The clinical application of spectral karyotyping (SKY™) in the analysis of prenatally diagnosed extra structurally abnormal chromosomes (ESACs)

Yuval Yaron1,2,3*, Erez Carmon2, Myriam Goldstein1,3, Nadia Yoskoboinik1, Yifat Ochshorn2, Zully Gelman-Kohan4 and Avi Orr-Urtreger1,3

1Prenatal Diagnosis Unit & Genetic Institute, Sourasky Medical Center, Tel Aviv, Israel
2Department of Obstetrics & Gynecology, Lis Maternity Hospital, 6 Weizmann Street, Tel Aviv, Israel
3Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
4Genetic Institute, Kaplan Medical Center, Rehovot, Israel

Objective The prenatal detection of de novo extra structurally abnormal chromosomes (ESACs) presents a challenge because the associated risk for congenital anomaly ranges from 100% to practically none, depending on the chromosomal origin. Despite the use of standard cytogenetic techniques and even fluorescence in situ hybridization (FISH), the origin of some ESACs often remains elusive. Spectral karyotyping (SKY™) is a molecular cytogenetic technique based on the simultaneous analysis of all chromosomes using a unique probe mix that allows the rapid identification of all chromosomes in 24 colors. The purpose of this study was to evaluate the use of SKY in the characterization of prenatally diagnosed de novo ESACs.

Methods This series includes five cases of de novo ESACs detected prenatally in routine amniocentesis samples performed for advanced maternal age. Cases of inherited ESACs or ESACs defined by standard cytogenetic techniques were excluded.

Results SKY analysis yielded valuable information, particularly in cases of nonsatellited ESACs: a der(18) and a ring(Y). In a case of a unisatellited der(15), SKY corroborated data obtained by standard cytogenetic techniques and FISH. Finally, in two cases of small bisatellited chromosomes, SKY was noncontributory.

Conclusions While SKY may be a valuable tool in some cases, especially nonsatellited and ring ESACs, it does have limitations and should be used judiciously in conjunction with other cytogenetic techniques.

INTRODUCTION

Extra structurally abnormal chromosomes (ESACs), also designated as marker, supernumerary, or accessory chromosomes, occur with a frequency of 0.14 to 0.72/1000 births (Warburton, 1991). In a large study of ESACs detected at amniocentesis in ~75 000 prenatal cytogenetic diagnoses, the rate of all ESACs was approximately 0.8/1000, with slightly more than half being de novo (Hook and Cross, 1987). Risks of fetal abnormalities associated with de novo ESACs span a wide spectrum, varying from as little as <2% for small bisatellited monocentromeric ESACs to as much as 100% for an isochromosome 18p (Warburton, 1991). Identifying the precise origin of an ESAC is thus of crucial importance for genetic counseling and parental decisions. Despite the recent developments in molecular cytogenetic techniques, accurate identification of de novo ESACs remains a challenge to the cytogenetic laboratory. Classical cytogenetic techniques using standard staining methods such as G-banding, C-banding, Ag-NOR staining, and so on are often incapable of determining the origin of de novo ESACs.

Recent developments in fluorescence in situ hybridization (FISH) techniques have enabled the detection of subtle cytogenetic aberrations, beyond the resolution of classical cytogenetics. While numerous FISH probes are widely available, the use of a specific probe requires prior knowledge or suspicion of a specific genomic locus for which the probe is specific. Alas, in the case of de novo ESACs, such knowledge is unavailable. To overcome this, a series of whole chromosome paints (WCPs) may be employed sequentially, until the chromosomal origin is, inadvertently, stumbled upon. However, this approach has several shortcomings, particularly in prenatal diagnosis: (1) there is a limited amount of sample material that may not allow multiple analyses, (2) the advancing gestational age poses serious time limitations for a sequential approach, and (3) analyses performed in parallel may not be cost-effective. Multicolored FISH (M-FISH) is a molecular cytogenetic technique that allows the simultaneous visualization of all 24 chromosomes in a single in situ experiment. With this technique, each chromosome is differentially labeled with a unique

