

# **Advanced genome-wide screening in human genomic disorders**

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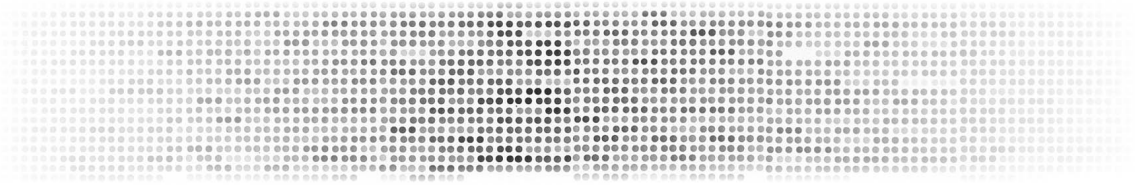
## Contents

Chapter 1:	Introduction	7
Chapter 2:	Insights from genomic microarrays into structural chromosome rearrangements <i>Am J Med Genet A (2005);132(1):36-40</i>	33
Chapter 3:	Array-CGH detection of micro rearrangements in mentally retarded individuals: clinical significance of imbalances present both in affected children and normal parents <i>J Med Genet (2006);43(2):180-6</i>	47
Chapter 4:	Diagnosis of genetic abnormalities in developmentally delayed patients: a new strategy combining MLPA and array-CGH <i>Am J Med Genet A (2007);143(6):610-4</i>	61
Chapter 5:	Array-CGH detection of a cryptic deletion in a complex chromosome rearrangement <i>Hum Genet (2005);116(5):390-4</i>	71
Chapter 6:	Ring chromosome formation as a novel escape mechanism in patients with inverted duplication and terminal deletion <i>Eur J Hum Genet (2007);15(5):548-55</i>	81
Chapter 7:	Molecular and clinical characterization of <i>de novo</i> and familial cases with microduplication 3q29: guidelines for copy number variation case reporting <i>Cytogenet Genome Res (2008);123 (DOI:10.1159/000184693)</i>	93
Chapter 8:	A homozygous deletion of a normal variation locus causes syndromic hearing loss in a patient from non-consanguineous parents <i>submitted</i>	117
Chapter 9:	Discussion	129
	Summary	137
	Samenvatting	139
	Curriculum Vitae	141
	List of publications	143



# Chapter 1

## Introduction





## **1.     *Genetics***

Genetics, the science of genes, heredity and variation of organisms, is one of the pillars of modern biology. Pioneering experiments of inheritance with pea plants were performed by Gregor Mendel between 1856 and 1863. He discovered that certain traits, like the color of seeds and the shape of pods, could be inherited in a dominant heterozygous and recessive fashion. Initially, Mendel's work received little attention; only after the rediscovery of the Mendelian inheritance, by Hugo de Vries and Carl Correns in 1900, his laws of inheritance became a basis for the understanding of genetics. Subsequently, the central role of DNA in inheritance was established in 1944, when DNA was shown to be the biochemical material responsible for transfer of genetic information in organisms.

## **2.     *Cytogenetics***

### **2.1    Chromosomes**

Karyotyping of human cells was developed in the 1950's, stimulated by the publication of Tjio and Levan in 1956 [1], who showed convincingly that human cells contain 46 chromosomes. The first constitutional genetic abnormality was found by Lejeune in 1959 [2]. He saw that the nuclei of patients with Down syndrome contained an extra small chromosome, which he linked to this syndrome. From that moment onwards, cytogenetic testing was boosted and became an important screening tool in clinical genetics. However, with no banding techniques yet available and no consensus on nomenclature, it was a difficult task. Based on morphological data, in the 1960's researchers could only classify the chromosomes into seven groups. It became clear that in congenital and prenatal disorders as well as in malignancies numerical and structural chromosome alterations are often present. Next to numerical chromosome alterations, called aneusomy, unbalanced translocations were detected causing an abnormal phenotype [3] and also deletions of chromosomes were found in patients with constitutional abnormalities [4,5]. This led to the conclusion that numerical chromosomal alterations and structural aberrations could be responsible for clinical phenotypes and encouraged the hunt for other alterations, a process that is still ongoing.

## 2.2 Chromosome banding

In 1968 Caspersson et al. [6] described banding of plant chromosomes with quinacrine dihydrochloride, quickly followed by the first banded human karyotype by the same group in 1970 [7]. After the development of this so-called fluorescent Q banding, several other banding techniques were developed independently of each other, including C-banding and G-banding [8]. They are based on staining with Giemsa dye and have the advantage of giving a non-fluorescent permanent staining of the chromosomes. G-banding, especially trypsin mediated G-banding, is nowadays one of the most widely used techniques for karyotyping. The technique does not require a fluorescence microscope and slides can be stored after staining for reevaluation.

Chromosome banding facilitated the recognition of individual chromosomes, as they could be identified more simply and more precisely. This led to the conclusion that the classification of chromosomes would have to change completely from the seven chromosome groups into a much more detailed system of naming chromosomes and their individual bands.

Leading researchers in human cytogenetics agreed upon a system of nomenclature, which was documented in the report of the Paris Conference (1971) [9]. This provided a basis for the description of structural and numerical rearrangements and variants. In time, a growing number of researchers and cytogeneticists joined the field of human genetics and the increasing amount of data generated resulted in updates of the nomenclature report, correcting errors and solving problems raised by increase of resolution using prometaphase chromosomes. Eventually this resulted in the publication of “An International System for Human Cytogenetic Nomenclature” (ISCN) in 1985 [10]. Continuous refining work, new findings and insights into for instance tumor cytogenetics were followed up with further updates in 1995 and 2005. The current version of the book also gives guidelines in nomenclature dealing with molecular cytogenetic techniques.

Discovery of chromosome banding has been the most important tool in cytogenetics for decades and allowed a serious leap forward in the diagnosis of genetic alterations. It is being used in the field of constitutional genetics in patients with mental retardation or developmental delay, growth retardation, congenital abnormalities and dysmorphisms, infertility and recurrent miscarriages [11]. The recognition of distinct chromosomal bands allowed scientists to discriminate between chromosomes and to make firm conclusions about trisomies, monosomies, deletions, duplications, inversions and translocations. The cause of syndromes could be pinpointed or better defined [12] and inherited traits resulting from balanced carriers were found.

To facilitate karyotyping, automated systems have been developed [13]. Complete systems combine automated metaphase finding and ranking of recorded images with segmentation and karyotyping of the chromosomes, after

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which the user can check and, if needed, correct the resulting karyogram. Simpler systems lack the possibility of metaphase finding. In routine diagnostic laboratories where substantial numbers of cases are screened, these systems have become standard equipment during the last two decades.

### 2.3 Limitations of chromosome banding

Banding technologies also have their downsides. They heavily depend on the accumulated experience and the subjective interpretation of the cytogeneticist. Moreover, it is a time consuming technique and success relies heavily on the quality of the cytogenetic preparations, mainly concerning the compactness and spreading of the metaphase chromosomes, as this influences the resolution. In general the resolution limit is estimated to be around 5 to 10 megabases (Mb). Another important issue is the fact that there is a limit in resolving complex karyotypes, leaving cryptic alterations undetected and markers unidentified in constitutional cytogenetics. To overcome a part of these limitations, novel genetic screening approaches have been developed.

## 3. *Molecular Cytogenetics*

### 3.1 Fluorescence *in situ* Hybridization

In the late 1960s methods for specific detection of RNA-DNA [14] and DNA-DNA hybrids [15] became available. These *in situ* hybridization techniques were at first performed using radioactive labeled probes. A series of technical improvements in labeling chemistry, microscopy and imaging lead in the 1980s to the development of fluorescence *in situ* hybridization (FISH) using fluorescently labeled probes [16,17]. FISH is a tool that enables the microscopic detection of specific genetic regions within a morphologically intact cell. The technique requires labeling of a DNA molecule with a fluorochrome or a hapten to which fluorescently labeled antibodies are developed. For this purpose several labeling techniques are available nowadays. In general they can be divided into enzymatic or chemical labeling strategies. Initially, enzymatic incorporation of biotinylated [18] or digoxigenin-bound [19] nucleotides was the favoured method. These haptens are subsequently visualized with fluorescently labeled avidin or antibodies. Nowadays, fluorescent nucleotides are used directly in the enzymatic labeling procedure, eliminating the need for secondary detection reagents [20]. Both interphase nuclei and metaphase spreads can serve as targets for a variety of fluorescently labeled probes. With the development of this technique, together with the diversity of specific DNA probes, FISH became an important additional tool for the cytogenetic diagnostic field. Structural alterations could be better mapped and sub-microscopic resolution alterations could be detected [21].

### 3.2 Labeling strategies

Early chemical labeling approaches used DNA binding molecules such as acetylaminofluorene and mercury. Mercurated nucleic acids were subsequently detected with sulphhydryl containing ligand carrying a hapten for indirect detection or a fluorochrome for direct detection [17]. Acetylaminofluorene binds to DNA covalently and acts directly as a hapten for immunocytochemical detection [22]. These techniques are not used anymore. Nowadays, chemical labeling is achieved using *cis*-platin labeling, also known as Universal Linkage System (ULS). In an aqueous solution at high temperatures the *cis*-platin molecule, to which a fluorochrome or hapten is coupled, predominantly binds coordinatively to the guanine-bases of DNA molecules [23]. A very diverse range of molecules can be coupled to these *cis*-platin molecules, for instance haptens, like biotin, dinitrophenol or digoxigenin, enzymes such as horseradish peroxidase as well as fluorescent dyes covering the spectrum of light.

Enzymatic labeling of DNA fragments has become the method of choice in most labs, for instance nick translation. Here the probe DNA is nicked with DNase I, and the resulting gaps are repaired using DNA polymerase I in the presence of a mix of labeled and unlabeled nucleotides [24]. Another enzymatic labeling reaction is random primed labeling, in which the probe DNA is denatured and random hexamers or octamers are allowed to anneal to the DNA. Using the Klenow fragment, a part of the DNA polymerase I enzyme, the random primers are extended in an isothermal reaction with a mix of labeled and unlabeled nucleotides [25]. Another enzymatic labeling strategy is to perform locus-specific polymerase chain reaction (PCR) or degenerate oligonucleotide primed (DOP)-PCR in the presence of a hapten or fluorochrome labeled nucleotide mix.

### 3.3 Types of probes

Broadly speaking, a chromosome consists of a centromere, telomeres and other repeat regions, with in between specific sequence. The total chromosomal DNA or parts of it can be used as a probe for FISH. A whole chromosome paint (WCP) or a partial chromosome paint (PCP) can for instance confirm a translocation found by G-banding. These probes are usually generated by flow sorting or microdissection of chromosomes that are subsequently randomly amplified using a polymerase chain reaction with random oligonucleotides (DOP-PCR) [26].

Usually, for chromosome ploidy determination alpha-satellite DNA specific for the centromeric region of chromosomes is used as a probe. These regions contain repetitive DNA which in most cases is unique for the chromosome to be investigated. Only the chromosomes 1/5/19, 13/21 and 14/22 cannot be distinguished using these probes, since these chromosome groups have highly homologous centromeric repeats. Instead, chromosome-specific sequences close to the centromere are selected, to minimize the chance of not detecting small

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marker chromosomes. For the hybridization of these euchromatic probes, unlike for centromeric probes, cohybridization of unlabeled C<sub>0</sub>t-1 is generally needed to suppress hybridization of repetitive sequences that are randomly present in all euchromatic DNA.

For diagnostic purposes an increasing number of commercially available, smaller sized, targeted probes have become available. Usually they consist of bacterial artificial chromosome (BAC) or cosmid vectors, containing a large insert (35-50 kb for cosmids and 100-200 kb for BACs) of specific human genomic DNA. These types of clones are used for locus-specific FISH and for detection and characterization of different chromosomal aberrations such as microdeletions, duplications and inter and intrachromosomal rearrangements such as inversions, insertions and translocations.

### 3.4 Types of FISH

Interphase FISH is a good screening tool for prenatal testing for the most common aneusomies like trisomy 13, 18 or 21 and aneusomies of the sex chromosomes, such as these aneusomies together form the vast majority of cases in clinical cytogenetics [27]. These whole chromosome imbalances are in general a result of nondisjunction in meiosis in one of the parents and can result in moderate to severe congenital abnormalities and delayed mental development. The development of the interphase FISH technique was a major improvement in prenatal screening of aneusomies, since it reduces the time from a laborious 7 to 14 days test with cell culture and G-banding to a rapid 24 hour test [28].

Interphase FISH can also be used for pre-implantation genetic diagnosis approaches prior to *in vitro* fertilization, both for aneusomy screening and for inheritance screening of the unbalanced results of balanced translocation carriers [29,30]. But because of the technical challenge of handling one or two cells, ethical issues and ongoing discussion with respect to governmental regulations, this procedure is not common practice in cytogenetic laboratories.

FISH on metaphase spreads is a powerful addition to conventional karyotyping. It can provide locus-specific information about alterations such as translocations, inversions, deletions and duplications. It is important to note that prior information is needed about the involved chromosomes to be able to perform these detections, except for multicolor FISH approaches that are discussed below. Metaphase FISH using whole chromosome paints can confirm translocations found with G-banding.

Locus-specific large insert clones can be used as a probe in metaphase and sometimes interphase FISH to genetically confirm suspected diagnoses in constitutional genetics. An example of this is the detection of a microdeletion syndrome like the Williams-Beuren syndrome [31], in which a part of 7q11.23 is deleted. Another application is the detection of cryptic imbalances of chromosome ends using probes specific for the gene-rich subtelomeric regions.

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These chromosome imbalances are estimated to account for ~5-10% of all unexplained mental retardation [32].

Metaphase chromosomes can also be used for reverse chromosome painting. With this technique, an unidentified marker chromosome is isolated using either flow sorting [33] or microdissection [34]. The isolated DNA material is amplified, for instance by DOP-PCR, after which the amplified material is fluorescently labeled and hybridized to normal metaphase chromosomes. Marker chromosomes of unknown origin can be typed quickly this way, but there are some disadvantages using this approach. Both isolation techniques are highly specialized, and need specific knowledge and specialized equipment. When reverse painting on metaphase spreads is used to map breakpoints, the resolution is determined by the compactness of the chromosomes as in conventional karyotyping. Also, if a marker chromosome consists of heterochromatic DNA sequence or of the satellite DNA of acrocentric chromosomes, this technique will not give a conclusive answer about the origin of the marker chromosome [35].

For certain complex rearrangements, in which G-banding analysis is insufficient to determine the karyotypes, multicolor FISH approaches have been developed. Combinations of probes and fluorochromes were used to allow more detection possibilities with the available fluorescent dyes and filters to separate the fluorescence signal. This so-called combinatorial labeling allows the distinction of  $2^n - 1$  targets with  $n$  fluorescent dyes [36].

Whole genome screening techniques require the differential labeling of all human chromosome paints with combinations of different fluorochromes. The main approaches are multicolor FISH, spectral karyotyping (SKY), and “combinatorial binary ratio labeling” (COBRA), resulting in 24 color FISH [37-39]. Subsequently the multiplicity of FISH was even further increased by introducing an extra fluorochrome to distinguish between the p and q arms of an individual chromosome [40]. In pre- and postnatal screening these multicolor approaches can facilitate the characterization of for instance unidentified or complex marker chromosomes [41,42].

A disadvantage of genome wide multicolor FISH and FISH of WCPs is that these techniques are incapable of detecting small intrachromosomal deletions, duplications, and paracentric inversions [43]. A second disadvantage is that, similar to G-banding, a limited spatial resolution of maximally about 3-5 Mb is achieved. While multicolor FISH, multicolor telomeric FISH [44] and multicolor centromeric FISH [45] can be used as whole genome scanning tools, implementation is difficult and not wide-spread because specialized equipment and analysis skills are needed and the throughput is low.

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### 3.5 Comparative Genomic Hybridization (CGH)

In the early 1990s a genome wide analysis method was developed based on the quantitative *in situ* comparison of the genome content of a test sample to a reference sample [46]. Using this technique, whole genomic DNA of a test sample and a normal control sample is differentially labeled with haptens or with fluorochrome labeled nucleotides. The two labeled samples are cohybridized to metaphase chromosomes of a normal human control, in the presence of C<sub>0</sub>t-1 DNA. The amounts of hybridized products relate linearly to the concentrations originally present in the samples, when hybridization equilibrium conditions are reached. Images of the metaphases are then analyzed and karyotyped using 4',6-diamidino-2-phenylindole (DAPI) counterstaining, followed by the computational measurement of the ratio of the fluorescence intensities along the whole chromosomes. A fluorescence ratio of 2:2 (=1) between the test sample and the reference sample reflects a normal copy number and is seen as a mixed color. The amount of 2 in the ratio calculation represents the copy number of a given locus or chromosome in a somatic cell. A gain or loss of a particular (part of a) chromosome will result in a 3:2 or a 1:2 readily detectable color ratio, compared to regions with equal copy number [47].

A main advantage of this genome wide screening technique is that there is no requirement for mitotic cells, which makes the technique very valuable for obtaining genotype information of certain tumors that are too complex to karyotype or too difficult to grow *in vitro* [48,49], and from paraffin-embedded tissue sections [50,51]. Furthermore a priori genetic information is not needed. The resolution of this technique depends mainly on the length of the normal metaphase chromosomes used to hybridize the samples to, and consequently resembles the resolution of G-banding studies at a limit of around 5 to 10 Mb for low copy gains or losses.

In constitutional cytogenetics, the value of this technique was appreciated as an addition to conventional karyotyping. Prenatal and postnatal cases with unsolved chromosomal aberrations as unbalanced translocations, insertions or markers of unknown origin could be correctly identified using a single CGH experiment [52,53]. It is noteworthy, however, that balanced rearrangements can not be detected using CGH.

### 3.6 Other molecular tools

In the field of clinical genetics obviously more molecular tools than discussed so far have been used in the past and are still being used in part. Examples are PCR and sequencing for mutation analysis and Southern blotting for copy number analysis, for instance in the fragile X syndrome [54]. Since these tools are mostly applied in region specific analysis, they will not be discussed further here.

#### 4. Diagnosis of patients with mental retardation

On average, about 1-3% of the human population has a developmental delay or mental retardation, with or without dysmorphic features [55]. Mental retardation is a neurological disorder which is noted by for instance developmental delay of motor function and speech in infants, slow learning skills, decreased skills of interaction with other people and a low intellectual capability. The cause of mental retardation is highly variable, it can be congenital or acquired through malnutrition, poisoning, infectious disease or trauma. Congenital forms of mental retardation can have a genetic, metabolic or environmental cause and can present with or without congenital abnormalities or dysmorphic features.

A phenotypic investigation of patients with idiopathic congenital mental retardation by pediatricians and dysmorphologists is often the first step in the elucidation of the cause. Generally, in about 40-60% of the investigated patients an etiological diagnosis could be made [55], depending on study-inclusion selection criteria. The importance of detailed clinical examination is illustrated by Van Karnebeek et al. [56] and Shevell et al. [57], who state that in around 60% of cases physical examination, including dysmorphological and neurological examination, was essential for achieving the diagnosis of the patient.

The most common form of inherited mental retardation is called fragile X syndrome [58]. The syndrome has a broad spectrum of developmental delay including forms of autism and is reported with and without congenital abnormalities. Because of the high variance and relative high frequency, patients with an idiopathic mental retardation are usually screened for fragile X syndrome. Prior to the finding of the molecular cause of the fragile X syndrome phenotype it was noted that most patients showed a fragile site on chromosome Xq27, when cells were cultured under folic acid stress conditions [59]. This type of culture was used as a diagnostic tool but proved to be unreliable [59]. In 1991 genetic instability of a trinucleotide repeat was detected at the site of the fragile X breakpoint [60]. It was noted that the amount of repeats played a critical role in the expression of the downstream gene. The common number of this repeat is between 6 to 44 copies, but when the number of repeats exceeds 200 in an individual, the downstream *FMR1* is transcriptionally silenced causing fragile X syndrome. In general, patients with fragile X syndrome have inherited the expansion of repeats from the mother with an instable intermediate status of repeats called premutation. For unknown reasons, inheritance from males with a premutation generally results in offspring with lower copy number of this repeat, whereas inheritance from females often results in expansion of the trinucleotide repeat [61].

Recurrent reports of patients with phenotypes comparable to each other has led to the identification of several distinct syndromes such as Down syndrome [62], Cri-du-Chat syndrome [63], Edwards syndrome [5], Cornelia de Lange syndrome [64] and Sotos Syndrome [65]. Since the first identification of chromosomal

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involvement in Down syndrome 50 years ago [2], many different genetic alterations have become known to play a role in embryonic and mental development. This knowledge sheds light on the spectrum of mental retardation and one may now conclude that the underlying genetics of mental retardation is extremely heterogeneous. With the emerging cytogenetic studies of patients with mental retardation and congenital abnormalities, genotype/phenotype correlations could be made for many syndromes, thereby proving the genetic cause of the syndrome.

As an example, Cri-du-Chat syndrome was found to have a partial deletion of chromosome 5p [63] and the Edwards syndrome was caused by a trisomy of chromosome 18 [5]. The correlation in the Sotos syndrome was harder to detect. In 2002 a Japanese group isolated the causal gene from the breakpoint of patients with Sotos syndrome and chromosomal translocations [66]. In the Cornelia de Lange syndrome (CDLS) it took even longer to find the genetic cause. In several reports of cytogenetic investigations of CDLS, chromosomal translocations and imbalances were found [67,68]. But heterogeneity in the phenotype caused confusion whether the described alterations were the main cause for CDLS [69]. Based on balanced translocations in CDLS patients Tonkin et al. [70] found the involvement of a gene called NIPBL and demonstrated mutations in this gene in more than half of their patients. Involvements of other genes in the same protein complex are now recognized to cause an X-linked [71] and a milder form of the CDL syndrome [72].

## **5. *Development of new screening tools***

### **5.1 *Array Comparative Genomic Hybridization (Array-CGH)***

In the last decade of the previous century researchers realized that improvement of the resolution of CGH could be established by using thousands or millions of DNA fragments each specific to a unique location of the genome immobilized on a glass surface as a target, instead of metaphase chromosomes. This technique was initially called matrix comparative genomic hybridization [73], nowadays the name microarray based or array comparative genomic hybridization (array-CGH) is generally used [74].

The basis of this technique was partly the Human Genome Project, a collective effort to sequence the whole human genome. As a spin-off large BAC and PAC clone libraries became available that contained well characterized and mapped human genomic DNA fragments of about 100 to 150 kb, covering nearly the whole genome. Selected clones were used for spotting on microscope slides. The hybridization principle of these printed array slides was comparable to classical chromosome based CGH hybridizations (Figure 1).

In the first protocols, the target DNA to be printed was isolated from bacterial cultures and after cleanup under the right conditions directly spotted on the

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array slides [73,74]. To generate enough material to comfortably print these arrays, linker adapter PCR [75] or degenerate oligonucleotide primed PCR (DOP-PCR) protocols were used [26,76] and were optimized [77]. In the latter paper Fiegler et al. suggested an underestimation of the fluorescence ratio of single copy changes that was seen in previous array-CGH publications was not mainly due to the incomplete suppression of repeat sequences, but was for a large part caused by the contamination of bacterial genomic sequence in the spotted clones. To overcome this problem, they designed degenerate oligonucleotide primers different from the commonly used 6MW DOP primer [26], which the help of the knowledge of the sequence from the human genome project.

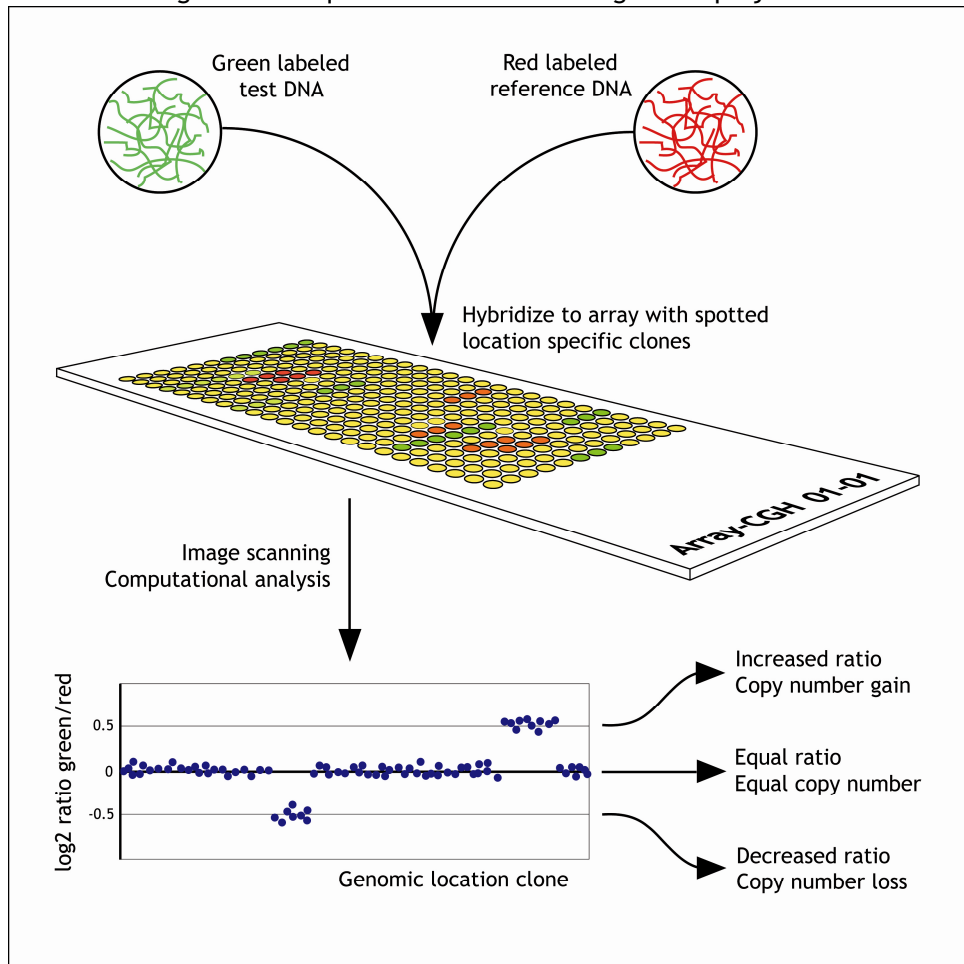


Figure 1. The principle of array-CGH

These DOP-PCR primers preferentially amplify human genomic DNA over *Escherichia coli* (*E. coli*) DNA because the primers have an averaged frequency of

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matches per kilobase of 0.58 based on the human genomic reference sequence, compared to an average of 0.03 in *E. coli* DNA. As a comparison, the original 6MW primer has an averaged frequency of matches per kilobase of 0.65 and 0.40 for human and *E. coli* DNA, respectively. Using this approach the DOP-PCR generates less background and produces a more evenly represented product.

