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BIO-TECHNICAL METHODS SECTION (BTS)

BTS Leukemia

Multicolor spectral karyotyping identifies novel translocations in childhood acute lymphoblastic leukemia

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We used a recently described molecular cytogenetic method, spectral karyotyping (SKY), to analyze metaphase chromosomes from 30 pediatric patients with acute lymphoblastic leukemia (ALL). This group included 20 patients whose leukemic blast cells lacked chromosomal abnormalities detected by conventional cytogenetics and 10 patients whose blast cells had multiple chromosomal abnormalities that could not be completely identified by G-banding analysis. In two of the 20 patients (10%) with apparently normal karyotypes, SKY identified three cryptic translocations: a t(7;8)(q34–35;q24.1) in one patient and a t(13;17)(q22;q21) and a der(19)t(17;19)(q22;p13) in another. Fluorescence *in situ* hybridization using subtelomeric probes proved the latter translocation to be a t(17;19). SKY analysis was also successful in defining the nature of the chromosomal abnormalities in four of the 10 patients with marker and derivative chromosomes. The identified abnormalities in the latter group included three novel translocations: a der(X)t(X;5)(p11.4;q31), a der(21)t(X;21)(p11.4;p11.2) and a t(X;9)(p11.4;p13). The presence of the t(X;9) was suggested by conventional cytogenetics. The application of fluorescence *in situ* hybridization using chromosome-specific painting probes and locus-specific probes complemented the SKY analysis by confirming the nature of the chromosome rearrangements defined by SKY and by identifying the amplification of the *AML1/CBFA2* gene in one patient with a duplicated 21q. Our study demonstrates the utility of SKY in identifying novel translocations and in refining the identity of chromosomal abnormalities in leukemias. *Leukemia* (2001) **15,** 468–472.
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Introduction

Acute lymphoblastic leukemia (ALL), the most common hematologic malignancy of children, accounts for one fourth of all cases of childhood cancer. Identification of chromosomal rearrangements by conventional cytogenetic approaches, such as G-banding, has become a routine part of the diagnostic work-up of patients with cancer and aids in determining the prognosis and in monitoring disease progression.[1] However, an important disadvantage of conventional cytogenetics is its inability to identify cryptic and complex translocations and to establish the identity of marker chromosomes. In addition, conventional cytogenetic and molecular methods do not detect chromosomal abnormalities in 10% to 20% of children with ALL. Although the underlying molecular lesions responsible for these leukemias are unknown, earlier studies have suggested that leukemic cells in some of these patients are likely to contain cryptic translocations. For example, the t(12;21) is not found by conventional cytogenetic methods but can be detected by reverse-transcriptase polymerase chain reaction (RT-PCR) and by fluorescence *in situ* hybridization (FISH).[2–5] For these reasons, more sensitive methods are needed to identify these potentially recurrent chromosomal aberrations and, in turn, to improve the ability to diagnose ALL and to predict the outcome of patients with cryptic chromosomal abnormalities.

Recently, multicolor spectral karyotyping (SKY) has become an important molecular cytogenetic technique in the identification of subtle translocations and marker chromosomes and in the delineation of complex chromosomal aberrations in studies of hematologic malignancies and solid tumors.[6–10] In SKY, the hybridization of 24 chromosome painting probes labeled with different fluorochromes allows the simultaneous and differential display of all pairs of human chromosomes. In this study, we used SKY to identify recurrent and cryptic chromosomal aberrations in pediatric patients with ALL who had normal chromosomes or complex translocations and marker chromosomes as detected by G-banding. We discovered three translocations in leukemic cells of two of the 20 patients with no previously seen chromosomal abnormalities: a t(7;8)(q34–35;q24.1) in one patient and a t(13;17)(q22;q21) and t(17;19)(q22;p13) in another patient. In addition, two other novel translocations, a der(X)t(X;5)(p11.4;q31) and a der(21)t(X;21)(p11.4;p11.2), were found by SKY in the group of patients with complex translocations or marker chromosomes. Except for the t(17;19), none of the four translocations identified in this study have been previously detected in patients with ALL.

