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RESEARCH ARTICLES

Detection of Unidentified Chromosome Abnormalities in Human Neuroblastoma by Spectral Karyotyping (SKY)

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Spectral karyotyping (SKY) is a novel technique based on the simultaneous hybridization of 24 fluorescently labeled chromosome painting probes. It provides a valuable addition to the investigation of many tumors that can be difficult to define by conventional banding techniques. One such tumor is neuroblastoma, which is often characterized by poor chromosome morphology and complex karyotypes. Ten primary neuroblastoma tumor samples initially analyzed by C-banding were analyzed by SKY. In 8/10 tumors, we were able to obtain additional cytogenetic information. This included the identification of complex rearrangements and material of previously unknown origin. Structurally rearranged chromosomes can be identified even in highly condensed metaphase chromosomes. Following the SKY results, the C-banding findings were reevaluated, and the combination of the two techniques resulted in a more accurate karyotype. This combination allows identification not only of material gained and lost, but also of breakpoints and chromosomal associations. The use of SKY is therefore a powerful tool in the genetic characterization of neuroblastoma and can contribute to a better understanding of the molecular events associated with this tumor.

INTRODUCTION

Neuroblastoma is the most common tumor in infants younger than 1 year and the second most common solid tumor of childhood (Grovas et al., 1997; Gurney et al., 1997). It is an embryonal cancer of the postganglionic sympathetic nervous system.

The clinical hallmark of neuroblastoma is heterogeneity. Some patients can be cured by surgery alone, but others have a fatal outcome. Furthermore, in some patients the tumor undergoes spontaneous regression or differentiation. This clinical diversity correlates with several molecular biologic features of neuroblastoma.

Established indicators of the aggressiveness of the tumor and poor outcome include the amplification of the *MYCN* gene (Brodeur et al., 1984, 1992: Seeger et al., 1985), deletion of the short arm of chromosome 1 (1p), a near-diploidy or near-tetraploidy (Ambros et al.. 1996), angiogenic phenotype (Meitar et al., 1996), the recently identified gain of chromosome arm 17q (Bown et al., 1999). and telomerase activity (Poremba et al., 2000). However, the presence of single copies of *MYCN*, a normal 1p, near-triploidy, the presence of an expanded $CD10^+$ cell population in the bone marrow (Mandel et al., 19941, absence of an angiogenic phenotype, and the expression of the *TRK* gene (Nakagawara et al., 1993) are associated with a favorable prognosis.

The karyotypic analysis of neuroblastoma tumors with conventional methods is often a difficult process that may be hindered by limited numbers of metaphase cells and poor chromosome morphology. often leading to only partial characterization of the chromosomal events. However, prior to the use of fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH), conventional cytogenetic analysis had identified a number of nonrandom abnormalities, including aberrations of 1p, 2p, 4p, 5q, 11q, 17q, and 19p (Franke et al.. 1986; Kaneko et al., 1987; Hayashi et al., 1989: Aver-Loiseau et al., 1995; Caron et al., 1996; Lastowska et al., 1997).

Spectral karyotyping (SKY) is a recently developed technique based on the hybridization of 24 fluorescently labeled chromosome painting probes (Garini et al., 1996). It has been shown in a number of studies that highly complex karyotypes with unidentified marker chromosomes can be defined by this method (Veldman et al.. 1997: Huang et al.,

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1998: Pulivarthi et al., 1998: Zattara-Cannoni et al., 1998; Fleischman et al.. 1999). We used SKY for the analysis of 10 primary human neuroblastoma tumors. which had previously been anal?-zed by conventional cytogenetics.

MATERIALS AND METHODS

Cytogenetics

Cases 1–9 were derived from a consecutive series of 17 primary neuroblastomas from which material was sent by various centers in Switzerland for cytogenetic investigation. The tumor material was processed as previously described (Betts et al., 1997). Multiple cultures were set up, although the most reliable culture employed Colcemid (final concentration, 0.005 g/ml; Life Technologies, Basel, Switzerland) added overnight on the day of sample receipt. Slides were made from fixed cell suspensions and were trypsin G-banded and Giemsa-stained. All other slides were aged before a full analysis was undertaken. Case 10 was described previously (Trakhtenbrot et al., 1999).