The first reports on array-CGH described a targeted array as a proof of principle [73,74]. Later, whole genome arrays were developed that covered the genome with a resolution of about one clone per megabase of euchromatin DNA, resulting in about 2400-3500 different clones per array [75,77], often printed in duplicate or triplicate. This development led to an increase of resolution of about 10 times compared to the conventional CGH technique, entering sub-microscopic detection levels of copy number changes in a genome wide fashion.

Imaging and analysis of the arrays was at first done with a CCD camera and ratios were manually calculated and presented in spreadsheet programs. Nowadays, scanning and analysis of arrays is almost fully automated. Hybridized arrays are mostly scanned using a laser scanner and the resulting images are then processed using commercial software. These software packages perform background corrections, remove outlier spots, normalize the data, calculate ratio values and display the data in comprehensive graphs. In general the ratio data is displayed as a  $2\log$  value, to better distinguish deletions from normal values.

In theory the resolution of array-CGH is unlimited, depending on the spacing of the used clones and their size. When the array-CGH technique was proven to work satisfactorily, several institutes developed large clone insert arrays tiling the whole euchromatic part of the genome [78-80]. Due to the performance of needle or inkjet printing techniques available, all these clones could be printed on one glass slide, but not in duplicate or triplicate. Consequently a second hybridization experiment is often needed to corroborate these results [79,80].

Spotted or immobilized oligonucleotides provide some advantages for the detection of copy number changes. They avoid the need for bacterial cultures and isolation of DNA and/or PCR amplification of large insert clones. Large oligonucleotide libraries are spotted and hybridized in a way comparable to other types of array-CGH [81], yielding similar results.

Commercial platforms for copy number analysis are now available, using oligonucleotides, photo-lithographically synthesized on the chips [82,83]. One type is the SNP-based genotype array platform that can be used for allelotyping and copy number analysis in the same experiment. An advantage of the system is the ability to detect copy number neutral alterations as uniparental disomy or copy number neutral homozygosity. A disadvantage of this platform is the lack of an internal quality control as a cohybridized reference sample, because these genotyping arrays are single color experiments. Other oligonucleotide platforms are hybridized using a similar test/reference principle as CGH and array-CGH. Results produced with these platforms need somewhat more statistical analysis compared to data produced with large insert clone arrays, such as binning of

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data points and a moving average to reduce false positive calls. With ongoing technical improvement and advances in statistics, oligonucleotide platforms are increasingly robust.

Targeted arrays have been specially designed for specific regions in the genome. For instance, in constitutional genetics several institutes that investigate the role of the X chromosome in mental retardation developed targeted tiling path arrays for chromosome X [84-86]. This X-linked mental retardation is of interest to researchers, since there is a clear overrepresentation of males in mental retardation of 30 to 40% suggesting involvement of the X chromosome in many cases.

## **5.2 Paired-end mapping**

In 2005 a DNA sequencing method was introduced [87], which allowed large-scale whole genome analysis of genomic DNA samples to screen for copy number alterations. The method is called paired-end mapping, in which genomic DNA was fragmented into pieces of about 3 kb. From these fragments the two ends were sequenced and mapped back on the reference sequence [88]. If the distance between the matches on the reference sequence was within certain cutoff values, no alteration was detected. If the distance between the matches in the reference genome was bigger than the high cutoff value, the analyzed sample had a deletion between the sequenced ends. When the distance was smaller than the low cutoff value, an insertion was detected in the tested genome. This approach allows detection of inversions as well, if the two matches mapped in different relative orientations on the reference genome. This feature offers a big advantage over array based whole genome screening, since these platforms cannot detect inversions. In the report of Korbelt et al. the whole genome was covered 2.1 fold for one sample and 4.3 fold for a second sample, so on average every fragment was analyzed at least two times. Similar techniques are expected to play a role in whole genome analysis in the near future.

## **5.3 Target specific screening tools**

In order to confirm the alterations found using whole genome screening tools as described before, and to investigate targeted regions for suspected specific copy number alterations as microdeletions and microduplications, several techniques have been used and developed. Although FISH has been the method of choice in many cytogenetic labs, different molecular techniques have been developed and standardized providing some advantages over FISH. Mostly, these techniques are based on quantitative amplification of DNA. Quantitative real-time polymerase chain reaction (qPCR) is based on the amplification of genomic DNA sequences with fluorescently labeled primers in a quantitative manner and the amplification is monitored in real-time. The number of cycles to reach a certain fluorescence

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level in a test locus and a reference locus in one sample is used to estimate a relative copy number [89].

Two other quantitative techniques called multiplex amplifiable probe hybridization (MAPH) and multiplex ligation dependent probe amplification (MLPA) are based on quantitative amplification of DNA fragments matching to genomic DNA. These fragments contain sequences 5' and 3' unrelated to human DNA, which are recognized with a universal primer pair. For MAPH, the fragments to be amplified are cloned parts of exons, for instance. They are hybridized to genomic DNA of a test sample which is cross-linked to a membrane. After a stringent wash only the hybridized fragments are available for quantitative PCR with the universal primer pair [90-92]. In MLPA the fragments to be amplified are generated using two probes adjacently annealed onto genomic test DNA of interest, followed by ligation to each other. Multiple probes recognizing different regions can be combined in one ligation reaction and only the ligation products can be used for amplification with universal primers [93]. Quantitative readout of the multiple fragments both for MAPH and MLPA is generally done with fluorochromes bound to the universal primers and a capillary sequencer.

Compared to FISH these techniques have the advantage that they can be performed using multiple probes to screen for multiple regions per sample (up to 60 regions with dual color MLPA [93]) on multiple samples per experiment.

Recent developments include efforts to analyze parts of the genome with high-throughput resequencing [87,94]. These sequencing methods are based on random sequencing short stretches of a pool of fragments representing specific parts of the genome and aligning those sequences back to the reference sequence. Different groups have applied this next generation sequencing to enriched DNA targets. Specifically designed oligonucleotide microarrays have been used to capture parts of the genome, such as genes and locus specific regions [95,96]. The yielded DNA was used for massive parallel sequencing and it proved to be feasible for mutation screening. For copy number analysis based on quantitative analysis of sequence reads per region, which in theory is possible, the current capture methods are not uniform enough [97]. Nevertheless, this type of detailed region-specific copy number analysis may play a role in the future.

## **6. *Applications of Array-CGH in constitutional genetics***

The development of the genome wide array-CGH technique opened new possibilities for genetic screening in constitutional genetics. In individuals with idiopathic mental retardation with or without congenital abnormalities often the causal genetic diagnosis remained unclear if the karyotype revealed no alterations in the G-banding resolution. Several feasibility studies showed the additional value of array-CGH to conventional cytogenetics [79,98-103], through gain of resolution and robustness. In a research setting this technique has a

proven detection rate of genetic alterations of 10 to 15%, depending on the selection criteria. Alterations found are mainly interstitial deletions or duplications not detected by classical cytogenetic screening [104]. The deletions and duplications appeared to be scattered over the genome with very little overlap, proving the heterogeneous nature of genetic causes for mental retardation. However, despite this heterogeneity the genetic cause for CHARGE syndrome could be unveiled because of detailed investigation of recurrent genetic alteration data in combination with a precise phenotypic description. Two unrelated patients with a similar phenotype described as CHARGE syndrome were found to have an overlapping microdeletion on chromosome 8q12. In subsequent investigations of patients with the same syndrome but showing no microdeletion, mutations in a gene called *CHD7*, located in the same genetic region, were found in almost 60% of cases [105].

To investigate the involvement of regions flanked by large segmental duplications in recurrent genomic disorders, Sharp et al. [106] designed an array containing 2007 BAC clones concentrated around 130 identified sites which have a unique sequence flanked by segmental duplications. It was shown previously that these regions may be involved in recurrent rearrangements because of large homologies between the flanking segmental duplications, causing non-allelic homologous recombination [107]. They found 16 pathogenic rearrangements of regions flanked by segmental duplication when applying this array to investigate 290 patients with mental retardation, including 4 patients with a similar deletion on 17q21.31. A similar deletion was also found by three other groups [108-110]. This microdeletion of about 600 kb in size causes a comparable phenotype with a moderate developmental delay, severe hypotonia, amiable behavior and characteristic facial features with a long face, a bulbous nasal tip and a broad chin. The genomic region of 17q21.31 contains at the site of the microdeletion a common 900 kb inversion polymorphism which is present in about 20% of the European, Icelandic and Middle Eastern population [111]. A direct consequence of this inversion polymorphism is the appearance of a directly oriented segmental duplication, causing this lineage to be subject to non-allelic homologous recombination. In the non-inverted lineage these repeats are reverse oriented which prevents recombination. As a result of the relative high frequency of inversion polymorphism carriers, this microdeletion is among the most common causes of a microdeletion syndrome with an estimated prevalence of around 1 in 13,000 to 1 in 20,000 [109] and an estimated frequency of around 1% in patients with mental retardation [112]. Finding the reciprocal duplication of this region is a relatively logical consequence of the presumed non-allelic homologous recombination mechanism [113].

Clinical studies of large numbers of patients using arrays specifically designed for certain genomic regions have been performed as well [114,115]. These specifically designed arrays contain probes for known microdeletion and subtelomeric regions, aiming to detect deletions and duplications which are

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clinically relevant. In contrast, high resolution arrays covering the whole genome, as previously described, can also detect alterations of unknown clinical consequence. The concept of not including detection of regions of unknown clinical consequence gives a clearer diagnosis in testing of patients with idiopathic mental retardation and also in patients with an atypical appearance of a syndrome linked to a known genetic rearrangement. This approach is very useful to confirm suspected genetic alterations as well, but the obvious disadvantage is that unsuspected disease-causing genetic alterations remain undetected.

## **7. Genetic variation**

The assumption that the genome of two unrelated individuals is 99.9% identical still leaves room for millions of different base pairs. Genetic variation can either be the cause of a disease, a predisposition to a disease, a defense against a disease or a normal variation. It may comprise variation at the nucleotide level, variation of repeat rich elements interspersed throughout the genome, copy number variations (CNVs) of for example segmental duplications or structural variations. Whether a detected variation is normal or predisposing to disease can be difficult to assess, since predispositions might be multifactorial, or they might have a low penetrance or a combination of both. Some variations of the genome which are not disease-causing at all for the individual itself like a Robertsonian translocation or most reciprocal translocations, can not be seen as normal variation, since there is a high chance that these balanced structural aberrations can have serious consequences for the next generation. In contrast, for instance the balanced inversion at chromosome 17q21.31 described earlier, from which about 20% of the European population is a carrier, is considered a normal variant. Although it predisposes to microdeletion or microduplication in the 17q21.31 region [109,110], the risk for the recombination event is in the same range as in other microdeletion/microduplication syndromes and is thus negligible.

### **7.1 Heteromorphisms**

Already prior to chromosome banding, investigators recognized chromosome variations between individuals, called heteromorphisms. At the time of the first conference on standardization in human cytogenetics in 1960 [116] the considerable variation in the length of the Y chromosome was already noted. Morphological variations present near the centromere of several chromosomes as well as in the short arms of D- and G-group chromosomes were found later. Through large studies on newborns it was concluded that these variations probably were normal heteromorphisms [117]. The first heteromorphism that was linked to a specific trait was a change in condensation of the part of the chromosome directly below the centromere of chromosome 1. It was noted in

three different families that the Duffy blood group type segregated with the chromosome 1 with relatively uncoiled chromatids in the paracentric region, leading to the conclusion that the gene for the Duffy blood group was probably located on this chromosome in the vicinity of this paracentric region [118].

In general, these microscopically visible heteromorphisms usually involve the heterochromatic regions and satellites of chromosomes. Less frequently, euchromatic variants are also reported [119]. These variants may have hidden phenotypic consequences which are not always directly evident.

## **7.2 Single Nucleotide Polymorphisms**

At the beginning of this century, it was generally believed that the biggest part of genetic variation between two individuals was based on single nucleotide polymorphisms (SNPs) [120]. Only recently submicroscopic copy number variation was proven to be a major contributor to genetic variation. It was estimated that there are at least 10 million SNPs in the human population, an average of 1 SNP every 300 base pairs. In 2001 an international SNP map working group had mapped 1.42 million SNPs [121] and in 2005 approximately 1.3 million SNPs were truly genotyped [122] in the HapMap collection. This is a collection of genomic DNA samples from four different populations: 30 parent-offspring trios of the Yoruba population of Nigeria, 30 trios of European descent from Utah, 45 unrelated Han Chinese from Beijing, China, and 45 unrelated Japanese from Tokyo, Japan. The International HapMap study is an extension of the Human Genome Project. The latter project generated the human reference sequence, giving information for the non-variant part of the genome, while the International HapMap study aims for cataloguing the nucleotides that can vary between individuals. More recently the consortium released an updated version of the map in which 3.1 million SNPs were mapped [123].

## **7.3 Copy Number Variation (CNV)**

Initially the frequency of larger genomic variations between individuals was expected to be low and generally disease-causing. Using novel emerging array-CGH techniques and SNP array platforms, submicroscopic variation of genomic fragments was detected [88,106,124-132]. The detected amount of variation causing no obvious phenotype was much larger than expected [133]. It was found that a considerable part of copy number variation was associated with the 5% of the genome that is present in segmental duplications in the genome. Not only were these CNV regions proposed to be involved in disease causing rearrangements through non-allelic homologous recombination [134], they were also associated with large scale normal variation [124,125] since many of these copy number variable regions contain genes with a variable expression that contributes to normal phenotypic variation [135,136].

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These findings stressed the importance of extensively investigating the human genome in several different populations to assess if a reported CNV could be benign or disease causing. Several groups studied genetic variations in HapMap samples or normal healthy men and women, with different platforms and techniques. To profit most from this data and to share all this information on variation with the rest of the research community, all data was collected in the Database of Genomic Variants (<http://projects.tcag.ca/variation>) [125]. Still growing, it has become a valuable tool to evaluate if a found CNV is assumed to be harmless or could be possibly causing a disease. Nevertheless care must be taken when using this database to filter out benign CNVs from disease-causing, even if the CNV was inherited from a phenotypically normal parent. A CNV could theoretically be disease-causing in one individual and be benign in another, for instance because of reduced penetrance, variation of expression of the unaffected allele through a different genetic background or because of a recessive trait.

## **8. Scope of this thesis**

Modern cytogenetics has experienced rapid technical changes and improvements since first determining the correct number of chromosomes in humans. This resulted in new methods for improvement of analysis of chromosomal alterations of which many have been described in the introduction of this thesis. Particularly, the last decade has had a big impact on the concept of cytogenetics with the introduction of whole genome molecular screening techniques. Chapter 2 of this thesis describes the implementation of array-CGH and its effect on the diagnosis of structural chromosome rearrangements. In chapter 3 the additional value of array-CGH to routine G-banding of patients with mental retardation and congenital abnormalities is described. A significant increase in the frequency of both inherited and *de novo* copy number variations in a group of 81 patients was found, in which no structural chromosome rearrangements were visible with conventional G-banding. This chapter and additional research combining array-CGH and MLPA, as described in chapter 4, have led to the insight that the current standard of routine whole genome screening with mainly G-banding needs to be reconsidered. What particular techniques will be adopted within diagnostics will depend on cost, quality and speed of the analysis procedure.

Chapter 5 of this thesis underlines the additional value of array-CGH to banding and FISH studies. It reveals the true complexity of a chromosome rearrangement that initially was thought to be a balanced three-way translocation. The mechanism of formation of a complex marker ring chromosome is explained in chapter 6. It was unraveled with array-CGH, combined with various other molecular techniques as FISH, MLPA and oligo array-CGH. With this investigation a novel translocation mechanism was shown to be involved in the formation of a ring chromosome.

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In chapter 7, the molecular and clinical characterization of a new microduplication 3q29 syndrome is described, showing that copy number variation of this region may cause a heterogeneous phenotype. Extensive documentation of disease association with copy number variation will be important to accurately distinguish normal variation from variation causing disease, as illustrated in chapter 8. It describes a case with a homozygous deletion of a normal variation locus causing hearing loss. Both chapter 7 and 8 also show the importance of international collaboration on collecting trusted copy number variation data in databases that will be accessible to the entire community.

In chapter 9 the future impact of the molecular techniques in clinical cytogenetic research and diagnostics is discussed.

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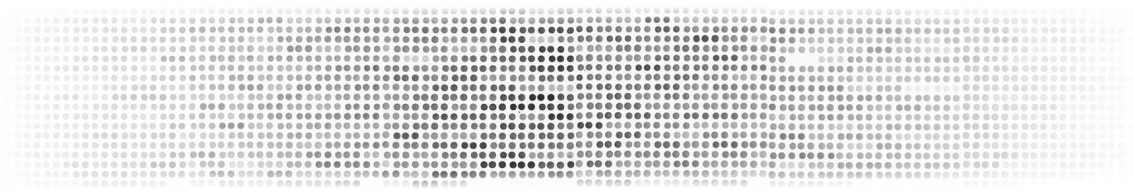
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## Chapter 2

### 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.

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## 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

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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

<i>Patient</i>	<i>Phenotype</i>	<i>G-banding karyotype</i>	<i>Karyotype after array CGH and FISH</i>
Patient A	Mental retardation and dysmorphisms	46,XX,del(11)(q14q21)	46,XX,del(11)(q14.3q22.3)
Patient B	Multiple congenital abnormalities (newborn)	46,XY,i(8)(q10)	46,XY,der(8)(qter→q21.3::p23.2→q ter) <sup>a</sup>
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::) <sup>b</sup>

<sup>a</sup> 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.

<sup>b</sup> 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 ophthalmologist 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).

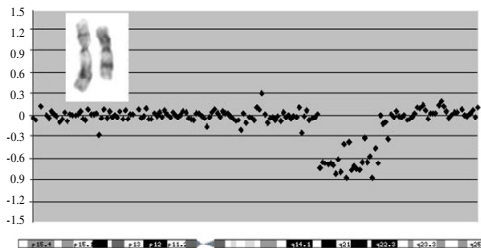


Figure 1. Deletion of chromosome 11 on patient A: The karyotype of the patient after G-banding analyses had been described as 46,XX,del(11)(q14-21), and an image of the del (11) (right) and its normal homolog (left) is shown on the insert. The array CGH profile shows the log<sub>2</sub> ratios of the 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.

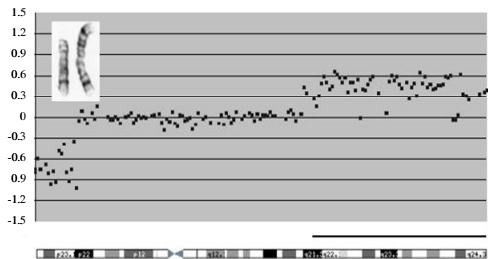


Figure 2. Analyses of the rearranged chromosome 8 from patient B. The insert shows the G-banding image of the rearranged chromosome 8 (right), originally interpreted as an isochromosome, and its normal homolog (left). The array profile displays the log<sub>2</sub> ratios of the clones plotted according 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 proband 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.

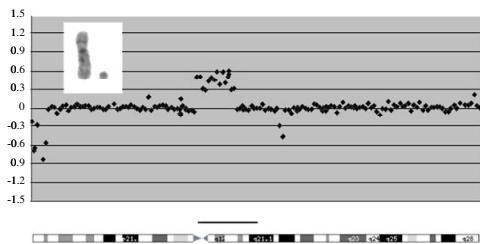


Figure 3. Ring(X) in a Rett syndrome patient. The insert shows a G-band image of the r(X) (right) and its normal homolog (left) in a patient with Rett syndrome. The array CGH profile shows the mapping of the ring chromosome. No Xp sequence was detected on the ring, indicating that the breakpoint was 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 Wellcome 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 Wellcome 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 log<sub>2</sub> ratios of the average of their replicates, and we considered sequences as amplified or deleted when outside the  $\pm 0.3$  range.

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## 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.

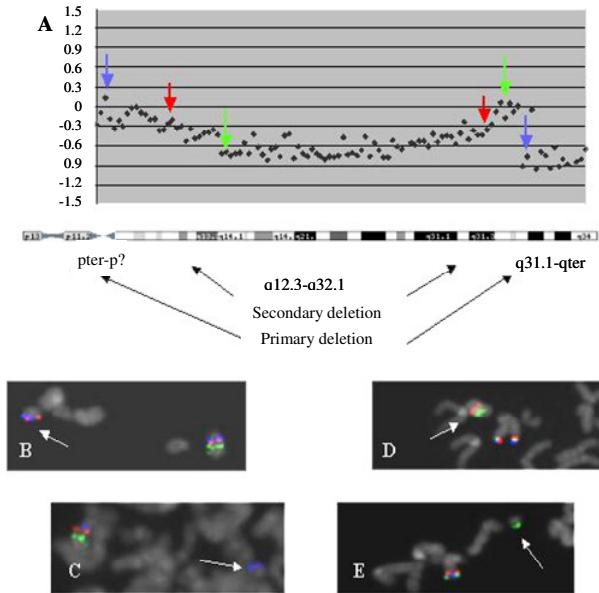


Figure 4. Ring chromosome on patient D. A: The array profile of chromosome 13 shows a primary deletion on the long arm (q31.1-qter). The presumptive deletion on 13p cannot be detected on arrays because of the repetitive nature of its sequences. The interstitial region presents a deletion of variable 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.

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## 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

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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.

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## Chapter 3

**Array-CGH detection of micro rearrangements  
in mentally retarded individuals:  
clinical significance of imbalances present both  
in affected children and normal parents.**

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## Array-CGH detection of micro rearrangements in mentally retarded individuals: clinical significance of imbalances present both in affected children and normal parents

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### ABSTRACT

**Background:** The underlying causes of mental retardation remain unknown in about half the cases. Recent array-CGH studies demonstrated cryptic imbalances in about 25% of patients previously thought to be chromosomally normal.

**Objective and methods:** Array-CGH with approximately 3500 large insert clones spaced at ~1 Mb intervals was used to investigate DNA copy number changes in 81 mentally impaired individuals.

**Results:** Imbalances never observed in control chromosomes were detected in 20 patients (25%): seven were de novo, nine were inherited, and four could not have their origin determined. Six other alterations detected by array were disregarded because they were shown by FISH either to hybridise to both homologues similarly in a presumptive deletion (one case) or to involve clones that hybridised to multiple sites (five cases). All de novo imbalances were assumed to be causally related to the abnormal phenotypes. Among the others, a causal relation between the rearrangements and an aberrant phenotype could be inferred in six cases, including two imbalances of the X chromosome, where the associated clinical features segregated as X linked recessive traits.

**Conclusions:** In all, 13 of 81 patients (16%) were found to have chromosomal imbalances probably related to their clinical features. The clinical significance of the seven remaining imbalances remains unclear. The limited ability to differentiate between inherited copy number variations which cause abnormal phenotypes and rare variants unrelated to clinical alterations currently constitutes a limitation in the use of CGH-microarray for guiding genetic counselling.

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Since karyotyping became a routine technique in clinical genetics, mental impairment, with or without other abnormalities, has often been found to be associated with chromosome rearrangements. However, in the majority of the patients, the G banded karyotype is normal, and in about half of them no obvious cause for the impairment is found (reviewed by Flint and Knight<sup>1</sup>). More recently, cryptic chromosome rearrangements have been reported in patients with an apparently normal karyotype and an unexplained abnormal phenotype. The best characterised rearrangements are the recurrent microdeletion syndromes, such as the Miller-Dieker lissencephaly (MIM 247200) and DiGeorge syndrome (MIM 188400). In addition, subtelomeric imbalances of variable sizes cause mental retardation in 5-7% of these cytogenetically "normal" cases.<sup>1-3</sup> In the last few years, genomic array (array-CGH) analysis has become available,<sup>4,5</sup> and appears to be a robust tool for detecting genomic imbalances in patients, with a much higher resolution than permitted by cytogenetic analyses based on chromosome banding (4-10 Mb).

Two recent studies using array-CGH with markers spaced on average at 1 Mb intervals across the genome have shown that about 25% of the patients with mental impairment associated with dysmorphisms and an apparently normal karyotype carried deletions or duplications below the level of resolution of classical cytogenetics.<sup>6,7</sup> About half the reported cases were de novo, and it is a reasonable assumption that the abnormal phenotype is causally associated with these imbalances. In the inherited cases, however, neither study succeeded in distinguishing between a true pathological or a chance association between copy number changes and abnormal phenotypes.

The precise contribution of microrearrangements to abnormal phenotypes has not been yet established, and in the ~1 Mb arrays used in these studies, imbalances smaller than 1 Mb would often be missed. Whatever the precise figure is, it appears large enough (>5%) to affect genetic counselling. The identification of imbalances in such families can lead to the detection of carriers and to prenatal diagnosis being offered.

Here, we report an array-CGH investigation of 81 patients with mental impairment accompanied by facial dysmorphisms and other congenital abnormalities. The significance of these findings and implication for genetic counselling are discussed.

## METHODS

### Patients

We studied 81 patients with mild to severe mental retardation associated with cranial/facial dysmorphisms and at least one additional dysmorphic feature, suggestive of the presence of a chromosomal abnormality. The karyotypes of all patients were considered normal after routine G-banding (~550 bands) and the

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cause of the abnormal phenotypes could not be determined. Family history and consanguinity were not taken into account as exclusion criteria.

The patients were ascertained in two genetic centres: (1) 61 patients from the Department of Human and Clinical Genetics, Leiden University Medical Centre, the Netherlands (KGCL-LUMC); (2) 20 patients from the Department of Genetics and Evolutionary Biology, Institute of Biosciences, University of São Paulo, Brazil (LGH-USP). This service is the largest reference centre for fragile-X syndrome diagnosis in Brazil, and receives an overrepresentation of male patients. As a result, 18 of the 20 patients were male. In all these patients, fragile-X syndrome had been ruled out on molecular and clinical grounds.

### **Comparative genomic hybridisation microarrays**

The array-CGH procedures were carried out as previously described.<sup>8</sup> Briefly, slides containing triplicates of ~3500 large insert clones spaced at ~1 Mb density over the full genome were produced in the Leiden University Medical Centre. The large insert clones set used to produce these arrays was provided by the Wellcome Trust Sanger Institute (UK), and information regarding the full set is available at the Sanger Institute mapping database site, Ensembl (<http://www.ensembl.org/>). Insert clones were isolated from the bacteria, using the Wizard SV 96 plasmid DNA purification system (Promega, Leiden, Netherlands) in combination with the Biomek 2000 laboratory automation workstation (Leiden Genomic Technology Centre (LGTC), Netherlands). DNA amplification, spotting on the slides, and hybridisation procedures were based on protocols previously described.<sup>9,10</sup> We used commercially available male and female genomic DNAs (Promega), which represent pools derived from at least seven same-sex individuals as reference samples. Test and reference DNA samples were labelled with Cy3- and Cy5-dCTPs (Amersham Bioscience, Roosendaal, Netherlands), respectively. After hybridisation, the slides were scanned with a GenePix Personal 4100A scanner, and the spot intensities measured using GenePix Pro 4.1 software (Axon Instruments, Westburg BV, Leusden, Netherlands). Further analyses were carried out using Microsoft Excel 2000. Spots outside the 20% confidence interval of the average of the replicates were excluded from the analyses. Target imbalances were determined on the basis of log<sub>2</sub> ratios of the average of their replicates, and sequences were considered as amplified or deleted when outside the  $\pm 0.33$  range.

