Advanced genome-wide screening in human genomic disorders

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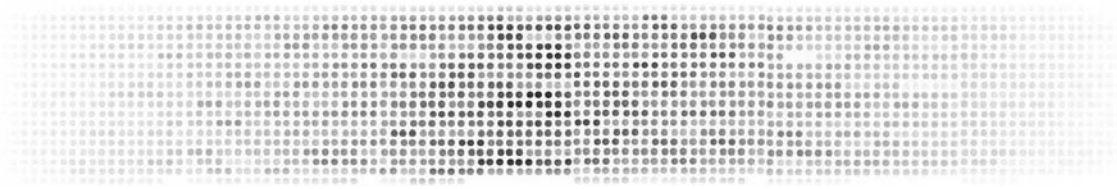
Chapter 2

Insights from genomic microarrays into structural chromosome rearrangements.



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Insights from genomic microarrays into structural chromosome rearrangements

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ABSTRACT

Array-based comparative genomic hybridization allows high-resolution screening of copy number abnormalities in the genome, and becomes an increasingly important tool to detect deletions and duplications in tumor and post-natal cytogenetics. Here we illustrate that genomic arrays can also provide novel clues regarding the structural basis of chromosome rearrangement, including instability and mechanisms of formation of ring chromosomes. We also showed that array results might impact the recurrence risks for relatives of affected individuals. Our data indicate that chromosome rearrangements frequently involve more breaks than current cytogenetic models assume.

INTRODUCTION

Chromosome replication errors resulting in exchange, duplication, or deletion of genetic material occur sporadically in both meiosis and mitosis, and may have implications for fertility, somatic, and cancer phenotypes. In routine clinical cytogenetics, structural chromosome alterations are interpreted as resulting from a minimum number of chromosomal breaks, followed by relocation and reunion of the chromosome segments, such that one chromosome break is required for a terminal deletion, two for interstitial deletions and reciprocal translocations, three for three-way translocations, and so on. Although these assumptions have been applied for decades, cytogeneticists have been aware that the actual number of chromosome breaks involved may be larger than assumed in these models, and a considerable amount of investigation has been done to determine the DNA bases of chromosome rearrangements [Park et al., [2002]; Stankiewicz et al., [2003]]. In fact, for a number of cases of terminal deletions, which were investigated at higher resolution, it was demonstrated that more than a break was involved in the origin of the rearrangement. These rearrangements were found to have been mis-classified as terminal deletions, and in fact represent either interstitial deletions or (half)-cryptic translocations [review in Kaiser-Rogers and Rao, [1999]].

One event that has been particularly intriguing, and has been investigated in some more detail in humans during the last two decades, is the formation of ring chromosomes.

Ring chromosomes are thought to originate from single breakages in both arms of a chromosome with subsequent fusion of the ends, and loss of the acentric segments. Molecular cytogenetic studies, however, have suggested that additional mechanisms for ring formation should exist, including transverse mis-division of the centromere [Callen et al., [1991b]] telomere fusion with no detectable loss of genetic material [Pezzolo et al., [1993]; Speevak et al., [2003]], and breakdown and rearrangement of a haploid complement shortly after fertilization in a triploid zygote [Beverstock et al., [2003]].

These analyses of chromosome rearrangements relied mostly on fluorescence in situ hybridization (FISH) data, which is an ideal methodology for investigating the presence or absence and approximate location of a limited number of chromosome targets, but is normally too focused to provide high-resolution information over extensive chromosome regions. In the last few years, comparative genomic hybridization to arrays (array CGH) has been incorporated into the repertoire of techniques yielding chromosome information [Solinas Toldo et al., [1997]; Pinkel et al., [1998]; Albertson et al., [2000]; Fiegler et al., [2003]]. It provides simultaneous information about copy number variation over a large number of loci and at greatly improved resolution (given by the spacing of the clones) compared to its precursor technique, chromosome CGH (10-20 Mb) [Kallioniemi et al., [1992]]. Array CGH has been proven particularly useful in the

study of tumors [Albertson and Pinkel, [2003]; Veltman et al., [2003]]. We describe here the use of genomic arrays combined with FISH analyses for re-evaluating chromosome rearrangements present in four human cell strains derived from karyotypically abnormal patients, including two ring chromosomes. This work illustrates that the interpretation based on G-banding often underestimate the complexity and number of breaks of the chromosomes.

