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Spectral karyotyping and fluorescence *in situ* hybridization detect novel chromosomal aberrations, a recurring involvement of chromosome 21 and amplification of the *MYC* oncogene in acute myeloid leukaemia M2

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Summary. Recurring chromosomal aberrations are of aetiological, diagnostic, prognostic and therapeutic importance in acute myeloid leukaemia (AML). However, aberrations are detected in only two thirds of AML cases at diagnosis and recurrent balanced translocations in only 50%. Spectral karyotyping (SKY) enables simultaneous visualization of all human chromosomes in different colours, facilitating the comprehensive evaluation of chromosomal abnormalities. Therefore, SKY was used to characterize 37 cases of newly diagnosed AML-M2, previously analysed using G-banding. In 15/23 patients it was possible to obtain metaphases from viably frozen cells; in 22 additional cases, fixed-cell suspensions were used. Of the 70 chromosomal aberrations identified by SKY, 30 aberrations were detected for the first time, 18 aberrations were redefined and 22 were confirmed. SKY detected two reciprocal translocations, t(X;3) and t(11;19). In five cases, eight structural aberrations resulted in partial gains of chromosome 21, six of which were undetected by G-banding. In 4/5 cases, these resulted in copy number increases for *AML1*. Amplification of *MYC* was detected in three cases. Using SKY and FISH, clonal aberrations were identified in 5/18 cases with a presumed normal karyotype; 3/5 aberrations were of known unfavourable prognostic significance. Karyotypes were entered into a custom-designed SKY database, which will be integrated with other cytogenetic and genomic databases.

Keywords: chromosomal aberrations, SKY, trisomy 21, AML-M2.

Acute myeloid leukaemia (AML) is a heterogeneous disease. The variability at the morphological level results from a differentiation block at various stages during haematopoietic maturation. The resulting morphological features have formed the basis for the definition of subgroups in the French–American–British (FAB) classification (Bennett *et al*, 1985; Löwenberg *et al*, 1999). Diversity is also present at the cytogenetic level, in which at least two thirds of the cases show clonal chromosomal aberrations at diagnosis (Heim & Mitelman, 1995). Recurrent reciprocal translocations are found in about 50% of AML cases (Look, 1997). Reciprocal translocations, such as the t(8;21) and t(15;17), and rearrangements, such as inv(16), lead to heterogeneity at the molecular level. These aberrations have proven to be of aetiological as well as of diagnostic, prognostic and therapeutic importance in AML. In addition, they have been shown to be specifically associated with FAB subgroups and, therefore, have been included in the new classification system of acute leukaemias by the World Health Organization (WHO) (Harris *et al*, 1999).

Despite the extensive cytogenetic data available on acute leukaemias, it was not until 1994 that chromosome painting techniques made the detection of the translocation 12;21 possible in childhood B cell acute lymphocytic leukaemia (B-ALL) (Romana *et al*, 1994). The indistinguishable banding pattern of the involved telomeric segments of

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Table I. Comparison of the G-banded karyotype with the SKY and FISH analysis.

Case	Material	G-banding analysis	SKY-analysis	FISH probes and analysis
1–5	C	Normal	Normal	I-FISH: 8, 11, 5p/5q, 7p/7q, *AML1*
6–13	F	Normal	Normal	I-FISH: 8, 11, 5p/5q, 7p/7q, *AML1*
14	C	46,XY[25]	46,XY[16]	Chr arm paints: 7p/7q; I-FISH: 8, 11, 5p/5q, 7p/7q, *AML1*; 5q × 1: 4%
15	C	46,XY[21]	46,XY, t**(11;19)(q23;p13.1)**[9]	Chr 11, 19 paint (confirmation)
16	C	46,XY,i(17)(q10)[1] 46,XY[24]	46,XY,**del(5)(q14q31)**[3] 46,XY[18]	Chr 5 paint, LSI 5p/5q (confirmation, breakpoint verification)
17	F	46XX[25]	46,XX,**der(15)t(14;15)**[1]‡,**dmin**[4][cp5] 45XX,**-18,der(21)t(18;21)(q11.2;p11.2)**[2] 46,XX[8]	LSI *MYC, TEL/AML1* (dmin origin), I-FISH CEP 18 × 1: 12·25%: der(21)
18	F	46XX[22]	45,XX,-**19**[3]/45,XX,-**21**[3] 46,XX[21]	I-FISH: LSI *AML1* × 1: 9·66%
19	C	46,XX,del(9)(q22q34)[5] 46XX[18]	46,XX,del(9)(q34)[3] 46,XX[11]	I-FISH *BCR/ABL*: *ABL* × 1: 7·5%
20	C	47,XY,+10[4] 46,XY[14]	47,XY,+10[5] 46,XY[17]	Chr paints: 5, 7
21	C	44,XY,r(5),dic(7;18)(q11;q11), i(8)(q10),-21,-22 45,XY,idem,+i(8)(q10) 44,XY,idem,der(17)t(5;17)(?; p11) 45,XY,idem,der(17)t(5;17),+mar 46,XY†	43–46,XY,del(5)(q11.2)[8] dic(7; 18)(q11.2;q11.2)[9],i(8)(q10)[7], +i(8)(q10)[7], **der(16)t(16;22)(q11.2;q11.2)**[9], der(17)t(5;17)(p11;p11.2)[8], **trp(21)(q21q22)**[9],-22[7][cp9]	Chr-, chr arm paints: 5, 9, 17, 21q (confirmation) CEP 8, LSI 5p/5q, *MYC, AML1*
22	C	No data	47,XY,+8	
23*	C	46–47,XY,-2,+r(?2)×2,-3,+del(?3)(q12)[4], 46–47,idem,add(16)(q?)[7]	44–49,XY,-2,+ace(2)[12],+ace(2)[10], del(3)(q12)[12],der(16)t(16;17)(q22;q21)[12], −**17[9]**[cp12]	Chr paints: 2, 3, 5 (confirmation)
24*	C	47,XX,+8[25]	47,XX,+8[20]	
25	C	46,XY,t(8;21)(q22;q22)[24] 46,XY[10]	46,XY,t(8;16;21)(q22;q13;q22)[7]	
26	F	46,XY,t(8;21)(q22;q22)[20]	46,XY,t(8;21)(q22;q22)[16]	
27*	F	47,XY,+8 [7] 46,XY[13]	47,XY,+8[2] 46,XY[5]	