Spectral Karyotyping

The cases investigated by SKY were selected on the basis that fixed suspension material was still availabie following cytogenetic and FISH investigations. Slides for SKY were prepared by use of chromosome preparations stored at −20°C. Chromosome labeling was performed with the SKY fluorescent labeling kit (Applied Spectral Imaging, Migdal HaEmek, Israel) according to the manufacturer's protocol. Chromosomes were counterstained with DAPI. Image acquisition was performed by use of an SD200 Spectracube (Applied Spectral Imaging) mounted on an Olympus BH-2 microscope using a custom-designed optical filter (SKY-1. Chroma Technology, Brattleboro, VT). .Automatic identification of chromosomes was based on the measurement of the spectrum for each chromosome.

Fluorescence In Situ Hybridization (FISH)

A range of probes (Vysis, Downers Grove, IL) was employed, including an *MYCN*-specific probe to test for the presence of *MYCN* amplification, and whole-chromosome painting probes for chromosomes 6. 12, 18. 21, and 22 were used to verify some of the translocations detected by SKY. All probes were hybridized according to the manufacturer's instructions.

RESULTS

SKY provided additional cytogenetic information in 8 of the 10 cases (Table 1). In cases 1 and 2, SKY supported the previously identified abnormalities determined by G-banding and FISH. By combining the SKY and G-banding/FISH results, an almost full definition of the tumor karyotypes was possible (Table 2). Two examples of SKY-classified karyotypes (cases 6 and 9) are presented in Figures 1A and 2A. The abnormalities that were newly determined or reclassified by SKY are shown in bold in both Tables 1 and 2. The majority of the changes identified by SKY were previously referred to as markers or add(v) chromosomes. In cases 4, 6, 9, and 10, a number of breakpoints could be defined more accurately by referring back to the G-banding analysis. In cases 5, 6, 7, and 10, five chromosome segments were reclassified.

The vast majority of rearrangements (19/27) detected were unbalanced and, in some cases, complex. An example of the complexity observed was the der(11) chromosomes present in case 9 (Fig. ZB).

In case 3, a possible jumping translocation (JT) was seen, involving 18q11 with recipient chromosomes 21q22 and 22q13. JT is defined as a translocation of the same chromosome segment on two or more recipient chromosomes in different cell clones of the same patient (Lejeune et al., 1979 Bernard et al., 2000). JT is a rare cytogenetic event reported in leukemias. lymphomas, and solid tumors (i :, carcinomas, renal tumors). To our knowledge, this could be the first report of such an event in neuroblastoma.

Not all rearrangements were defined after identification by SKY. That could be the combination of three reasons: additional material detected by G-banding was too small to be detected by SKY the rearrangement is a complex internal event that SKY will not detect; and different cultures were analyzed by SKY and G-banding, and different subclones were analyzed. An example is add(1)(q32) and add(11)(p15) in case 4. Both were found only in a clone not detected by G-banding. In case 7, the additional segment on add(1)(q42) and on add(13)(q32) was not detected by SKY because of its small size or due to similarity of spectral characteristics between the chromosome and the additional segment.

A further strength of SKY was that a greater number of metaphase cells could be fully or partially analyzed than is typically possible with the average G-banding analysis, principally because SKY was able to provide information on metaphase

TABLE 1. Reclassification of Abnormalities Detected Originally by G-Banding by SKY*