Materials and methods

Patients

Of the 800 patients studied cytogenetically, we identified 28 patients with B-lineage ALL and two with T-cell ALL for which fixed cell pellets were available. The ages of the patients

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ranged from 1 year to 15 years. Leukemic cells from 28 patients with B-lineage ALL were screened by RT-PCR to detect any t(9;22), t(4;11), t(1;19), and t(12;21). None of these translocations were detected by RT-PCR in these patients.

Cytogenetic analysis

Cytogenetic analysis of bone marrow samples from all patients was performed at the time of diagnosis. Informed consent for the bone marrow aspiration was obtained from the parents or guardians and, when appropriate, from the patients. Cytogenetic analysis was performed according to the methods of Raimondi *et al*.[11] Karyotypes were designated according to the International System for Human Cytogenetic Nomenclature (ISCN).[12]

Spectral karyotyping

Slides for SKY were prepared by using the cell suspensions that were stored at –20°C after the cytogenetic analysis. The human SKY probes (Applied Spectral Imaging (ASI), Carlsbad, CA, USA) were generated by PCR amplification of flow cytometry-sorted human chromosomes. This amplified DNA was then labeled with different fluorochromes. Denaturation, hybridization, and detection procedures were performed according to the manufacturer's protocol. Chromosomes were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Spectral images were acquired with the SD200 Spectracube system (ASI) attached to a Nikon Eclipse 800 microscope (Nikon, Melville, NY, USA). We obtained a single exposure of the image by using a custom-designed triple-band pass filter (Chroma Technology, Brattleboro, VT, USA) that permitted the simultaneous excitation of all dyes. An interferometer measured the spectrum at each pixel of the image. A discrete color was assigned to all pixels with identical spectra according to a spectrum classification system, and the pixels were then displayed in the assigned colors. Images were captured and analyzed according to the manufacturer's instructions. Usually, five to eight metaphase chromosome spreads from each patient were analyzed by SKY. The DAPI images and G-banding results were used in the identification of the chromosome bands and the assignments of the breakpoints.

FISH

Whole chromosome painting probes, alpha satellite probes, and subtelomeric (TelVysion) probes (Ventana, Tucson, AZ, USA; Vysis, Downer's Grove, IL, USA) were used to confirm the structural and numerical abnormalities identified by SKY. The cosmid 10455 (*IgJH*) (obtained from Dr WM Kuehl, National Cancer Institute, Bethesda, MD, USA) was labeled with digoxigenin by nick translation and was used as a probe to confirm the t(8;14). The probe used to evaluate the *AML1* amplification was purchased from Vysis, and the *MLL* probe was purchased from Ventana.

Results

SKY has been shown to be a useful molecular-based method that can identify chromosomal rearrangements that are not visible by routine light microscopy. To assess the utility of SKY in identifying cryptic chromosomal rearrangements in pediatric patients with ALL, we analyzed a select group of leukemic samples.

SKY analysis of leukemic cells with apparently normal chromosomes

This group of 20 patients lacked chromosomal abnormalities by conventional cytogenetics. SKY analysis found no chromosomal aberrations in leukemic cells of 18 of 20 patients. However, in two patients in which cytogenetic methods detected no chromosomal abnormalities, SKY identified three cryptic translocations (Table 1): a t(7;8)(q34–35;q24.1) in a 13-year-old male patient with T-lineage ALL (Figure 1a and b) and a t(13;17)(q22;q21) and a der(19)t(17;19)(q22;p13) in a 6-year-old girl with B-lineage ALL (Figure 2a and b). FISH with painting probes for the appropriate chromosomes confirmed these translocations (Figure 1c and 2c). The reciprocal nature of the t(17;19) could not be identified by either SKY or FISH using whole chromosome painting probes. However, FISH using a 19p subtelomeric probe confirmed the presence of the reciprocal t(17;19)(data not shown). The two translocations t(7;8) and t(13;17) have not been previously identified in patients with ALL, probably because of the subtle nature of these changes.