We defined as abnormal a copy number change that we had not previously detected in around 100 normal control observations for each chromosome pair. The control data compiled chromosome information from DNA hybridisations of the following:

- a. Normal to normal individuals.
- b. Normal individuals to individuals previously diagnosed by G-band karyotype to carry partial or complete monosomies or trisomies.

- c. Normal individuals to individuals having a clear chromosome alteration detected by array (a minimum of five altered consecutive clones).
- d. Normal individual to the parents of patients in item c.

We excluded from the analyses the abnormal chromosomes detected by G-banding or CGH arrays in patients, as well as the corresponding chromosomes in their parents, irrespective of their carrier status. The imbalances detected by G-banding were also used to verify the ability of the arrays to ascertain copy number changes and to detect clones mapped to wrong chromosomes. On the basis of this set of normal chromosomes, we determined the average hybridisation ratio for each clone, and excluded from the analyses the 3% clones with an SD >0.073, totalling 110 clones. The remaining ones had an average SD of 0.039. In the hybridisations between normal to normal individuals, or normal to individuals carrying chromosomal alterations (items a and b above), we did not observe changes in the profiles using dye swap (inverted combination of fluorochromes for test and reference DNAs). Based on these results, we only undertook more than one hybridisation per individual in those experiments in which more than 3% of the clones were excluded owing to low intensities of the spots or a noisy background. The threshold of 0.33 for duplications and deletions was empirically chosen because it represented the lowest combined false positive and negative rates in control hybridisation testing of normal DNA and DNA from autosomal trisomies/monosomies.

#### **Fluorescence in situ hybridisation**

Fluorescence in situ hybridisation (FISH) experiments were carried out by standard techniques to validate the presence of deletions and duplications identified by microarray analyses. When an alteration was confirmed by FISH, hybridisations using the same probes were done to investigate whether the parents carried the rearrangement present in the child, either in balanced or unbalanced form. Aliquots of the same amplified DNAs used to spot the arrays were employed as probes for the FISH experiments. Clones mapping to the unbalanced chromosome regions were hybridised to metaphases derived from patients' blood lymphocytes. In cases of duplication, interphase nuclei were also analysed. At least 25 cells were analysed per hybridisation. A region was considered as duplicated when, in interphase nuclei, the corresponding clone produced three FISH signals accompanied by two signals in a different colour from a non-duplicated adjacent clone, used as a control. We considered a chromosome region to be partially deleted when the FISH signal from the corresponding clone on one of the chromosomes was consistently less intense ( $\leq 25\%$  intensity) than on its homologue.



### Multiplex amplifiable probe hybridisation

A study of genomic imbalances by multiplex amplifiable probe hybridisation (MAPH) technique<sup>11</sup> in 188 patients with mental retardation has recently been reported<sup>12</sup>: 162 loci were screened, comprising chromosome regions known to be involved in mental retardation (subtelomeric/pericentromeric regions and the genes involved in microdeletion syndromes), as well as interstitial genes randomly spaced throughout the genome. Although the MAPH patient sample partially overlapped the patients reported here (48 of the Dutch patients), importantly, the two studies were carried out independently and in parallel, and the MAPH results were unknown to those performing array-CGH. Patients 1, 2, 8, 18, and 20 listed in table 2 were part of the overlapping sample.

Table 1. Distribution of patients according to type and inheritance of imbalance

<i>Imbalances</i>	<i>De novo</i>	<i>Inherited</i>	<i>Unknown</i>	<i>Number of patients</i>
Interstitial	4	7	3	14
Deletions	3	5	1	9
Duplications	1	2	1	4
Deletion/duplication	0	0	1	1
Terminal	3	2	1	6
Deletions	2	0	1	3
Duplications	1	1	0	1
Deletion/duplication	0	1	0	1
Total number of patients	7	9	4	20

## RESULTS

In this array-CGH analysis, imbalances not previously observed in our control samples were detected in 20 patients: six had duplications, 12 had deletions, and two carried both deleted and duplicated chromosome segments. Six other imbalances detected on arrays were not included among the imbalances because either (a) a presumptively deleted clone yielded two FISH signals of apparently similar intensities at the expected location on both homologues (one case), or (b) a BAC, supposedly in altered copy number, yielded multiple sites of FISH hybridisation in normal individual metaphases (five cases). It is unclear why we obtained disagreement between array-CGH and FISH results in the deletion case (a), but it emphasises that false positive results may occur in our test. On the other hand, clones that are known to hybridise to multiple sites are excluded from our array.

Table 2. Summary of copy number changes, associated clinical findings, and inheritance

Patient (institution) and inheritance	Array-CGH imbalances	Confirmation	Clinical summary	Maximum size of imbalances
<b>De novo</b>				
1 (LUMC)	Deletion of 5 clones at 1pter-1p36.31 (GS-232-B23 to RP11-49J3)	Confirmed by FISH	Female, MR, epilepsy, facial dysmorphism, shortening of metacarpals and metatarsals, hirsutism	7.2 Mb
2 (LUMC)	Deletion of 18 clones at 18q22.3qter (RP11-45A1 to GS-75-F20)	Confirmed by FISH	Female, MR, short stature, hearing loss, congenital heart defect (total anomalous pulmonary venous return), mild facial dysmorphisms, narrow and long fingers	8.3 Mb
3 (LUMC)	Deletion of 12 clones at 3p24.3-p24.1 (RP11-27J5 to RP11-103N21)	Confirmed by FISH	Female, mild MR, facial dysmorphism, club feet, triphalangeal thumbs, mild anaemia	10.7 Mb
4 (LUMC)	Deletion of 2 clones at 13q32.3 (RP11-118F16 to RP11-564N10)	Confirmed by FISH	Male, MR, tall stature, corpus callosum agenesis, hearing loss, facial dysmorphism	3.2 Mb
5 (USP)	Deletion of 7 clones at 17p11.2 (RP11-524F11 to RP11-121A13)	Confirmed by FISH	Female, MR, behavioural problems including self aggression, hyperactivity, sleep disturbances, decreased pain sensitivity; midface hypoplasia, upward slanting of palpebral fissures (diagnosis: Smith-Magenis syndrome, OMIM 1822900)	5.7 Mb
6 (LUMC)	Duplication of a single clone at 17pter-p13.3 (GS-68-F18)	Confirmed by interphase FISH	Female, mild MR, facial dysmorphisms	0.8 Mb
7 (LUMC)	Duplication of a single clone at 6p12.3 (RP3-442L6)	Confirmed by interphase FISH	Female, mild MR, hypotonia, joint hyperlaxity, facial dysmorphism, strabismus	1.7 Mb
<b>Inherited</b>				
8 (LUMC)	Deletion of 4 clones at 6q27qter (RP11-351J23 to GS-57-H24). Duplication of 13 clones at 20q13.31qter (RP11-46O6 to bB152O15)	Confirmed by FISH [t(6,20)(q27;q13.31)mat]	Male, MR, hypotonia, microcephaly, brain anomalies, mild facial dysmorphisms	4.7 Mb deleted; 7.5 Mb duplicated
9 (LUMC)	Duplication of 2 clones at 3q29-qter (GS-196F4 and GS-56H22)	Confirmed by interphase FISH; paternal	Female, moderate MR, facial dysmorphism, ataxia	0.4 Mb
10 (LUMC)	Partial deletion of a single clone at 15q15.3 (RP11-263I19)	Confirmed by FISH; paternal	Female, mild MR, hypotonia, facial dysmorphism, cloudy cornea	0.9 Mb
11 (LUMC)	Deletion of a single clone at 15q13.1 (RP11-408F10)	Confirmed by FISH; maternal	Female, mild MR, short stature, microcephaly, minor facial dysmorphism, premature breast development	2.2 Mb
12 (USP)	Deletion of a single clone at 10q21.1 (RP11-430K23)	Confirmed by FISH; maternal (mother with learning difficulties and similar dysmorphisms as the patient)	Male, mild MR, hyperactivity, facial dysmorphism, prominent ears, long digits, hyperextensibility of joints	2.5 Mb
13 (USP)	Duplication of a single clone at Xq28 (RP5-1087L19)	Confirmed by MAPH; maternal; two affected first cousins born to maternal aunts; duplication also present in the investigated cousin	Male, MR, hypoplasia of cerebellar vermis, Dandy-Walker anomaly, large prominent ears, high-arched palate, abdominal obesity, flat feet.	1.3 Mb
14 (USP)	Partial deletion of a single clone at Xp11.23 (RP1-54B20)	Confirmed by FISH; maternal; similarly affected males referred in the maternal family	Male, severe MR, short stature, microcephaly, prominent ears, deep set eyes, short filtrum, early onset puberty	1.8 Mb
15 (LUMC)	Partial homozygous deletion of a single clone at 2p12 (RP11-89C12)	Confirmed by FISH; first cousin; heterozygous father and homozygous mother	Female, MR, microcephaly, cleft palate, congenital cataract, microphthalmia; equally affected sibling carries same homozygous deletion	1.0 Mb
16 (LUMC)	Duplication of a single clone at 8p11.1 (CTD-2115H11)	Confirmed by FISH; maternal	Male, mild MR, short stature, facial dysmorphisms	1.3 Mb
<b>Unknown</b>				
17 (LUMC)	Deletion of 6 clones at 6pter-p25.2 (GS-62-L11 to RP11-15N12)	Confirmed by FISH; mother not a carrier*	Male, mild MR, hearing loss, iris dysplasia, eccentric pupil, hypertelorism	5 Mb
18 (USP)	Deletion of a single clone at 16p11.2 (RP11-74E23)	Confirmed by FISH; mother not a carrier*	Female, mild MR, severe speech delay, facial dysmorphism	1 Mb
19 (USP)	Duplication of a single clone at 22q11.21 (XX-91c)	Confirmed by MAPH and array-CGH tile-path of chromosome 22; mother not a carrier*	Male, mild MR, turriccephaly, convergent strabismus, myopia, high and narrow palate, large upper first incisors	3.9 Mb
20 (LUMC)	Deletion of a single clone at 22q11.21 (XX-p273a17); duplication of a single clone at 22q11.21 (XX-91c); deletion of 5 clones at 22q12.1 (CTA-390B3 to RP11-329J17)	Confirmed by MAPH and array-CGH tile path of chromosome 22**	Male, mild MR, hearing loss, microcephaly, cataract, cleft palate, double set of teeth	1.1 Mb deleted; 3.9 Mb duplicated; 3.9 Mb deleted

\*Father deceased or unavailable.

\*\*Parents deceased.

LUMC, Leiden University Medical Centre; MR, mental retardation; USP, University of São Paulo.

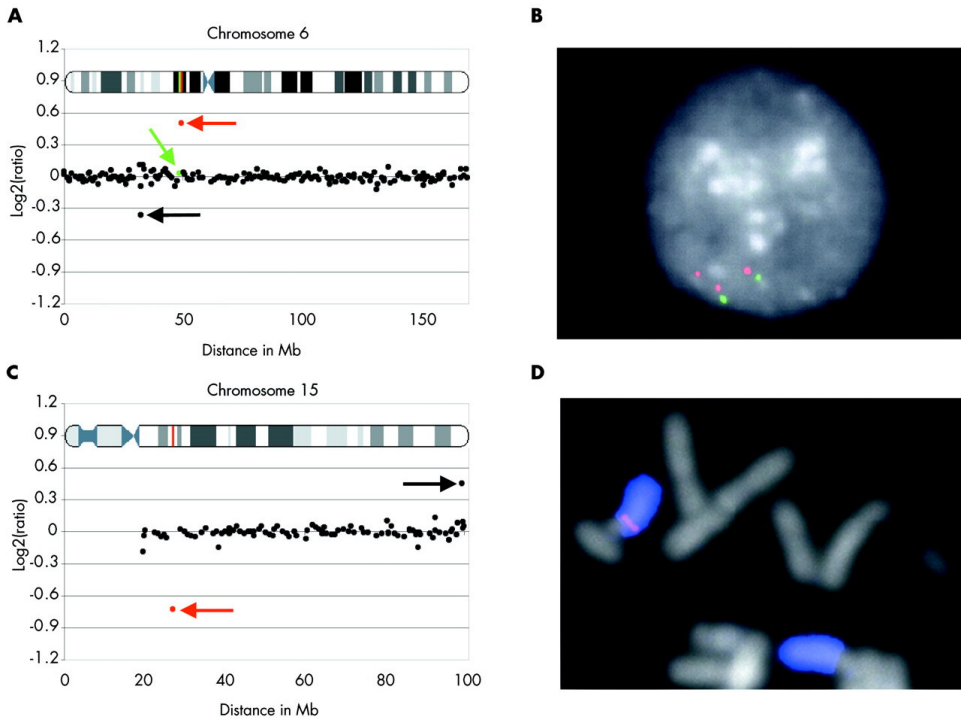


Figure 1 Examples of duplications and deletions ascertained by array-CGH and confirmed by FISH. Unbalanced and control insert clones are represented in red and green colours, respectively, in the array profiles and corresponding ideograms. The black arrows show large insert clones which are also found altered in normal controls. (A) Chromosome 6 array-CGH profile from patient 7, showing a duplication of a single clone at 6p12.3 (RP3-442L6). (B) Three interphase-FISH signals of the duplicated PAC RP3-442L6 (red) and two signals of the adjacent non-duplicated BAC RP11-334H12 (green) confirm this duplication. (C) Deletion of a single clone at 15q13.1 (RP11-408F10) in patient 11. (D) FISH to a metaphase showing the presence of two chromosomes 15 (whole-chromosome 15 library in blue), but a single signal for BAC RP11-408F10 (red). BAC, bacterial artificial chromosome; CGH, comparative genomic hybridisation; FISH, fluorescence in situ hybridisation.

Table 1 shows the distribution of the copy number changes according to the type and inheritance of the imbalances, and table 2 presents the copy number changes, clinical data, and family analysis.

Fourteen of the imbalances were interstitial and six were terminal. Deletions and duplications were confirmed by FISH; case 13 was confirmed by MAPH, and for chromosome 22 (cases 19 and 20) an array of overlapping sequencing tile path clones<sup>13</sup> allowed us to delineate the duplication and deletions (Kriek et al, unpublished data). Figure 1 shows examples of array-CGH deletions and duplications, and FISH confirmation. Among the 16 patients whose parents were available for examination, seven carried de novo imbalances and nine had inherited rearrangements. Among the rearrangements, one patient (case 15) had a homozygous deletion inherited from heterozygous father and homozygous mother (fig 2).

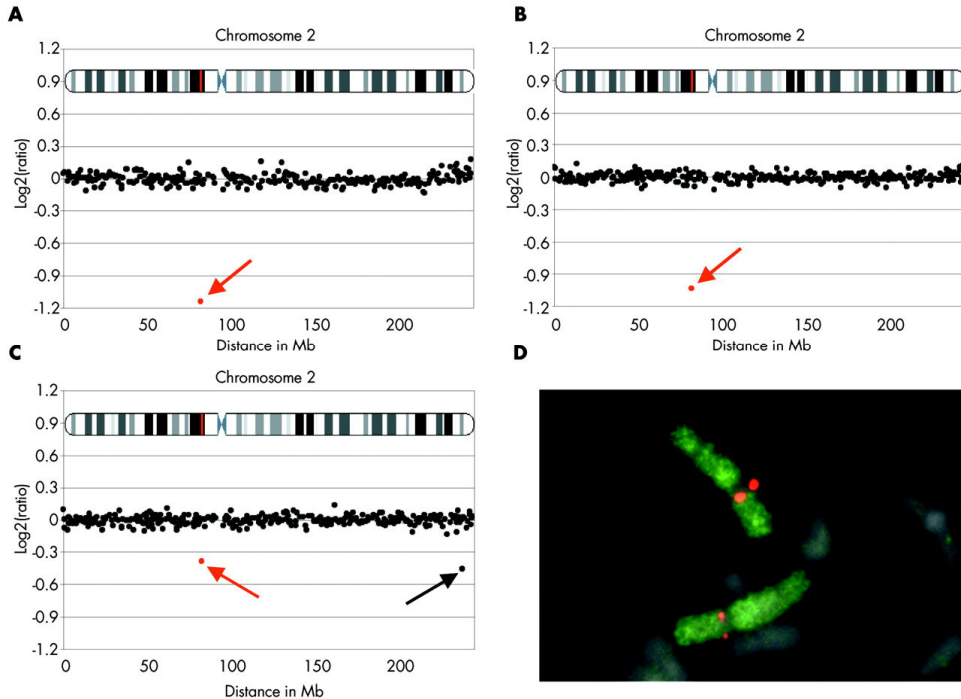


Figure 2 Chromosome 2 imbalances in the family of patient 15. Chromosome 2 array-CGH profiles of the proband (A) and his mother (B) show a deletion of BAC RP11-89C12 (red arrow) at 2p12. The log<sub>2</sub> ratios around -1.0 indicate that the deletion is homozygous. (C) Chromosome 2 array-CGH profile of the father shows a deletion of the same BAC (red arrow) in a ratio compatible with heterozygosity. (D) FISH using BAC RP11-89C12 (red) to the father's metaphase shows that the signal on one chromosome 2 (whole chromosome library in green) is less than one quarter the size and intensity of the homologue. BAC, bacterial artificial chromosome; CGH, comparative genomic hybridisation; FISH, fluorescence in situ hybridisation.

## DISCUSSION

We used array-CGH to investigate DNA copy number imbalances in 81 individuals presenting with mental retardation, dysmorphic features, and an apparently normal karyotypes, and detected unique alterations in 25% of these. In addition, 33 clones contained in our array (>0.1%) exhibited copy number alterations detected at least once in the control analysis, and were considered as normal variants. These genomic imbalances might well represent some of the large scale copy number variations of DNA segments recently described in humans and mice.<sup>14-16</sup>

More than half the imbalances comprised one clone or less (partial deletions). The two largest alterations (patients 2 and 3) encompassed between 8 and 11 Mb and, after CGH-array results were known, they could retrospectively be visualised on G-banded chromosomes. The frequency of imbalances detected in our study is similar to the 24-25% found in previous array-CGH studies of mentally impaired individuals.<sup>6,7</sup> However, the patients in ours and in the two previous

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array-CGH studies were selected not to be solely representative of undiagnosed mental retardation, but rather to include patients whose dysmorphic features in conjunction with mental retardation were suggestive of chromosomal abnormalities. It is well known that the frequency of chromosomal abnormalities is increased when mental retardation is associated with congenital abnormalities or dysmorphic features. While these studies prove the value of array-CGH for uncovering cryptic rearrangements, further studies in categorised samples of mentally retarded individuals will be required to establish the frequency of imbalances which have a pathological consequence giving rise to mental retardation, and to differentiate them from rare variants picked up serendipitously in the screening. Among the 20 chromosome rearrangements that we detected, six (7.4%) were subtelomeric and 14 (17.3%) were interstitial. Similarly to the two previous studies with array-CGH, the frequency of cryptic interstitial rearrangements was two to three times greater than the frequency of terminal imbalances.

When patients carry de novo imbalances, either interstitial or terminal, it is reasonable to assume that the copy number change is the cause of the phenotype. On the other hand, inherited subtelomeric and interstitial rearrangements have different implications for genetic counselling. Inherited subtelomeric rearrangements result from the segregation of a balanced translocation in one of the normal parents,<sup>1</sup> and their detection allows genetic counselling and prenatal diagnoses to be provided. In contrast, inherited interstitial rearrangements detected by array-CGH seem to be equally imbalanced in a normal parent, and no carriers of balanced interstitial rearrangements were detected in ours or in the previous array-CGH studies. Therefore, these inherited interstitial rearrangements pose a new situation in genetic counselling, because the normal parent apparently carries the same imbalance as the affected child. We observed various different situations that suggest that rare inherited copy number variations can either affect the phenotype or represent "normal" variants. The X chromosome imbalances (patients 13 and 14) were associated with clinical features showing an X linked pattern of inheritance—that is, other affected males were related to the probands by their phenotypically normal mothers. An intriguing case is that of patient 15: he and his equally affected sibling are homozygous for a partial deletion of one clone at 2p12. Their clinically normal parents are first degree cousins, and the father is heterozygous for the same deletion, while the mother is homozygous (fig 2). This deletion therefore appears as a rare variant segregating in the family, and the abnormal phenotype of the children is likely to be caused by homozygosity for another recessive mutation. Furthermore, this family is of Turkish descent, and the frequency of the deletion in this population is unknown.

In four cases (patients 17 to 20), one or both parents could not be investigated for the presence of imbalances. In patient 17, although the de novo status could

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not be proved, the patient had the typical features of the deletion 6pter syndrome,<sup>17,18</sup> thus indicating that the deletion was causative. Among the infrequent DNA segment imbalances found in our patients, there was a single map location overlap—namely, the duplication of cosmid XX-91c on chromosome 22 which is present in patients 19 and 20 (table 2). The tiling path analysis of chromosome 22 in patient 19 showed that this duplication encompasses ~1.7 Mb, and overlaps the often deleted region in the DiGeorge/velocardiofacial syndrome (DG/VCFS). Two recent papers reported on patients with duplication of this region, suggesting that dup22q11.2 is an emerging syndrome<sup>19,20</sup>; the learning abilities of the carriers ranged from normal to severely impaired, and associated clinical features were extremely variable, including normal individuals ascertained through affected relatives. Our patient had only mild mental retardation and some unspecific dysmorphic features, which may be present in different syndromes, including patients with dup22q11. As the frequency of the duplication 22q11.2 is significantly increased among mentally retarded patients, we considered that the alteration in our patient was probably causative of his phenotype. Patient 20 carries two deletions on chromosome 22 in addition to this recurrent duplication. Furthermore, this patient had specific congenital abnormalities, some of which do not seem to be associated with the dup22q11.2 syndrome, and might be caused by at least one of the deletions (Kriek M et al, unpublished data). Unfortunately, further investigation could not be undertaken because not all the parents of these two patients were available.

Thus, among the 20 rearrangements detected in our patients (table 2), we were able to identify 13 as causative of the abnormal phenotypes: those which were de novo (patients 1-7), the imbalanced rearrangement inherited from a balanced parent (patient 8), the two familial X chromosome alterations (patients 13 and 14), the deletion 6pter (patient 18), the dup22q (patient 19), and the rearrangements of chromosome 22 in patient 20, which were too large and complex not to be the probable cause of the phenotype. In fact, the rearrangements involving two or more clones were all de novo (mean (SD) maximum average size, 5.4 (3.6) Mb), while the rearrangements present in normal carriers tended to be smaller (maximum average size 1.6 (0.8) Mb). It is not itself surprising that rearrangements involving large segments of DNA have a smaller probability of being present in normal carriers.

Among the 13 cases with copy number imbalances considered causative of the phenotype, five were terminal and eight were interstitial, showing a similar contribution of these rearrangements to mental retardation associated with other clinical features. However, in patients who inherited an apparently identical interstitial imbalance from their parents, we cannot disregard the possibility that imprinting, incomplete penetrance, and loss of heterozygosity for a detrimental recessive gene contribute to the different effect in parents and affected children, as has been reported for dup22q11.2.<sup>19,20</sup>

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The frequent occurrence of rare genomic imbalances in affected children and their normal parent represents a complicating factor in the interpretation of array-CGH results. Among our patients we found inherited imbalances that were appeared clearly associated with a pathological effect, while others most probably represented genomic variants not contributing to the abnormal phenotype. Recent initiatives such as those of the Sanger Institute ([www.sanger.ac.uk/PostGenomics/decipher/](http://www.sanger.ac.uk/PostGenomics/decipher/)) and the European Cytogeneticists Association (<http://www.ecaruca.net/>) to create platforms for compiling molecular cytogenetic data from clinical genetic studies will hopefully provide a base for understanding the role of different DNA copy number variations in genetic diseases. Collecting and understanding larger sets of data will improve our ability to determine which copy number variations contribute to abnormal phenotypes, and eventually result in a more consistent application of CGH-microarray for genetic counselling.

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#### FOOTNOTES

Conflicts of interest: none declared

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## Chapter 4

# Diagnosis of genetic abnormalities in developmentally delayed patients: A new strategy combining MLPA and array-CGH.

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# Diagnosis of Genetic Abnormalities in Developmentally Delayed Patients: A New Strategy Combining MLPA and Array-CGH

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## To the Editor:

Developmental delay (DD) affects ~3% of the general population and the underlying cause remains unknown in about half of the cases. G-banded karyotyping is the most common approach for the detection of genomic alterations, however, despite its indisputable success, this tool has limited resolution, usually being unable to detect genomic changes ~3-5 Mb. It is known that micro alterations that escape detection by classical cytogenetics contribute substantially to the etiology of DD [Flint et al., 1995; Vissers et al., 2003]. This limitation has been partly overcome by fluorescence in situ hybridization (FISH) with a resolution of 5-500 kb, however, it has a limited possibility for multiplexing, for example, in most of the routine practice only 2-3 regions can be analyzed simultaneously. Therefore, candidate probes (especially for microdeletion syndromes) need to be selected a priori for FISH investigation, based on the patient's phenotype.

Recent technological developments, such as array-based comparative genomic hybridization (array-CGH) [Pinkel et al., 1998; Antonarakis, 2001; Snijders et al., 2001] and Multiplex Ligation-dependent Probe Amplification (MLPA) [Schouten et al., 2002], are efficient methods for screening for copy number imbalances in multiple genomic regions simultaneously. MLPA especially has already found its way into the diagnostic laboratories for several indications (e.g., BRCA1 gene and NFI gene screening); however, the standard of practice for the assessment of

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developmental delay does not currently include MLPA and array-CGH testing. In this article, it is argued that both techniques are extremely valuable tools for the diagnostic setting in DD patients, and the implementation of both techniques should be considered.

Data regarding the robustness of both techniques have been provided previously [Price et al., 2005; Rooms et al., 2005]. In the case of array-CGH, thousands of sites can be simultaneously investigated in one patient, allowing partial or total coverage of the genome. The number of targets that can be screened by MLPA is limited to <60 loci per assay, however, 96 samples can be simultaneously tested at a cost less than one array-CGH hybridization. As MLPA analysis requires relatively little hands-on time (Table I), it is more suitable for the initial screening of large patient numbers.