Case number	G-banding analysis	Reclassification by SKY	FISH verifications with painting probes
3	I. add(4)(p14), add(12)(q24), add(7)(p22) +2 ~ 6mar [15]/	I. der(4)t(4;19)(p14;q13), der(12)t(12;21)(q24;q21), +der(7)t(Y;7)(q11;p22), der(22)t(18;22)(q11;q13) [6]/	p18 and p22
	II. clone not detected	II. 65 ~ 69. X, −X. −Y, idem but instead of der(22) t(18;22)(q11;q13) there is der(21)t(18;21) (q11;q22)[9]	p18 and p21
4	add(4)(q31), +mar[9]	der(4)t(4;8)(q26;q21)der(5)t(5;18)(p11;q21), der(6)t(6;12)(?;?)[12]	p6 and p12
5	der(1)t(X;1)(q24;p34), add(16)(q22), der(21)t(?1;21)(p34;p11)[7]	der(1)t(1;2)(p34;?), der(16)t(X;16)(q26;q22), der(21) t(19;21)(q13;p11)[14]	
6	I. add(1)(p36), add(6)(q23~25), add(7) (p21), der(16)t(16;?;17)(p13;?;q21), der(17)t(X;17)(q21;q10) [10]	I. der(1)t(1;7)(p36;?), der(6)t(6;14)(q21;q11)der(7) t(6;7)(p21.1;p22), der(16)t(16;17)(p13;q21)t(9;17) (q21;q25), der(17)t(4;17)(q26–28;p11)[6]/	
	II. clone not detected	II. 73–91, XXYY, idem to (I)×2[3]/	
	III. clone not detected	III. 91, XXYY, idem to (II), but instead of der(16) t(16;17)(p13;q21)t(9;17)(q21;q25)×2 there are der(16)t(16;17)(p13;q21)×2,[1]/92. XXYY, idem, t(4;7)(?;?)×2[1]	
7	der(16)t(16;17)(q12;q21), +1~5mar [10]	der(19)t(17;19)(q21;p13)[13]	
8	I. +2~3mar [10]	I. +del(7)(q31),der(11)del(11)(p12–q13)[19]/	
		II. 87–89, idem to (I),+del(7)(q31), +del(7)(q31) [4]	
9	add(1)(p36), add(6)(q16~22), +8–14mar[10]	der(1)t(1;17)(p36;q21), der(6)t(6;12)(q21;q14), der(11)(7qter→7q11.2::11p13→11q22::7q11.2→ 7qter), der(11)(8qter→18q11.2::7q3?→7q21::11p15→11q22::7q11.2→7qter), der(12)t(5;12) (?;?),der(14)t(14;17)(p11;q21)[20]	
10	I. del(1)(?p34)×2 der(16)t(?5;16)(?q31; q24), der(19)t(19;?)(q13;?)[33]/	I. der(1)t(1;12)(p13;q11–12)×2, der(16)t(16;17) (q22;q11), der(19)t(8;19)(?;q13)[45]/	
	II. 47, idem, der(10)t(10;?)(q26;?)[12]	II. 47, idem to (I),der(10)t(4;10)(q22;q26)[15]	

*Abnormalities detected only by SKY are shown in bold.

cells that would not be analyzable by G-banding. This provided further clues to the clonal evolution of tumors and made the analysis of polyploid metaphases possible. The advantage of the latter was shown in case 6, where one of the polyploid metaphase cells contained a der(16)t(16;17)(p13;q21) instead of the der(16)t(16;17)(p13;q21)t(9;17)(q21;q25) that characterized the diploid and an additional polyploid clone (Fig. 1B). This demonstrated the presence of divergent clonal evolution in this tumor.

All cases were investigated by FISH with a probe for *MYCN*, and amplification of the *MYCN* region was shown to be present in cases 1, 2, 4, 6, 8. and 10. which in all cases was in the form of double minutes (dmin). In cases 4 and 10. only the signal pattern of the *MYCN* probe, in both interphase and metaphase cells. allowed the identification of dmin, as they were apparently too small to be visualized under a light microscope. In case 3, despite the presence of dmin, no *MYCN* amplification was demonstrated, thereby implying amplification of another chromosomal region. In three cases (1, 6, and 8), **SKY** detected the presence of double-minute chromosomes, painted with the color of chromosome 2. In cases 2–4, SKY did not detect the dmin. probably due to either their small size or low copy number.

SKY was successful in demonstrating five cases with 1p deletion; all were the results of unbalanced translocations (cases 1, 5, 6, 9, and 10). In two cases. this involved chromosome arm 17q, and the other three involved chromosomes 2, 7, and 12.

DISCUSSION

Neuroblastoma is often characterized by gains and losses of genetic material and complex chromosomal abnormalities (Biedler et al.. 1973: Bro-