SKY analysis of leukemic cells with structural and numerical chromosomal abnormalities

To extend our study, we examined the ability of SKY to help in defining the derivative or marker chromosomes observed by conventional cytogenetics. Ten cases were selected that had either structural or numerical abnormalities. In these patients, conventional cytogenetics detected 29 acquired clonal abnormalities, which included seven unidentified chromosomal material and four deletions. SKY confirmed the structural and numerical abnormalities in cells from four of these 10 patients, whereas in the remaining patients, SKY obtained additional information (Table 1). In most cases, the results of SKY were confirmed by FISH using whole chromosome painting probes.

In one patient (Table 1, patient No. 3), the original karyotype as determined by G-banding was 47,X,add(X)(p22.2),del(5)(q32),+mar (Figure 3a). The additional material on the X chromosome was identified as material from chromosome 5, but no reciprocal translocation was observed (Figure 3b). SKY showed that the marker chromosome resulted from a translocation between chromosomes X and 21 (Figure 3b). Dual-color FISH using painting probes for chromosomes X, 5, and 21 confirmed the SKY results (Figure 3c). The redefined karyotype was as follows: 47,X,der(X)t(X;5)(p11.4;q31),del(5)(q32),der(21)t(X;21)(p11.4;p11.2).

In patient No. 4 (Table 1), SKY confirmed a new translocation: t(X;9)(p11.4;p13) (Figure 4a–c) that was suggested by conventional cytogenetics.

In another patient (Table 1, patient No. 5), both cytogenetic analysis and SKY revealed a +X,+10,del(11)(q23) and a qdp(21)(q11.1q22) (Figure 5a and b). Because of the presence of the *AML1* (*CBFA2*) gene on 21q22, we performed FISH with the *AML1* locus-specific probe and found that the *AML1* gene was amplified (Figure 5c). The amplification represented the duplication of the 21q22 segment. The significance of *AML1* amplification and of extra copies of 21q in the patho-

Table 1 Summarized results of cytogenetic, SKY, and FISH analyses of leukemic cells from patients with ALL and chromosomal abnormalities

Clinical features				*Laboratory findings*		
Patient No.	*Age (Year)*	*WBC mm³*	*Marrow blasts %*	*Cytogenetic analysis*	*SKY*	*FISH*
Group 1 – Apparently normal karyotypes						
1*	13	172.5	98	normal	t(7;8)(q34-35;q24.1)	confirmed
2	6	1.3	97	normal	t(13;17)(q22;q21),	confirmed
					der(19)t(17;19)(q22;p13)	t(17;19)
Group 2 – Abnormal karyotypes						
3	5	173.6	99	add(X)(p22.2)	der(X)t(X;5)(p11.4;q31)	confirmed
				del(5)(q32)	del(5)(q32)	confirmed
				mar	der(21)t(X;21)(p11.4;p11.2)	confirmed
4	15	14.0	93	t(X;9)(p11.4;p13),+11	confirmed	confirmed
5	12	1.7	93	+X,+10	confirmed	ND
				del(11)(q23)	confirmed	*MLL* deleted
				qdp(21)(q11.1q22)	confirmed	*AML1* amplified
6	5	16.5	97	add(4)(p16)	der(4)t(4;8)(p16;q13)	confirmed
				t(8;14)(q11.2;q32)	del(8)(q11.2)	Ig *JH* locus translocated
					der(14)t(8;14)(q11.2;q32)	to 8q11.2
				+21	confirmed	ND
7	13	9.3	99	t(8;14)(q11.2;q32)	del(8)(q11.2),	Ig *JH* locus translocated
					der(14)t(8;14)(q11.2;q32),	to 8q11.2
				+21	confirmed	ND
8*	1.3	12.2	94	t(1;2)(p36.1;p21)	confirmed	confirmed
				add(5)(q35),del(10)(q24)	t(5;10)(q35;q24)	confirmed
				inv(14)(q11.1q32)	not observed	ND
				del(16)(q24)	confirmed	ND
				add(17)(p11.2)	der(17)t(9;17)(q13;p11.2)	confirmed
9	6	20.7	97	+?X	+5	confirmed
				mar1	der(20)t(9;20)	ND
				mar2	der(9)del(9)(p11.2)del(9)(q11.2)	confirmed
				+21	confirmed	ND
10	4	103.2	99	–Y,	confirmed	confirmed
				der(20)t(1;20)(q21;q13.3)	confirmed	
11	10	3.0	94	–?13	confirmed	ND
12	3	5.9	91	t(7;8)(p15;p23)	del(7)(p15)	no translocation
				+21c,+X	confirmed	ND