PATIENTS, MATERIALS, AND METHODS

Cell Strains and Controls

Four fibroblast cell strains containing structural rearrangements as determined by G-banding karyotype were selected for this study, three of them from the fibroblast cell repository of the Department of Medical Genetics Utrecht (University Medical Hospital, Utrecht, the Netherlands), and one from a patient with Rett syndrome previously reported by us [Rosenberg et al., [2001]]. These cell strains were selected for presenting unbalances of different chromosome regions to allow verification of our CGH array protocols. The karyotype of the cell strains as originally defined with G-banding, and following verification with array CGH data are presented in Table I.
As reference for our array hybridizations, we used commercially available male or female DNAs (Promega, Leiden, the Netherlands) that represent DNA pools derived from at least seven same-gender individuals.

Table I. Summary of the Patients Investigated, and Their Cytogenetic Findings Before and After Molecular Cytogenetic Studies

Patient	*Phenotype*	*G-banding karyotype*	*Karyotype after array CGH and FISH*
Patient A	Mental retardation and dysmorphisms	46,XX,del(11)(q14q21)	46,XX,del(11)(q14.3q22.3)
Patient B	Multiple congential abnormalities (newborn)	46,XY,i(8)(q10)	46,XY,der(8)(qter→q21.3::p23.2→q ter)[a]
Patient C	Rett syndrome	46,X,r(?)	46,X,r(X)(p10q21.1)
Patient D	Fetus (5th month pregnancy) presenting at US oligohydramnion, nuchal belb, and no detectable kidneys	46,XX,r(13)	46,XX,r(13)(::p11→ q12.3~q14.13::q22.2~q32.2→31.1::)[b]

[a] Later on, the mother was found to carry an inversion and, therefore, the rearranged chromosome was described as rec(8)dup(8q)inv(8)(p23.1q21.2)mat.
[b] This patient presents ring instability, as evidenced by the CGH array and FISH results. Therefore, the patient is a mosaic in which the r(13) presents interstitial deletions of variable size.

Patient A

This patient was born in 1974, and was referred for cytogenetic evaluation at the age of 8 years, because of mental retardation and dysmorphisms. Clinical examination at this age revealed a height at 10th centile and an occipito-frontal circumference (OFC) at the 50th centile. She had an apparent hypertelorism, upturned nose, ptosis, protruding ears, uvula bifida, a cleft of the soft palate, lumbal hyperlordosis, pedes plano valgi, bilateral sandal gaps, and borderline

mental retardation. Also at this time, the following limited information about the patient clinical history was obtained. At birth, her weight was reported to be 2,000-2,500 g, and she was hospitalized after few days because of feeding problems. Her medical history revealed continuous feeding problems, consultation of an opthalmologist because of ptosis, and hearing problems, probably related to middle ear infections for which ear tubes were inserted. The mother of the proband had a history of drug-addiction and prostitution.
Chromosome analysis in lymphocytes and fibroblasts of the patient revealed a 46,XX,del(11)(q14-21) karyotype (Fig. 1).

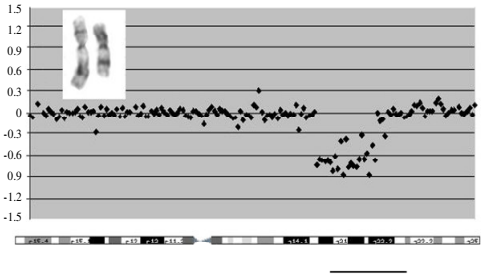

Figure 1. Deletion of chromosome 11 on patient A: The karyotype of the patient after G-banding analyses had been described as 46,XX,del(11)(q14-21), and an image of the del (11) (right) and its normal homolog (left) is shown on the insert. The array CGH profile shows the log2 ratios of the clones (test/reference DNAs) plotted according to their positions on chromosome 11 (from pter to qter). These results allowed to redefine the breakpoints on del(11) as q14.3-q22.3.