TABLE 2. Final Tumor Karyotype Combining G-Banding and SKY Results*

Case	Age (Y, M)	Stage	Survival (M)	Final tumor karyotype	*MYCN* verification
1	3, 2	IV	46+	46,XY,der(1)t(1;17)(p36.1;q12),der(22)t(17;22)(q21;p12),50~100dmin[8]/46,idem,del(11)(q23.1q23.3)[12]	dmin, *MYCN*
2	0.4	I	15+	46,XX,der(3)t(3;17)(p13;q23),10~100dmin[cp16]	dmin, *MYCN*
3	6.10	III	13	66~69,XXY,-3,der(4)t(2;4)(p21~22;q33),der(4)t(4;19)(p14q13),+der(7)t(Y;7)(q11;p22),-10,der(12)t(12;21)(q24;q21),+13,+17,-19,+20,-21,-21,der(22)t(18;22)(q11;q13),10~20dmin[cp20]/65~69,X,-X,-Y,-3,der(4)t(2;4)(p21~22;q33),der(4)t(4;19)(p14;q13),+6,+der(7)t(Y;7)(q11;p22),-10,der(12)t(12;21)(q24;q21)+13,-19,+20,-21,der(21)t(18;21)(q11;q22),+der(?)(?;17)(?;q12),10~20dmin[cp10]/130~138,idem×2[cp3]	dmin, *MYCN*-
4	3, 11	IV	10	56~61(3n),XX,-X,der(4)t(4;8)(q26;q21),der(5)t(5;18)(p11;q21),der(6)t(6;12)(?;?),-8,-9,-10,-11,-14,-16,-21[cp10]/56~61,idem,add(1)(q32),+del(2)(p16),+7,add(11)(p15),-13,-15,-18,-19,-20[cp15]	*MYCN* 2–15 copies
5	2, 5	IV	11+	46,-X,der(X)t(X;7)(q22;q11.2),der(1)t(1;2)(p34;?),der(4)t(4;17)(p12;q21),der(16)t(X;16)(q26;q22),+17,der(21)t(19;21)(q13;p11)[7]/46,idem,del(11)(q23)[3]/47,idem,+18[3]	*MYCN*-
6	1, 6	IV	11 days	46,XY,der(1)t(1;7)(p36;?),der(6)t(6;14)(q21;q11),der(7)t(6;7)(p21.1;p22),der(16)t(16;17)(p13;q21)t(9;17)(q21;q25),der(17)t(4;17)(q26~28;p11),20~100dmin[cp21]/92,idem×2[2]	dmin, *MYCN*
7	6.9	III	14+	70~72,XY,-X,add(1)(q42)x2,del(1)(q32q42),-3,+8,-11,del(11)(q14q23),+13,-14,-15,-16,+18,+18,+18,+18,-19,der(19)t(17;19)(q21;p13)[cp13]/70~72,idem,+7,-9,der(9)t(6;9)(p11;p11).+10,-12,add(13)(q32),+17,+18,+18,-19,+20,-22[cp10]/46,XY,del(11)(q14–q23)c[1]	*MYCN*-
8	4.0	IV	34+	87~89,XX,-Y,-Y,-3,-5,+del(7)(q?31),-9,-10,der(11)del(11)(p12)del(11)(q13),+20,20~200dmin[cp33]/46,XY[5]	dmin, *MYCN*
9	2.7	IV	31+	83~89,XXX,-X,del(1)(p34),der(1)t(1;17)(p36;q21).-3,-4,-5,der(5)t(5;12)(?;?),der(6)t(6;12)(q21;q1?4),+i(6)(p10),-9,der(11)(7qter→7q21::11p15→11q22::7q21→7qter),der(11)(18qter→18q11.2::7q3?→7q21::11p15→11q22::7q21→7qter),der(14)t(14;17)(p11;q21),-19,-21,der(22)t(17;22)(q11.2;q13)×2[cp20]	ins(?;2)(?;p23~24) *MYCN*
10	5, 5	IV	6	47,XX,der(1)t(1;6)(q4;?),der(1)t(1;12)(p13q11–12)×2,der(6)t(16;17)(q22;q11),der(19)t(8;19)(?;q13)[45]/47,idem,der(10)t(4;10)(q22;q26)[15]	*MYCN*

*Abnormalities facilitated by SKY are shown in bold.

deur et al., 1977). The interpretation of the full karyotypic picture can be complicated further by the presence of divergent clonal evolution and nonclonal rearrangements. We have used SKY combined with classical G-banding to characterize a series of primary neuroblastomas karyotypically.

Classical cytogenetic investigation of neuroblastoma is notoriously problematic, and unidentified chromosomal regions will often remain even after analysis by a skilled cytogeneticist. SKY is a novel technique that, when used alone, allows the identification of chromosomes involved in numerical and structural aberrations in the investigated tumor. However, when the SKY results are used in conjunction with G-banding, specific chromosomal segments and breakpoints can be defined, thereby allowing an almost full characterization of the karyotype.