*The patient had T-lineage ALL.
ND, not done.

genesis of leukemia is not known. FISH with the *MLL* probe identified the deletion of one allele of the *MLL* gene in this patient. In addition, FISH using an 11q subtelomeric probe did not identify any translocation to any other chromosome; this finding confirmed the del(11)(q23) (data not shown).

Conventional cytogenetic analysis identified an add(4)(p16) and a t(8;14)(q11.2;q32), whereas SKY showed the add(4)(p16) to be a der(4)t(4;8)(p16;q13) and the t(8;14) to be a der(14)t(8;14)(q11.2;q32) and a del(8)(q11.2) in patient No. 6 (Table 1). Although a reciprocal translocation was not detected by SKY, FISH with probes for the *JH* region of the immunoglobulin locus showed that the *JH* gene segments were translocated to the der(8) (data not shown); thus, confirming the reciprocal nature of the translocation. In another patient (Table 1, patient No. 7), G-banding identified a similar t(8;14)(q11.2;q32) and a +21. However, SKY did not detect the reciprocal translocation, but a signal from chromosome 8 was observed on chromosome 14. As in the previous case, FISH with the *JH* probe confirmed the presence of a reciprocal translocation.

Cytogenetic analysis identified a t(1;2), add(5)(q35), del(10)(q24), inv(14)(q11.1q32), del(16)(q24), and add(17)(p11.1) in the T-leukemic blast cells of one patient (Table 1, patient No. 8). The findings of SKY showed that the add(5) and del(10q) resulted from a reciprocal translocation of chromosomes 5 and 10, and that the add(17) contained DNA from chromosome 9 and formed the unbalanced der(17)t(9;17)(q13;p11.2). All translocations were confirmed by FISH (data not shown), but the inv(14) could not be seen by using SKY. In another patient (Table 1, patient No. 9), cytogenetic analysis found a +?X,+21, and two marker chromosomes. SKY identified +?X as chromosome 5, and one marker as a der(20)t(9;20), whereas the other was shown to be a der(9)del(9)(p11.2)del(9)(q11.2). In patient 10, the results of G-banding and SKY were concordant. A questionable finding (–?13) was confirmed by SKY (Table 1, patient No. 11). In another patient (Table 1, patient No. 12), a +X,t(7;8)(p15;p23) and +21c were identified by conventional cytogenetics; however, the results of SKY and FISH could not confirm the t(7;8). Instead, both methods detected a deletion of 7p. Because no additional material was available, we could not confirm the balanced nature of this subtle rearrangement by FISH with probes for the subtelomeric regions of these chromosomes.