Table I. *continued*

Case	Material	G-banding analysis	SKY-analysis	FISH probes and analysis
28*	F	46,XY[9] 79–92,XXYY[3] 75–92,XXY,der(3)t(3;5)(q12;?)×2,+del(3)(p12),(14;?)(p?12;?),add(17)(q?)×2[8]	35–46,XY,**der(2)t(2;21)(q11.2 q11.2)**[1], der(3)*t*(3;5)(p12; p1?3)[12],**-5**[11],**-7**[11],**-10**[11], **der(12)t(12;17)(p13;q21)**[13], **der(13)t(13;21)(p11; q11.2)del(21)(q22.1)**[9], + **idic(13)(q14)**×**2** − **6**[12], **der(15)t(8;15)(p11.1–11·2;?)**[1]‡, **der(15)t(15;21)(p13;q11.2)**[12], +**der(15)t(15;21)(p13;q11.2)**[2], **der(16)t(3;16)(p21;q13)**[14],**-17**[9], der(17)t(10;17)(q11.2;q25)dup(10)(q21)[14], **-19**[4],**-21**[10][cp16] 79–81,XXYY, <−4n >, **der(2)t(2;21)(q12;q11.2)**[2] der(3)t(3;5)(p12;p1?3)×2[2],**-5**[2],**-5**[2],**-7**[2], **-10**[2],**-10**[2],-12[2], der(14)t(14;17?)(p11.2;q11.2)[2], **der(16)t(3;16)(p21;q13)**×**2**[2],**-17**[2],**-17**[2], der(17)t(10;17)(q11.2;q25)dup(10)(q21)×2 [2],**-19**[2],**-21**[2][cp2]	Chr paints: 3, 5, 10, 13, 16, 18 (confirmation) LSI *AML1* × 3: 11·7%, LSI *AML1* × 4: 7%, cosmid *AML1*
29*	F	47,XY,+8[25]	47,XY,+8[10] 32,XY,**der(18)del(18)(p11.2)** **t(1;18)(q21;q23)**,inc[1] 46,XY[2]	Chr paints: 1, 18: **clonal aberration**, additional aberrations involving chr.1q
30	F	46,XY,der(18)t(18;21)(p11;q11) 46,XY†	46,XY,der(18)t(18;21)(p11.2;q11.2)[16] 46,XY[3]	Chr-, chr arm paints: 18, 18p, 21 (breakpoint verification), CEP18:der(18) LSI *AML1*, cosmid *AML1*
31	F	45,X,-X,t(8;21)(q22;q22)[17] 46XX[3]	45,X,-X,t(8;21)(q22;q22)[20]	
32	F	45,XY,der(2)t(2;11)(p25;?),del(6)(q15), der(11;12)ins(12;11)(p11.2;p11q13) t(6;12)(q?;p13),der(18)t(11;18)(?;q23)[20]	45,XY,der(2)t(2;11)(p23; p13), der(6)t(6;12)(q21;p12),-11, der(12)t(6;12;11;12)(q21;p12;q12;q13), der(18)t(11;12?;18;12)(q21;?;q21;?)[19],	Chr paints: 2, 3, 6, 11, 12, 18 (confirmation, breakpoint verification)
33	F	45,X,-Y,t(8;21)(q22;q22), t(11;16)(q13;p13.1)[23] 46,XY[5]	45,X,-Y,t(8;21)(q22;q22), t(11;16)(q13;p11.2)[14] 46,XY[2]	Chr arm paints: 11p, 11q, 16p, 16q (breakpoint verification),LSI *CyclinD1*
34	F	47,XX,+14[12] 46XX[9]	47,XX,+14[16]/46,idem,**–19[4]** 46,XX[5]	

Table I. *continued*

Case	Material	G-banding analysis	SKY-analysis	FISH probes and analysis
35	F	44,XY,-17,der(19)t(17;19)(q21;q13.4), −21[2]	41–44,Y,t(X;3)(q24;p21)[12], **dup(8)(q24)**[13],+**der(9)t(9;17)(p12;p11.2)**	Chr paints: X, 3, 7, 17, 19, 21 (confirmation, breakpoint verification),
		44,idem,add(X)(q28)[19]	[1],+**der(9)t(9;21)(p12;q11.2)**[1],**ace(9)**[3], -17[12],der(19) t(17;21;19;21;19)(q21;q21:?:q11.2;q13.4) [13],-21[12][cp13]	Chr paints: 9, 17, 21: **clonal aberrations**, additional 9q aberrations, Chr paint 8, P1 *ETO*, LSI *MYC*: **ish dup(8)(q24)(MYC amp)** LSI *AML1*, cosmid *AML1*
36	F	45,X,-Y,t(8;21)(q22;q22)[20]	45,X,-Y,t(8;21)(q22;q22)[13]	
37	F	46,XX,del(9)(q22)[6] 46XX[14]	46,XX,del(9)(q22)[3]/47,idem,+**8**[1] 46,XX[3]	I-FISH: CEP 8 × 3: 17%

*The asterisk marks patients with a preceding history of MDS.
†Number of metaphases analysed was not available in this case.
‡New aberrations seen in only one metaphase in which the clonality could not be established. These aberrations were not included in the overall count.
New aberrations detected by SKY are shown in bold. Redefined aberrations are underlined.
C, metaphases obtained after culture of viably frozen material were used for SKY-and FISH-analysis. F, metaphases from fixed-cell suspensions prepared at diagnosis were used for analysis.
FISH analysis: I-FISH, interphase FISH (200–500 nuclei were counted); CEP, chromosome enumeration probe; LSI, locus specific probe.

chromosomes 12p and 21q had prevented the characterization of this translocation using conventional banding methods. This translocation is now known to be the most common genetic lesion in paediatric ALL, and it identifies a distinct subgroup with a favourable prognosis (Shurtleff *et al*, 1995). It is therefore conceivable that additional cryptic translocations may be revealed in AML when molecular cytogenetic techniques, such as combinatorial multifluorescence *in situ* hybridization (M-FISH) or spectral karyotyping (SKY), are used (Schröck *et al*, 1996; Speicher *et al*, 1996). Furthermore, new recurrent translocations and translocations involving hitherto unknown fusion partners for genes that play a key role in leukaemogenesis continue to be discovered (Rowley, 1999).

SKY is a recently developed technique that enables one to visualize all 24 human chromosomes in different colours in a single metaphase by utilizing combinatorial probe labelling, fluorescence microscopy, spectroscopy, CCD-imaging and spectral image analysis (Garini *et al*, 1996; Schröck *et al*, 1996). Therefore, SKY is especially useful for identifying chromosomal aberrations leading to colour changes, including translocations or insertions, as well as the identification of marker chromosomes and homogeneously staining regions (hsr). Because of the complexity of chromosomal aberrations in malignant cells and their frequent poor quality, SKY has become a valuable tool in tumour cytogenetics (see Knutsen & Ried, 2000 for a review).