We showed that the majority of rearrangements detected were unbalanced (Table 2), particularly so in the case of 1p and 17q. Deletions of 1p have been associated with a poor prognosis in neuroblastoma (Franke et al., 1986; Fong et al., 1989 Caron et al., 1996; Schleiermacher et al., 1996). The five cases with a 1p abnormality in this series were all the result of an unbalanced translocation. This observation would suggest that the majority of 1p deletions in neuroblastoma result from such an event rather than solely from a deletion event. Two of the del(1p) cases resulted from translocations involving 17q. Such a rearrangement has previously been suggested to be the most common rearrangement event in neuroblastoma (Franke et al., 1986). Chromosome arm 17q was found to be involved in unbalanced translocations, in addition to 1p, with five chromosomal regions: 4q26–28, 9q21, 16p13,

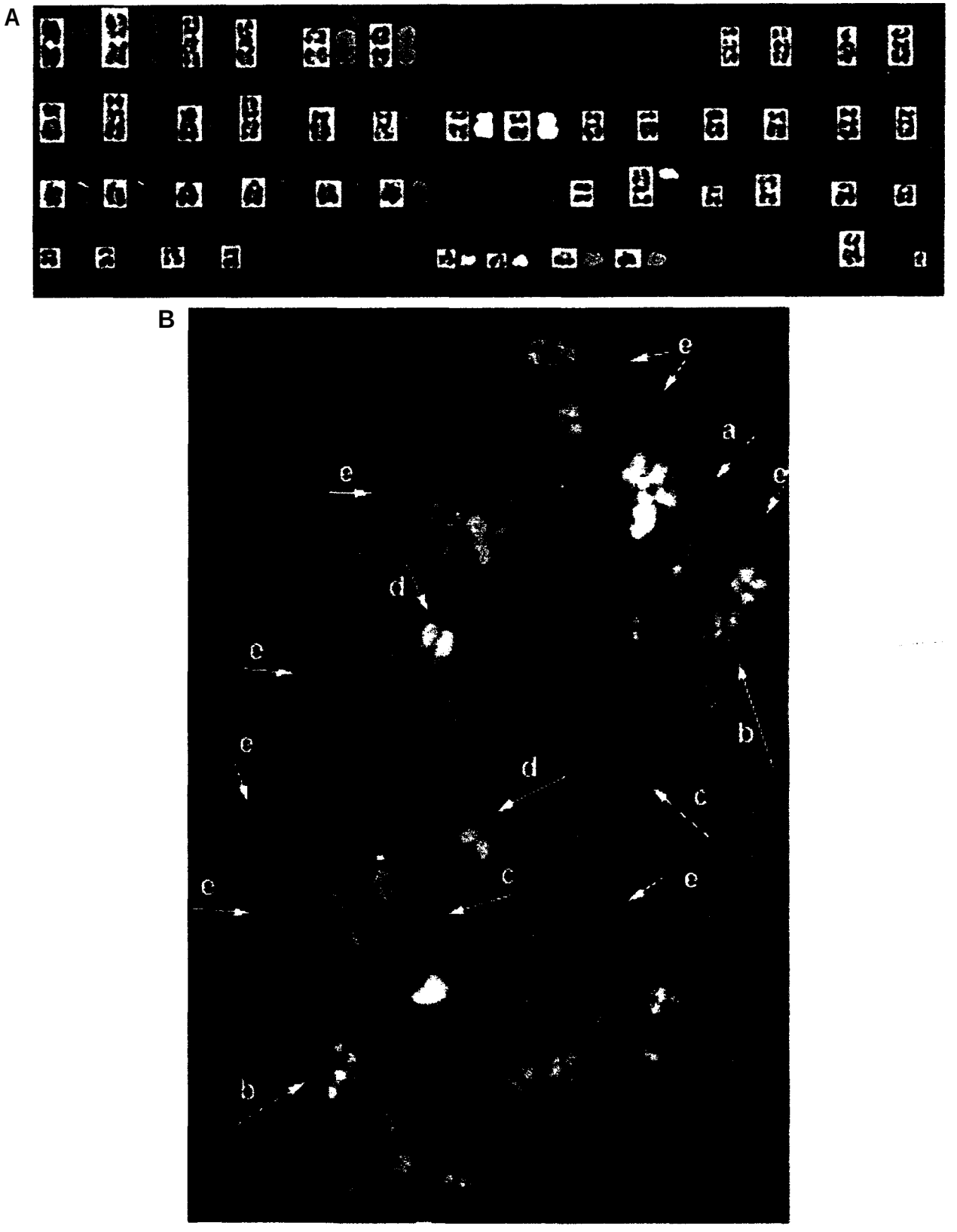

Figure 1 Case 6 A SKY spectra-based colors classification colors of chromosomes 13 14, 15, and 21 centromeres differ from the rest of the chromosome due to nonspecific sequences in those regions The following chromosomal abnormalities are seen clearly in the karyogram der(1)t(1;7)(p36;?) der(6)t(6;14)(q21;q11). der(7)t(6;7)(?p21 1;p22) der(16)t(16;17)(p13;q21)t(9 17)(q21;q25) and der(17)t(4;17)(q26-28 p11) B Polyploid metaphase cell in RGB display colors SKY can detect markers ever in polyploid cells where full analysis cannot be aone The arrows indicate rearranged chromosomes a der(7)t(6;7)(?p21 1;p22) b der(16)t(16 17)(p13;q21)t(9 17)(q21;q25) c der(17)t(4;17)(q26-28;p11) d der(6)t(6 14)(q21;q11) e dmins-*MYCN*–positive ir the color of chromosome *1*