*Correspondence to: Yuval Yaron, Genetic Institute, Sourasky Medical Center, 6 Weizmann Street, Tel Aviv 64239. E-mail: yyaron@tasmc.health.gov.il

Copyright © 2003 John Wiley & Sons, Ltd.
combination of fluorescent dyes, which allows the rapid identification of all chromosomes in 24 colors. The analysis may be performed by the sequential use of specific filters for each fluorochrome or by the use of spectral karyotyping (SKY), which is based on the simultaneous spectral analysis of all fluorescent dyes in the probe mix (Schröck et al., 1996).

The purpose of this study was to evaluate the use of SKY in the elucidation of prenatally diagnosed de novo ESACs of unknown origin and its relevance for genetic counseling and parental decision-making.

### MATERIALS AND METHODS

#### Patients

The study included five cases with a de novo ESAC of unknown origin, detected by second trimester amniocentesis performed for advanced maternal age. Following the initial finding of the ESAC, couples underwent genetic counseling, and parental karyotyping was performed to rule out the presence of the ESAC in one of the parents. Cases with inherited ESACs as well as ESACs of known origin were not included in the study. In all cases, targeted ultrasound and fetal echocardiography were recommended.

#### Cytogenetic analysis

Initial chromosome analysis of amniotic fluid samples was performed by standard G-banding of metaphase chromosomes from the amniotic fluid cultures. Karyotypes were established according to the International System for Human Cytogenetic Nomenclature (ISCN), 1995 (Mitelman, 1995). To further define the origin of the ESACs, additional cytogenetic staining techniques included, in all cases, silver staining for nucleolar organizing regions (Ag-NOR) to assess the presence of satellite stalks of acrocentric chromosomes. In addition, FISH for specific chromosome regions was used, as appropriate, according to manufacturers’ instructions. Usually, FISH was performed with a chromosome 15 probe (LSI D15S10, Vysis), because nearly half of all ESACs are inverted duplications of the short arm of chromosome 15 (Warburton, 1991). The D15S10 probe is designed to identify deletions of the D15S10 locus and the UBE3A gene located within the 15q11.13 region, which is associated with Angelman/Prader–Willi syndrome (AS/PWS). The probe contains additional controls for the short arm of chromosome 15 (15p11.2, SpectrumGreen CEP D15Z1) and the long arm of chromosome 15 (15q22, SpectrumOrange LSI PML).

Other FISH probes were used as appropriate to confirm or rule out the involvement of a specific chromosome or chromosomal region, including the Y chromosome-specific probes (Yhet, Ycent (Vysis), Amely (Oncor), and the chromosome 22–specific probe 22LSI DiGeorge/VCF (Vysis).

### Spectral karyotyping

Spectral karyotyping (SKY™) was performed in all cases according to the manufacturer’s procedure [Applied Spectral Imaging (ASI) Ltd, Migdal Ha’Emek, Israel] (Schröck et al., 1996), to refine standard cytogenetic findings. Briefly, this technique employs combined Fourier spectroscopy, charge-coupled device (CCD) imaging, and optical microscopy. The SKY probe mix is composed of chromosome-specific composite libraries, generated by polymerase chain reaction (PCR) from flow-sorted human chromosomes. The chromosome-specific probes are conjugated to a specific combination of five different fluorophores: Cy2, Spectrum green, Cy3, Texas Red, and Cy5. The probe mix is hybridized with a slide containing metaphase spreads prepared from material stored in fixative according to the protocol recommendations by the manufacturer (ASI Ltd). The metaphases are captured and analyzed using the SD200 system (ASI Ltd).

### RESULTS

Clinical data, cytogenetic analysis of amniotic fluid samples, and SKY results are presented in Table 1. In all cases, a de novo ESAC with variable degrees of mosaicism was detected. In all cases, parental karyotype analyses were normal. A detailed description of each case is provided here.