The focus of this study was aimed at the detection of novel, recurring chromosomal aberrations in AML-M2, one of the most frequent subgroups of AML. Although the t(8;21)(q22;q22) is specifically associated with AML-M2, this translocation is found in only 30–40% of the M2 cases with chromosomal aberrations, and correlates with a phenotypic subgroup within AML-M2. We did not exclude cases with a translocation 8;21 from our study because there is evidence that additional genetic events are required for leukaemogenesis, and secondary cytogenetic abnormalities can influence the generally favourable prognosis associated with t(8;21) (Schoch *et al*, 1996; Friedman, 1999).

In the present study, we applied SKY to 37 AML cases of the FAB subgroup M2 and demonstrated that retrospective cytogenetic analysis of viably frozen cells is possible.

PATIENTS AND METHODS

Viably frozen bone marrow cells (23 patients) or fixed cell suspensions prepared from bone marrow at diagnosis (22 patients) were available for this study. All 45 patients (23 women, 22 men; age 16–84 years) were newly diagnosed with AML-M2 and were treated according to the study protocols and guidelines of the German AML Co-operative Group (AMLCG). None had received prior cytostatic therapy. Five patients had a previous history of myelodysplastic syndrome (MDS). The diagnosis of AML, FAB subtype M2, was reviewed and confirmed as established within the AMLCG. Conventional cytogenetic analysis using G-banding was performed at the time of diagnosis prior to cytostatic

treatment. The results were either performed or reviewed by the reference laboratories, and were available for all but one patient (case 22, Table I).

Preparation of metaphases from viably frozen bone marrow cells. The cells had been separated using Ficoll centrifugation, frozen with dimethyl sulphoxide (DMSO) and stored in liquid nitrogen for up to 9 years. Specimens from 15 of these 23 patients (65%) yielded metaphase spreads after short-term *in vitro* culture.

For the preparation of metaphase spreads, cells were thawed and cultivated for 24, 48 and 72 h in either (a) Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRL, Gaithersburg, MD, USA) or (b) RPMI-1640 supplemented with a cocktail of growth factors: 10% giant-cell-tumour-conditioned medium, GCT, Origen (Igen, Gaithersburg, MD, USA); (c) 10% GCT-medium, stem cell factor (SCF) (Boehringer Mannheim, Indianapolis, IN, USA), and Flt3-ligand (R & D Systems, Minneapolis, MN, USA); or (d) 10% GCT-medium and interleukin 3 (IL-3) (Boehringer Mannheim, Germany). Chromosome harvest and metaphase spreads were performed according to standard procedures (Barch *et al*, 1997).

Spectral karyotyping (SKY). Metaphases from 37 patients were available for analysis. SKY was performed as previously described (Schröck *et al*, 1996). Twenty-four differentially labelled chromosome-specific painting probes were simultaneously hybridized onto metaphase chromosomes. Probe preparation, slide pre-treatment, hybridization and detection were carried out as per established protocols (Macville *et al*, 1997). Image acquisition was performed using the SpectraCube SD200 (Applied Spectral Imaging, Carlsbad, CA, USA) connected to an epifluorescence microscope (DMRXA, Leica Microsystems, Wetzlar, Germany). The SKYVIEW v.1·2 or 1·2.04 software was used for image analysis. Images were acquired of all metaphases in the hybridization area. Seven to 27 SKY images and corresponding inverted 4,6-diamidino 2-phenyl-indole (DAPI) images were completely analysed for each patient. Cytogenetic nomenclature adhered to the guidelines of the ISCN (Mitelman, 1995). Aberrations were characterized as 'new' when they had not been detected by G-banding; they were designated as 'redefined' when structural aberrations proved to be more complex than observed with G-banding alone, when additional material was identified or when breakpoints were assigned or changed. Karyotypes were designated as being complex when three or more unrelated aberrations were present.

Fluorescence in situ hybridization (FISH). To confirm SKY results, further delineate complex aberrations or to better define chromosomal breakpoints, FISH was performed on metaphase chromosomes and interphase nuclei according to standard protocols, using chromosome painting probes, chromosome arm-specific painting probes, as well as BAC-, cosmid- and P1-clones. Furthermore, FISH was used to establish the clonality of aberrations seen only in single metaphases by SKY, as well as to identify hidden aberrations in cases with reputedly normal karyotypes.

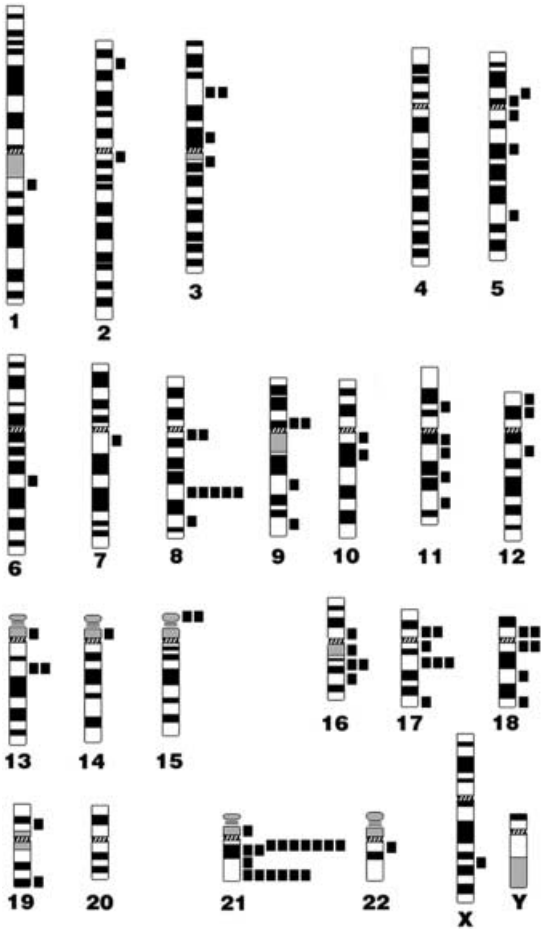

Fig 1. Breakpoint map. All breakpoints identified in the 37 cases analysed are indicated by black squares at the respective bands in the chromosome ideogram. Five cases with a t(8;21) were analysed in our series. The cluster of breakpoints at 8q22 and five breakpoints at 21q22 correspond to this translocation. Other breakpoint clusters were detected at the centromere of chromosome 17 as well as 17q21, at the centromere of chromosome 18, and on chromosome 21, bands 21q11.2 and 21q22.