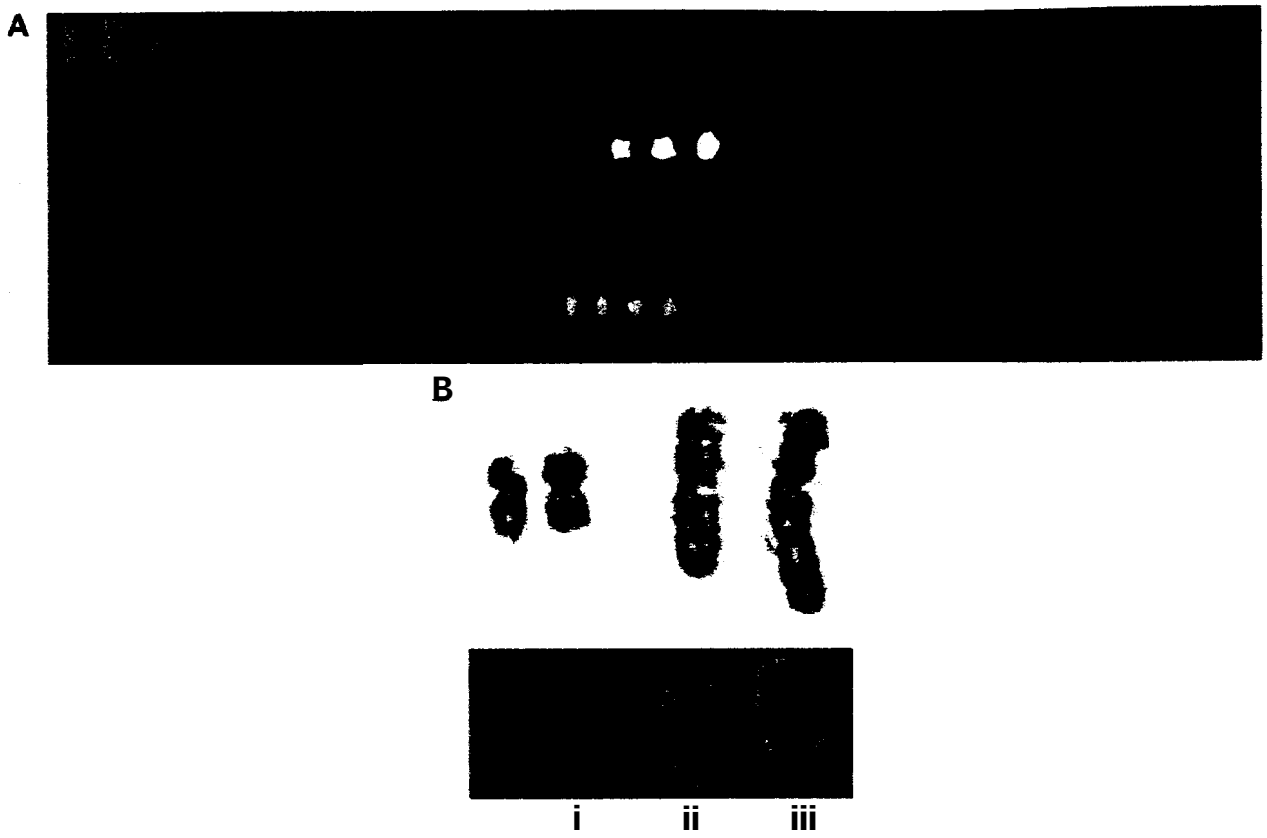

Figure 2. Case 9. *A* Chromosomal aberrations identified by SKY are seen in chromosomes 1, 5, 6, 11, 14, and 22: der(1)t(1;17)(p36;q21), der(5)t(5;12)(?;?), der(6)t(6;12)(q21;q1?4), der(11)(7qter→7q21::11p15→11q22::7q21→7qter), der(11)(18qter→18q11.2::7q3?→7q21::11p15→11q22::7q21→7qter), der(14)t(14;17)(p11.2;q[illegible]), and der(22)t(17;22)(q11.2;q13)x2. B: Normal and aberrant chromosomes 11, after G-banding and SKY. i, normal chromosome 11; ii, der(11)(7qter→7q21::11p15→11q22::7q21→7qter); iii, der(11)(18qter→18q11.2::7q3?→7q21::11p15→11q22::7q21→7qter).

16q22, and 19p13. Interestingly, some cases were found to have multiple 17q rearrangements, with different breakpoints on chromosome arm 17q. Indeed, further use of SKY in the identification of partner chromosomes in these types of prognostically important rearrangements may ultimately lead to a refinement of the clinical implications of these events.

MYCN amplification is a common event in neuroblastoma and one that has great importance in the clinical outcome (Brodeur et al., 1992: Yoshimoto et al.. 1999). SKY was able to detect only 3/4 cases with dmin-*MYCN* amplification. previously verified by FISH. This illustrates that, although SKY is a powerful technique, it still has its limitations. Thus. small dmins may not always be detected. A further current limitation is that intrachromosomal rearrangements such as inversions will not be detected. showing that SKY alone is insufficient.

In addition to the previously described involvement of 1p and 17q, a number of other chromosomal bands were involved in more than one tumor: 4q26, 7p22, 7q11.2, 18q11.2, and 19q13. It should also be noted chat there was frequent partial gain through structural rearrangements of chromosome 7. The involvement of 4q, 7/7q, and 18q could be significant because gains of these segments have also been reported frequently in cytogenetic and/or CGH studies in neuroblastoma (Altura et al., 1997; Van Roy et al., 1997). The contribution of genes in these regions to neuroblastoma tumorigenesis should be the target of further investigation.