#### Case 1

In this 43-year-old primigravida, cytogenetic analysis of amniotic fluid cells revealed a mosaic karyotype with a de novo ring ESAC of unknown origin, present...
in 10/29 cells. The compact configuration of the ring ESAC did not lend itself to analysis of the banding pattern. SKY analysis revealed that the ESAC originated from chromosome Y (Figure 1). Additional studies by FISH using the Y chromosome probes, Yhet and Ycent (Vysis) and Amely (Oncor), confirmed the presence of all these chromosomal regions within the ESAC. The final karyotype was 47,XY,+r(ISH t(Y)(wcpY+, Yhet+, Ycent+, Amely+)]10]/46,XY[19].

Case 2

A 38-year-old multigravida, who had two normal children from a previous marriage, underwent amniocentesis in a twin gestation. Cytogenetic analysis revealed a normal male karyotype (46,XY) in one co-twin. In the other co-twin, however, a mosaic karyotype was detected with a de novo ESAC of unknown origin present in 11/14 cells. Ag-NOR staining was negative, as was FISH with the LSI probe D15S10 (Vysis). SKY analysis demonstrated that the ESAC was a derivative of chromosome 18 (Figure 2).

Given the poor prognosis, the parents opted for selective reduction. During the procedure, fetal blood
was obtained for confirmation, which corroborated the initial karyotype 47,XX,+der(18)[5]/46,XX[35].

Case 3

In this 37-year-old multigravida, who has two healthy children, cytogenetic analysis of amniotic fluid cells revealed a mosaic karyotype with a de novo ESAC of unknown origin, present in 10/34 cells. Ag-NOR staining demonstrated that it is a unisatellited ESAC. FISH analysis with the LSI probe D15S10 (Vysis) revealed that the ESAC is, in fact, a derivative of chromosome 15, but does not contain the 15q11–13 region, which is associated with the Prader–Willi/Angelman syndromes. The ESAC was positive only for the 15q11.2-specific probe (D15Z1), representing the short arm of chromosome 15 (Figure 3D). SKY analysis confirmed that the ESAC originated from chromosome 15. The final definition of the karyotype is 47,XY, +ish der(15)(D15S10 +)[10]/46,XY[19]. Ultrasound scans and fetal echocardiography were normal. After extensive genetic counseling, the parents elected termination of pregnancy. Pathological examination of the abortus did not demonstrate any structural malformations.

Case 4

This 45-year-old primigravida had a long-standing history of primary infertility. On amniocentesis, a mosaic karyotype was found, with a de novo ESAC of unknown origin present in 37/55 cells. Ag-NOR staining revealed a small (chromosome 21), bisatellited ESAC. FISH analyses with the LSI probe D15S10 for 15q11.2 and the chromosome 22–specific 22LSI DiGeorge/VCF probe were both negative. SKY analysis was inconclusive as the small marker chromosome was present in only some of the cells, and spectral analysis classified it differently in each one of the metaphases analyzed. The bisatellited nature of the ESAC suggested that it is a derivative of an acrocentric chromosome, excluding chromosome 15. Additional studies were not possible because all amniocyte cultures were exhausted. Ultrasound scans and fetal echocardiography were normal. Given the overall good prognosis and their long-standing infertility, the couple elected to continue with the pregnancy. A healthy girl was born after a normal pregnancy and delivery. She is currently 26 months of age, with normal psychomotor development and full language skills. She was unavailable for further testing.