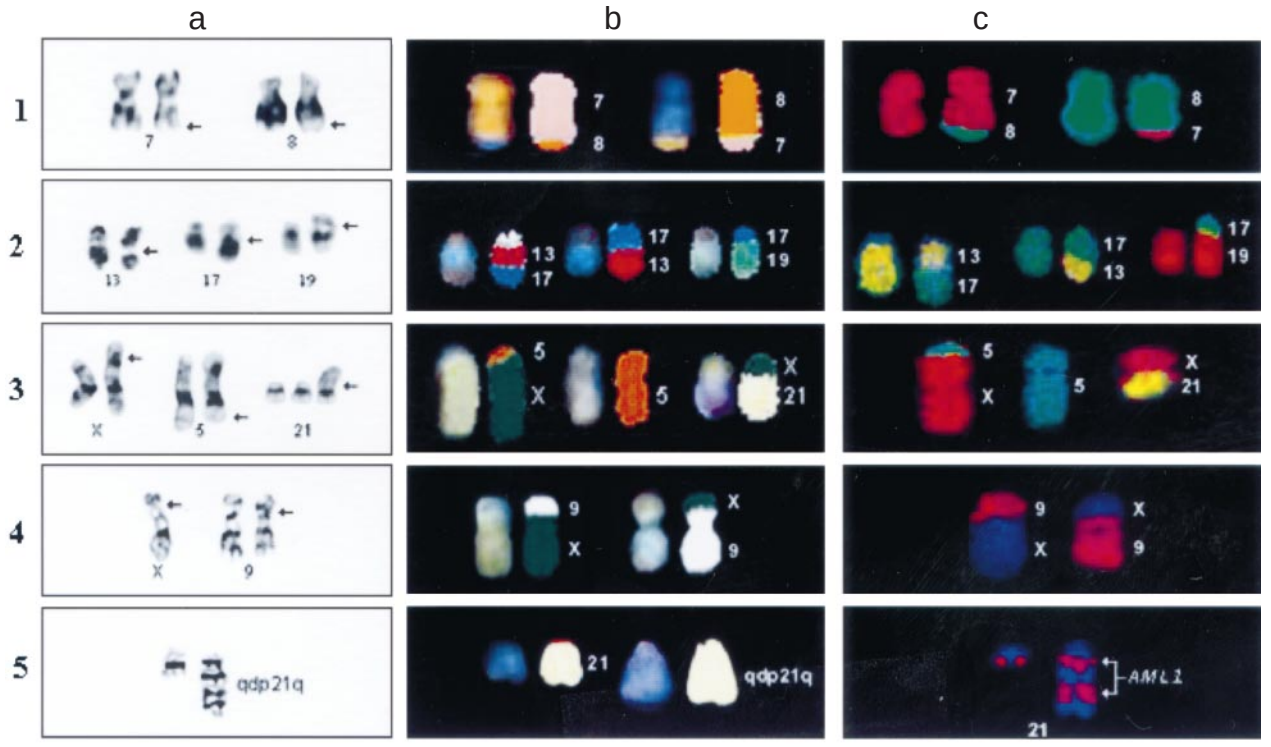

Figure 1 Identification of the t(7;8) by SKY (case No. 1). G-banding (a) and SKY results (b). Display colors are shown on the left; spectral classification colors are shown on the right. (c) Confirmation of the t(7;8) by FISH using painting probes for chromosomes 7 and 8. The probe for chromosome 7 was labeled with digoxigenin, and the probe for chromosome 8 was labeled with biotin. Normal chromosomes 7 and 8 are shown to the left of the translocated chromosomes.

Figure 2 Identification of the t(13;17) and the der(19)t(17;19) by SKY (case No. 2). G banding (a) and abnormal chromosomes 13, 17 and 19 identified by SKY (b). The signal from chromosome 19 was not observed on the other homolog of chromosome 17 by SKY. Display colors are shown on the left, and the spectral classification colors are shown on the right. (c) Confirmation of the t(13;17) and t(17;19) by FISH. A combination of digoxigenin- and biotin-labeled chromosome 13 probe, biotin-labeled chromosome 17 probe and digoxigenin-labeled chromosome 19 probe was used to confirm the t(13;17) and der(19)t(17;19). The reciprocal nature of the t(17;19) could not be identified either by SKY or by FISH using whole chromosome painting probes. Display and spectral colors for the abnormal chromosomes are shown on the left and right side respectively. However, FISH with the 19p subtelomeric probe confirmed the balanced t(17;19) (data not shown).

Figure 3 Identification of der(X)t(X;5), del(5)q32), and der(21)t(X;21) by SKY (case No. 3). G-banding (a) and SKY results (b). Display colors are on the left, and spectral classification colors are shown on the right. (c) Confirmation of der(X)t(X;5), del(5)(q32), and der(21)t(X;21) by FISH. Probes for chromosomes X and 5 were labeled with digoxigenin and biotin, respectively. For detection of chromosome 21, we used a combination of digoxigenin- and biotin-labeled probes.