A coordinate association between the loss of 3p and 11q has recently been described by Breen et al. (2000). In this present study, only one case (case 2) had a deletion of 3p, with a further three having loss of the whole chromosome (cases 7, 8, 9). Five cases also had deletions of 11q or loss of the whole chromosome (cases 1, 5, 7, 8, 9). Therefore, our results can be said neither to support nor disprove the association. The regions 3p and 11q probably have an important role to play in the tumorigenesis

of neuroblastoma, but whether their combined loss is nonrandom requires further proof.

Our work shows the capability of the SKY technique to identify novel marker chromosomes, to clarify complex chromosome aberrations, and to describe clonal evolution in neuroblastoma. The combination of conventional karyotyping and the SKY technique allows an improved understanding of solid tumors. The contribution of SKY to the field of neuroblastoma cytogenetics is potentially of great value, because more precise definition of chromosomal rearrangements may lead to new information concerning genetic events in neuroblastoma oncogenesis and to the identification of prognostic markers.

In summary, the full strength of SKY is observed when it is used in combination with conventional banding techniques, resulting in the following gains and thus leading to further clarification of neuroblastomas: reconfirmation of the G-banding results; identification of partially characterized rearrangements; definition of markers of unknown origin; reclassification of G-banding further refinements of breakpoints originally determined by G-banding; and analysis of both polyploid and poor-morphology metaphase cells, which typically cannot be fully analyzed by classic Cytogenetics.

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