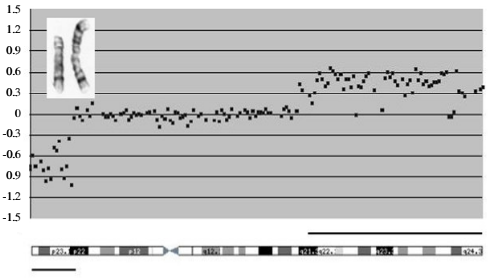

Figure 2. Analyses of the rearranged chromosome 8 from patient B. The insert shows the G-banding image of the rearranged chromosome 8 (right), originally interpreted as an isochromosome, and its normal homolog (left). The array profile displays the log2 ratios of the clones plotted according to their position on chromosome 8 (from pter to qter), and shows an 8p terminal deletion (p23.2-pter) and 8q terminal duplication (q21.3-qter).

Patient B

The proband was the 4th child of healthy parents. He was born after an uneventful pregnancy of 37 weeks with a birth weight of 2,210 g and an OFC of 31 cm. He had a broad nasal bridge, periorbital fullness, retrognathia, webbing of the neck, hypospadias, prominent heels, overriding 2nd toes, and mild syndactyly of 2nd and 3rd toes of both feet. On further investigations, he appeared to have a tetralogy of Fallot, cysts in the left kidney, a somewhat small cerebellum, and a small corpus callosum. The G-banding karyotype of the lymphocytes at that time was interpreted as 46,XY,i(8)(q10) (Fig. 2) and the karyotypes of the parents were regarded as being normal. Due to the bad prognosis, treatment was withdrawn and the patient died at 11 days of age. Cultured fibroblasts from a post mortem skin biopsy confirmed the karyotype as observed in lymphocytes.

Patient C

The propositus is the 2nd child of healthy non-consanguineous parents. Her sister and half-brother are normal. The patient was born after an uneventful pregnancy, with a weight of 3,500 g and length of 50 cm. At the age of 3 months, convulsions occurred, and were found to be associated with periods of hypoglycemia without hyperinsulinemia. Extensive screening for inborn errors of metabolism and endocrine disturbances did not reveal any consistent abnormality. The subsequent course was characterized by severe motor and mental retardation, the development of epilepsy, and failure to thrive. Her height from 2 years onwards was at or below the 5th centile. At the age of 5 years the patient was referred for syndrome evaluation and genetic counseling.

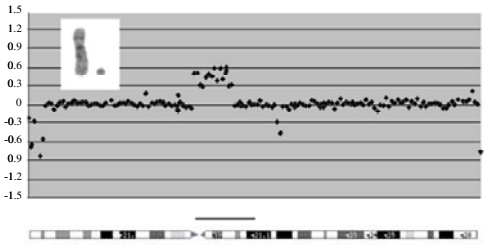

Figure 3. Ring(X) in a Rett syndrome patient. The insert shows a G-band image of the r(X) (right) and its normal homolog (left) in a patient with Rett syndrome. The array CGH profile shows the mapping of the ring chromosome. No Xp sequence was detected on the ring, indicating that the breakpoint was very near or at the centromere. The DNA of the patient was hybridized against a normal 46,XY DNA, and the under-represented sequences are regions of X/Y homology.

On the basis of psychomotor regression after a period of normal development, severe mental retardation, growth deceleration, loss of purposeful hand skills with appearance of stereotypical hand movements, epilepsy, and microcephaly, the diagnosis of Rett syndrome was made. G-banding karyotype showed a 46, X,r(?) karyotype (Fig. 3). The ring was later shown by molecular cytogenetic methods to be X-derived, and being always inactive [Rosenberg et al., [2001]].