A cosmid clone developed for the t(8;21), which detects the first five exons of the *AML1* (*RUNX1*) gene (encompassing the DNA-binding domain, Runt homology domain), and a P1-clone for *ETO* were courtesy of Dr N. Sacchi (Johns Hopkins University, Baltimore, MD, USA; Sacchi *et al*, 1995). Centromere probes for chromosomes 8, 11 and 18, and loci-specific probes for 5p15.2/5q31, 7p11.1-q11.1/7q31, *BCR/ABL*, *CYCLIN D1*, *MYC* and *TEL/AML1*, were obtained from Vysis (Downers Grove, IL, USA) and used according to the manufacturer's instructions. Images were acquired using the LEICA Q-FISH software (Leica Imaging Systems, Cambridge, UK). Interphase FISH (I-FISH) signals were enumerated in 200–500 nuclei per sample. To establish the cut-off level, at least three karyotypically normal samples were analysed and three times the standard deviation was added to the mean count. The term amplification was applied when five or more signals were detected with a locus-specific probe.

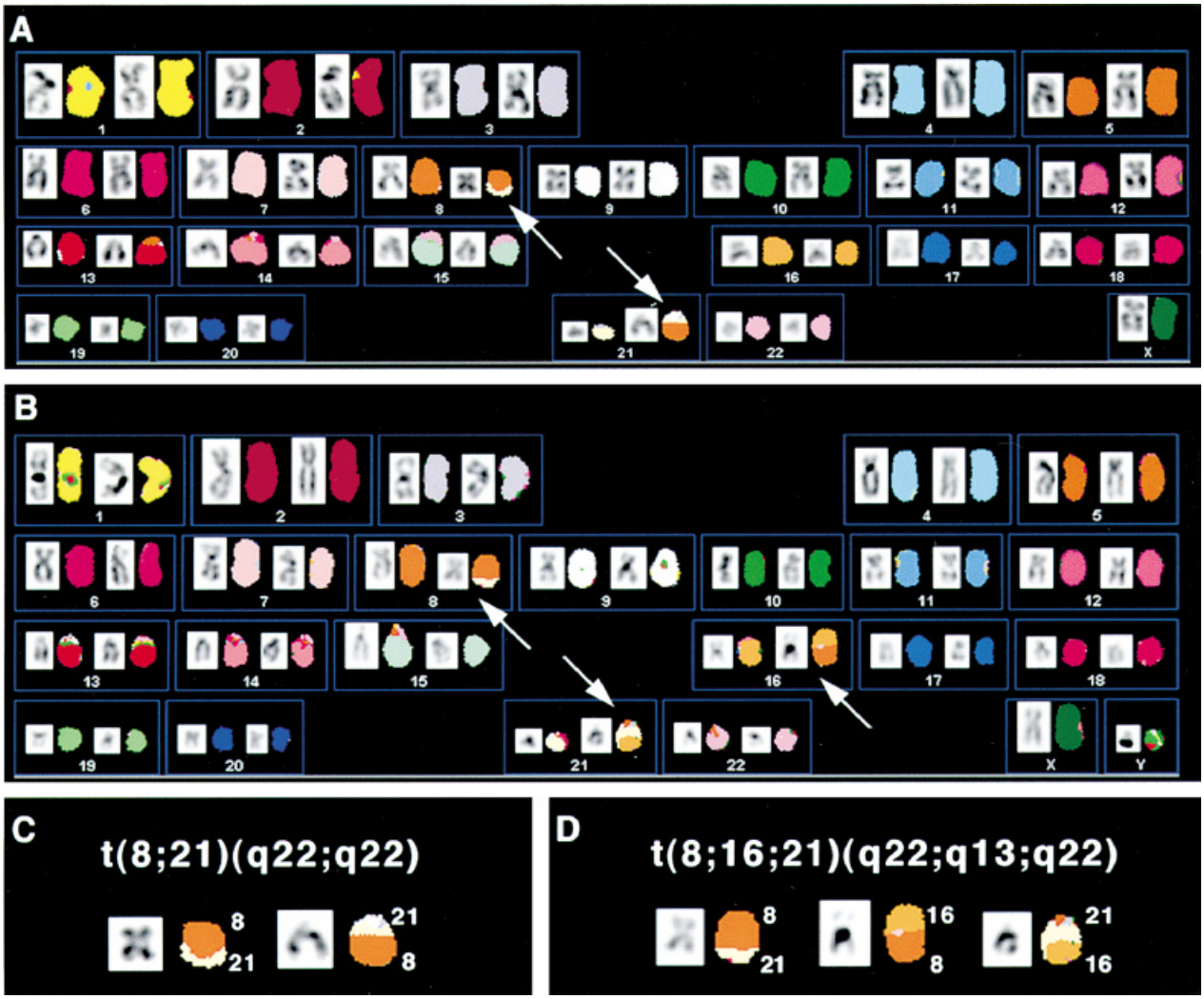

Fig 2. A translocation t(8;21) was redefined as three-way translocation t(8;16;21) after SKY analysis. (A) depicts the karyotype of case 31. Shown are the inverted DAPI image of each chromosome arranged side by side with the SKY classification. The arrows delineate the t(8;21). (B) shows the full karyotype of case 25. The arrows indicate the three-way translocation t(8;16;21). (C) and (D) demonstrate the t(8;21) and the t(8;16;21) in more detail.

RESULTS

We performed SKY and FISH on metaphase chromosomes from 37 patients, newly diagnosed with AML-M2. The comparison of G-banded and SKY karyotypes as well as the supplemental FISH experiments are shown in Table I.

Initially, SKY results of metaphases obtained under different culture conditions were compared. Although in general, metaphase yields from cultures supplemented with growth factors were higher, no differences in the aberrations identified could be detected.

Forty-three structural and numerical aberrations were identified using conventional cytogenetic analysis, whereas 70 aberrations were detected using SKY, 48 of which were structural aberrations. Thirty new aberrations (43%) were detected, 18 aberrations (26%) were redefined and 22 (31%) were confirmed. In 10/37 cases, only a single

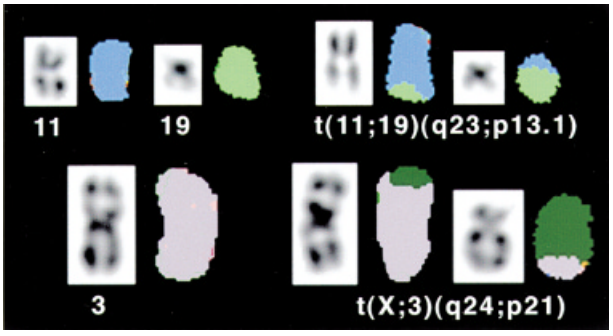

Fig 3. Two balanced translocations detected using SKY: translocation t(11;19)(q23;p13.1) in case 15 and t(X;3)(q24;p21) in case 35. These translocations were not identified in the G-banded karyotypes. The inverted DAPI image and the SKY classification are shown for the normal (left) as well as the rearranged chromosomes (right).

Table II. Resulting chromosomal gains and losses in cases with complex karyotypes.