Case 5

In this 40-year-old multigravida, cytogenetic analysis of amniotic fluid cells revealed a mosaic karyotype with a de novo ESAC of unknown origin present in 8/10 cells. Ag-NOR staining revealed a small (chromosome 21), bisatellited ESAC. Further analysis by C-banding demonstrated only heterochromatin in the ESAC. FISH analysis with the LSI probe D15S10 (Vysis) for 15q11.2 (D15Z1) was negative. As in Case 4, SKY analysis was inconclusive (Figure 4). It was concluded that the ESAC is a derivative of an acrocentric chromosome excluding chromosome 15. Ultrasound scans and fetal echocardiography were normal. The parents decided to continue with the pregnancy and gave birth to a healthy, phenotypically normal girl who is currently three months old and developing well. Postnatal chromosomal analysis demonstrated a mosaic of 70 to 80% of the ESAC in the placenta and neonatal blood sample. While it is still early to conclude, the child appears to be normal.

Figure 3—Spectral karyotyping analysis of a metaphase spread from Case 3. (A) Inverted DAPI image of one of the metaphase spreads analyzed. Arrow indicates the ESAC, marked as ‘mar’. (B) Presentation of the same metaphase spread in display colors. Arrow indicates the ESAC, marked as ‘mar’. (C) Presentation of spectral analysis–based classification pseudocolours. The ESAC is shown to originate from chromosome 15. (D) FISH analysis using multiple chromosome 15 probes. The D15S10 probe for 15q11.13 region and the PML probe for 15q22 are both in SpectrumOrange, while the D15Z1, specific for the short arm of chromosome 15 (15p11.2), is in SpectrumGreen. Note that both normal chromosome 15s are positive for all 3 loci, while the marker is positive only for D15Z1 in SpectrumGreen, indicating that it is a derivative of 15p.
DISCUSSION

Extra structurally abnormal chromosomes (ESACs) are a heterogeneous group with regard to prognosis. Warburton et al. estimated that the risk of abnormality associated with a de novo ESAC is 15% for a nonsatel-lited ESAC and 11% for a satellited ESAC (Warburton, 1991). However, and as pointed out by Gardner and Sutherland, 'Lumping ESACs into a group is rather like putting Down, Edwards, and Patau syndrome into a single trisomy category' (Gardner et al., 1996). The actual risk varies to a great extent. Small der(Y) or small monocentromeric bisatellited ESACs have an associated risk of less than 5%, whereas isochromosomes i(18)p, i(12)p or the isodicentric chromosome 22 of the cat-eye syndrome are associated with a 100% risk of abnormality of varying severity (Warburton, 1991). Thus, general risk figures are of limited use for counseling physicians and families, alike. This underscores the importance of precise definitions of de novo ESACs of unknown origin.

SKY allows the simultaneous detection of all 24 different chromosomes by labeling each with a different combination of fluorescent dyes. This technique has been extensively used in the elucidation of complex chromosomal rearrangements that are otherwise unresolved by classical cytogenetic techniques and/or FISH alone. SKY has been mostly used in the study of chromosomal aberrations associated with cancer. Many human malignancies, in particular hematological cancers as well as bone and soft-tissue sarcomas, demonstrate chromosomal aberrations. Some cancer-associated aberrations are well defined and confer a specific disease phenotype or predict a response to therapy or outcome (Singh et al., 2001; Tsushima et al., 2001; Zielenksa et al., 2001).

SKY has also been used to determine the origin of postnatally detected chromosome aberrations associated with phenotypic and developmental abnormalities. Huang et al. analyzed a newborn with multiple congenital anomalies and a de novo ESAC, subsequently shown by SKY to originate from the distal long arm of chromosome 15 (Huang et al., 1998). Morelli et al. reported a child with congenital malformations, previously associated with trisomy 17p. SKY analysis indeed revealed a partial trisomy of the short arm of chromosome 17 (Morelli et al., 1999). Numabe et al. used SKY to demonstrate 7q distal trisomy syndrome resulting from an unbalanced translocation in a five-year-old girl (Numabe et al., 2001).

In the case of prenatally detected de novo ESACs, there is a limited amount of tissue available for analysis and a need for rapid results. It may theoretically be possible to reuse the same slide by re-denaturation and sequentially employ a range of FISH probes. However, the reliability of each subsequent attempt is expected to decrease and such a sequential approach may also prove to be costly and time-consuming.