To assess their value in clinical diagnosis, we have independently tested 58 developmentally delayed (DD) patients using both array-CGH and MLPA. This study was reviewed and approved by the Institutional Review Board of the Leiden University Medical Center, conforming to Dutch law and the World Medical Association Declaration of Helsinki. The patients had, in addition to DD, either dysmorphic features or congenital malformations or both (DD “plus” patients). All patients had a normal karyotype and, where tested (the vast majority of the patients), had tested negative for Fragile X syndrome. The array-CGH results were partly reported elsewhere [Rosenberg et al., 2006] without the comparative analysis with MLPA.

The array used in the study contained ~3,500 large genomic insert clones spaced at ~1Mb intervals over the genome, meaning that the resolution of the arrays used is 0.3-3 Mb. Array-CGH testing was performed as described by [Knijnenburg et al., 2005]. The clones were provided by the Wellcome Trust Sanger Institute (UK), and information regarding the full set is available at the Ensemble web site.

The MLPA probe design and assay was performed as described previously [White et al., 2004]. It included a set of synthetic probes designed for 71 regions known to be frequently altered in DD patients (probe sequences are available on request). This set targets 42 chromosome ends (except for the p-arms of the acrocentric chromosomes), five pericentromeric regions on the q-arm of acrocentric chromosomes (the regions tested included the first gene-specific unique sequence near the centromere on the q-arm) and 24 probes (Table II) containing microdeletion syndrome-related sequences. The size of the probes used was between 75 and 125 bp, and the number of sites investigated by MLPA corresponds to ~2% (71/3,500) of all regions tested by array-CGH.

Seventeen alterations were detected by array-CGH analysis, of which 14 were verified using either FISH or MLPA (14/58=24%). (The MLPA probes were specifically designed for confirming these alterations. They were not part of the screening set.) As far as was tested the remaining three changes could not be confirmed using FISH or MLPA.

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MLPA analysis identified eight alterations, all of which were confirmed by FISH, MAPH or sequencing (8/58=14%). Table III provides an overview of the alterations found. The eight alterations found solely by array-CGH were all located in regions not covered by MLPA probes. In contrast, the two alterations detected by MLPA only were too small to be detected by array-CGH analysis. One of these alterations was a point mutation near the ligation site of the MLPA probe, which disturbed the ligation and appeared as a deletion. The point mutation (that was never reported before) has been proven by bi-directional sequencing. It is a silent mutation, and it was also present in one of the parents. Therefore, it was considered to be a single nucleotide polymorphism (SNP). Although all MLPA probes have been designed outside the sequences containing known SNPs, theoretically, a low frequency SNP could be present at or near the ligation site. Therefore, it is necessary to confirm copy number variations by a second MLPA probe covering an adjacent sequence or by sequencing.

Of the eight alterations detected by MLPA, we considered six to be probably causative as the phenotype of the patients agreed with the clinical features described in literature for those chromosome alterations. All these rearrangements were also detected by array-CGH. In two of these six cases, however, we could not confirm that the rearrangement was *de novo*. Two of the eight alterations detected by MLPA are likely to be polymorphic variants, as they are also present in unaffected family members.

TABLE I. A Comparison of the Man-Hours and Material Required for Both Karyotyping and MLPA Analysis

	<i>Karyotyping</i>	<i>MLPA</i>
Number of samples performed per week	12	5x96 wells plate
Total time before result per sample	32-40 hr	8 hr <sup>a</sup>
Materials needed	Cell culture, reagents	DNA reagents, probe set

This table shows that MLPA is suitable for the screening of copy number variations in a large number of patients within relatively short time. Compared to karyotyping, this technique is much faster and requires less hands-on time. As it is also possible to analyze a part of a fragment run or use a DNA sequencer with less throughput capacity, it is not necessary to wait for 96 patient samples requiring MLPA testing.

<sup>a</sup>Recently, it was shown that MLPA analysis can be performed within 8 hr (Kalf et al. in preparation).

Nine of the fourteen confirmed rearrangements detected by array-CGH are probably pathogenic, four alterations might be polymorphic variants as they are present in unaffected family members. The clinical consequences of the remaining alteration are currently unknown, because the patients' parents were unavailable for testing. This latest FISH confirmed array-CGH finding which was not detected by MLPA, was located near the chromosome end of the long arm of chromosome 10. The corresponding "subtelomeric" MLPA probe in our study mapped proximal to the altered BAC.

**TABLE II. Overview of the Microdeletion Syndrome Related Probes Used by MLPA Screening**

Disorder	Chromosome band	Gene
Alagille syndrome	20p12.2	JAG1
Angelman syndrome	15q12	UBE3A
Cat eye syndrome	22q11.1	CECR2
DiGeorge syndrome	22q11.2	DGCR2
DiGeorge syndrome	22q11.2	HIRA
DiGeorge syndrome	22q11.2	TBX1
DiGeorge syndrome	22q11.2	UFD1L
DiGeorge syndrome like region	10p14	CUGBP2
Extostosis	8q24	EXT1
Jacobsen syndrome	11q25	HNT
Miller-Dieker syndrome	17p13.3	LIS 1
Mowat-Wilson syndrome	2q22	SIP1
Prader-Willi syndrome	15q12	SNRPN
RETT syndrome	Xq28	MECP2
Rubinstein-Taybi syndrome	16p13,3	CBP
Smith-Magenis syndrome	17p11.2	RAI1
Smith-Magenis syndrome	17p11.2	COP3
Smith-Magenis syndrome	17p11.2	DRG2
Sotos syndrome	5q35	NSD1
Trichorhinophalangeal syndrome	8q23.3	TRPS1
William-Beuren syndrome	7q11.23	ELN
William-Beuren syndrome	7q11.23	FKBP6
Wolf-Hirschhorn syndrome	4p16.2	MSX1
X-linked hydrocephalus	Xq28	L1CAM

Based on the data on the human genome variation database, the region involved might be polymorphic. Moreover, the clinical features of the patient do not resemble those corresponding with previously described 10q chromosome end alterations [Waggoner et al., 1999]. The sizes of the reported alterations, however, are larger than the one obtained in this study.

The comparison between the screening results for detecting copy number variations using the different approaches shows the reliability and specific strengths of both techniques. In summary, using  $\approx 2\%$  of the loci tested by array-CGH, MLPA detected 50% (8/16) of all alterations. Three potentially pathogenic alterations were not detected using MLPA, as they were localized outside the regions tested.

Based on the outcome of this parallel screening and costs considerations, we suggest the following strategy for diagnostic purposes: when a patient presents with DD of unclear

etiology and the G-banding karyotype is normal, the first screening will use MLPA for the commonly altered regions in DD patients (currently, chromosome ends and microdeletion syndrome-related regions). Subsequently, when MLPA is negative and the patient's phenotype is suggestive of a chromosome abnormality, array-CGH follows.

Alternatively, the order of testing could be reversed. MLPA using subtelomeric probes is capable of detecting trisomies as well as the vast majority of the unbalanced translocations, both of which comprise a substantial part of the alterations diagnosed using cytogenetic tools. Table I shows that MLPA requires

less manpower (hence is cheaper) and is considerably faster compared to karyotyping, and thus, it seems more effective to use MLPA as an initial screening tool. In addition to the time- and cost-effectiveness, MLPA has a much higher resolution for detecting copy number variations compared to karyotyping, and therefore, this technique is capable of detecting copy number variations that remain undiagnosed using this cytogenetic tool. Applying MLPA testing first will even be more effective when a MLPA probe set encompassing the most frequent microdeletion related regions is added. In a diagnostic setting, it is preferable to have at least two MLPA probes per regions of interest (instead of one as was used in this study) to limit false positive and false negative results as much as possible. Implementing microdeletion syndromerelated regions and two probes per region will increase the costs related to MLPA screening, however, this will also reduce the necessity of performing FISH for the detection of microdeletion syndromes, and the need for additional confirmation tests (with the exception of sequencing, see above).

It is obvious that balanced translocations and inversions will not be detected using this or other molecular techniques (unless they are specifically designed to detect breakpoints). Also, for a proportion of the samples with a positive outcome using the initial MLPA screening, subsequent karyotyping is essential for localization of these structural rearrangements. These include, for example, aneusomies for which Robertsonian translocations have to be excluded. Based on these arguments, karyotyping will maintain its essential role in a diagnostic process, however it will only be implemented for selected samples.

TABLE III. Copy Number Variations Detected by Two Techniques Independently

	Only by a-CGH			Only by MLPA			By a-CGH and MLPA			Total
Altered	11			2			6			19
Confirmed	8 <sup>a</sup>			2 <sup>b</sup>			6			16
	De novo	Present in parents	Unknown	De novo	Present in parents	Unknown	De novo	Present in parents	Unknown	
Deletion	2	3	1 <sup>c</sup>	0	1 <sup>d</sup>	0	2 <sup>c</sup>	0	1 <sup>c,e</sup>	10
Duplication	1	1	0	0	1 <sup>d</sup>	0	1 <sup>c</sup>	0	0	4
del./dup.	0	0	0	0	0	0	0	0	1 <sup>d,e</sup>	1
UT	0	0	0	0	0	0	1 <sup>c</sup>	0	0	1
Confirmed total	3	4	1	0	2	0	4	0	2	16

An overview of the results obtained by screening of 58 DD patients using array-CGH and MLPA. All rearrangements were not detected by routine karyotyping.

UT, unbalanced translocation.

<sup>a</sup>These regions were not covered by MLPA analysis.

<sup>b</sup>These alterations were too small to be detected by array-CGH.

<sup>c</sup>Alterations localized at the chromosome ends.

<sup>d</sup>Alterations present in regions related to micro-deletion syndromes.

<sup>e</sup>(One of) the patient's parents were (was) unavailable for testing. The phenotype of the patient, however, resembles that described in literature. Therefore, this alteration is thought to be pathogenic.

After MLPA testing, additional array-CGH can be performed for patients with a clinical phenotype suggestive for chromosomal alterations. Although this will increase the cost, it will also increase the number of copy number variations detected.

Array-based techniques are evolving rapidly. Several reports have described the results of testing developmentally delayed patients tested using a 3,000-clone array [Visser et al., 2003; Tyson et al., 2005; Menten et al., 2006; Rosenberg et al., 2006; Shaw-Smith et al., 2006]. In addition, de Vries et al. used an array with 32,000 clones for the detection of copy number variations. Recently, SNP-based arrays have successfully been used to detect genome-wide copy number variations [Friedman et al., 2006]. These type of arrays have an even higher resolution than the array used in de Vries et al. Future comparative studies will help to determine which array platform is the most appropriate to implement.

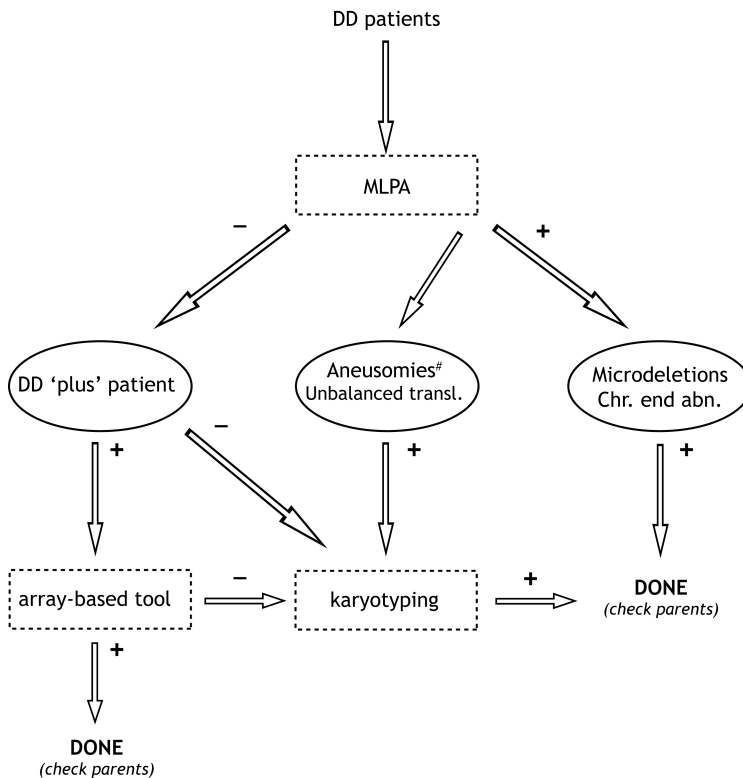


FIG. 1. This flow chart summarizes the alternative diagnostic approach for screening developmentally delayed patient samples. In this approach, karyotyping will only be requested for a selected group of samples: (1) Samples that had tested negative for MLPA (and array-based tool in the case of DD “plus” patients). (2) Samples for which information about the location of the structural rearrangement is essential for clinical practice. These include aneusomies for which a Robertsonian translocation should be excluded (acrocentric chromosomes (#)), unbalanced translocations and some of the alterations detected by array-CGH. Chr. end abn.: chromosome end abnormality, DD “plus” patients are patients with dysmorphic features and/or congenital malformations in addition to DD. These patients are suggestive for chromosomal imbalances.



In short, the alternative diagnostic approach would include MLPA for DD samples, with subsequent array-based testing (for DD “plus” patients that had tested negative for MLPA). Karyotyping could then be used to locate structural rearrangements for selected cases and for samples that showed no alteration using MLPA (and array-CGH) (Fig. 1). In this way, the screening of DD samples will be more effective in relation to the probability of finding a disease-causing rearrangement, which will improve the basis for counseling.

## ACKNOWLEDGMENTS

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## Chapter 5

### Array-CGH detection of a cryptic deletion in a complex chromosome rearrangement.

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# Array CGH detection of a cryptic deletion in a complex chromosome rearrangement

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## Abstract

Balanced complex chromosome rearrangements (CCR) are extremely rare in humans. They are usually ascertained either by abnormal phenotype or reproductive failure in carriers. These abnormalities are attributed to disruption of genes at the breakpoints, position effect or cryptic imbalances in the genome. However, little is known about possible imbalances at the junction points. We report here a patient with a CCR involving three chromosomes (2;10;11) and eight breakpoints. The patient presented with behavioural problems as the sole phenotypic abnormality. The rearrangement, which is apparently balanced in G-banding and multicolour FISH, was shown by genomic array analysis to include a deletion of 0.15-1.5 Mb associated with one of the breakpoints. To explain the formation of this rearrangement through the smallest possible number of breakage-and-reunion events, one has to assume that the breaks have not occurred simultaneously, but in a temporal order within the span of a single cell division. We demonstrate that array comparative genomic hybridisation (CGH) is a useful complementary tool to cytogenetic analysis for detecting and mapping cryptic imbalances associated with chromosome rearrangement.

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## Introduction

Complex chromosome rearrangement (CCR) is a general term designating any structural rearrangement involving more than two breakpoints and/or chromosomes (Pai et al. 1980). Individuals with balanced CCRs are most often ascertained through fertility problems, recurrent miscarriages, or by congenital anomalies in newborn offspring. These individuals are otherwise normal, and their problems are a direct consequence of either meiotic failure or imbalanced chromosome segregation. However, some carriers of apparently balanced complex translocations present phenotypic abnormalities and/or mental retardation (Batanian and Eswara 1998; Batista et al. 1994; Joyce et al. 1999; Phelan et al. 1998). The abnormal phenotype in these cases is thought to originate from disruption of genes or cryptic imbalances in the genome (Batista et al. 1994). While disruption of genes at breakpoints is well documented and has been instrumental in the mapping of several disease genes, such as, DMD, NF1 and mesomelic dysplasia (Ledbetter et al. 1989; Spitz et al. 2002; Zatz et al. 1981), cryptic deletions or duplications in the genome in apparently balanced translocations have only sporadically been demonstrated (Borg et al. 2002; Kumar et al. 1998).

Here we report a 5-year-old patient who exhibited behavioural changes and delay in speech development as the only phenotypic abnormalities, and was found to carry an apparently balanced CCR on the basis of Giemsa banding and multicolour FISH. The rearrangement, which involved three chromosomes and at least eight breakpoints, was studied at high resolution by multicolour FISH and genomic array, and a cryptic imbalance was detected.

## Clinical description

The patient is a Caucasian 5-year-old male, the first child from healthy and non-consanguineous parents. Pregnancy was unremarkable and the Apgar score at birth was 8/9. At 19 months of age, delayed speech, hyperactivity and attention deficit were noted, and he underwent a neurological evaluation. Results of EEG, audiogram and screening for inborn metabolic errors were all in the normal range.

At 5 years of age, he was referred for genetic and cytogenetic evaluation. His intelligence was apparently normal (IQ evaluation was denied), but hyperactivity and attention deficit were noted. The child is still under treatment for psychological and speech difficulties. However, because the child is raised in a tri-lingual environment, the significance of the speech delay is difficult to evaluate.

## Materials and methods

### Molecular and classical cytogenetic studies

Peripheral blood lymphocytes from the patient and his parents were cultured for 72 h according to standard procedures. Cytogenetic analysis was performed on GTG-banded chromosomes, and 25 cells from the patient and 20 cells of each parent were fully analysed.

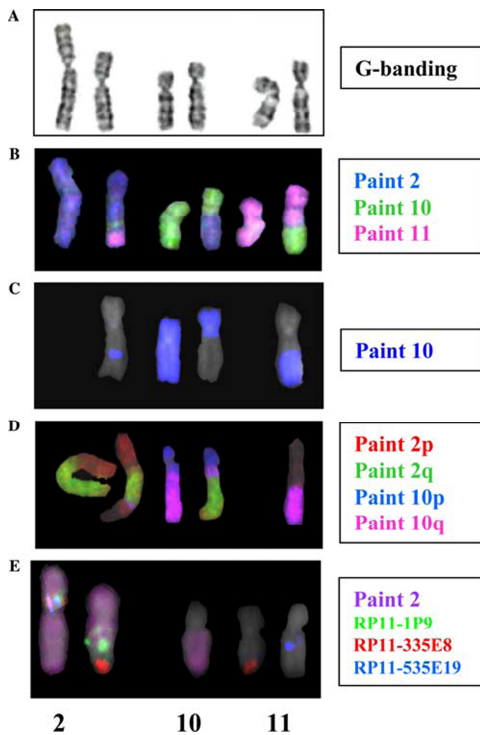


Fig. 1a-e Rearranged chromosomes and their normal homologues present in the patient carrying a complex chromosome rearrangement. a G-banding image shows a three-way translocation (2;10;11) and an insertion of 2p in der(2)(q); b 24-colour hybridisation confirms the exchange of material between (2;10;11), and identifies an additional weak signal on der(2)(q). c Painting with a chromosome 10 library shows that the small insertion on 2q is derived from chromosome 10. d Hybridisation with chromosome arm paintings for chromosome 10 and 2 shows that the material from 10q into der(2)(q) maps proximal to 2p material. e Chromosome 2 painting hybridised together with three BAC probes on 2p. The three signals are present on the normal chromosome 2. The other green and blue signals map to der(2)(q) and der(11), respectively (see map in Fig. 2). The red-labelled BAC is deleted and does not present other signals on 2p material. Note that the red-labelled BAC also presents a secondary signal on 11qter

Metaphases of the patient were analysed by the combinatorial binary ratio labelling (COBRA-FISH approach; Tanke et al. 1999), allowing the identification of each arm by a different colour combination. Labelling, hybridisation and immunostaining were performed as previously described (Tanke et al. 1999). For chromosomes that were found by multicolour FISH to be rearranged, libraries labelled with single fluorochromes were hybridised to metaphases of the patient to confirm the identity of the rearranged chromosome segments.

### Array comparative genomic hybridisation (CGH)

Slides containing triplicates of ~3,500 BAC/PAC DNA targets spaced at ~1 Mb intervals were produced in the Leiden University Medical Center. The BAC set used to produce these arrays was received from the Wellcome Trust Sanger Institute (UK), and information regarding the full set is available at the Sanger Center mapping database site, Ensembl (<http://www.ensembl.org/>). Protocols for BAC DNA preparation and amplification, spotting on the slides and hybridisation were previously described (Knijnenburg et al. 2005), and based on protocols optimised by the group of Dr. N. Carter (Carter et al. 2002; Fiegler et al. 2003; Tanke et al. 1999). Slides were scanned with a GenePix Personal

4100A scanner, and the spot intensities measured by the GenePix Pro 4.1 software (Axon Instruments, Westburg BV, Leusden, The Netherlands). Further analyses were performed using a home-made routine developed in Microsoft Excel 2000, and spots outside the 20% confidence interval of the average of the replicates were excluded from the analyses. Unbalances of the targets were determined based on log 2 ratios of the average of their replicates, and we considered sequences as amplified or deleted when outside the  $\pm 0.3$  range.

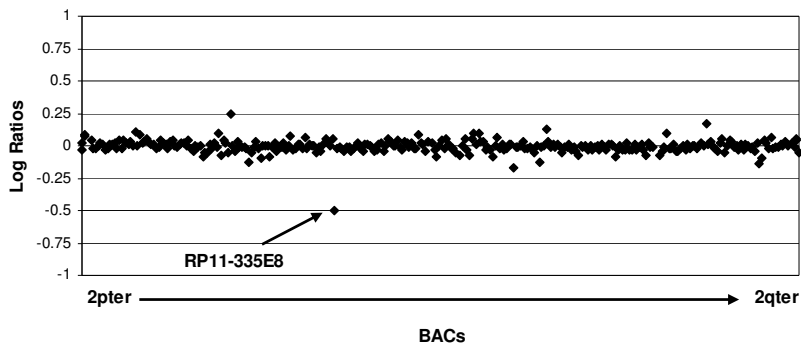


Fig. 2 Array CGH profile of chromosome 2. The graphic shows the log 2 values of the array CGH test/reference ratios for BACs in chromosome 2. The arrow shows the deleted BAC on 2p

## Results

G-banding analyses revealed a CCR, which involved a three-way translocation  $t(2;10;11)(q23;q22;q23)$ , plus an additional intrachromosomal rearrangement involving the translocated chromosome 2 and resulting in the insertion of segment 2p11.2-p15 into 2q22 (Fig. 1a). Karyotypic analysis of the parents was normal.

Multicolour FISH analyses confirmed the insertion of 2p material into the long arm of chromosome 2, and revealed that the exchange between chromosomes 2, 10 and 11 was more complex than a straight three-way translocation: in addition to the previously detected segment of 11q on the der (2) long arm, a small insertion of 10q material was also present (Fig. 1b-d).

Array CGH results revealed a cryptic deletion on 2p involving BAC RP11-335E8, whose localisation was cytogenetically compatible with the proximal breakpoint of the insertion (Fig. 2). To confirm this deletion and investigate how it related to the rearrangement, we hybridised both the deleted and its two flanking probes to metaphase spreads of the patient. One signal of each one of the three probes localised to the proximal short arm of the normal chromosome 2. In accordance with the array results, RP11-335E8 did not produce any other signal on 2p material. However, RP11-335E8 produced secondary signals on 11qter, which is typically seen in normal controls (data not shown): in the cells of this



patient, one of these secondary signals localised to the normal chromosome 11, and the other to the terminal region of der(2) (red-labelled, Fig. 1e). The two flanking probes hybridised also to the long arms of der(2), (RP11-1P9, green-labelled, proximal BAC) and der(11) (blue-labelled, distal BAC: RP11-535E19), respectively (Fig. 1e). Accordingly, the karyotype of the patient is currently regarded to be as follows (Mitelman 1995): 46,XY,der(2)(2pter → 2p15::2p11 → 2q22::10q22 → 10q23::2p13 → 2p15::11q23 → 11qter),der(10)(10pter → 10q22::2q22 → 2qter),der(11)(11pter → 11q23::2p12 → 2p12::10q23 → 10qter). Figure 3 shows a map of the rearranged region on chromosome 2p, the location of the FISH probes on chromosome 2, and indicates to which of the derivatives these probes map.

A representation of the rearranged chromosomes, and a proposed sequence of events invoking the minimum number of exchanges leading to this karyotype within a single cell cycle, are depicted in Fig. 4.

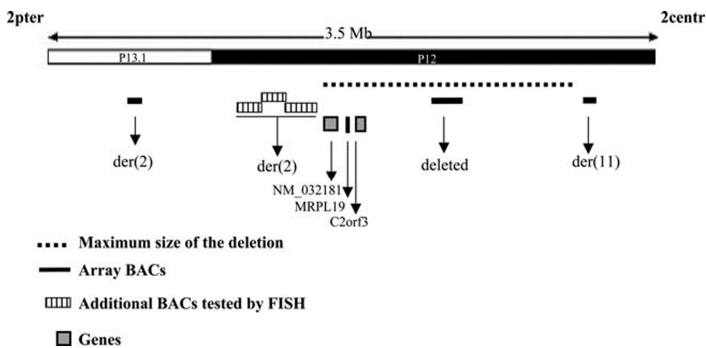


Fig. 3 Scheme of the rearranged region on 2p. The figure is a representation of the 2p region containing the deleted BAC and the breakpoint between der(2) and der(11). The figure shows the BACs present on the array, the additional BACs investigated by FISH, the maximum possible size of the deletion, and the genes that might be absent

## Discussion

Initially, the complex rearrangement described here was interpreted as balanced, and derived from a three-way translocation between chromosomes 2, 10 and 11, and an insertion from the short into the long arm of der(2). However, 48-colour chromosome arm painting revealed that the rearrangement was far more complex than originally estimated, involving a larger number of breaks. Furthermore, array CGH analyses showed a cryptic deletion associated with one of the breakpoints. Given the ~1 Mb resolution of the array used, the occurrence of further imbalances of small size cannot be excluded. Until recently, the detection of imbalances associated with apparently balanced chromosome rearrangements depended on sequencing across the breakpoints, and have only sporadically been described (Borg et al. 2002; Kumar et al. 1998). The

combination of array CGH and FISH, as described here, enormously facilitates such analysis.

It is unclear how the phenotype of our patient relates to his chromosome rearrangements, but it is likely that either the 2p deletion, or one of the many breakpoints, or a combination of these, had a role in this. None of the breakpoints present in our patient are compatible with gene locations believed to be involved in attention deficit-hyperactivity disorder (ADHD), except maybe ADRA2 (10q25.2) (<http://www.ensembl.org/>), which is relatively close to the cytogenetically estimated breakpoints on 10q (10q22 and 10q23). The BAC on 2p that is deleted in our patient does not contain any identified gene, but the surrounding area that might also be deleted (in between the two adjacent BACs) includes a number of genes (<http://www.ensembl.org/>). We showed by FISH that the majority of them map to the der(2) (Fig. 3), and only few of them map to the possibly deleted area, namely NM\_032181, MRPL19 and C2orf3. NM\_032181 is a gene of unknown function, but C2orf3 is known to be a regulator involved in transcription repression (Johnson et al. 1992), while MRPL19 codes for the 60S ribosomal mitochondrial protein L19 (<http://www.ensembl.org/>). Although the phenotypic effect of hemizyosity for these genes is unknown, the gene functions are broad enough to consider their possible involvement in the abnormal phenotype of the patient.