Figure 4 Identification of t(X;9) by conventional cytogenetics and SKY (case No. 4). G-banding (a) and SKY results (b). The display colors are shown on the left, and the spectral classification colors are shown on the right. (c) Confirmation of the t(X;9) by FISH. The probe for chromosome 9 was labeled with digoxigenin, and the identity of the X chromosome was determined by the inverted DAPI images.

Figure 5 Identification of *AML1* amplification by FISH (case No. 5). G banding of quadruplicated 21q (a), SKY results (b), and detection of the *AML1* amplification (c). Normal chromosome 21 is to the left of the quadruplicated 21q chromosome.

Discussion

To date, only two studies have used SKY to identify cryptic chromosomal abnormalities in patients with hematologic malignancies and normal karyotypes: one study included patients with myelodysplastic syndrome,[13] and the second involved patients with T cell leukemias.[14] Although neither study found cryptic abnormalities, SKY confirmed or clarified complex rearrangements. Our study is the first to use SKY to detect abnormalities in pediatric patients with ALL in whom no chromosomal abnormalities have been found by conventional cytogenetic or molecular methods. SKY identified cryptic translocations in two of 20 normal cases (10%). Furthermore, SKY helped to characterize the nature of complex chromosomal abnormalities that could not be fully defined by conventional cytogenetics. In six cases, SKY findings complemented those of conventional cytogenetics, whereas in the rest, the results of cytogenetic analysis were either clarified or modified by those of SKY.

We identified four novel translocations by SKY alone: t(7;8)(q34–35;q24.1), t(13;17)(q22;q21), der(X)t(X;5)(p11.4;q31), and der(21)t(X;21)(p11.4;p11.2); an additional novel translocation, t(X;9)(p11.4;p13), was detected by conventional cytogenetics and SKY. These translocations have not been previously described in patients with ALL.[15]

Conventional cytogenetics and SKY in combination with FISH identified the amplification of the *AML1* gene in one patient with quadruplicated 21q. Chromosome 21, the chromosome on which *AML1* is located, is the most frequently duplicated chromosome, occurring in approximately 15% of patients with ALL; these patients have a favorable prognosis.[16–18] *AML1* amplification has been described in adult patients with acute myeloid leukemia and myelodysplastic syndrome, and in pediatric patients with ALL.[10,19,20] Also, other genes located on chromosome 21 may be implicated in the pathogenesis of leukemia. The significance of *AML1* amplification in patients with ALL is not clear at this time.

Only three of the breakpoints in the newly identified trans-

locations in the present study harbor candidate genes associated with known malignancies. The translocation in the patient with T cell ALL may involve the T cell receptor–β gene (*TCR*β) on 7q34–35 and the *MYC* gene on 8q24. The Xp11 breakpoint has been shown to be involved in synovial sarcoma, and the 17q21 harbors the retinoic acid receptor–α gene (*RARA*), which is involved in acute promyelocytic leukemia.

Although several studies have shown that SKY analysis can identify cryptic rearrangements in leukemias and solid tumors, few studies have shown that subtle deletions and inversions may not be detected by painting probes used in SKY.[21] Our study of patients with ALL whose chromosomes were found to be normal by conventional cytogenetic methods shows that SKY can identify novel cryptic translocations. Also, the results of our study make it clear that SKY may not detect inversions and may fail to determine the balanced origin of subtle translocations. This lack of detection is probably due to the absence of subtelomeric sequences in commercially available probes used for SKY and whole chromosome painting assays. The best approach for identifying all the chromosomal rearrangements in a tumor cell is the combined use of conventional cytogenetics and FISH using whole chromosome painting probes and chromosome-specific subtelomeric sequences.[22] Thus, SKY, when combined with G-banding, has great potential for identifying cryptic translocations and for resolving complex karyotypes and marker chromosomes in leukemias and solid tumors.

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