Case	Chr 5	Chr 7	Chr 8	Chr 16	Chr 17	Chr 18	Chr 21
32						−18q21→qter	
21	−5q11→qter	−7q11.2→qter	+8q×5 (*MYC* amp)	−16q11.2→qter	−17pter→p11.2	−18q11.2→qter	+21q21→q22 (*AML1* × 3)
28	−5p13→qter −5p13→qter	−7 −7		−16q13→qter −16q13→qter	−17pter→q21 −17pter→q11.2 −17q25→qter		+21q11.2→q22.1 −21pter→q11.2 (*AML1* × 3:11·7%)
35			+8q24→qter (*MYC*amp)		−17pter→q21 −17q11.2→qter		+21q22 (*AML1* × 3*)
23				−16q22→qter	−17pter→q21		

*Using the cosmid clone for *AML1*.

aberration was present; in seven cases, two chromosomal aberrations were identified; and in six cases, three or more unrelated aberrations were detected and were therefore deemed to have a complex karyotype. All breakpoints designated were compiled in a breakpoint map (Fig 1) and all SKY karyotypes were catalogued in a custom-designed database presently under development (The SKY/CGH Database, 2001).

Reciprocal translocations

In five cases, the t(8;21)(q22;q22) initially identified using G-banding was readily detected by SKY (Fig 2A). In case 25, this translocation was redefined as a three-way translocation t(8;16;21) (Fig 2B). Two balanced translocations were identified using SKY: in case 15 a t(11;19) was newly detected, and in case 35 the add(X)(q28) was redefined as t(X;3) (Fig 3). Furthermore, in case 33 a translocation 11;16, present in addition to a t(8;21), was confirmed and the breakpoint for chromosome 16 was redefined.

Unbalanced structural aberrations

Overall, 40 unbalanced structural aberrations (deletions, duplications and triplications, dicentrics, isodicentrics, isochromosomes, and unbalanced translocations) were detected, seven of which were confirmed, 16 were redefined and 17 newly identified using SKY analysis. Twenty-three of the 40 structural aberrations were unbalanced translocations: 10 were revealed using SKY, 12 were redefined aberrations and one was confirmed (Table I). Although deletions, small duplications and para- or pericentric inversions are not readily detected using SKY because they do not lead to a colour change of the aberrant chromosome, a deletion of 5q and a duplication of 8q were recognized in case 16 and 35, respectively, owing to the change in size of the chromosome arm.

Whereas aberrations of chromosome 5 (case 16), chromosome 9 (19, 37), chromosome 18 (17, 29, 30) and chromosome 21 (17, 30) were present in cases with simple karyotypes, unbalanced aberrations involving chromosomes 7, 16, and 17 were only found in cases with complex karyotypes in this series (21, 23, 28, 35). The chromosomal gains and losses resulting from unbalanced aberrations

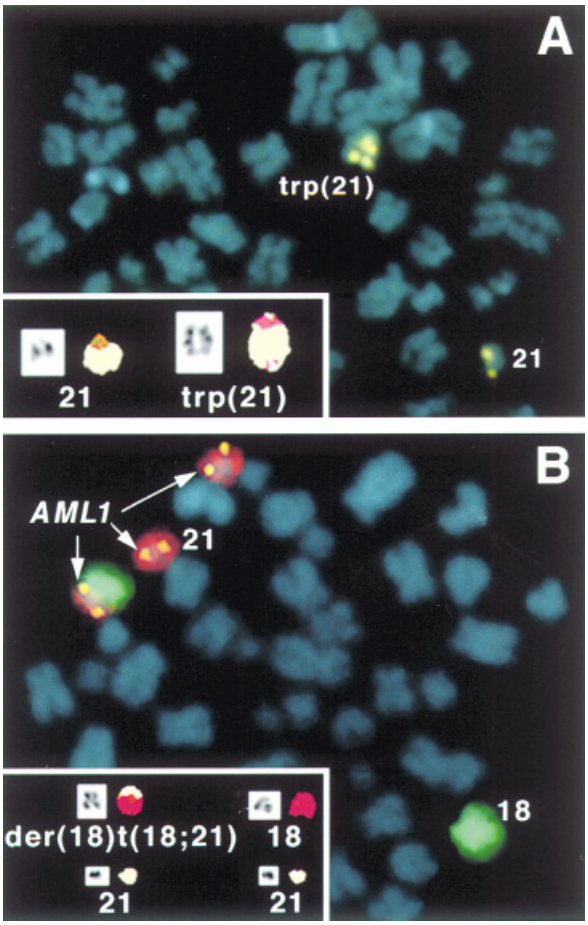

Fig 4. Gain of chromosome 21. Metaphases from two of the four cases with partial trisomy 21 are shown. (A) shows a metaphase from case 21. In this patient, SKY identified a partial triplication of chromosome 21 and a duplication of bands q21-q22 as shown in the insert at the bottom left corner. Hybridization with a locus-specific probe revealed the duplication of *AML1* (yellow signals). (B) shows a metaphase from case 30. The unbalanced translocation der(18)t(18;21) detected using G-banding was confirmed by SKY. FISH-analysis showed that the partial gain of chromosome 21 (red) involved the region of the *AML1* gene (yellow signals).