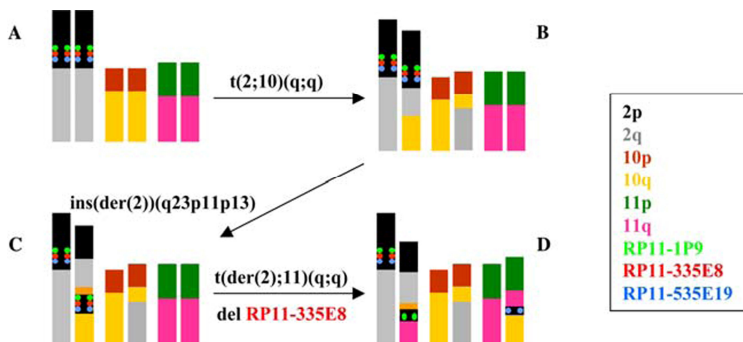


Fig. 4a-d Proposed model for the formation of this complex chromosome rearrangement. The proposed sequence of events invokes a minimum number of exchanges leading to the reported rearrangement. a-d Each of the steps in temporal order, from the normal karyotype (a) to the observed configuration of the rearranged chromosomes after G-banding, multicolour FISH and array CGH analysis (d). For the present model, we have to assume that the recombination events have occurred in a chronological order within the span of a single cell division

The sequence of events that gave rise to the CCR in our patient is not easy to determine. We propose a chronological sequence in Fig. 3 that basically involves two translocations and one insertion, which appears to be the minimum number of events required for such rearrangement. It is reasonable to conceive that a “catastrophic” event simultaneously producing multiple chromosome breakages gave rise to this and other CCRs. A complication of our model for the present

rearrangement, is that the events proposed must have occurred sequentially; i.e. first, a translocation, followed by an insertion, and finally by another translocation, in which a segment would be lost in the breakpoint region. In cancer, complex chromosome rearrangements are assumed to result from alterations accumulated during many cell divisions. However, we found no evidence of the presence of different clones containing precursor karyotypes in our patient, suggesting that his CCR originated either at gametogenesis or at fertilisation. Because of the absence of detectable mosaicism, we are forced to assume that the recombination events have probably occurred in a temporal order within the span of a single cell division.

The present study illustrates that array CGH, combined with other molecular cytogenetic methodologies, will not only improve the description of rearranged chromosomes, but also challenge our interpretation of their mechanisms of origin.

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## Chapter 6

# Ring chromosome formation as a novel escape mechanism in patients with inverted duplication and terminal deletion.

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## Ring chromosome formation as a novel escape mechanism in patients with inverted duplication and terminal deletion

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Keywords: ring chromosome, array-CGH, inverted duplication

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### Abstract

Ring chromosomes are rare cytogenetic findings and are associated at phenotypic level with mental retardation and congenital abnormalities. Features specific for ring chromosome syndromes often overlap with the features of terminal deletions for the corresponding chromosomes.

Here we report a case of a ring chromosome 14 which was identified by conventional cytogenetics and shown to have a terminal deletion and an additional inverted duplication with a triplication by using large insert clone and oligo array-Comparative Genomic Hybridization (array-CGH), FISH and Multiplex Ligation-dependent Probe Amplification (MLPA).

The combination of an inverted duplication with a terminal deletion in a ring chromosome is of special interest for the described syndromes of chromosome 14. The presented findings might explain partly overlapping clinical features described in terminal deletion, duplication and ring chromosome 14 cases, since these rearrangements can be easily overlooked when performing GTG-banding only.

Furthermore, we suggest that ring chromosome formation can act as an alternative chromosome rescue next to telomere healing and capture, particularly for acrocentric chromosomes.

To our knowledge this is the first time an inverted duplication with a terminal deletion in a ring chromosome is identified and characterized using high resolution molecular karyotyping. Systematic evaluation of ring chromosomes by array-CGH might be especially useful in distinguishing cases with a duplication/deletion from those with a deletion only.

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## Introduction

Ring chromosomes are uncommon findings in pre- and postnatal cytogenetics. Inheritance of ring chromosomes has been reported<sup>1-3</sup> but the majority of ring chromosomes are *de novo*. They have been reported for all human chromosomes and are known to cause multiple congenital anomalies and mental retardation. Ring chromosomes are generally believed to result from distal breakage of the short and long arm of a chromosome and rejoining of the ends.<sup>4</sup> There are also reports with no apparent deletion of the telomeric ends, thus resulting in complete ring chromosomes.<sup>5</sup> Patients with ring chromosomes often exhibit a general overlap in phenotype, which has coined the term “ring syndrome”.<sup>6</sup> Growth failure with no or minor anomalies is found to be the major abnormality in patients with complete ring chromosomes and is thought to be the result of cell death during development. Indeed, sister chromatid exchange during the cell cycle may cause mechanical interference of the cell division because of disruption, breakage, entangling or doubling of rings, resulting in aneuploidy and possible death of the daughter cells. In a study of 207 patients carrying a ring chromosome,<sup>6</sup> one fifth showed to be affected with this general ring syndrome. When both telomeric ends and coding sequences are deleted, the phenotype of the patient is in general more severe, often with specific characteristics related to the chromosome involved.

Here we report the characterization of a ring chromosome 14 containing a terminal deletion and an inverted duplication with a triplication by using molecular cytogenetic tools such as array-CGH, MLPA and FISH. The existence of a duplication with a terminal deletion in a ring chromosome similar to other duplication/deletion cases<sup>7,8</sup> might have clinical consequences in patients with ring chromosome 14 syndrome. This finding might explain the overlapping clinical features in patients with a ring chromosome 14 compared to patients with a terminal duplication of chromosome 14, since existing duplications in ring chromosomes can be easily overlooked at the cytogenetic level. Accordingly the large overlap in clinical features between published patients with a distal duplication and those with a distal deletion suggests that some duplication patients have an accompanying distal deletion, similar to the patient discussed in Chen et al.<sup>9</sup>

Here we show the importance of using combined molecular cytogenetic techniques in the characterization of chromosomal alterations, in particular in patients with ring chromosomes.

## Case report

The proband is an 8-year-old girl of healthy, non-consanguineous parents. She has two sisters and two brothers, all normal. Ultrasound examination at 34 weeks of gestation showed generalized growth retardation and one umbilical artery. Birth

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was at 37 weeks, birth weight was 1970 gram (p30), head circumference was 30.5 cm (<p3).

Examination at 4 months revealed craniofacial dysmorphisms namely hypertelorism, upturned nose, broad nasal bridge, malformed helices and mild micrognathia. A single palmar crease bilaterally and abnormally implanted toes were noted. At 9 months a patent ductus arteriosus was surgically closed. At present she is severely developmentally delayed. She is microcephalic (<p3), has short stature (<p3) and low weight (<p3). Growth hormone (GH) and Insulin-like Growth Factor 1 (IGF1) levels were normal. She suffers from recurrent upper and lower airway infections, eczema, scoliosis and retinitis pigmentosa. She has hypogammaglobulinemia (IgG/M and A) and normal numbers of peripheral B lymphocytes. She has sleeping difficulties, poor feeding and seizures. Informed consent was obtained from parents and patient according to routine LUMC procedure.

### Materials and methods

Conventional cytogenetic analysis on GTG-banded chromosomes from cultured lymphocytes of the patient and the parents was performed according to standard techniques. From the proband and the parents 100 and 50 cells were analyzed respectively.

Fluorescence in situ hybridization (FISH) was performed according to standard protocols on metaphase chromosomes or interphase nuclei of the proband, using Cy5-ULS or D-Green-ULS labeled Whole Chromosome Painting probe (WCP) #14 (Kreatech biotechnology, Amsterdam, the Netherlands), half-YAC clone yRM2006 and Vysis® LSI® IGH/CCND1 combined probe (for 11q13 and 14q32.33, respectively) (Abbott Molecular, Hoofddorp, the Netherlands). Three BAC clones that mapped at 14q32.12 and two at 14q32.33, namely RP11-258D14, RP11-489D22, RP11-371E8, RP11-73M18 and RP11-417P24 respectively (table 1), labeled with Cy3-dUTP (GE Healthcare, Diegem, Belgium) or FITC-dUTP (Roche diagnostics, Almere, the Netherlands) were used for further confirmations.

Array-CGH was performed using ~1.0 Mb spaced whole genome large insert clone arrays, which were made available by the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk>). The clones were grown, amplified and spotted as previously described.<sup>10,11</sup> Genomic DNA of the patient was isolated using standard techniques, and 500 ng was labeled with Cy3-dCTP (GE Healthcare, Diegem, Belgium) using the BioPrime® DNA Labeling System (Invitrogen, Breda, the Netherlands). As a reference DNA, 500 ng female human genomic DNA (Promega, Leiden, the Netherlands) was labeled using Cy5-dCTP. Hybridization and slide washing was performed without prehybridization on a HS400 hybridization station (Tecan, Giessen, the Netherlands). The arrays were scanned with a GenePix 4100A scanner (Axon Instruments, Union City, CA) and the images were processed using GenePix Pro 4.1 software. Final analysis of the intensity ratios of the

hybridized DNA was done using Microsoft Excel according to published standards.<sup>10</sup>

For further high resolution analysis of duplications and deletions, oligo array-CGH was performed using the Agilent Human Genome CGH Microarray Kit 244K (Agilent, Amstelveen, the Netherlands) according to the manufacturer's instructions following protocol version 4.0. Data analysis was performed with the CGH Analytics 3.4 software platform (Agilent, Amstelveen, the Netherlands).

MLPA was performed as described by White et al.<sup>12</sup> The selected probes were ordered from Invitrogen (Breda, the Netherlands), sequences are available as online supplementary data. Quantitative readout was done using the ABI 3730

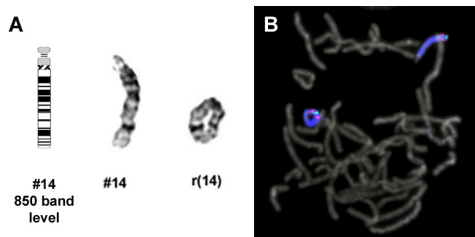


Figure 1: The ring chromosome 14 of the patient and its normal homolog. (a) GTG-banded images (b). FISH applied to a metaphase cell with BAC RP11-73M18 (red), BAC RP11-371E8 (green) and whole chromosome paint for chromosome 14 (blue), showing the inverted duplication in the ring chromosome.

DNA analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). The accompanying Genescan 3.5 software was used for peak analysis and further downstream normalization and calculations were performed in Microsoft Excel as described before<sup>12</sup>.

Quantitative fluorescent polymerase chain reaction (QF-PCR) was performed using polymorphic short tandem repeat (STR) markers for allelotyping. Markers were chosen to cover the distal part of chromosome 14q, from 14q24.2 to 14qter.<sup>9</sup>

Table 1: Genomic location of BAC clones used in confirmatory FISH and genomic location of oligos around the breakpoints found with oligo array-CGH.

<i>BAC clone</i>	<i>Locus</i>	<i>Region<sup>a</sup></i>	<i>Involvement</i>
RP11-258D14	14q32.12	92079320-92288476	in proximal side of triplication
RP11-489D22	14q32.12	92428426-92630687	in distal side of triplication
RP11-371E8	14q32.13	92565641-92758891	in proximal side of duplication
RP11-73M18	14q32.33	103217347-103382885	in distal side of duplication
RP11-417P24	14q32.33	105267358-105437117	in proximal side of deletion
<i>Oligo</i>	<i>Locus</i>	<i>Region<sup>a</sup></i>	<i>Involvement</i>
A_14_P101212	14q32.12	91679319-91679378	in proximal side of triplication
A_16_P02972545	14q32.12	92360710-92360769	in distal side of triplication
A_14_P109278	14q32.12	92371629-92371688	in proximal side of duplication
A_16_P02989318	14q32.33	103716114-103716166	in distal side of duplication
A_16_P02989342	14q32.33	103727207-103727260	in proximal side of deletion

<sup>a</sup>according to Ensembl v42 database, [http://www.ensembl.org/homo\\_sapiens/index.html](http://www.ensembl.org/homo_sapiens/index.html)

## Results

Cytogenetic analysis resulted in a ring chromosome 14 in 95 of the 100 analyzed cells from the patient (figure 1a) while the remaining 5 cells showed a complex rearrangement involving endoreduplication of the ring chromosome. Both parents presented a normal karyotype. FISH using the yRM2006 probe demonstrated the absence of the subtelomeric region of 14q in the ring chromosome. In a later stage, when the proband presented with recurrent infections, the deletion was shown to encompass the IgH gene using the LSI<sup>®</sup> IGH/CCND1 combined probe, which is located at 14q32.33 at about 1 Mb from the telomere (data not shown). Array-CGH was performed to further map the deletion. The result revealed an additional duplication of 14q32.12 to 14q32.32 (figure 2) next to the already detected deletion of 14q32.33 to 14qter. The size of the duplication was found to be 10.8 to 13.5 Mb, from BAC clone RP11-73M18 to RP11-371E8, whereas the size of the deletion was 1.1 to 3.0 Mb, from BAC clone RP11-417P24 to CTC-820M16. These findings were confirmed by FISH, whereby the duplicated clones were chosen most proximal (RP11-73M18) and distal (RP11-371E8) to the centromere. The FISH showed the duplication to be inverted (figure 1b). BAC clone RP11-417P24, located within the deleted area, was used to confirm the deletion (not shown). FISH using the same BAC probe sets showed no alteration (gain, loss or inversion) in any of the parents.

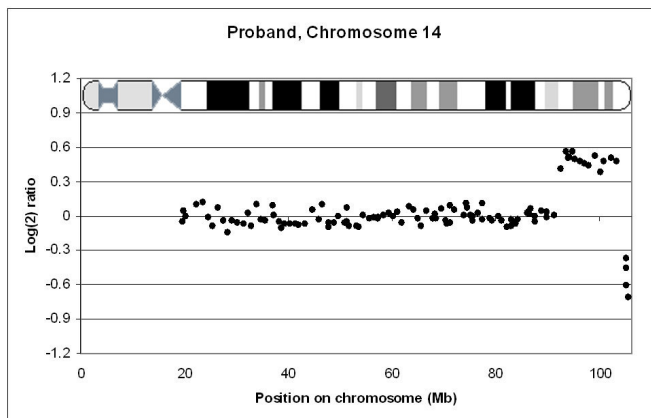


Figure 2: Array-CGH log<sub>2</sub> ratio plot of chromosome 14 of the proband at about 1 Mb resolution, showing a distal duplication and a terminal deletion of chromosome 14 material. The dotted horizontal line at -0.3 and 0.3 represents the threshold line for deletions and duplications, respectively.

Analysis with polymorphic markers that map to the deletion, duplication and a normal part of the chromosome, proved the duplication to be intrachromosomal. The marker analysis showed a duplication of one allele of markers D14S557 and D14S543 (Table 2). Parental analysis revealed that the ring chromosome is of paternal origin.

Additional high resolution oligo array-CGH confirmed the previous findings, the size of the amplified region was estimated to be 11.344 Mb and the size of the deletion was estimated to be 2.641 Mb. The genomic locations of the oligos

Table 2: Location of short tandem repeat (STR) markers and genotypic results in the proband and her parents.

Markers	Locus	Region <sup>a</sup>	mother	father	proband
D14S620	14q24.2	72401116-72401231	114,114	114,118	114,118
D14S739	14q31.1	81336367-81336556	184,184	176,184	176,184
D14S616	14q31.3	84263655-84263874	211,215	223,223	211,223
D14S128	14q31.3	85450372-85450704	336,363	332,344	332,336
D14S617	14q32.12	91272543-91272683	139,161	161,165	139,165
D14S557	14q32.32	102189847-102190162	304,320	275,287	287,287,304
D14S543	14q32.33	103658598-103658852	244,252	241,249	249,249,252

<sup>a</sup> according to Ensembl v42 database, [http://www.ensembl.org/homo\\_sapiens/index.html](http://www.ensembl.org/homo_sapiens/index.html)

around the breakpoint are found in table 1. Interestingly, this assay revealed a triplicated 681 kb region in the proximal duplication region, belonging to band 14q32.12 (figure 3). A confirmatory MLPA test using probes designed for all altered regions (triplicated, duplicated, deleted and normal) proved the presence of all alterations, including the triplication of the region in band 14q32.12 (figure 4) and it showed that the parents of the proband have a normal copy number for all tested regions. FISH using BAC's RP11-258D14 and RP11-489D22 showed that the triplicated region is located within the ring, at the distal side of the rearranged q-arm (not shown).

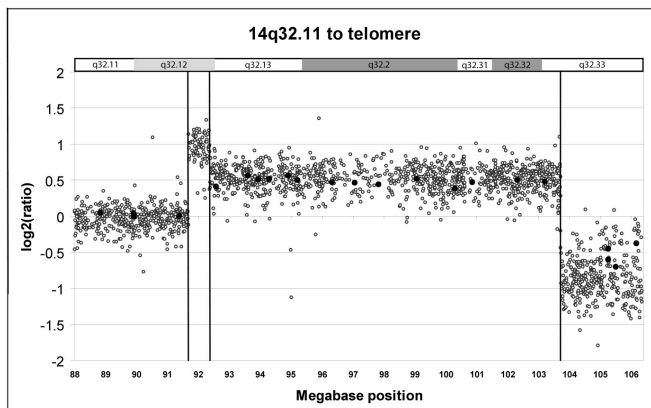


Figure 3: A combined display of the oligo array-CGH (open circles) and the  $\pm 1$  Mb resolution large insert clone array-CGH (closed circles), log<sub>2</sub> ratio plots of the chromosome 14q32.11-qter region of both experiments on the proband are shown. The oligo array shows a triplication, a duplication and a deletion of the distal region of chromosome 14. The clones of the 1 Mb array fall just adjacent to the triplication.

## Discussion

Using the combination of FISH and array-CGH in genetic analysis has demonstrated that seemingly simple rearrangements are often more complex than defined by GTG-banding.<sup>10,13,14</sup> After conventional cytogenetic screening (figure 1a) the karyotype of the proband was described as 46,XX,r(14)(p12q32). To further map the breakpoint, array-CGH was performed with a resolution of about 1 Mb (figure 2). Next to the expected deletion of band 14q32.33 an additional duplication of 14q32.12 to 14q32.32 was revealed. FISH using the first

and the last duplicated BAC clone confirmed this finding and showed the duplication to be inverted (figure 1b), which originated from one paternal chromosome based on STR-allelotyping (table 1).

An inverted duplication associated with a terminal deletion was first described for distal chromosome 8p,<sup>7,8</sup> followed by 1q,<sup>15</sup> 4q,<sup>16</sup> 15q,<sup>17</sup> and, more recently, also for chromosome 14q.<sup>9</sup> A mechanism for the formation of inverted duplications associated with terminal deletions has been described both for intrachromosomal duplications, based on normal parental chromosomes<sup>18</sup> and for intra- and interchromosomal duplications based on a parental inversion carrier.<sup>19</sup> These proposed mechanisms assume that an intrachromosomal U-type recombination during meiosis I, or a loop formation combined with one or two recombination events between homologous alleles, has occurred. The consequential recombinant dicentric chromosome would be deleted beyond the distal recombination site. At meiosis II, the two linked chromatids can segregate to the opposite poles resulting in a breakage between the two centromeres. If this breakage occurs asymmetrically, the two resulting recombinant products are a derivative with an inverted duplication and a deleted 14q derivative.

When chromosomal rearrangements take place, either constitutional or tumorigenic, broken chromosome ends need to be stabilized to prevent end-to-end fusions and exonucleolytic degradation. Telomere healing can be accomplished by addition of human telomeric tandem repeat sequence to broken chromosome ends<sup>20</sup> or by telomere capture, which in fact is actually subtelomeric translocation to the broken chromosome end, resulting in an extra duplicated subtelomeric region. This latter mechanism is proven in melanoma and other cancer cell lines and in irradiated lymphoblastoid and fibroblast cells.<sup>21</sup> Examples of constitutional telomere capture are less common and is only reported a few times.<sup>22,23</sup> Among the several inverted duplication/deletion events reported,<sup>7-9,15-19,23</sup> in a single case the broken chromosome was proven to be repaired with telomere capture.<sup>23</sup>

On the contrary, in our case the healing of the broken end may have been mediated by ring formation. In an acrocentric chromosome, an additional break involving the short arm probably does not lead to an additional loss of coding sequence and consequently to impaired cell proliferation. The fact that ring chromosome formation in 47% of the reported cases<sup>6</sup> involves acrocentric chromosomes, supports this notion.

If the recombination were based on non allelic homologous recombination (NAHR), then a fragment with a normal copy number would still be present between two recombined low copy repeats, between the duplicated and the deleted region at 14q32.32-14q32.33. Since, neither the 1 Mb BAC array nor the STR-allelotyping was conclusive to locate a possible fragment with a normal copy number between the duplicated and deleted region, an oligo array was performed. The 244k Agilent oligo array platform was chosen because between the distal duplicated and proximal deleted BAC clones there were 271 oligo

reporters available, while for example in the 500k SNP based chips from Affymetrix 120 SNP elements were present with an uneven coverage of the region of interest, including some gaps of 150 kb.

Table 3: Comparison of the clinical features in published cases with distal chromosome 14 alterations and the presented case.

	Van Karnebeek et al. <sup>25</sup>				Chen et al. <sup>9a</sup>		Our proband	
	Linear 14q deletions q32-distal	Ring 14 deletions (in %)	Ring 14 deletions q32-distal	Ring 14 deletions (in %)	14q duplications q31-qter	Inv dup/del case	Inv dup/del ring case	
No. of cases	12		20		9			
Gender (males)	3		8		5			
Mental retardation	11/11	100	20/20	100	7/7	100	+	
Seizures	1/10	10	19/20	95			+	
Hypotonia	8/12	67	11/19	58	5/9	56	+	
Prenatal growth retardation	5/12	42	7/16	44			+	
Postnatal growth retardation	5/12	42	9/17	53	7/8	88	+	
Microcephaly	4/12	33	14/18	78	6/9	67	+	
Dolichocephaly	3/8	38	5/12	42				
High forehead	10/10	100	7/12	58				
Prominent forehead	7/10	70	9/12	75				
Hypertelorism	3/10	30	7/15	47	7/9	78	+	
Strabismus	3/9	33	4/15	27	1/7	14	-	
Blepharophimosis	6/10	60	10/13	77	0/7	0	+	
Ptosis	4/11	36	2/14	14	0/7	0	-	
Downslanting palpebral fissures	6/11	55	8/13	62	7/9	78	+	
Epi-/telecanthi	8/12	67	12/17	71	2/9	22	+	
Broad/flat nasal bridge	10/11	91	10/13	77			+	
Anteverted nares	2/9	22	5/12	42			+	
Dysmorphic nose					6/9	67	+	
Short bulbous nose	5/10	50	6/11	55			-	
Long philtrum	4/9	44	4/11	36			-	
Broad philtrum	8/10	80	8/11	73			-	
Thin upper lip	6/8	75	5/11	45				
Small, fish shaped mouth	6/10	60	6/13	46			-	
Highly arched palate	9/10	90	7/13	54			-	
Abnormal dentition	2/6	33	2/10	20				
Low set ears	3/9	33	7/8	88			-	
Malformed helices	5/8	63	3/8	38	7/9	78	+	
Micrognathia	6/11	55	5/12	42	8/9	89	-	
Pointed chin	4/10	40	1/12	8			-	
Short neck	1/6	17	7/14	50				
Webbed neck	2/6	33	1/11	9			-	
Congenital heart defect	3/9	33	1/15	7	6/9	67	+	
Single palmar crease	6/7	86	1/15	7			+	
Brachydactyly	0/3	0	3/9	33			-	
Clinodactyly	2/7	29	1/8	13	7/9	78	+	
Tapering fingers/arachnodactyly	2/5	40	2/9	22			-	
Retinitis pigmentosa	0/5	0	8/16	50			+	
Scoliosis							+	

<sup>a</sup> and references herein.

Despite the fact that the oligo array did not detect a region of normal copy number between the duplicated and the deleted fragment, the region of the distal breakpoint could be narrowed down to ~11 kb.

There was no evidence for the presence of LCR's in this interval (Ensembl, release 42), but this region does encompass a 2.8 kb chained self alignment fragment having homology within its own region. Possibly this region can cause hairpin or loop formation and can mediate recombination or a double strand break, eventually leading to the U-type of translocation.

Moreover, the oligo array showed an extra copy number gain of the first 600 kb of the duplicated region (figure 3), at 14q32.12, which was confirmed with MLPA (figure 4). Based on FISH results with a BAC probe from this region, it was found that the extra 600 kb fragment is inserted at the distal side of the chromosome, the part that is fused to the p-arm side of the centromere. In complex chromosome rearrangements, often more breaks are found than actually needed to explain the rearrangements on cytogenetic level.<sup>10,13,14</sup> The extra amplification of this fragment at the distal side of the chromosome is probably a secondary event, following the inverted duplication and the distal break of the dicentric chromosome, illustrating the struggle to rescue the chromosome. The finding of this additional triplication stresses the importance of introducing high resolution techniques in investigating genetic aberrations in patients. Due to the large overlap in the clinical features of cases with rearrangements within the distal region of chromosome 14 (table 3), ring chromosome 14, 14q deletion, and chromosome 14 distal duplication syndromes should be discussed together.

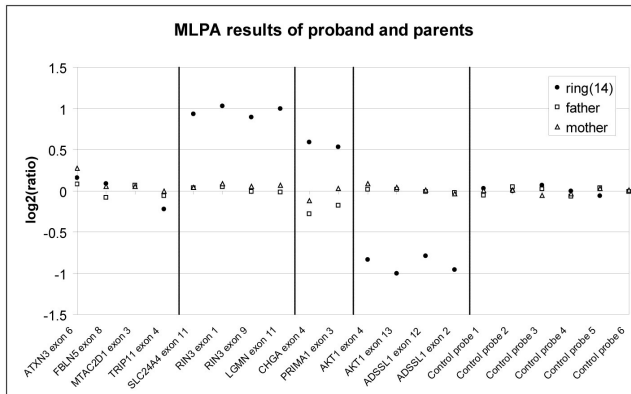


Figure 4: Log<sub>2</sub> ratio plot of the confirmatory MLPA. The proband (closed circles) shows triplication, duplication and deletion of the expected probes. Both parents show no copy number change in the tested regions. Three control probes were tested in duplo (control probe 1-6) and are located in the EXT1 gene on chromosome 8q24.11, in the CREBBP gene on 16p13.3 and in the p300 gene on chromosome 22q13.2.