Patient D

This case was a fetus of a mother in her 5th pregnancy. Her first pregnancy resulted in the birth of a son with a single umbilical artery and a small kidney that in other respects appeared to be healthy. The subsequent three pregnancies ended in a spontaneous early abortion.
The present pregnancy was uneventful until 14 weeks of pregnancy. At that time, the fetus appeared to be normal with a normal amount of amniotic fluid. At 18 weeks of pregnancy ultrasound revealed severe oligohydramnion, nuchal bleb, and no detectable kidneys. For further diagnostic evaluation amniocentesis was performed. Cytogenetic analysis of cultured amniocytes revealed a 46,XX, r(13) karyotype. The breakpoints of the ring 13 were not determined. The pregnancy was terminated at 22 weeks. Weight of the fetus was 250 g, length 25.5 cm, and placental weight 90 g. She had a nuchal bleb and low set ears. Post mortem examination was mentioned, but results were not available in medical records.

CGH Arrays

Slides containing 3,500 BACs were produced in the Leiden University Medical Center. The particular BAC set used to produce these arrays is distributed to academic institutions by the Welcome Trust Sanger Institute (UK) at no cost, and contains targets spaced at 1 Mb density over the full genome, a set of subtelomeric sequences for each chromosome arm, and a few hundred probes selected for their involvement in oncogenesis. Information regarding the full set is available in the Cytoview window of the Sanger Center mapping database site, Ensembl (http://www.ensembl.org/). BAC DNAs were isolated from the clones, using the Wizard SV 96 Plasmid DNA Purification System (Promega, Leiden, the Netherlands) in combination with the Biomek 2000 Laboratory Automation Workstation (Leiden Genomic Technology Center facilities, LGTC, the Netherlands). This DNA purification kit is designed to isolate DNA from plasmids and results in small amounts of DNA (100 ng DNA from 1ml culture) when used for BAC isolation. However, in our experience, this system was easier to implement using robotics than usual protocols for BAC DNA isolation. The resulting DNA had low levels of contamination from the host E. coli, and was suitable for DNA amplification and subsequent array production. Amplification of the DNA, spotting on the slides and hybridization procedures were based on protocols optimized by the group of Dr. N. Carter (Sanger Institute, UK), and presented in a workshop supported by the Welcome Trust [Carter et al., [2002]]. This set of BACs and protocols are described in detail [Fiegler et al., [2003]]. In parallel to the production of amplified DNAs for spotting on the arrays, we also produced DNA aliquots of every BAC for FISH. The FISH probes were produced to confirm rearrangements detected by the micro-array analysis, to determine the structural organization of the rearrangements, and visualize rearrangements in their balanced form (in normal carriers).
Slides hybridized with Cy3- and Cy5-dCTPs (Amersham Bioscience, Roosendaal, the Netherlands) labeled DNAs were scanned either with an Agilent DNA microarray scanner (Agilent Technologies, Amstelveen, the Netherlands) or a GenePix Personal 4100A scanner (Axon Instruments, Westburg BV, Leusden, the Netherlands). The spot intensities were measured by GenePix Pro 4.1 software. Within this software, spots in which the reference DNA intensity was either below five times the average of the background or presented more than 3% saturated pixels were excluded from further analyses. The test/reference ratios were normalized for the median of the ratios of all features. The triplicates of the features were averaged in a homemade routine developed in Microsoft Excel 2000, and spots outside the 20% confidence interval of the average of the replicate were excluded. Only those targets presenting at least two spots within 20% confidence interval of their average were used. Unbalances of the targets were determined based on log2 ratios of the average of their replicates, and we considered sequences as amplified or deleted when outside the ±0.3 range.

Fluorescence In Situ Hybridization (FISH)

Based on array results, BACs representing chromosome regions with different copy numbers in the same chromosome were selected to confirm by FISH, the array findings. BAC DNAs were directly labeled with FITC-, Cy3-, or Cy5-conjugated dUTPs by nick translation, and hybridized according to standard protocols.