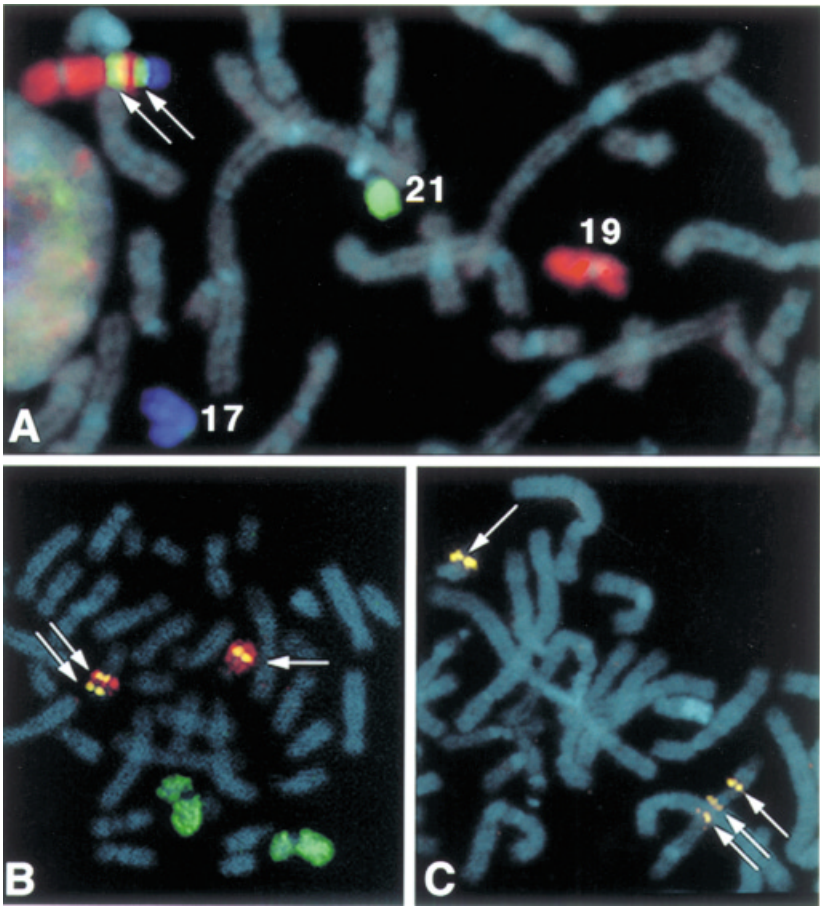

Fig 5. Involvement of *AML1* in an unbalanced chromosomal aberration. In case 35 the der(19)t(17;21;19;21;19) was redefined using SKY. FISH analysis with chromosome painting probes (chr. 17 blue, chr. 19 red, chr. 21 green) confirmed the complex rearrangement (A). (B) Further analysis with a cosmid-clone mapping to the first five exons of *AML1* showed duplication on the der(19) (material from chromosome 21 painted in red, *AML1*: yellow signals). Nevertheless, analysis with a locus-specific probe encompassing the entire *AML1* gene revealed three signals (yellow) on the der(19), indicating possibly an additional break within the gene (C).

involving chromosomes 5, 7, 8, 16, 17, 18 and 21 within complex karyotypes are summarized in Table II. Five cases (21, 23, 28, 32, 35) displayed aberrations involving these chromosomes in different combinations (Table II).

Only one case with three unrelated aberrations (33, Table I) did not involve a gain or loss of material of any of these chromosomes; this case had a t(8;21) and two secondary aberrations, -Y and t(11;16).

Aberrations involving chromosomes 5 and 7. In cases 16, 21 and 28, aberrations involving chromosome 5 were detected or redefined using SKY. Whereas a deletion of chromosome 5q was the sole anomaly in case 16 (Table I), in cases 21 and 28 aberrations of chromosome 7 were also seen (Table II).

Aberrations involving chromosomes 16 and 17. We detected three unbalanced aberrations involving chromosome 16. These unbalanced translocations occurred only in cases with complex karyotypes (21, 23 and 28) and resulted in a loss of chromosome arm 16q. The smallest region lost was 16q22 16qter (Table II).

In four cases (21, 23, 28 and 35) with complex karyotypes, we found seven unbalanced translocations involving chromosome 17. Five aberrations led to loss of 17p (smallest overlapping region 17pter→p11.2), whereas two led to a loss of 17q (17q25→qter) (Table II). The breakpoints on chromosome 17 clustered around the centromere and band 17q21 (Fig 1).

Aberrations involving chromosome 18. In five of our cases aberrations involving chromosome 18 were observed. These aberrations led to a loss of 18p (smallest region lost: p11.2→pter; 17, 29, 30) and loss of 18q (smallest overlapping region: 18q23→qter) in three cases (21, 29, 32 respectively). Whereas cases 21 and 32 had complex karyotypes, cases 30, 17 and 29 had one or two unrelated aberrations (Table I). Both cases 17 and 30 had whole-arm unbalanced translocations involving 18q and 21q.

Aberrations involving chromosome 21. Not including the cases with a t(8;21), chromosome 21 was involved in eight structural aberrations in five patients (cases 17, 21, 28, 30 and 35), resulting in a partial trisomy in four of the five cases (21, 28, 30 and 35), and a trisomy for the *AML1* gene in three of the five cases (21, 28 and 30) (Fig 4). In case 28, three different unbalanced aberrations involving chromosome 21 were detected using SKY: der(2)t(2;21), der(13)t(13;21)del(21)(q22.1) and a der(15)t(15;21). In contrast to all other aberrations involving chromosome 21, the der(13)t(13;21) did not involve band 21q22, which was deleted. In case 35, chromosome painting confirmed two insertions of chromosome 21 material into the der(19) t(17;21;19;21;19). Hybridization with a 500-kb probe encompassing the *AML1* gene showed three signals on the der(19) on metaphase chromosomes of higher resolution. However, using the cosmid clone for the first five exons of the *AML1* gene, only two signals were detected on the

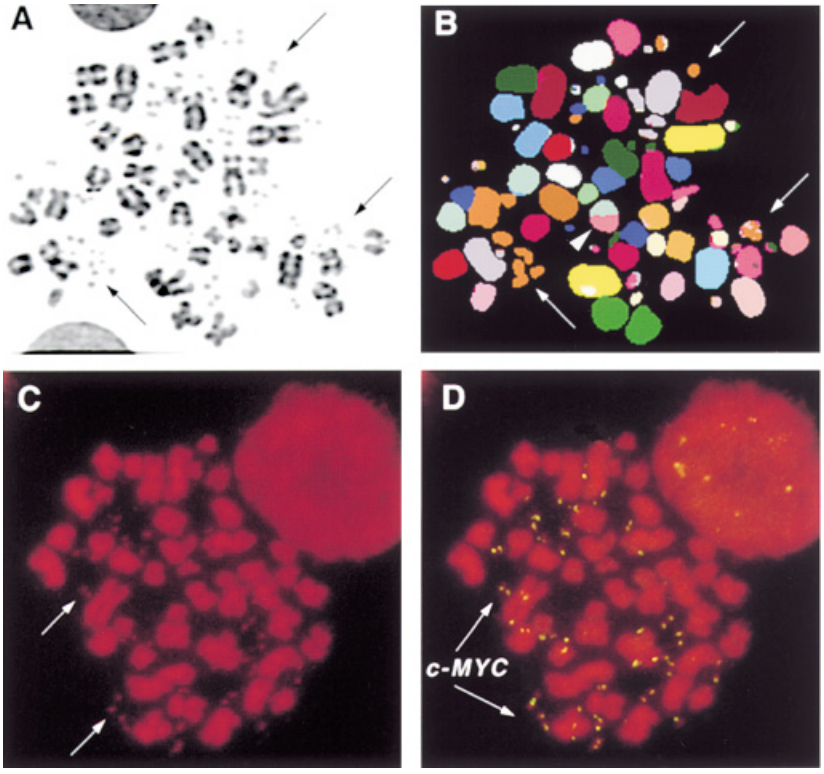

Fig 6. Analysis of double minute chromosomes (dmin). The inverted DAPI image of a metaphase of case 17 in which dmin were present is shown in (A). (B) shows the same metaphase after SKY-classification. The dmin classified as material derived from chromosomes X, 3, 8, 12, 20, and 21. The arrowhead at the centre of the metaphase identifies the non-clonal der(15)t(14;15). (C) shows another metaphase with dmin pseudo-coloured in red. FISH analysis with a probe for *MYC* revealed signals (yellow) localizing to all dmin.

der(19) (Fig 5). In addition, chromosome 21 was involved in the unbalanced translocation der(9)t(9;21) in this case. Breakpoints in the chromosome 21 aberrations clustered in bands 21q11.2 and 21q22 (Fig 1).

