of metaphase FISH revealed that one fusion signal was located on der(15) and the other fusion was located on der(7). Taken together, the chromosomal rearrangements in our case are probably formed by a 4-point break mechanism (Fig. 4B). Two sequential reciprocal translocations occurred among the long arms of chromosome 7, 15 and 17. The involved 17q participated in both translocations with two very close breaks. The final outcome was a transposition of genetic

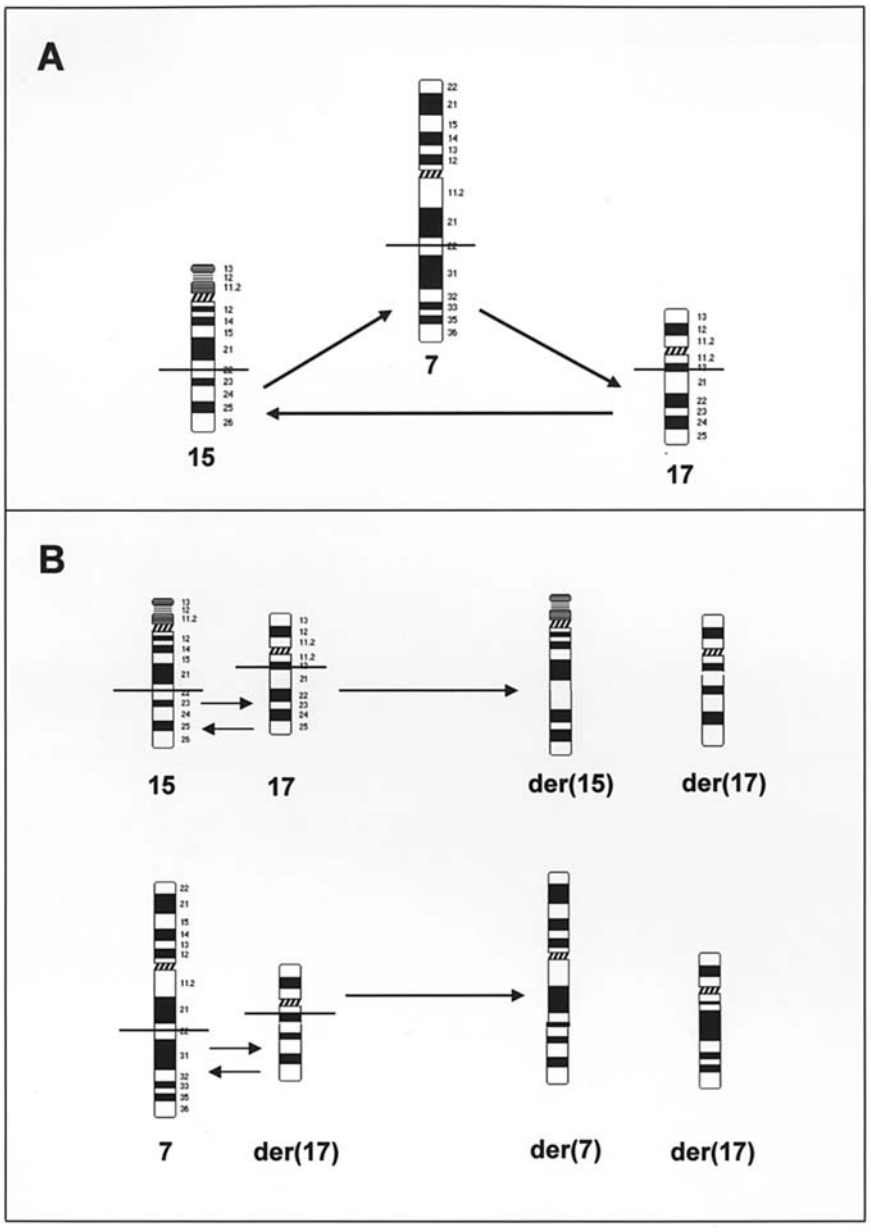

Figure 4. Ideogram showing different mechanisms of the formation of three-way translocation. (A) A typical 3-point break three-way translocation. (B) A 4-point break three-way translocation of case 52.

material from chromosome 17 to 15 to form the *PML/RARα* fusion on der(15) and a joining of genetic material from chromosomes 17 and 15 to form the reciprocal *RARα/PML* fusion gene on der(7). This produced the *PML/RARα* fusion on der(15) and *RARα/PML* fusion on the translocated 17q arm on der(7) (Fig. 3A).

Loss of DNA around the breakpoints of translocation has been observed in hematologic malignancies, notably the 9q34 deletion in chronic myelogenous leukemia with t(9;22)(q34;q11.2) (11). None of the 52 APL patients showed a detectable loss of DNA around translocation breakpoints by FISH, which manifests as loss in fusion signals. DNA loss around translocation breakpoints of APL was not detected in the 52 APL patients reported by Brockman *et al* (8) either.

We have shown D-FISH as a highly sensitive (100%) method to detect the *PML/RARα* and *RARα/PML* genes in APL. However in 7 out of 52 cases (14%) atypical patterns were present which can lead to diagnostic difficulties. We propose that D-FISH results should always be interpreted together with conventional cytogenetics and in atypical patterns further supplemented with studies using S-FISH and metaphase FISH. Interestingly, t(15;17)(q22;q12) not associated with APL has also been reported in other acute myeloid leukemias (AML) (12,13).

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