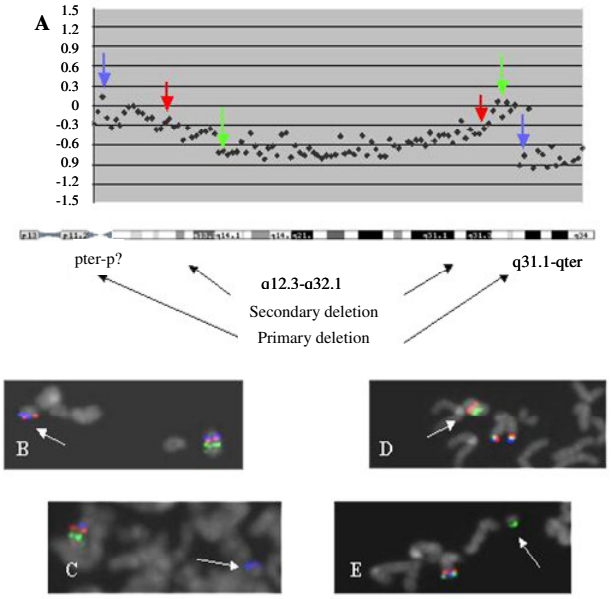

Figure 4. Ring chromosome on patient D. A: The array profile of chromosome 13 shows a primary deletion on the long arm (q31.1-qter). The presumptive deletion on 13p cannot be detected on arrays because of the repetitive nature of its sequences. The interstitial region presents a deletion of variable size and copy number (secondary deletion), and the arrows indicate the probes that were used for FISH to investigate the proximal and distal breakpoints. The FISH results for the green, red, and blue probe combinations on the proximal breakpoint are shown in (B-C), while the results for the distal probe set are displayed on (D-E). In each image, the normal chromosome 13 and the ring chromosome (white arrow) are shown. Note the different configurations of the ring 13 in each of the investigated breakpoints.

RESULTS

Table I and Figures 1-4 summarize the karyotype of the cell strains as originally defined with G-banding, and following verification with array CGH and FISH data. Patient A had been diagnosed as carrying a deletion of chromosome 11, which comprised mostly band q14. The results of the genomic array showed that the size of the interstitial deletion was 15 MB (between clones RP11-268B20 and RP11-569A20), and revealed that the breakpoints map more distal than originally estimated by G-banding (q14.3-22.3). Figure 1 shows a G-banding image of the rearranged chromosome 11 and its normal homolog, and the corresponding array CGH profile of chromosome 11.

Based on G-banding analyses, the karyotype from patient B carried a rearranged chromosome 8, which was interpreted as an isochromosome 8. CGH-array hybridization, however, has shown that chromosome 8 presented balanced, deleted, and amplified segments, which was incompatible with the diagnosis of an isochromosome. FISH results using probes located within regions of distinct copy numbers on chromosome 8 (data not shown) confirmed the array findings, and also revealed the structure of the der(8). Based on these results, the karyotype of the patient was described as 46,XY,der(8)(qter?q21.3::p23.2?qter. Figure 2 shows the array CGH profile of chromosome 8 in this patient, and the insert shows a G-banded image of the der(8) and its normal homolog (right and left, respectively). The families of patients carrying isochromosomes have no increased risk of recurrence, but the rearrangement presented by this patient might have originated from a pericentric inversion in one of the parents. The parents had been previously analyzed and no abnormality were detected. However, after we re-initiated this study, the karyotype of the mother was re-examined, and she was found to carry an inv(8)(p23.1;q21.2), which is morphologically similar to a normal chromosome 8.

Patient C has been previously reported by us to carry a r(X) [Rosenberg et al., [2001]]. Our array CGH results show that the r(X) is formed by a continuous segment of chromosome, with no suggestion of ring mosaicism or instability. However, every target from Xp represented on our array was found to be absent on the ring, indicating that the breakpoint was at or very near to (<600 kb) the centromere. A G-banded image of the r(X) and its homolog, and the correspondent array CGH profile are shown in Figure 3.

Patient D was reported to carry a r(13), with unknown breakpoints. The array CGH profile from chromosome 13 on patient D evidenced a terminal deletion on 13q. Because of its repetitive nature, no probes were available for the short arm of chromosome 13, but it is reasonable to assume that terminal deletions on both arms followed by fusion of the chromosome ends, were the primary events that originated the ring structure. The areas immediately adjacent to the breakpoints showed normal copy number as expected, but the ring presented, in addition, an interstitial deletion, which is here designated as secondary deletion because it did not originate the ring structure (Fig. 4A). The interstitial deletion showed a gradient in copy number varying from deleted to balanced, suggesting that the patient presents a mosaicism for the r(13), with variable sizes of the secondary deletion.