Amplification of MYC. Double minute chromosomes (dmin), which are rare in AML (Heim & Mitelman, 1995), were detected in case 17 in this series. The SKY classification of the dmin was ambiguous and identified the dmin material as being derived from chromosomes X, 3, 8, 12, 20 and 21. Further FISH analysis with gene-specific probes for *MYC*, *AML1* and *TEL* revealed the amplification of *MYC* in the dmin (Fig 6). Amplification of *MYC* was also detected in two other cases with complex karyotypes (21 and 35; Table II). Whereas the two copies of the isochromosome i(8q) in case 21 were identified using G-banding, the dup(8)(q24) [ish dup(8)(q24)(*MYC* amp)] in case 35 has not been described previously (Table I).

Cases with normal karyotypes using G-banding analysis

Seventeen of 36 cases (47%) had a normal karyotype as established by G-banding analysis at the time of diagnosis. One additional case (16) had a non-clonal marker, an i(17)(q10). In four (15, 16, 17 and 18) of these 18 cases (22·2%), new structural and numerical aberrations were detected using SKY (Table I). We identified a cryptic balanced translocation, 11;19 (case 15), and an unbalanced translocation, der(21)t(18;21) (case 17). In case 16, the i(17)(q10) was not found, but instead an unrelated clone with a del(5q) was revealed and confirmed with FISH. In case 18, two minor clones with numerical aberrations, i.e. loss of either chromosome 19 or chromosome 21, were detected using SKY. The loss of chromosome 21 was ascertained with I-FISH using a probe for *AML1*: in 9·66% of the interphase cells analysed only one signal was detected.

I-FISH analysis. To exclude the possibility that malignant clones had gone undetected in the population of metaphase cells in cases with a normal karyotype, we performed interphase cytogenetic analysis with centromere probes for chromosomes 8 and 11, with dual-colour probes for 5p and 5q, 7p and 7q and a locus-specific probe for the *AML1* gene on all remaining cases with normal karyotypes. A loss of 5q was detected in a minor clone in case 14. Overall, using SKY and FISH, clonal aberrations were detected in five of the 18 cases with a presumed normal karyotype.

Clonal aberrations detected using FISH

In cases 29, 35 and 37, SKY analysis detected three structural and one numerical aberration in single metaphases respectively. FISH analysis revealed that these aberrations were in fact clonal (Table I). In case 29, a FISH experiment identified a jumping translocation involving chromosome 1q in four metaphases. In case 35, SKY showed a der(9)t(9;17) and der(9)t(9;21), leading to a trisomy for chromosome 9q. FISH revealed additional translocations involving chromosome 9. In case 37 the clonality of a numerical aberration, a trisomy 8, was verified using interphase FISH.

Structural aberrations in single metaphases were detected using SKY in two additional cases, a der(15)t(14;15) in case 17 (Fig 6) and a der(15)t(8;15) in case 28. Nevertheless, not enough material was available in these cases to confirm

the clonality using FISH and, therefore, they were not included in the number of aberrations detected using SKY.

DISCUSSION

We analysed 37 cases of AML-M2 using SKY and FISH and compared the results with the karyotypes established at diagnosis using G-banding. Thirty novel aberrations (43% of all aberrations) were detected, 18 aberrations (26%) were redefined and 22 (31%) were confirmed. As has been previously reported, SKY makes possible the identification of cryptic translocations, complex aberrations and additional material as well as marker and ring chromosomes (for review see Knutsen & Ried, 2000).

Balanced aberrations, such as the t(8;21), are detected in 10–12% of all AML cases. Thus, our series should have been large enough to detect new recurrent reciprocal aberrations of similar frequency. The fact that no new recurrent translocations were identified in our study indicates that it is unlikely that additional hidden recurring chromosomal aberrations are present in AML-M2. The notion that cryptic translocations are indeed rare in AML is strengthened by the data of Kearney *et al* (1999) who used a 12-colour multiplex telomere assay (M-TEL) in 15 AML cases but failed to detect cryptic translocations.

Although we identified no new recurrent translocations, two balanced translocations were detected using SKY which were not observed using G-banding, t(11;19) and t(X;3)(q24;p21). Kakazu *et al* (1999) and Zhang *et al* (2000) also reported detecting cryptic 11;19 translocations using SKY in one case of myelodysplastic syndrome (refractory anaemia with excess blasts, in transformation) [MDS (RAEB-T)] and one of AML respectively. These findings suggest that the incidence of t(11;19) in AML/MDS may have been underestimated in the past. To the best of our knowledge, t(X;3)(q24;p21) has only been described previously in a case of Ph+ chronic myeloid leukaemia (Swolin *et al*, 1983).

In contrast to the two balanced translocations identified using SKY, 17 new and 16 redefined unbalanced structural aberrations were detected. This high number of unbalanced aberrations emphasizes the need to establish their pathogenetic role in leukaemogenesis. Whereas 5q abnormalities occurred as single aberrations, unbalanced aberrations of chromosomes 16 and 17 were only detected within complex karyotypes, making them more likely to represent secondary aberrations. The unbalanced translocations involving chromosome 16 resulted in loss of 16q, with breakpoints at 16q11.2, q13, and q22. Deletions of 16q13 and 16q22 have been reported as recurrent aberrations in AML (Mitelman *et al*, 1997). One of our seven unbalanced aberrations involving chromosome 17 was a der(17)t(5;17), an infrequent but recurrent abnormality in myeloid disorders (Wang *et al*, 1997). The breakpoints on chromosome 17 clustered in the centromere region and in band 17q21, a chromosomal region known to be frequently involved in non-random structural aberrations in AML-M1 and M2 (Longo *et al*, 1990). Five of the aberrations detected led to loss of 17p and, therefore, loss of *TP53* at 17p13.

Reports of unbalanced translocations of chromosome 18 in AML are not frequent in the literature (Mitelman *et al*, 2000). Therefore, it is surprising that five of our cases displayed translocations resulting in the loss of 18p and distal 18q. To our knowledge, no tumour suppressor genes in these regions have been implicated in leukaemogenesis. Interestingly, two cases had whole-arm translocations involving chromosomes 18 and 21. Whereas both cases led to a deletion of 18p, only one of these rearrangements led to trisomy 21q.