The ring chromosome 14 syndrome was delineated by Schmidt et al.,<sup>24</sup> and it was further described by van Karnebeek et al.<sup>25</sup> A distal duplication of chromosome 14 results in a variable clinical picture that is mainly depending on the size of the duplication.<sup>9,26</sup> Main features that are found in distal duplications of 14q31→qter are mental and growth retardation, microcephaly, hypertelorism, abnormal ears, micrognathia and congenital heart defects. In the case of Chen et al., an additional terminal deletion was demonstrated using FISH, next to the duplication. The small terminal deletion, which in itself can lead to a severe phenotype,<sup>25</sup> or an additional duplication in a ring chromosome, is sometimes only detectable by molecular techniques such as FISH and array-CGH. It emphasizes the significance of high resolution molecular karyotyping for the establishment of accurate phenotype/genotype correlations.

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Supplementary information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)

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## Chapter 7

# **Molecular and clinical characterization of *de novo* and familial causes with microduplication 3q29: guidelines for copy number variation case reporting.**

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## Molecular and clinical characterization of *de novo* and familial cases with microduplication 3q29: guidelines for copy number variation case reporting

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### ABSTRACT

Microdeletions of 3q29 have previously been reported, but the postulated reciprocal microduplication has only recently been observed. Here, cases from four families, two ascertained in Toronto (Canada) and one each from Edinburgh (UK) and Leiden (Netherlands), carrying microduplications of 3q29 are presented. These families have been characterized by cytogenetic and molecular techniques, and all individuals have been further characterized with genome-wide, high density single nucleotide polymorphism (SNP) arrays run at a single centre (The Centre for Applied Genomics, Toronto). In addition to polymorphic copy-number variants (CNV), all carry duplications of 3q29 ranging in size from 1.9 to 2.4 Mbp, encompassing multiple genes and defining a minimum region of overlap of about 1.6 Mbp bounded by clusters of segmental duplications that is remarkably similar in location to previously reported 3q29 microdeletions.

Consistent with other reports, the phenotype is variable, although developmental delay and significant ophthalmological findings were recurrent, suggesting that dosage sensitivity of genes located within 3q29 is important for eye and CNS development. We also consider CNVs found elsewhere in the genome for their contribution to the phenotype. We conclude by providing preliminary guidelines for management and anticipatory care of families with this microduplication, thereby establishing a standard for CNV reporting.

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## INTRODUCTION

Contiguous gene syndromes involving small chromosomal duplications are typically less frequently reported in comparison to their microdeletion counterparts. Although these rearrangements can both arise from a common mechanism involving non allelic homologous recombination with regionspecific low copy repeats (Lupski 2004), microduplication syndromes are usually less commonly recognized, possibly due to ascertainment bias, milder and more variable phenotype, and technical limitations of cytogenetics and fluorescent in situ hybridization (FISH). Well characterized chromosomal regions shown to involve these reciprocal duplication and deletion events include duplication of 17p11.2 causing a phenotype associated with moderate mental retardation and behavioural disturbances (Potocki et al. 2000), with the reciprocal microdeletion resulting in Smith-Magenis syndrome; microduplication of 22q11.2 (Ensenauer et al. 2003) having a somewhat variable phenotype with cardiac malformation and features similar to the classical microdeletion 22q11.2 syndrome; microduplication of 15q11-q13 characterized by developmental delay and autism, reciprocal to deletions causing Prader-Willi/Angelman syndromes (Dimitropoulos and Schultz 2007), and microduplication of 7q11.23, which has been related to severe expressive language delay (Somerville et al. 2005; Orellana et al. 2008; Torneiro et al. 2008; Merritt and Lindor 2008), while the corresponding deletion causes Williams-Beuren syndrome (Osborne et al. 1996). Most recently, copy number variations (CNVs) in the form of microdeletions and microduplications of chromosome 16p11.2 have also been observed in autism spectrum disorder (Kumar et al. 2008; Marshall et al. 2008; Weiss et al. 2008).

With the use of microarray-based techniques, increasing numbers of novel copy number variants are being discovered both in apparently healthy control individuals (Redon et al. 2006; Pinto et al. 2007), and in patients with genetic disorders such as autism (Autism Genome Project Consortium 2007; Sebat et al. 2007; Marshall et al. 2008) and schizophrenia (Walsh et al. 2008; Xu et al. 2008). Improved resolution of these microarray platforms is resulting in greater power to detect ever smaller events, well below the level of resolution of conventional cytogenetic examination (Feuk et al. 2006; Carter 2007).

A microdeletion syndrome on chromosome 3q29 was originally described in six patients (Willatt et al. 2005). The common phenotypic features included a long narrow face, short philtrum, high nasal bridge, developmental and significant speech delay. The microdeletion was approximately 1.5 Mb in length and was between identical low copy repeat sequences on either side of the deletion breakpoints. This suggests that this region is susceptible to nonallelic homologous recombination, which could result in reciprocal exchange events at chromosome 3q29. Two recent reports describe the apparent reciprocal microduplication event: the first, in the heterozygous state in five individuals of a three-generation pedigree (Lisi et al. 2008), and the second including 19 cases, five of which appear to be the reciprocal event with the remainder overlapping this region (Ballif et al. 2008). Here, we describe index cases from four pedigrees (Case 1 apparently de novo, Case 2 a mother-child inheritance, Case 3 a nuclear family with multiple members carrying the duplication, and Case 4 an adopted

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child from whom information about the biological parents is unavailable). These cases all have microduplication of chromosome 3q29, validated by fluorescent in situ hybridization (FISH), array-CGH, MLPA and/or high-resolution DNA SNP microarrays. Regardless of the initial discovery and validation techniques, we have also analyzed these individuals with genome-wide Affymetrix 500k SNP arrays in order to provide fine-map duplication breakpoints and ascertain other CNV events in their genomes. The clinical phenotypes of these patients are described in detail. Of interest, two have significant ophthalmological findings and developmental delay was frequent, suggesting that dosage sensitivity of genes located within 3q29 might be important for eye and cognitive development.

## CLINICAL REPORT

### Case 1 (Toronto):

This patient is a 23 month old girl (Figure 1a), who was born to healthy, non-consanguineous parents. The family history was negative for congenital anomalies (See pedigree, Figure 2a). The pregnancy was complicated by hyperemesis for the first five months and hypertension for the last two weeks. There were no known teratogenic exposures. Fetal ultrasounds at 9 and 20 weeks of gestation were reportedly normal. The patient was born at 36 weeks gestation via spontaneous vaginal delivery. Labour and delivery were uncomplicated with no neonatal resuscitation required. Apgars were nine at one and five minutes. The birth weight was 2,580g (50th-75th centile), length was 50 cm (90th centile), and head circumference was 31.5 cm (25th centile). Multiple congenital anomalies noted at birth included a large anterior fontanel, a high forehead with bitemporal narrowing, a downslanting right palpebral fissure, simple low-set ears, a broad nasal root and slitlike nares, a deeply grooved philtrum, thin upper lip and short neck with redundant nuchal skin (Figure 1a). She had a U-shaped cleft of the secondary palate. Extensive ophthalmologic abnormalities included bilateral microphthalmia, a right iris coloboma, right corneal clouding consistent with a Peter's anomaly, and a cataract of the left eye. There was a 2 cm umbilical hernia. The anus was simple and anteriorly displaced. An abdominal ultrasound revealed a cyst of unknown etiology located at the right crest of the diaphragm. Examination of the extremities revealed partial 2-3 toe syndactyly bilaterally, sandle-gap bilaterally, and camptodactyly of the toes. A skeletal survey in the newborn period revealed bilateral proximal radial-ulnar synostosis. MRI of the brain at birth revealed absence of the inferior cerebellar vermis with an enlarged cisterna magna, consistent with a Dandy-Walker variant. There were also multiple small cystic changes of the periventricular white matter within the frontal horns of the lateral ventricles. An echocardiogram at birth was reported as normal; however re-evaluation at approximately one month of age for a persistent murmur revealed an 8 mm secundum atrial septal defect with left to right shunting, which has remained asymptomatic since birth. Abdominal ultrasound at 5 weeks of age further defined her abdominal cyst as arising from the stomach wall and wrapping around the inferior vena cava. The cyst was resected and she had an unsuccessful attempt at umbilical hernia

repair. She has had severe gastroesophageal disease and feeding difficulties since birth, requiring multiple high dose antireflux medications. Conductive hearing loss was detected at 6 months of age and required the insertion of myringotomy tubes. She underwent a right corneal transplant and left cataract excision at 4 months of age. With the use of a contact lens in the left eye, her visual acuity was 20/190 in the left eye and 20/960 in the right eye. A 2.7x2.8 cm subcutaneous mass was noted on the posterior right thigh. CT scan of the mass suggested that it was likely a hemangioma. No medical intervention was required. Growth parameters at 8 months of age revealed weight less than the 3rd centile, length at the 25th centile, and head circumference just below the 50th centile. Her physical features, including microphthalmia, were similar to her newborn exam. There was central hypotonia. At 18 months of age, the patient's first tooth erupted. Tooth shape was normal. A repeat attempt at surgical repair of the umbilical hernia and her extensive diastasis recti was successful at 20 months of age.

Developmental concerns were noted in the first year of life as she had significant hypotonia and visual impairment. She was smiling at 3 months of age, reaching and grasping at 8 months of age. She began rolling over at eight months. Following her surgery at 20 months, she began to sit independently, crawl and stand with support. She had a formal communication assessment at 15 months of age which indicated that her receptive language abilities were in the 7-9 month old range, and her expressive skills were in the 5 month old range. She was babbling at 23 months but did not yet have specific words. She receives occupational therapy, speech therapy, and is enrolled in an infant development program.

**Case 2 (Edinburgh):**

This girl was the first child of non-consanguineous parents. She was born by spontaneous vaginal delivery weighing 3,080g (12th percentile) at 41 weeks of gestation. An increased nuchal translucency was noted during the pregnancy but no invasive testing or detailed ultrasound examination was carried out. She was noted to be hypotonic soon after birth and was admitted to the neonatal intensive care unit. A cardiac ultrasound demonstrated an atrioventricular septal defect. She was thought to have facial dysmorphism compatible with a diagnosis of Down syndrome but chromosome analysis revealed a 46,XX apparently normal female karyotype. At this point she was reviewed by a clinical geneticist (DRF) who noted significant craniofacial dysmorphisms including upslanting palpebral fissures, large anterior fontanelle, brachycephaly, hypoplastic supraorbital ridges and a depressed nasal root (Figure 1b). Her eye examination was remarkable with a left sided iris "coloboma" caused by segmental aniridia with no evidence of an optic fissure closure defect. Her occipito-frontal circumference at 1 week of age was 34cm (25th percentile). She had minor digital dysmorphisms with 5th finger clinodactyly and mild syndactyly of the 2nd and 3rd toes on the left foot. She had unusual buttock folds. At this point she had further investigations including FISH for deletion 22q11.2 and Smith-Magenis syndrome, a full skeletal survey, a diasiallotransferrin assay for congenital disorders of glycosylation, and quantitative plasma amino acid urinary organic acids analysis, all of which were

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normal. She had an emergency admission for four different infective episodes during the first five months of life: bronchiolitis, adenovirus pneumonia, pneumococcal conjunctivitis and *Clostridium difficile*. At the age of seven months she had an elective repair of her AVSD and a secundum atrial septal defect. She had a prolonged recovery in intensive care and required continuous inotropic support for 38 days following the operation.



Figure 1. Clinical presentation photographs. Consent for publication was obtained in all cases. a) Case 1, ascertained in Toronto, Canada (newborn, left panel; at age 23 months, right panel). b) Case 2, ascertained in Edinburgh, UK. Detail of left eye abnormalities shown (lower panel). c) Case 3, ascertained in Leiden, The Netherlands. d) Case 4, ascertained in Toronto, Canada, at age 11 (top panels). Details of ears are shown (bottom panels).

She had a Griffiths assessment at the age of 10 months and 24 days which showed global developmental delay with a developmental age equivalence for locomotor 2.75 mo., personal & social 3.5 mo., hearing & language 6.5 mo., eye and hand coordination 4.5 mo. and performance 3.5 mo. She was noted to have a mild ataxia and a brain MRI at the age of 4.3 years showed a small cerebellar vermis. When last reviewed at the age of 8,6 years her height was  $-3.3SD$ , weight  $21^{st}$

percentile and OFC  $-3.2$  SD. She is a very pleasant and friendly girl who was in good general health. She remains hypotonic and mildly ataxic. She speaks in sentences and has no behavioural problems. She attends a special educational establishment where she is making progress with all aspects of her development but she has significant global cognitive impairment.

The proband's mother and 19-year-old maternal half-sister were both healthy (See pedigree, Figure 2b). The mother had a subsequent pregnancy that resulted in a termination for multiple fetal anomalies identified on antenatal ultrasound scanning. An autopsy on this fetus showed esophageal atresia with a tracheoesophageal fistula, a ventricular septal defect, truncus arteriosus, bilateral renal agenesis, bilateral radial aplasia, bilateral postaxial polydactyly of the feet and bilateral syndactyly of the 2nd/3rd and 3rd/4th toes. The mother's full sister, who is healthy, had a child who died as a result of a complex cardiac defect. The proband's maternal half uncle had been well until the age of 45 years when he was diagnosed with renal cell carcinoma.

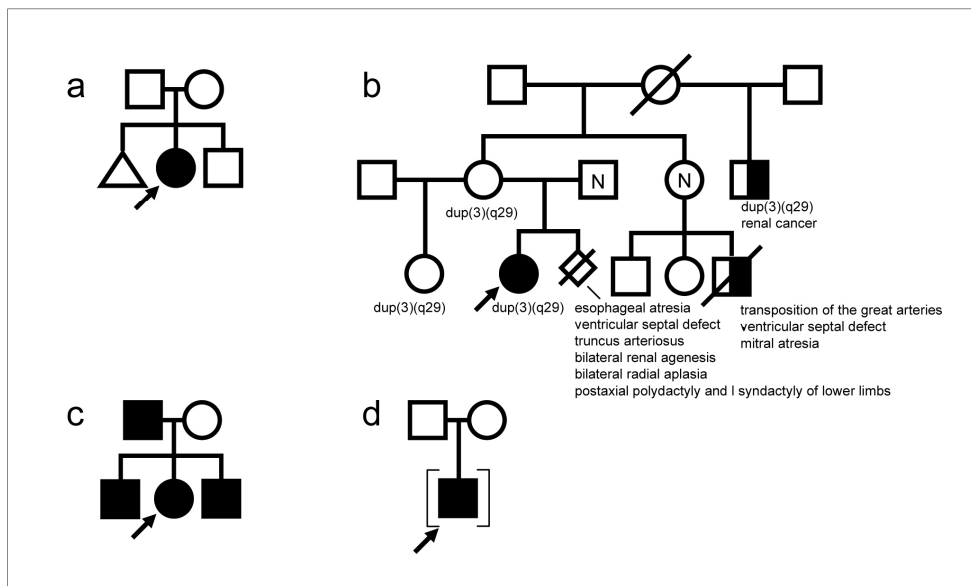


Figure 2. Pedigrees of cases. a) Case 1. b) Case 2. c) Case 3. d) Case 4.

### Case 3 (Leiden):

This 16 year old girl was born at term with normal birth weight (Figure 1c). There were no neonatal feeding problems or hypotonia. Motor development was slightly retarded. She was able to walk at two years of age. There was a more severe delay in speech development. At 10 years of age her vocabulary covered 40 words. MRI at 12 years of age showed no brain abnormalities. At 16 years of age the girl was not toilet-trained. When walking she would easily stumble over. She was obese and had a small, narrow forehead, straight eyebrows, narrow palpebral fissures, hypotelorism, open mouth appearance, crowding of the teeth and low posterior hairline. There was profound mental retardation.



Both brothers of this girl attended special schools because of learning difficulties (See pedigree, Figure 2c). The father of the girl lives in an institution. His IQ is 64. He is unable to read or write. He has straight eyebrows, deep set eyes and narrow palpebral fissures.

#### Case 4 (Toronto):

The proband is an adopted male who was thirty years of age at the time of last examination. He was the product of first pregnancy for a then 16 year old mother, who gave him up for adoption soon after birth. He was born at full term via vaginal delivery in breech presentation with a birth weight of 2,150 g (below 3rd centile). He was noted at birth to have micrognathia, significant limb reduction defects of four extremities, congenital right hip dislocation, grade 1 hypospadias and left cryptorchidism. At 17 months of age his weight was 5.4 kg (well below the 3rd centile), head circumference 45.5 cm (-2 SD); he had mild dysmorphisms described as a hypoplastic mandible with overbite as well as mild developmental and significant speech delay. Cardiac evaluation revealed a grade 2/6 systolic murmur, but his EKG was normal. His hearing was tested at two years of age and was low-normal, with very mild conductive hearing loss in the left ear. ENT evaluation at 4 years of age (Figure 1d) revealed a narrow, high vaulted palate with submucous cleft palate and very mild tongue coordination difficulties. He was assessed by ophthalmology at eight years of age and was found to have slight nystagmus, visual acuity of 20/20 and no structural eye defects. At age 11 (Figure 1d), he was assessed by the craniofacial service because of severe class II malocclusion and underwent extensive orthodontic treatment and surgery including LeFort 1 to intrude the maxilla, mandibular sagittal split advancement and vertical reduction with advancement genioplasty. At 15 years of age he had left inguinal exploration that revealed an atrophic testis that was removed.

The patient has mild developmental delay and learning disabilities. His milestones were delayed and he did not sit by himself until 2 years of age. At 34 months of age he was performing at the level of a 20 month old, with prominent speech delay. The patient received therapy and was able to attend regular school with additional help due to learning disabilities affecting his reading comprehension. He finished high school, obtained a college degree, and now lives independently and works in customer care services.

At last examination at 30 years of age his head circumference was 58 cm (+2SD); his features include a broad nasal bridge, high arched palate; ears that are normally placed but have simple, pointed pinnae with a thin upper border. He has increased adipose tissue and has developed multiple stria in the torso and abdomen. His extremities show significant transverse reduction defects. His most well developed limb is his upper right arm which includes a normal humeral arm segment and a partly developed forearm that extends 20 cm below the elbow and ends on a blind stump. The left arm and both legs consist only of proximal segments. All extremities have dermatoglyphic patterns at the tips, suggesting at least partial development of the hands and feet. However, no digits or metacarpals are appreciated.

Table 1: Comparison of clinical features in four patients with duplication of 3q29

Clinical Feature	Case 1	Case 2	Case 3	Case 4
	(Toronto; 23 months)	(Edinburgh; 8 years)	(Leiden; 16 years)	(Toronto; 30 years)
Birth Weight	2,850g (50 <sup>th</sup> -75 <sup>th</sup> centile)	3,080g (12 <sup>th</sup> centile)	3,200g (25 <sup>th</sup> centile)	2,150g (<3 <sup>rd</sup> centile)
Microcephaly	-	+	not reported	-
Generalized Obesity	-	-	+	+
Ocular anomalies	+	+	not reported	-
Palpebral fissure anomalies	+	+	+	-
Cleft Palate	+	-	-	+
Dental anomalies	+	-	+	+
Conductive hearing loss	+	-	not reported	+
Structural brain anomaly	+	+	-	-
Developmental delay	+	+	+	+
Hypotonia	+	+	-	-
Congenital Heart Disease	+	+	-	-
Muskuloskeletal anomalies	+	-	not reported	+

## MATERIALS AND METHODS

**Case 1 (Toronto):** PHA-stimulated lymphocytes from peripheral blood were cultured for 72 hours with thymidine synchronization. GTG-banding analysis was performed on peripheral blood lymphocytes using standard cytogenetic techniques. G-banded karyotypes at 500 band resolution were prepared for the patient and both of her parents. The de novo change in our patient was further evaluated using fluorescent in situ hybridization (FISH). FISH was performed on cultured lymphocytes using the following probes: a chromosome 3q subtelomeric probe (Oncor, Gaithersburg, MD), and BAC clones RP11-159K3 and RP11-962B7, directly labeled with Spectrum Orange and Spectrum Green, respectively (Figures 3 and 4). Hybridized metaphase spreads were analyzed using a Zeiss Axioplan 2 epifluorescence microscope. Images were captured by an Axiocam MRm Camera (Imaging Associates, Bicester, UK) and analyzed using an imaging system with MetaSystems Isis Software version 5.1.110 (Boston, MA).

**Case 2 (Edinburgh):** The 3q29 duplication in the proband was initially discovered with the BlueGnome CytoChip V1.1 1Mb BAC-CGH array (BlueGnome Ltd., Cambridge, UK), which has contig coverage of microdeletion regions. BAC array-CGH was performed on the proband and both parents where genomic DNA from each case was labeled by random priming. Hybridization and washes were performed on an HS 400TM Pro hybridization station (Tecan Ltd., UK). Each subarray was prehybridized for 45 minutes at 37° C with 1.5 µg of herring sperm DNA (Sigma-Aldrich, UK) in 75 µl of hybridization buffer (50% formamide, 7% dextran sulphate, 2x saline sodium citrate (SSC), 10mM Tris-HCl pH 7.5, & 0.1% (v/v) Tween 20). Test and reference samples were mixed, co-precipitated, and resuspended in a 75 µl hybridization solution that also contained 2.5 µg/µl Cot-1 DNA (Invitrogen), denatured at 75° C for 15 minutes, incubated for two hours at 37° C to block repetitive sequences, and hybridized for 21 hours. Post-hybridization washes were performed using 3 wash cycles in each of PBS/0.05%Tween at 37° C, 0.1x SSC at 54° C, 1x PBS at 37° C, and a final wash in PBS/0.05%Tween at 23° C. Slides were dried using high purity nitrogen. Arrays were scanned using a GenePix Pro 5.0 array scanner (Axon Instruments, UK) and

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analysed using BlueFuse for Microarrays analysis software version 3.4 (BlueGnome Ltd, UK).

The proband, an unaffected sister (age 19) and mother, as well as an uncle who has renal cancer at the age of 49 and a maternal aunt and her child who died with complex congenital heart disease were also assayed by MLPA. Confirmatory MLPA was performed using both P036B and P070 human telomere assays (MRC Holland, Netherlands), which contain two independent probes for the 3q29 region. The P036B probe is situated in the BDH gene on 3q. The proximal probe sequence was GCCACCGGGAGGAAGTGGGCCAT and the distal probe sequence TCTAACACCCGTTGCTACCATGCTGGCCACCCGCCTCTCCAGA. The second probe on 3q, P070, is located in KIAA0226 and has a proximal probe sequence 5'-CTCTTTCTCCAGGTCAGTGCCTGGAGGACAG and distal probe sequence 5'-ATGTGCCGTCTTGTCCCTGCTTTCACATCAGCATAGGATCA. MLPA products were processed using an ABI 3100 Genetic Analyzer with ABI GeneScan™ ROX500™ size standard. Quantitative data analysis was obtained using the SoftGenetics® Gene Marker® v1.4 software.

**Case 3 (Leiden):** Conventional cytogenetic analysis on GTG-banded chromosomes from cultured lymphocytes of the index case was performed according to standard techniques. Array-CGH was performed on all five family members using the ~1.0 Mb spaced whole genome large insert clone arrays, for which the clones were kindly made available by the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk>). The clones were grown, PCR amplified and spotted as previously described (Knijnenburg et al. 2005; Fiegler et al., 2003). Genomic DNA of the patient was isolated using standard techniques, and 500 ng was labeled with Cy3-dCTP (GE Healthcare, Diegem, Belgium) using the BioPrime® DNA Labeling System (Invitrogen, Breda, the Netherlands). As a reference DNA, 500 ng female human genomic DNA (Promega, Leiden, the Netherlands) was labeled using Cy5-dCTP. Hybridization and slide washing was performed without prehybridization on an HS400 hybridization station (Tecan, Giessen, the Netherlands). Arrays were scanned with a GenePix 4100A scanner (Axon Instruments, Union City, CA) and images were processed with GenePix Pro 4.1 software. Final analysis of the intensity ratios of the hybridized DNA was as previously described (Knijnenburg et al. 2005).

Confirmatory MLPA was performed on the index case as described (White et al. 2004). The selected probes were located in the NCBP2 gene. The proximal sequence was 5'-GCCCGCGGAATTTCGATTGGTGATGTTCTTCAGCAAATTCAA-CAGGCCAAAGGAGTGTTC and the distal sequence was 5'-GTCAGTACA-GAGCTCTCACTCACACTAGTGAATTCGCGGC.

Quantitative readout was performed with an ABI 3730 DNA analyzer. The accompanying Genescan 3.5 software was used for peak analysis and further downstream normalization and calculations were performed as described (White et al. 2004). Two-colour interphase FISH confirmation of the duplication in the proband was performed with clones CTC-196F4 at 3q29, partly overlapping the DLG1 gene (as in Willatt et al. 2005), and 3p subtelomeric clone GS-1186B18 as a control.

**Case 4 (Toronto):** Routine cytogenetic workup was as for Case 1, above. The initial karyotype report of 46, XY was followed up with chromosomal microarray analysis (Kleberg cytogenetics Laboratory, Baylor College of Medicine, Houston TX USA; CMV version 5.0).

**Table 2. CNVs Detected in Patient Families With Affymetrix 500k Microarrays**

Cytoband	Estimated Size (bp)	Type	Status In Children <sup>(1)</sup>	Gene(s) involved
<b>Family of Case 1</b>				
<b>Proband</b>				
3q29	2,399,433	gain	<i>de novo</i>	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
6q16.1	60,058	loss	inherited (paternal) <sup>(2)</sup>	no genes; overlaps known CNVs
8p23.1	407,187	gain	inherited (maternal)	FAM86B1, DEFB130, LOC44005; region of segmental duplications and known CNVs
<b>Mother</b>				
8p23.1	202,167	gain	-	no known genes; numerous cDNAs; region of segmental duplications and known CNVs
10q11.1-q11.21	906,591	gain	-	ZNF33B, BMS1L and numerous cDNAs; region of segmental duplications and known CNVs
11q22.1	626,031	loss	-	cDNA AK128111; overlaps known CNVs
15q11.2	1,908,357	loss	-	OR4N2, OR4M4, POTE15, LOC283755 and multiple cDNAs
22q11.23	211,233	gain	-	LRP5L and multiple cDNAs; region of segmental duplications and known CNVs
<b>Father</b>				
6q16.1	60,058	loss	-	no genes; overlaps known CNVs
10q11.22	124,801	gain	-	no genes; region of segmental duplications and known CNVs
14q11.2	153,147	gain	-	OR4N2, OR4K2, OR4K5, OR4K1 (odorant receptor gene cluster)
<b>Family of Case 2</b>				
<b>Mother</b>				
3q29	2,086,988	gain	-	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
7q11.23	428,467	gain	-	POMZP3, UPK3B, cDNA BC043544, intron of cDNA BC013192
14q21.1	357,718	loss	-	cDNA BX248273; encompasses small CNV
<b>Proband</b>				
3q29	2,086,988	gain	inherited (maternal) <sup>(2)</sup>	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
7q11.23	422,126	gain	inherited (maternal) <sup>(2)</sup>	POMZP3, most of UPK3B, cDNA BC043544, intron of cDNA BC013192
14q11.2	219,459	gain	unknown	OR4Q3, OR4M1, OR4N2, OR4K2, OR4K5, OR4K1 (odorant receptor cluster)
14q21.1	368,345	loss	inherited (maternal) <sup>(2)</sup>	cDNA BX248273; encompasses small CNV
15q11.2	1,662,281	gain	unknown	OR4N2, OR4M4, POTE15, LOC283755 and multiple cDNAs
17q21.31	183,068	gain	unknown	5' end of KIAA1267, and cDNAs BC018467 and BC000924
<b>Family of Case 3</b>				
<b>Proband</b>				
3q29	1,893,889	gain	inherited (paternal)	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
8p23.1-p23.2	176,963	gain	inherited (paternal)	5' end of MCPH1, and cDNAs including AK025595
15q11.2	1,378,020	loss	<i>de novo</i> <sup>(3)</sup>	OR4N2, OR4M4, POTE15, LOC283755 and multiple cDNAs
19q13.42	456,306	gain	inherited (maternal)	cDNA BX248273; encompasses small CNV
<b>Mother</b>				
12q24.31	114,901	gain	-	3' ends of P2RX7 and CAMKK2, and all of P2RX4
19q13.42	996,692	gain	-	multiple genes
<b>Father</b>				
3q29	1,893,889	gain	-	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
4q24	556,763	gain	-	TACR3
8p23.1-p23.2	224,359	gain	-	5' end of MCPH1, and cDNAs including AK025595
10q11.22	848,700	gain	-	SYT15, GPRIN2, PPYR1, ANXA8L1
14q11.2	222,787	gain	-	OR4Q3, OR4M1, OR4N2, OR4K2, OR4K5, OR4K1 (odorant receptor gene cluster)

Table 2. Continued from previous page.