We selected three probes representing different copy numbers for each of the breakpoint regions of the interstitial deletion, and these two probe sets were separately hybridized by FISH to metaphase spreads from patient D. The arrows on Figure 4A represent the position and labeling colors of the six selected probes, and FISH images from the proximal and distal breakpoint regions are presented in Figure 4B-C and 4D-E, respectively.

DISCUSSION

We used genomic arrays combined with FISH analyses for re-evaluating the chromosome rearrangements present in four human cell strains derived from karyotypically abnormal individuals. In each of these cases, the results of the new investigation corrected or complemented the original karyotype description.
The array CGH results from patient A confirmed the presence of the interstitial deletion, but demonstrated that the breakpoints mapped distal to the locations estimated by G-banding. It is not surprising that the determination of the breakpoints by G-banding visual assessment carries a degree of imprecision, and for most families of patients with cytogenetically detectable abnormalities, the precise mapping of the structurally rearranged chromosomes has little impact. For diagnosis of the patient, determination of the carrier status of relatives, and eventual pre-natal diagnosis, it is mostly the presence or absence of the rearrangement in relatives and fetus that determines the recurrence risk, rather than its precise structure. Patient B, however, is an exception: the karyotype of the patient as originally defined by G-banding reported the presence of an i(8), which does not suggest increased risk of chromosomal abnormalities among relatives. Array CGH results revealed that the rearranged chromosome 8 was not an isochromosome but instead, presented terminal deletion and duplication of the short and long arms, respectively. Rearrangements presenting both terminal deletion and duplication, such as the one present in our patient, can be originated from crossing-overs within pericentric inversion regions. If such inversion is present in one of the parents, an increased recurrence risk for other relatives exists. In fact, after we re-initiated this study, the karyotype of the mother and her brother were re-examined, and both of them were found to carry an inv(8)(p23.1q21.2).
Patient C was diagnosed with Rett syndrome, and has been earlier reported by us to carry an r(X) [Rosenberg et al., [2001]]. The CGH array data showed that every sequence from Xp represented in our array was deleted on the r(X), indicating that the breakpoint was at, or very close (<600 kb) to the centromere. Most rings are thought to originate from terminal deletions and subsequent fusion of both arms of a chromosome. However, Callen et al. [Callen et al., [1991a]] reported that some rings lack specific satellite DNA sequences from one side of the centromere, and proposed that these rings originated from a transverse mis-division of the centromere combined with a U-type exchange of one of the chromosome arms. It is possible that some centromere mis-division caused the (peri) centromeric break in our ring. However, the transverse mis-division of the centromere proposed by the authors should first originate a chromosome in which every sequence will either be deleted or duplicated, such as in an isochromosome, and will then be further deleted by the U-type exchange. The array results from this patient do not suggest that any sequence on the ring is

present in more than one copy and, therefore, is unlikely to have been formed by the proposed mechanism.

The ring from chromosome 13 on patient D was found by array CGH to present an interstitial (secondary) deletion, in addition to the terminal deletions that originated the ring structure. This interstitial deletion shows a gradient in copy number (Fig. 4a), which we demonstrated by FISH to reflect a mosaicism of different configurations of the ring (Figs. 4B-E). This pattern suggests that the internal deletion became gradually larger, probably associated to instability of the ring. Ring instability is thought to result from sister chromatid exchanges that, because of the ring structure, may result in interlocking and dicentric rings, which break and rearrange during segregation [review in Kaiser-Rogers and Rao, [1999]].

We illustrate here that the complementation of the G-banding karyotype with array data can provide insights on the structure of rearranged chromosomes, and may sometimes impact genetic counseling. Array CGH provides a new base to understand and visualize the mechanisms of chromosome rearrangements.

Acknowledgements

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