Of the eight structural aberrations involving chromosome 21 detected by SKY (in five cases), six had not been observed by G-banding. In four of the five cases, these aberrations led to a partial gain and trisomy of chromosome 21(Table II). Although trisomy 21 is the second most common numerical aberration in AML, occurring both as a primary and a secondary event, unbalanced structural aberrations that result in copy number increases for chromosome 21 are infrequent (Cortes *et al*, 1995; Mitelman *et al*, 1997, 2000). The overall prognostic significance of trisomy 21 in AML is not clear, but trisomy 21 as a single abnormality seems to confer a poor prognosis (Cortes *et al*, 1995; Wan *et al*, 1999). Of note, in our patient 30 the der(18) t(18;21)(p11.2; q11.2), which resulted in a gain of 21q, was the sole abnormality; this patient relapsed 14 months after achieving a complete remission. This same translocation was observed by Kakazu *et al* (1999) in a patient with MDS (RAEB-T). Therefore, the prognostic significance of structural aberrations leading to a gain of chromosome 21q would appear to be worthy of further evaluation.

In three of four cases, the partial trisomy 21 involved band 21q22, as shown by the increase in copy number for the *AML1* gene. Despite the fact that this region has been termed the 'Down Syndrome-critical region', no specific gene in this region has been identified as being responsible for the increased risk of leukaemia in this syndrome. It is unclear if *AML1* is really the target gene or whether another gene or genes on 21q are involved in our cases. Nonetheless, the transcription factor *AML1* is one of the most frequent target genes for translocations and mutations in acute leukaemia (Osato *et al*, 1999; Lutterbach & Hiebert, 2000) and the wild-type protein itself has transforming capacity (Kurokawa *et al*, 1996). Furthermore, recent evidence has shown that enforced expression of *AML1* leads to a shortened G1-phase of the cell cycle (Strom *et al*, 2000). Therefore, it is not inconceivable that an overexpression or untimely expression of wild-type *AML1* through trisomy 21 could have a biological effect.

A new, previously unknown partner gene for *AML1*, *AMP-19*, was recently cloned by Hromas *et al* (1999) from an AML-M2 patient with t(19;21)(q13.4;q22); this balanced translocation led to an out-of-frame fusion and therefore probably to the expression of a truncated *AML1* protein with abrogated function. In our case 35, a der(19)t (19;21;19;21;17) with the same breakpoint (19q13.4) as that seen in the patient of Hromas *et al* (1999) led to a partial trisomy 21 and, specifically, a trisomy of the part of

AML1 encompassing the DNA-binding (Runt homology-) domain. As a fusion gene created by an unbalanced translocation has not been described, this aberration could lead to an overexpression of a truncated protein and thereby to transcriptional repression.

In our cases, the breakpoints for the unbalanced aberrations of chromosome 21 clustered in band 21q11.2. This breakpoint is involved in the rare but recurrent balanced translocation t(11;21)(q23.3;q11.2) described in MDS (Wlodarska *et al*, 1999). Furthermore, in AML three reciprocal translocations involving the breakpoint 21q11 [t(17;21)(p13;q11), t(21;21)(p11;q11) and t(21;22)(q11;q13)] have been described in a small number of cases (Mitelman *et al*, 1997). A putative gene responsible for the transient abnormal myelopoiesis (TAM) occurring in some newborn infants with Down syndrome has been mapped to 21q11.2 (Shen *et al*, 1995). The relevance of this gene to leukaemogenesis remains to be established.

Dmin represent an infrequent phenomenon in AML and are found in only about 1% of cases (Heim & Mitelman, 1995). Whereas most cases have shown an amplification of *MYC* in the dmin, three cases have had amplifications of *MLL* and one case had amplification of the 11q region, which contained the genes *ETS1*, *FLI1*, *SRPR*, *NFRKB*, *KCNJ5*, *IFNβ1* and *CDKN2A* (Ariyama *et al*, 1998; Crossen *et al*, 1999; Streubel *et al*, 2000). In our case, the SKY classification of dmin was ambiguous and indicated material from several chromosomes. Nevertheless, FISH analysis demonstrated that all dmin showed a signal for *MYC*. A possible explanation for the ambiguous SKY classification is a low euchromatin content of the dmin. Similar problems with small markers and dmin, which were also resolved with FISH, have been reported by other investigators (Veldman *et al*, 1997; Zhang *et al*, 2000). Although the prognosis of patients with dmin and *MYC* amplification without the presence of complex karyotypes is not clear, the prognosis of patients with *MYC* amplification and a complex karyotype is poor (Bruckert *et al*, 2000). In our study, two additional cases (21, 35) with complex karyotypes showed amplification of *MYC*.

In three of our cases that showed normal karyotypes using G-banding but clonal abnormalities using SKY and FISH analysis, the metaphases had been obtained from viably frozen cells. Zhang *et al* (2000) noted a similar phenomenon in two cases of AML and suggested that there might be a different growth pattern after freezing; they also speculated that a sampling bias might explain the discrepancy between G-banding and SKY-data. Chromosomal aberrations detected in our cases included del(5q) and t(11;19), and in the series of Zhang *et al* (2000) a t(11;19) and monosomy 7, all of which are of known negative prognostic value.

Of five structural aberrations determined by SKY to be non-clonal, three were demonstrated to be clonal by FISH (cases 29 and 35). In both cases, FISH detected additional aberrations leading to copy number gain of chromosome 1q and 9q, respectively, in metaphases of poor morphology. These findings indicate that aberrations detected in single metaphases may not necessarily represent 'cytogenetic noise.' They may actually be jumping translocations, a type of aberration that has been associated with a poor prognosis (Bernard *et al*, 2000).

To collect the emerging SKY data, a database is currently under development. The design of the database (The SKY/CGH Database, 2001) will allow the immediate correlation of chromosomal aberrations detected using SKY with previously reported cytogenetic abnormalities. It will also, via the linkage of the cytogenetic and physical maps of the human genome as part of the Cancer Chromosome Aberration Project, CCAP (Kirsch *et al*, 2000), enable the high resolution mapping of chromosomal breakpoints and, eventually, the assignment of breakpoints to the sequence of the human genome.

In our study, the number of aberrations detected clearly demonstrates the value of combining SKY and G-banding for the comprehensive evaluation of cytogenetic changes in AML. As some of the aberrations detected are of established significant prognostic value, and the karyotype at diagnosis is one of the most important independent prognostic factors for survival, such findings are of relevance and can influence disease management (Büchner *et al*, 1999). Nevertheless, the prognostic value of these aberrations detected in small clones using SKY needs to be confirmed prospectively. The compilation of SKY data in the database should expedite the identification of both new balanced and recurrent unbalanced aberrations, as well as the identification of new subgroups of leukaemia.

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