Cytoband	Estimated Size (bp)	Type	Status In Children <sup>(1)</sup>	Gene(s) involved
<b>Family of Case 3</b>				
<b>Brother 1</b>				
1q31.3	142,667	loss	inherited (paternal) <sup>(4)</sup>	CFHR3, CFHR1 and 5' end of CFHR4
3q29	1,893,889	gain	inherited (paternal)	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
8p23.1-p23.2	221,809	gain	inherited (paternal)	5' end of MCPH1, and cDNAs including AK025595
10q11.22	1,087,629	gain	inherited (paternal)	GPRIN2, PPYR1, ANXA8L1, ANXA8, and multiple other cDNAs
12q24.31	79,614	gain	inherited (maternal)	3' ends of P2RX7 and CAMKK2, all of P2RX4
14q11.2	229,920	gain	inherited (paternal)	OR4Q3, OR4M1, OR4N2, OR4K2, OR4K5, OR4K1 (odorant receptor gene cluster)
15q11.2	1,662,281	gain	de novo <sup>(3)</sup>	OR4N2, OR4M4, POTE15, LOC283755 and multiple cDNAs
19q13.42	857,878	gain	inherited (maternal)	multiple genes
<b>Brother 2</b>				
3q29	1,893,889	gain	inherited (paternal)	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
4q24	831,405	gain	inherited (paternal)	TACR3
8p23.1-p23.2	198,636	gain	inherited (paternal)	5' end of MCPH1, and cDNAs including AK025595
10q11.22	848,700	gain	inherited (paternal)	SYT15, GPRIN2, PPYR1, ANXA8L1
15q11.2	1,662,281	gain	de novo <sup>(3)</sup>	OR4N2, OR4M4, POTE15, LOC283755 and multiple cDNAs
19q13.42	541,228	gain	inherited (maternal)	multiple genes
<b>Case 4</b>				
2q27.3	81,134	loss	unknown	multiple cDNAs; encompassed by known CNV
3q29	2,041,109	gain	unknown	multiple genes; overlapping with 3q29 microdeletion syndrome (Willatt et al. 2005)
6q24.2-q24.3	2,637,073	gain	unknown	3' end of UTRN, EPM2A, GRM1, RAB32, FLJ44955, FBX030, SHPRH, various cDNAs; encompasses several small CNVs
14q11.2	249,343	loss	unknown	OR4N2, OR4K2, OR4K5, OR4K1, OR4K13, OR4K14, OR4K15 (odorant receptor gene cluster; encompasses known CNV and segmental duplications)
Xp11.23	67,327	loss	unknown	ZNF630, SSX6; encompasses known CNV and segmental duplications

(1) Parental origin is inferred assuming Mendelian inheritance of CNV events. In Case 2, these are apparently maternal; however, the father's sample was not available for analysis. (2) Parental origin of losses in Case 1 and Case 2 were confirmed by examination of SNP genotypes in these regions. (3) The 15q11.2 region is very complex and apparently de novo events may actually be inherited; in particular, the loss in Case 3 contains multiple heterozygous SNPs and is therefore not a simple hemizygous deletion. (4) SNP genotypes are consistent with paternal inheritance of the 1q31.3 loss in this individual. The corresponding CNV in the father was identified by only one algorithm and thus is not reported.

### Affymetrix Genome-Wide SNP Array and Copy Number Analyses

For CNV analysis, we adhered to recommended guidelines (Scherer et al. 2007). In order to maximize consistency between samples collected at the three sites (Toronto, Leiden and Edinburgh), all samples were characterized with the Affymetrix 500k array set at The Centre for Applied Genomics in Toronto. Each sample was genotyped with the GeneChip® Human Mapping Nspl and Styl Arrays (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's instructions and as described previously (Kennedy et al. 2003). For copy number determination, we used three approaches: DNA Chip Analyzer (dChip) (Li and Wong 2001; Lin et al. 2004; www.dchip.org), CNAG (Nannya et al. 2005) and GEMCA (Komura et al. 2006). The first two algorithms were applied separately to each 250k array, and GEMCA was applied to combined 500k array data. CNVs were scored if they were detected in the same individual either a) on both arrays, or b) by two of the algorithms. In our hands, these criteria result in high confidence CNV calls that are >95% likely to be confirmed by an independent technology such as qPCR (Pinto et al. 2007; Marshall et al. 2008). In the cases of

copy number losses, SNP genotypes were examined in order to determine parent of origin.

## RESULTS

All phenotype and CNV data are entered in the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER; <http://decipher.sanger.ac.uk/>).

**Case 1 (Toronto):** A subtle cytogenetically detectable difference at 3q29 was detected at a G-band resolution of 500 bands in Case 1. This alteration was not observed in her parents' chromosomes at the same resolution (data not shown). The chromosomal difference was determined to be interstitial as the chromosome 3q subtelomeric probe revealed two normal signals in the correct position in this patient (Figure 3c). Further investigation using the Affymetrix 250K NspI Array revealed a 2.4 Mb duplication of 3q29 (Figure 4; Table 2). The duplication was determined to be de novo, as neither parent revealed a CNV at this locus.

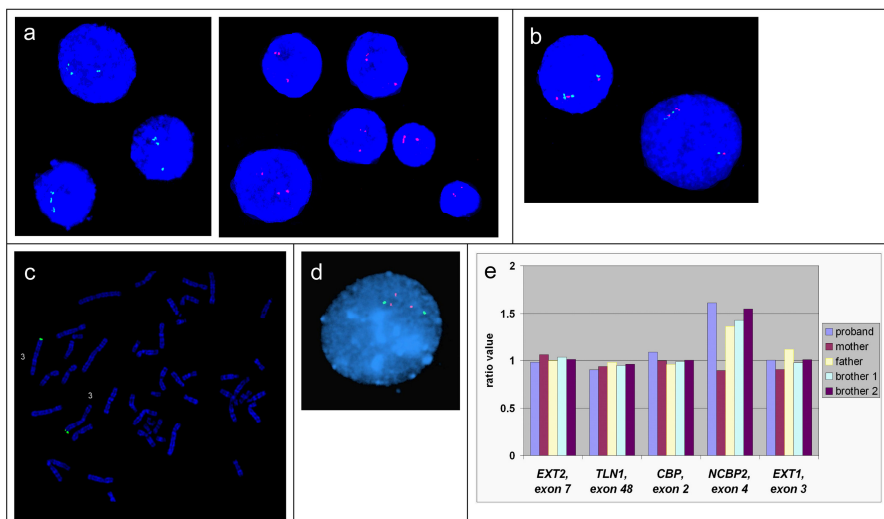


Figure 3. Confirmatory studies of duplications. a) FISH confirmation in Case 1, showing duplication of BAC probes RP11-962B7 (green) and RP11-159K3 (red). b) FISH in case 1. Cohybridization of RP11-962B7 and RP11-159K3, demonstrating a tandem, directly oriented duplication. c) FISH in Case 1, showing two copies of the 3q subtelomeric probe (green). d) FISH confirmation in Case 3, showing duplication of probe CTC-196F4 (red) but not of control probe GS-1186B18 (green). e) MLPA confirmation in Case 3 and family members. The MLPA probe in the NCBP2 gene shows a 3:2 ratio in the proband, the father and both brothers, while the control probes located elsewhere in the genome show a normal 2:2 ratio. The mother shows no duplication of the NCBP2 MLPA probe.

The patient also had a 60 kb loss at 6q16.1 and a 407 kb gain at 8p23.1 (Table 2). The 6q16.1 locus contains no known genes and overlaps numerous known segmental duplications and CNVs, and the 8p23.1 region is a locus of known copy-number polymorphisms and segmental duplications in the vicinity of the beta-defensin gene DEFB130. This CNV overlaps with the proximal end of the region of

8p23.1 duplication reported by Barber et al. 2007 (see Discussion). Interphase FISH analysis of the 3q29 region using BAC clone probes RP11-159K3 and RP11-962B7 revealed three signals for each probe, confirming the duplication (Figure 3a). Clone RP11-962B7 is located approximately in the middle of the region identified as a duplication by microarray, while clone RP11-159K3 is located approximately 600 kb distal to RP11-962B7, also within the duplicated region in this patient (Figure 3). Co-hybridization of the two BAC clone probes suggested that the structure of the rearrangement was a tandem, direct duplication (Figure 3b). The parents of this patient had the normal two signals for each probe, confirming that the duplication occurred *de novo* in our patient (data not shown). These same FISH probes were hybridized to metaphase spreads which confirmed their localization to 3q29 only, in both the patient and her parents (data not shown).

In order to rule out non-paternity (and thus the possibility that the 3q29 microduplication was in fact inherited, rather than *de novo*), we used PedCheck (O'Connell and Weeks 1998) to detect markers incompatible with the pedigree. Of the 262,264 SNPs on the Nspl array, 271 autosomal and 20 X chromosome SNPs were inconsistent either between either the father or mother and the proband. Of the autosomal SNPs, 117 were inconsistent between mother and child, 115 between father and child, and 39 were consistent between the child and each parent separately, but not as a pair. Since the proportion of incompatible markers is low (~0.1%), the pedigree is consistent with the SNP data. Moreover, the rates of inconsistency between mother and child (43.2%: 117/271) versus father and child (42.4%: 115/271) are nearly identical, indicating that these are due to random genotyping errors and ruling out non-paternity in this family.

**Case 2 (Edinburgh):** High resolution SNP array analysis confirmed a duplication at 3q29 of 2.08 Mbp in size in the proband as detected originally using a 1 Mb BAC array platform. Notably, the distal boundary was identical to that of Case 1, although the proximal breakpoint was slightly farther distal, accounting for the difference in size (2.08 Mbp vs. 2.4 Mbp in Case 1; see Figure 4). However, the unaffected mother and a maternal half-sister carry the apparently identical duplication, as does a maternal halfuncle who has renal cancer at the age of 45. No imbalance of the 3q29 region was evident in the proband's father as seen by BAC array-CGH or MLPA. The mother and proband were evaluated with Affymetrix 500k SNP arrays and their duplications were found to be identical in extent (Table 2). Unfortunately there was no material from the subsequent affected fetus of this mother to test for the duplication. A maternal aunt, and her child who died with complex congenital heart disease, do not have the duplication (as assayed by MLPA; data not shown; see pedigree, Figure 2b) and thus this heart disease in the extended family does not appear related to the 3q29 duplication. CNVs detected in the proband (at 7q11.23, 14q11.2, 14q21.1, 15q11.2 and 17q21.31) and mother (7q11.23 and 14q21.1) are previously reported polymorphic CNVs found in apparently healthy individuals (Table 2) and are thus unlikely to contribute to the phenotype seen in this patient. The ~360 kb loss at 14q21.1 and 420-430 kb gain at 14q21.1 are also present in the proband's unaffected mother.

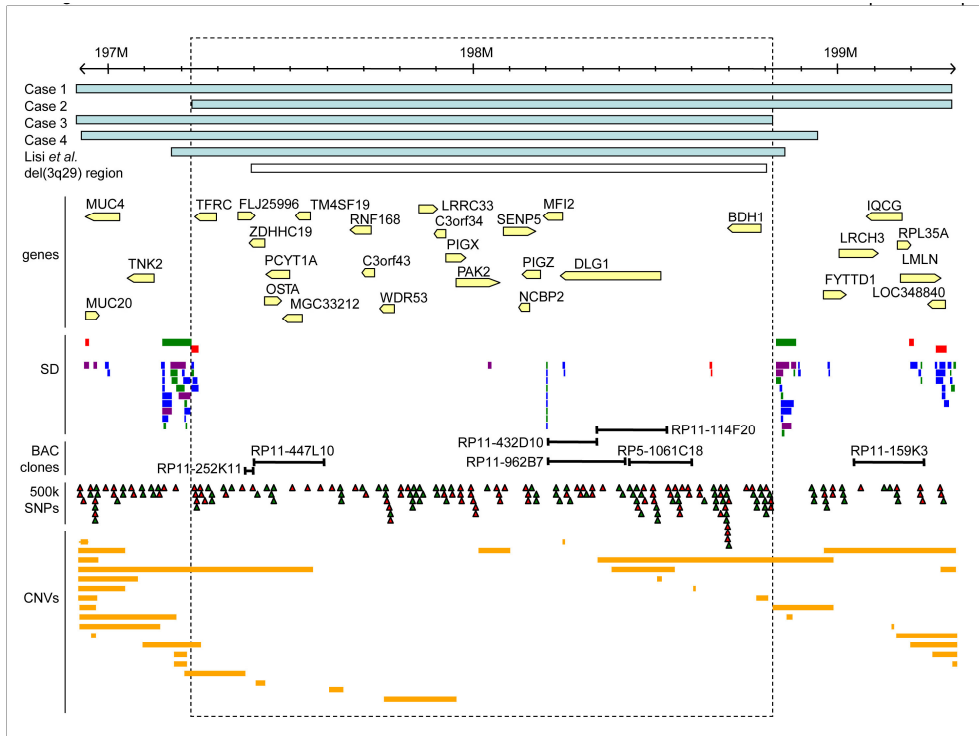


Figure 4. Microduplication region at 3q29. Scale at the top is in millions of base pairs (NCBI Build 35). The duplicated regions in four cases are shown (blue bars). The approximate location of the duplication described previously is shown (Lisi et al. 2008). The region of recurrent 3q29 microdeletion described in Willatt et al. 2005 is also indicated (hatched bar); this corresponds to the duplication reported in Ballif et al. 2008. Feature tracks from the Database of Genomic Variants are shown below: known genes (arrow indicating direction of transcription), segmental duplications (SD), SNPs on the Affymetrix 500k array set (red triangles: Nspl array; green triangles: Styl array) and previously reported copy number variants (CNVs; orange bars). Locations of BAC clones used for FISH mapping (Case 1) or duplicated on BAC-CGH array (Cases 3, 4) are also indicated. Duplicated probe CTC-196F4 in Case 3 is located within BAC clone RP5-1061C18 (shown here; see also Willatt et al. 2005).

**Case 3 (Leiden):** BAC-CGH analysis confirmed that the proband, her father and two affected brothers carry duplications of BAC clones RP11-252K11 and RP11-114F20 (data not shown; see Figure 4). SNP microarray analysis demonstrated an identical 1.9 Mbp duplication at 3q29 in these individuals (Table 2). The proximal boundary was identical to that of Case 1, and the distal boundary was somewhat more proximal (Figure 4). Other CNVs detected in this family include loci at 1q31.3, 4q24, 7q11.23, 10q11.22, 12q24.31, 14q11.2, 14q21.1, 15q11.2, 17q21.31, 19q13.42, all of which overlap known CNVs found in apparently healthy individuals (Table 2). An apparent de novo loss at 15q11.2 in the proband was not supported by examination of genotype data, as 16 SNPs in this region were heterozygous (data not shown); as this is in a complex region including multiple similar odorant receptor genes, this CNV may be a false positive, or may represent a loss to two copies as compared with higher copy number in other members of this family. One additional CNV in this family, at 8p23.1-p23.2, is also in a region of known CNVs and is proximal to the microduplication 8p23.1



region described by Barber et al. 2008 (see Discussion). The duplication in the proband was confirmed by two-colour interphase FISH (Figure 3d). The MLPA probe in the NCBP2 gene also confirmed the duplication (3:2 ratio as compared with control), the father and both brothers, while control probes located elsewhere in the genome show a normal 2:2 ratio. The mother showed no duplication of the NCBP2 MLPA probe (Figure 3e).

**Case 4 (Toronto):** BAC-CGH array analysis revealed a gain in copy number of two clones (RP11-447L10 and RP11-432D10; Figure 4) located at cytogenetic band 3q29, with no other sites of copy number change detected. Microarray analysis demonstrated a duplication of 2.0 Mbp at 3q29 (Table 2). The proximal boundary was similar to those of Cases 1 and 3, and the distal boundary just slightly distal to that of Case 3 but still within a cluster of segmental duplications (Figure 4). Other CNVs detected in this individual that are also seen in healthy individuals were at 2q27.3, 14q11.2 and Xp11.23 (Table 2). One additional large (2.6 Mbp) CNV at 6q24.2-q24.3 encompasses several small known CNVs but also results in a previously unreported copy number gain of a number of known genes including Utrrophin (UTRN), the Lafora progressive myoclonus epilepsy gene EPM2A, a metabotropic glutamate receptor (GRM1), Ras oncogene family member RAB32, two genes apparently involved in protein ubiquitination (SHPRH and FBXO30) and an expressed repetitive element (FLJ44955).

## DISCUSSION

Duplication 3q syndrome (dup3q) has been described in the literature, consisting of dysmorphic features including microcephaly, low-set ears, downturned corners of the mouth, bushy eyebrows and long eyelashes, along with eye, palate, renal and cardiac anomalies (Steinbach et al. 1981, Aqua et al. 1995). The phenotype has been said to partially overlap that of Brachmann de Lange/Cornelia de Lange syndrome (OMIM#122470). Many groups have described the cytogenetic critical region associated with the 3q duplication syndrome as involving 3q26 (Aqua et al. 1995, Rizzu et al. 1997, Faas et al. 2002); however Battaglia et al. (2006) suggested that it was 3q29. The four index patients which we have presented do not have a phenotype consistent with Brachman de Lange syndrome, suggesting that 3q29 is unlikely to be involved in the previously described “Duplication 3q syndrome”.

Prior to the past year, there were few cases described with pure duplications of chromosome 3q. Faas et al., in 2002, described three patients with cytogenetically visible chromosome 3q duplications that extended to include 3q29. The duplications in these patients included more proximal cytogenetic bands in addition to 3q29, whereas our reported cases had small duplications localized within 3q29; however, features common to both groups included mental retardation and ocular anomalies.

As molecular cytogenetic techniques have advanced over the recent years, we have been able to better detect and more precisely define microdeletions and microduplications in this region. The presence of microdeletions (Willatt et al. 2005) and the abundance of segmental duplications in this region (in particular at

approximately 196.6 Mbp and 199 Mbp) suggested that the reciprocal microduplications might exist, and two recent reports (Lisi et al. 2008; Ballif et al. 2008) and the cases reported here support this hypothesis.

As demonstrated by our cases, there appears to be a variable clinical phenotype associated with this duplication (see Table 1), characterized by ocular and cardiac anomalies, hypotonia, developmental and speech delay. However, the phenotype is variable with reduced penetrance; the mother and maternal half sister of Case 2 are unaffected, and the two brothers of Case 3 have milder phenotypes. Case 4 demonstrates the wide spectrum of

Table 3. Genes in minimal region of 3q29 duplication

Gene	Function	Known Syndromes/Diseases	MIM
BDH1	3-hydroxybutyrate dehydrogenase, type 1; interconversion of acetoacetate and (R)-3-hydroxybutyrate during fatty acid catabolism	none	-
C3orf43	unknown	none	-
C3orf34	unknown	none	-
DLG1	homologue of Drosophila tumour suppressor gene; putative lymphocytespecific TSG	homologous to X linked mental retardation gene DLG3 (Willatt et al. 2005); eye anomalies in knockout mouse (Nguyen et al. 2003)	601014
FLJ25996	unknown	none	-
LRRC33	leucine rich repeat containing 33	none	-
MF12	melanoma cell surface glycoprotein; similar to transferrin	none	155750
MGC33212	TCTEX1D2, Tctex1 domain containing 2	none	-
NCBP2	nuclear cap-binding protein 2	none	605133
OSTA	organic solute transporter alpha subunit; basolateral bile acid and steroid transporter	none	-
PAK2	p21 activated kinase homologous to X linked mental retardation gene PAK3 (Willatt et al. 2005)	605022	-
PCYT1A	phosphate cytidyltransferase 1, choline, alpha isoform; phosphatidyl choline synthesis	none (essential for survival in cultured cells)	123695
PIGX	phosphatidyl inositol glycan, class X; synthesis of glycosylphosphatidylinositol in the endoplasmic reticulum	none	610276
PIGZ	phosphatidyl inositol glycan, class Z; synthesis of glycosylphosphatidylinositol	none	611671
RNF168	ring finger protein 168	none	-
SENP5	SUMO1/sentrin specific peptidase 5; required for mitosis and/or cytokinesis	none	-
TFRC	transferrin receptor 1	cellular receptor for New World hemorrhagic fever arenaviruses; knockout results in severe anemia and neurologic abnormalities in mouse model	190010
TM4SF19	transmembrane 4 L six family member 19	none	-
WDR53	WD repeat domain 53	none	-
ZDHHC19	homologue of palmitoyltransferase of NRAS and HRAS (ZDHHC9)	homologous to ZDHHC9 causing X linked mental retardation with Marfanoid habitus	-

phenotypic differences, although it is possible that his tetramelia may be unrelated to the duplication (see below). The previously reported family (Lisi et al. 2008) also included individuals with some similar features, notably frequent developmental delay and some with palpebral fissure anomalies. The cases

reported by Ballif et al. (2008) had duplications varying in size from 0.2 to 2.4 Mb, but only five of these had the apparent reciprocal duplication of the previously reported deletion (Willatt et al. 2005). Of these, clinical information was reported for three, with mild to moderate developmental delay as the only common feature, and craniosynostosis, high palate, seizures and a ventricular septal defect occurring in two cases each (Ballif et al. 2008).

Notably, the duplication events in all four of the families presented here overlap, and those of Cases 2, 3 and 4 each share one of the boundaries of Case 1, the largest of the duplications. Together, these define a minimum critical region of approximately 1.58 Mbp in size, encompassing the region from the TFRC to BDH1 genes. The proximal and distal ends of this minimal region correspond to clusters of segmental duplications (Figure 4), indicating a possible recombination-mediated mechanism for the formation of these duplications. The region is similar in size to the 3q29 microdeletions previously reported (Willatt et al. 2005), which extend from BAC clone RP11-252K11 (proximal end at 197.40 Mbp) to RP11-535N19 (distal end at 198.81 Mbp) (see Figure 4), further supporting the hypothesis that these are reciprocal products of a deletion/duplication event mediated by non-allelic homologous recombination at segmental duplications (Lupski 2004; Ballif et al. 2008; Lisi et al. 2008). This region contains 20 known genes (Table 3).

DLG1 seems to be a good candidate for the ocular aspects of the dup(3)(q29) phenotype (microphthalmia in Case 1, partial aniridia in Case 2). DLG1 is expressed in the developing lens and retinal pigment epithelium, and a DLG1 gene-trap homozygous mouse has overgrowth of the lens epithelium as one part of the phenotype (Nguyen et al. 2003). With regards to developmental delay, Willatt et al. 2005 in their description of microdeletions of 3q29 in this region (see Figure 3) point out that two of the genes in this region, PAK2 and DLG1, are homologues of the X-linked mental retardation genes PAK3 and DLG3. We also note that this region contains the ZDHHC19 gene, a homologue of the ZDHHC9 palmitoyltransferase, mutations of which have been shown to cause X linked mental retardation with Marfanoid habitus (OMIM 300646). Importantly, all of these are increased in copy number in the 3q29 duplication patients described here, whereas they are reduced to haploidy in 3q29 microdeletion patients. Possibly, these or other genes in the region are sensitive to both increases and decreases in gene dosage, either of which might disrupt normal development.

The other CNVs detected in these patients overlap previously reported polymorphic CNVs present in the general population (reflected by their presence in the Database of Genomic Variants; Iafrate et al. 2004), with two exceptions. The most obvious potentially pathogenic CNV is at 8p23.1 in Case 1, overlapping the proximal end of the region seen in 8p23.1 duplication syndrome patients (Barber et al. 2008). This patient shares some clinical features with the patients of Barber et al., notably a high forehead, cardiac malformations, low-set ears and partial 2/3 toe syndactyly. The Family 1 proband of Barber et al. also had a high arched palate which could be compared to the cleft palate of our Case 1. We cannot rule out that some of these features seen in our Case 1 may be due to the overlap of the 8p23.1 CNV with the proximal end of the 8p23.1 microduplication region; however, there is a relatively small region of overlap

(407 kb of ~3.75 Mb total) containing only the beta-defensin gene DEFB130 and two genes of unknown function (FAM86B1, and LOC440053 which has homology to zinc finger protein genes). Furthermore, our Case 1 and Case 2 probands share common anomalies and phenotypic features, and Case 2 was not found to have a 8p23.1 CNV, suggesting that their similarities are more likely a result of their common 3q29 duplication. In contrast, the 8p23.1-p23.2 CNV seen in the family of our Case 3 is distinct, being located distal to this microduplication region. Although Barber et al. ran a custom Agilent 44k genome-wide oligonucleotide CGH array on their patients (<http://www.ngrl.org.uk/Wessex/array.htm>), they report no results indicating whether changes in the 3q29 region (or anywhere else in the genome other than 8p23.1) were observed.

The second potentially pathogenic CNV is the gain at 6q24.2-q24.3 seen in Case 4. This 2.6 Mbp region might contribute to the tetramelia seen only in this patient, possibly due to the involvement of the Utrophin/Dystrophin-like protein (UTRN) gene, or the putative protein-ubiquitin ligases SHPRH and FBXO30. However, there is currently no experimental evidence that gains of activity or genomic copy number of these or the other genes in this region (GRM1, EPM2A, RAB32 and FLJ44955) can affect limb development, although extensive work characterizing UTRN knockouts has been performed. Given its role in normal muscle development, further characterization of overexpression of Utrophin in limb development may shed some light on this patient's phenotype.