Alternatively, SKY has been suggested for these purposes as a rapid and simultaneous tool of high precision. Interestingly, however, there are only a few reports in the medical literature describing the use of SKY in prenatal diagnosis, as partly reviewed by Bayani and Squire (Bayani and Squire, 2001). Phelan et al. reported the use of SKY in the elucidation of a prenatal case with a complex chromosomal rearrangement involving nine breakpoints on four different chromosomes (Phelan et al., 1998). Likewise, Peschka et al. described the use of SKY in the prenatal elucidation of a de novo complex chromosome rearrangement involving eight breakpoints on four different chromosomes (Peschka et al., 1999). Haddad et al. described the use of SKY in the evaluation of eight de novo chromosomal markers, only one of which was diagnosed prenatally, demonstrating that extra material of unknown origin attached to the chromosome 21q originated from chromosome 4 (Haddad et al., 1998). Ning et al. described the first use of SKY in the prospective prenatal diagnosis of a mosaic, de novo, nonsatellited ESAC of unknown origin. In their case, SKY led to the determination that the ESAC originated from chromosome 14, which was later confirmed by
In this case, SKY had a minor contribution in the counseling and parental decisions. In Case 1, the parents had seriously considered termination of pregnancy because of the vague nature of the ring chromosome. The finding by SKY that the ring chromosome was a Y-derivative offered reassuring risk estimates that allowed the parents to reach a well-informed decision and to continue with the pregnancy.

In Case 2, an ESAC was detected in one co-twin but the parents were reluctant to intervene, weighing the overall risk of 15% for a fetal anomaly against the risk of losing the entire pregnancy after second trimester selective reduction (~5%) (Yaron et al., 1998). In this case too, SKY revealed that the ESAC is, in fact, a der(18), which would be associated with ~100% risk for very poor prognosis.

In Case 3, SKY confirmed the results obtained by FISH analysis using the LSI probe D15S10, demonstrating that the ESAC is a derivative of chromosome 15. Nonetheless, and despite the relatively good prognosis, the parents chose to terminate the pregnancy. Thus, in this case, SKY had a minor contribution in the counseling and management.

In both Cases 4 and 5, a small de novo, bisatellited ESAC was found. FISH analysis with the LSI probe D15S10 ruled out chromosome 15 as a possible origin. In both cases, the SKY procedure did not yield any significant results and the working hypothesis was that the ESAC is a derivative of an acrocentric chromosome excluding chromosome 15. These two cases demonstrated that the resolution of SKY is limited in the cases of ESACs, which are derivatives of acrocentric chromosomes.

Fan et al. concluded that the resolution of SKY in determining interchromosomal translocations is in the range of 1 to 2 Mbp (Fan et al., 2000). However, SKY may also have limited value in cases of ESACs derived from the short arms of the acrocentric chromosomes. This may be due to the fact that the SKY probe mix does not correctly assign the chromosomal origin of the short arms of the acrocentric chromosomes. This is often manifested in routine SKY analyses wherein the short arms of acrocentric chromosomes display different colors from the rest of the chromosomes, appearing as ‘pseudotranslocations’. Perhaps, the newly described AcroM-FISH technique, which consists of probes for all acrocentric chromosomes, centromere probes for chromosomes 13/21, 14/22, 15, and a probe specific for rDNA, may offer specific benefits for such cases (Langer et al., 2001). In conclusion, SKY is a valuable tool, especially in cases of de novo non-satellited or ring ESACs, when used in conjunction with other standard cytogenetic techniques.

ACKNOWLEDGEMENTS

We thank Yaffa Nevo Ph.D. and Nava Furman M.S. for their assistance in the cytogenetic analysis.

REFERENCES


Phelan MC, Blackburn W, Rogers RC, et al. 1998. FISH analysis of a complex chromosome rearrangement involving nine breakpoints on chromosomes 6, 12, 14 and 16 [In Process Citation]. Prenat Diagn 18: 1174–1180.