In summary, we have described four new cases with 3q29 duplications, with a minimum region of overlap of 1.6 Mbp corresponding in location to the previously reported 3q29 microdeletions. The phenotype in these families reveals variable expressivity and reduced penetrance. Global developmental delay was the most consistent feature in our cases and two other studies (Lisi et al., 2008; Ballif et al., 2008). Other features common to some of the patients included ocular anomalies, congenital heart defects, structural brain anomalies and hypotonia. Further phenotypic characterization of these patients, in combination with improved molecular understanding of the 3q29 duplicated region, will better delineate potential dosage sensitive genes in this genomic interval and their possible roles in cognitive and ocular development.

### **Management and Anticipatory Care**

Management guidelines for duplication of chromosome 3q29 have not been previously published. Our recommendations are based on the phenotypes described in our newly reported cases and those of the cases recently described by Lisi et al. and Ballif et al. in 2008. Importantly, as we discuss in this paper, the CNV content at other sites should also be considered in performing genotype and phenotype correlations.

Infancy and initial diagnosis:

- a) Ophthalmologic evaluation
- b) Echocardiogram
- c) Brain imaging
- d) Developmental assessment by 6 months of age, and continuing every 1-3 years as needed

- 
- e) Hearing evaluation
  - f) Skeletal survey
  - g) Early intervention services
  - h) Offer cytogenetic/molecular testing to parents to determine if the duplication of 3q29 is de novo or familial. Refer parents for genetic counseling if contemplating future pregnancies.
  - i) Family support

#### Childhood:

- a) Ongoing developmental services and therapy. Individualized educational plan if appropriate. Children will likely benefit from speech therapy, occupational therapy, and physical therapy.
- b) Referral to paediatric dentistry
- c) Encourage physical activity and balanced diet, given reported obesity in some children/adults with duplication 3q29

#### Adolescence and Adulthood:

- a) Annual medical examination as per standard medical practice
- b) Ongoing developmental services, individualized educational plan, and counseling regarding work placement appropriate for level of development.
- c) Education regarding sexual development and recurrence risk in offspring if appropriate for level of development

These recommendations will be updated as we learn more about the natural history and variable phenotype of this condition, as well as the impact of CNVs at different sites in the genome.

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## WEB RESOURCES

The Database of Genomic Variants (DGV): <http://projects.tcag.ca/variation/>  
Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER): <http://decipher.sanger.ac.uk/>  
Online Mendelian Inheritance in Man (OMIM):  
<http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM>

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## Chapter 8

**A homozygous deletion of a normal variation locus causes syndromic hearing loss in a patient from non-consanguineous parents.**

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## A homozygous deletion of a normal variation locus causes syndromic hearing loss in a patient from non-consanguineous parents

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**Keywords:** normal copy number variation; homozygous deletion; segmental duplication; hearing loss; contiguous gene syndrome

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### ABSTRACT

#### Background:

International databases with information on copy number variation of the human genome are an important reference for laboratories working with high resolution whole genome screening platforms. Genomic deletions or duplications which have been detected in the healthy population and thus marked as normal copy number variants (CNVs) can be filtered out using these databases when searching for pathogenic copy number changes in patients. However, a potential pitfall of this strategy is that reported normal CNVs often do not elicit further investigation, and thus may remain unrecognized when they are present in a (pathogenic) homozygous state. The impact on disease of CNVs in the homozygous state may thus remain undetected and underestimated.

#### Methods and results:

In a patient with syndromic hearing loss, array comparative genomic hybridization (array-CGH) and multiple ligation-dependent probe amplification (MLPA) revealed a homozygous deletion on 15q15.3 of a normal variation region, inherited from hemizygous carrier parents. The deletion is about 90 kilobases and contains four genes including the *STRC* gene, which is involved in autosomal recessive deafness (DFNB16). By screening healthy control individuals we estimated the frequency of hemizygous deletion carriers to be about 1.6%

#### Conclusion:

A homozygous deletion of a CNV region causing syndromic hearing loss was characterized by a panel of molecular tools. Together with the estimated frequency of the hemizygous deletion these results underline the potential clinical relevance of the 15q15.3 locus for patients with (syndromic) hearing impairment. This example shows the importance of not automatically eliminating registered CNVs from further analysis.

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## INTRODUCTION

Since the implementation of techniques for the detection of copy number variations of the human genome, such as array comparative genomic hybridization (array-CGH), single nucleotide polymorphism (SNP-) or oligo-arrays and paired-end mapping, it has become evident that copy number variations (CNVs) contribute for a large part to the total genetic variation.[1-12] The majority of this data is being pooled now in databases such as the Database of Genomic Variants (DGV, <http://projects.tcag.ca/variation/>).[8,13] Many of these CNV regions harbor genes. Wong et al.[2] found that 1673 RefSeq-genes overlapped 546 of 800 found CNVs. In the study of Redon et al.[1] 2908 RefSeq-genes were found in a total of 1447 CNV regions. The function of the genes located in CNV regions are often not related to primary development, but are in general more related to sensory perception.[1,2] In this way, they probably contribute to normal population variation.

The increased resolution of genome wide molecular karyotyping tools has led to the daunting task of classifying CNVs as pathogenic or non-pathogenic in the clinical setting. To facilitate the interpretation of detected copy number alterations, genome browsers and linked databases such as the DECIPHER database (<http://www.sanger.ac.uk/PostGenomics/decipher/>)[14] and the DGV database are widely used. The DGV database is often used as a reference template to quickly filter benign variants out of the substantial amount of CNVs found in screening patients. Similarly, the inheritance status of CNVs is used to determine whether they may be pathogenic, based on the assumption that copy number alterations inherited from a healthy parent are less likely to be disease-related or have phenotypic consequences.[15] However, this procedure of selection must be done prudently, as important information may be too easily disregarded.

In the recently described deafness-infertility syndrome (DIS) [MIM #611102] hearing loss and infertility are caused by a recessive contiguous gene deletion on chromosome 15q15.3.[16,17] The region consists of a segmental duplication, with four known active genes in the proximal region: the cation channel *CATSPER2* gene [MIM \*607249], which has a function in sperm motility[16], *STRC*, coding for stereocilin [MIM \*606440], which is expressed in the sensory areas of the inner ear[18], the inositol phosphate kinase *HISPPD2A* [MIM \*610979] and creatine mitochondrial kinase-1A (*CKMT1A*). Homozygous mutations in *STRC* have been described in non-syndromic autosomal recessive sensorineural hearing loss linked to the *DFNB16* locus [MIM #603720].[16,18] Only *CKMT1A* has a functional homologue in the duplicated region, named *CKMT1B*, while all others are pseudogenes with inactivating mutations. Recombination of the segmental duplication on 15q15.3, possibly mediated by non-allelic homologous recombination (NAHR), may lead to loss or gain of the repeats.

By using different molecular genetic screening tools we ascertained a patient with a homozygous deletion of the proximal repeat region of 15q15.3, containing the active genes. Detailed characterization of the deletion proved that the deletion was inherited from non-consanguineous parents who appear to be heterozygous carriers. To further assess the nature and frequency of the

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rearrangements in this area, a control panel from the normal hearing population, a panel of patients with sensorineural hearing loss and the HapMap samples with reported variation in this region and their available family members (according to the DGV) were tested with the multiplex ligation dependent probe amplification (MLPA) set developed for this genomic segmental duplication region on chromosome 15q15.3. The HapMap[19] samples are a set of cell lines from healthy volunteers from various ethnic backgrounds, used to study human variation. Here we prove that a reported normal variant can have serious phenotypic consequences in a recessive fashion which emphasizes the need for careful interpretation of copy number variation data.

## METHODS

The patient described here is a 10-year-old boy from non-consanguineous, healthy parents. He has slowly progressive bilateral sensorineural hearing loss, disharmonious mental retardation (IQ 56), short stature and dysmorphic features. Dysmorphic features include small palpebral fissures, synophrys, a high nasal bridge, brachydactyly and puffy hands and feet. The audiogram of the patient at the age of 7 years shows a moderate hearing impairment of all frequencies (fig 1). Informed consent was obtained from the parents and the proband.

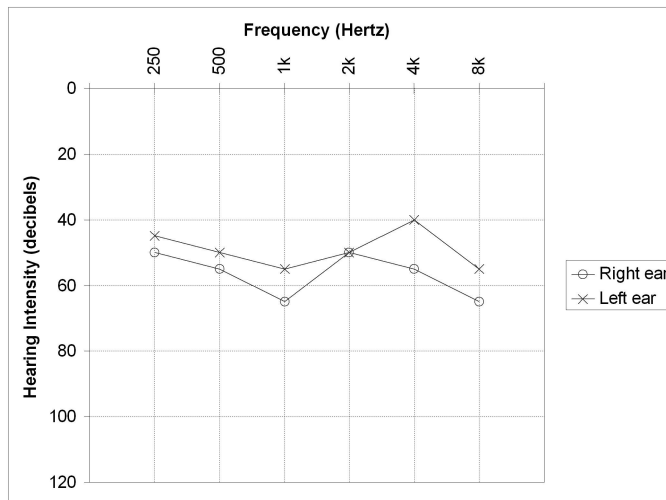


Figure 1. Audiogram of the proband showing moderate bilateral hearing loss.

Routine GTG-banding of cultured lymphocytes was performed using standard cytogenetic analysis. DNA was isolated from whole blood using standard isolation techniques. Routine amplification and sequencing for gap junction protein, beta-2 (*GJB2*) [MIM \*121011] coding mutations was performed as described previously.[20]

Array-CGH was performed using ~1.0Mb spaced whole genome large insert clone arrays, which were made available by the Wellcome Trust Sanger Institute, according to published methods.[21-23]

A HumanHap300-duo BeadChip SNP-array experiment (Illumina B.V., Eindhoven, the Netherlands) was performed according to procedures as suggested by the manufacturer. The SNP array, consisting of 317,000 oligonucleotide probes, was analyzed with the BeadStudio v3.0 software provided.

MLPA probes were designed to delineate the deletion further, because the coverage of the SNP platform in this 15q15.3 region was poor. MLPA was used to confirm the results, determine the extent of the deletions, and further analyze this region in other patient groups. Despite the fact that there is over 98% homology between the segmental duplicons, for probe design it was possible to distinguish between the proximal and distal repeat by using the small regions of non-homology or paralogous sequence variants (PSVs), known from the reference sequence. This resulted in a set of 23 probes specific for the active proximal and the inactive (containing pseudogenes) distal region, with a resolution of approximately 10 kb, located between 41.63 Mb and 41.83 Mb on chromosome 15q15.3. Three probes were localized within the active *STRC* gene. Probes were ordered from Operon (Cologne, Germany). The sequences are available in the online supplemental table 1. MLPA was performed as described previously,[24] with the adaptation that the ligation was performed at an annealing temperature of 60°C.

Long-range PCR and sequencing reactions were used to characterize the breakpoints of the maternally and paternally derived deletions in the patient. PCR primers were designed at PSVs region according to the reference sequence, between the location of the MLPA probes that showed a normal ratio and a homozygous deletion. Theoretically, fragments from both the distal and the proximal repeat could be amplified with these primers. PCR and subsequent sequencing was performed with the PCR primers on the patient and both parents. The sequences were aligned to the human reference sequence, NCBI Build 36.1, using the online BLAT tool of the UCSC genome browser (<http://genome.ucsc.edu/>).[25]

## RESULTS

Routine GTG-banding revealed a normal male karyotype (46,XY). Routine screening for mutations in *GJB2*, the most common pathogenic gene in hearing loss, revealed no mutations in the coding sequence.

Analysis of genomic DNA of the patient on ~1Mb spaced large clone insert array-CGH revealed two single clone deletions on chromosome 15. One deletion was found in band 15q26.2, involving bacterial artificial chromosome (BAC) clone RP11-315L6. When mapping this clone back to a genome browser and the DGV, this clone localized to a complete gene desert region which is described to be involved in normal variation. The other deletion found on chromosome 15 involved BAC clone RP11-263I19 (fig 2A) located at 15q15.3. Although this BAC clone is located in an area also previously reported to be involved in normal variation[1,9,10,12], this region has also been described to be involved in sensorineural hearing loss[18] as well as the recently published deafness-infertility syndrome.[16,17]

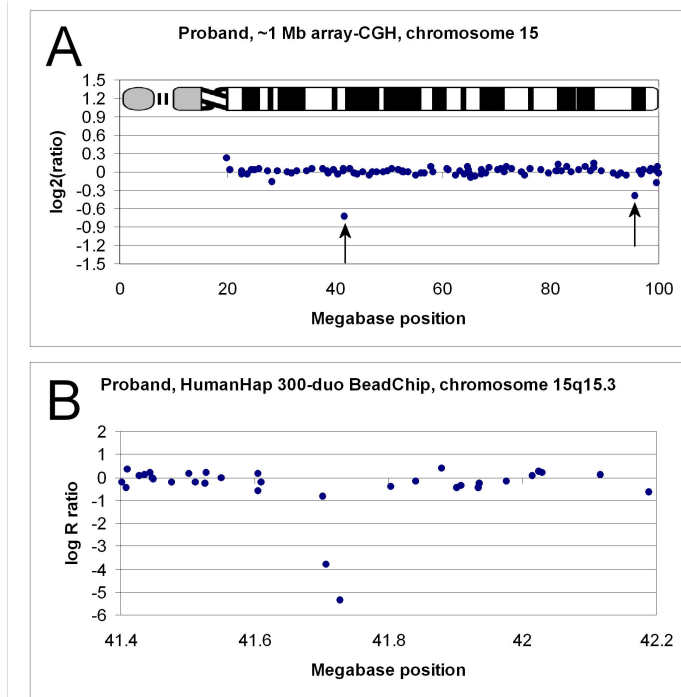


Figure 2. Micro-array profiles. (A) Large-insert clone array-CGH profile of the proband of chromosome 15 showing the deletion of BAC RP11-263119 located around 41.7 Mb and BAC RP11-315L6 around 95.6 Mb (indicated by the black arrows). (B) SNP array profile of chromosome 15q15.3 of the proband showing the deletion of SNPs rs2927071 and rs8042868 corresponding to RP11-263119 region.

To further characterize this deletion, a HumanHap300-duo BeadChip SNP-array experiment was performed. The results ruled out any unknown consanguinity of the parents or uniparental disomy in the patient, since the SNP profiles did not show large blocks of homozygous alleles in the patient. The hybridization showed a homozygous loss of SNPs rs2927071, rs8042868 and rs8038068, which were all located in a stretch of 25 kb, inside the proximal repeat sequence containing the four active genes (fig 2B). The deletion at 15q26.2 was also detected on the Illumina platform and was in size and location exactly comparable to the reported copy number variant (CNV) reported by Redon et al.[1]

Because of poor coverage of the SNP platform in this particular area, MLPA probes were designed to delineate the deletion further. The proband showed a homozygous deletion from 41.66 to 41.74 Mb. The deletions on both alleles involve the majority of the proximal repeat region, deleting or disrupting the active genes. The deletion size was at least 80 kb and a matching heterozygous deletion was found in both parents (fig 3).

Figure 4 shows the sequencing result of a conclusive PSV between the MLPA probes where breakpoints on the paternal and maternal allele had to have taken place. This sequencing result revealed proof of one heterozygous call in the patient and proved that the sequence of the maternally derived allele in the patient only consisted of the distal repeat and the paternally derived allele only

of the proximal repeat (fig 4). Sequencing of other regions containing PSVs with regards to breakpoint mapping was inconclusive.

To assess the frequency and the type of alteration of this locus, the developed MLPA set was tested on a panel of 64 normal hearing persons and 20 in-house control samples. Also with the same MLPA panel, a group of 45 patients with non-syndromic sensorineural hearing loss was screened for genetic alterations. This group of patients was proven by sequencing to have no mutation in the *GJB2* gene.[20] The MLPA test revealed that none of these samples had a contiguous deletion the size of the duplicon region. One sample in the patient group and two in the control group were found to have a duplication of the proximal repeat part, with a size comparable to the size of the deletion of the patient described herein, and the previously published consanguineous families.[16,17]

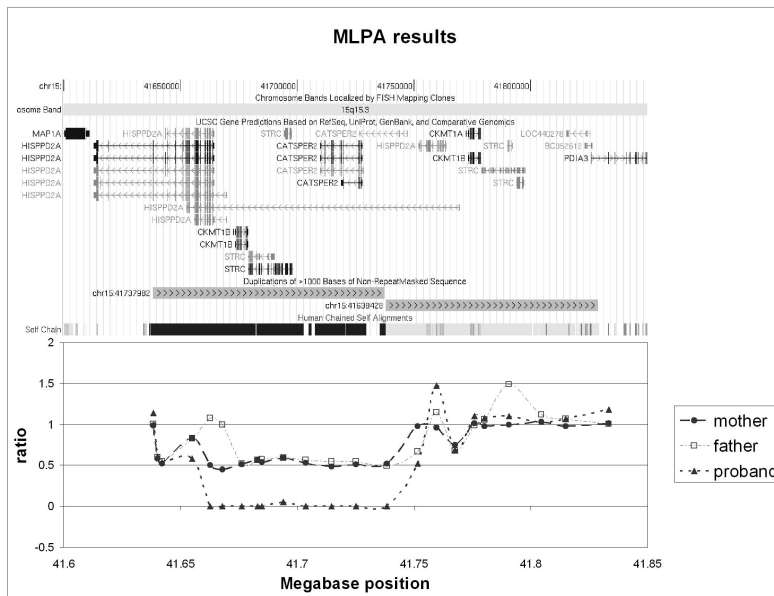


Figure 3. MLPA results on the proband and both parents. The upper panel shows the involved region using the UCSC browser, in which the involved genes and the two large duplicon blocks are displayed. The lower panel shows the ratio profiles of normalized MLPA data. Each data point represents a specific MLPA probe that discriminates between the proximal and distal repeat region. The homozygous deletion in the proband of the largest part of the proximal duplicon and the beginning of the distal duplicon is indicated by the closed triangles. The parents both show the heterozygous deletion in the region.

To confirm alterations involving this region reported in the DGV, 21 samples of the HapMap collection were tested with the developed MLPA set. Of these samples, 10 were reported with either a gain or a loss in the 15q15.3 region[1,10] and the remainder 11 were the closest family members, if they were available. Two additional samples with a duplication as reported in the DGV were unavailable for analysis. Six of the 10 available samples were described as having a deletion size of at least one duplicon. All six deletions were confirmed and they could be narrowed down to the contiguous deletion of the size of one duplicon.



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In all six cases the deletion involved the largest part of the proximal duplicon, deleting or disrupting the active genes on that allele. The four other samples had the smaller alterations reported,[10] as discussed below. All MLPA data is available in the online supplemental table 2.

## DISCUSSION

In this study we describe the detailed molecular characterization of a homozygous deletion of a seemingly normal variation locus at 15q15.3, inherited from non-consanguineous parents. Using array-CGH, a deletion at chromosome 15q15.3 containing a 100 kb tandem segmental duplication region was found. SNP-array analysis ruled out possible consanguinity of the parents and showed the deletion to be homozygous. The rearrangement deletes the proximal repeat sequence at chromosome 15q15.3 on both alleles. It is important to note that only three SNP were located in this area, so current standard diagnostic reporting algorithms will not detect the deletion. This might result in false negative calls for the involvement of this region in diagnostic settings.

Homozygous deletion of the 15q15.3 region has been reported in the DIS syndrome. However, mental retardation and/or structural congenital abnormalities were not reported in those cases. [16,17] The clinical overlap with our patient is thus the hearing loss and potentially the infertility. The more severe phenotype in our patient may represent one end of a broader phenotypic spectrum associated with homozygous deletion of 15q15.3, as was noted before in other syndromes.[26,27] Alternatively, the mental retardation and dysmorphic features are unrelated, or only partially related to his 15q15.3 homozygous deletion.

MLPA testing characterized the homozygous deletion in detail. Based on the assumption that the deletions are mediated by NAHR during meiosis a deletion of a full repeat-size is expected, which is about 90 kb (fig 3) and fits the MLPA results. The MLPA probe at 41.75 Mb shows a heterozygous deletion ratio for the patient and the father and a normal ratio for the mother. Consequently, the distal breakpoint of the father is located slightly more telomeric than in the mother and assuming NAHR as the responsible recombination mechanism, the proximal breakpoint in the father should also be more telomeric (fig 4).

The results also show that the father of the described proband has two copies of the region detected by two MLPA probes around 41.67 Mb, while the proband did not inherit any of these two copies (fig 3). These probes are located in a non-coding area. Likely the father has two copies of this region on the non-transmitted, intact allele. Similar copy number variation was also observed by the MLPA test performed on control samples and HapMap samples. Some more small CNVs in the most proximal part of the proximal duplicon is noted throughout the sample sets, involving MLPA probes in intronic regions in and around the *HISPPD2A* gene. These variations might represent smaller genomic changes similar to the four small alterations in the HapMap cases described by Perry and colleagues.[10] Currently, there are no cost effective methods to screen for structural variants smaller than ~3 kb in a genome wide approach.[11]

The relevance of the non-contiguous variations found in intronic regions with the developed MLPA set is thus unclear.

In order to estimate the frequency of this deletion we tested the genomic DNA of 45 persons with non-syndromic sensorineural hearing loss and 75 control persons. Neither heterozygous nor homozygous deletions were found. Interestingly, one sample in the patient group and two samples in the control group were found with a duplication of the proximal repeat part, with a size comparable to the observed deletion. This leads to the conclusion that having an extra copy of the four active genes does not present a phenotype and also supports the theory of NAHR as the main mechanism of recombination of this repeat region.

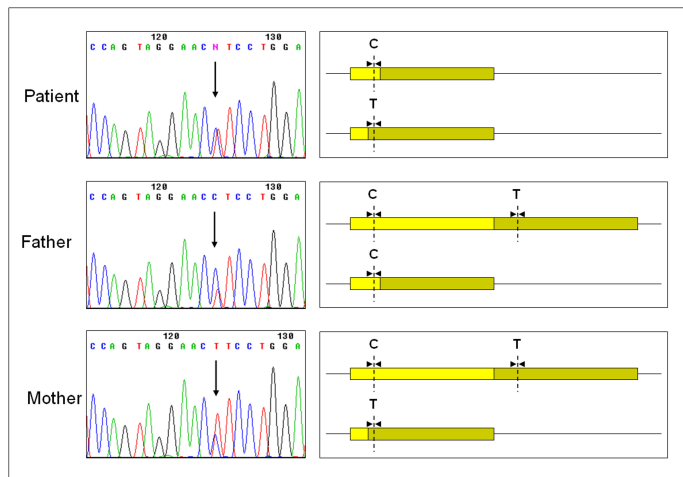


Figure 4. Sequence results of the paralogous sequence variant at base pair 41652116 of chromosome 15 in the proband and both parents. Sequence graphs show an equal ratio in the proband between the PSVs on both alleles, while the mother shows a 2:1 ratio for the C-allele and the father shows a 2:1 ratio for the T-allele (black arrows). The schematic drawing next to the sequence results show the inheritance of the different alleles that result in the ratio values of the PSV. The bright yellow bar is the proximal duplication, the dark yellow bar is the distal duplication and the arrowheads are the primers used to amplify the sequenced fragment. It also shows that in the maternal allele NAHR should have taken place proximal to the PSV, while in the paternal allele NAHR should have taken place distal to the PSV.

The investigated region on chromosome 15q15.3 was identified to be involved in normal variation (both gain and loss) in separate studies. [1,9,10,12] In the HapMap collection[19] that is analyzed in a part of these studies, 12 samples had a CNV reported at this location. Eight of these samples had a CNV of a continuous region greater than 30 kb in size, which indicates a full a deletion or duplication. This was confirmed with our MLPA test in the six available samples with the bigger alteration. Of these samples with alterations the size of a duplication, 4 HapMap samples showed inheritance of the 15q15.3 CNV with either paternal or maternal transmission, while the remaining cases were individual samples. With regard to ethnic distribution, the 15q15.3 CNVs were present in the CEPH population, the Yoruban population from Nigeria and the Chinese and Japanese population. In our study we confirmed all these CNVs by using the targeted MLPA set. The existence of these deletions in different ethnic populations supports

that the presence of this variation is not restricted to one region or one founder family.

Pooling all in-house whole genome screened samples (n=300), locus specific data (n=109) and normal variation data published in the literature and summarized in the DGV database (n=320), a hemizygous deletion frequency of ~1.6% and a duplication frequency of ~0.7% (n=729) is found for this genomic region. This proves that the involvement of a normal variation locus with a hemizygous deletion frequency of 1.6% and a serious phenotype in the homozygous state is of importance for genetic testing. The presented data show that potentially important information may be missed when disregarding CNVs based on inheritance status and reported involvement in normal variation.

The fact that both pathogenic point mutations in the *STRC* gene[18] and homozygous deletions of the 15q53.3 region[16,17] have been reported to be associated with hearing loss, emphasizes the importance of considering the chromosome 15q15.3 locus in screening for the genetic cause of hearing loss.

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**Patient consent:** Informed consent was obtained of the patient and his parents described in this article.

**Supplemental Data:** Two tables are available online.

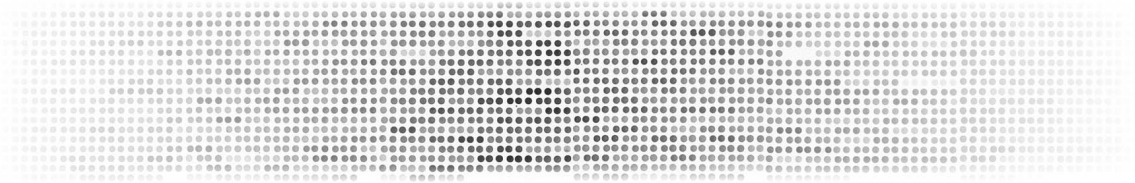
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# Chapter 9

## Discussion





Before the introduction of genome wide molecular screening techniques, structural alterations in chromosomes have been found mainly by G-banding, allowing the detection of aneusomies and structural alterations of at least 5-10 Mb in size. They have been commonly identified based on the principle of “phenotype first”, in which patients with the same phenotype have been found to have similar structural or numerical chromosomal alterations. Cryptic alterations were occasionally revealed by the detection of a patient with a balanced translocation in a patient group with similar phenotypic characteristics and no apparent visible structural alterations. The translocation breakpoints in the index case could then pinpoint the region causative for the phenotype and the patient group could subsequently be screened for involvement of this genetic region with FISH or mutation detection by sequencing.

With the introduction of molecular techniques for whole genome analysis a new era has started in clinical cytogenetics. Array-CGH, SNP arrays and massive parallel sequencing provide information on unbalanced structural chromosomal alterations in much higher resolution and with more precision than conventional banding methods. Massive parallel sequencing techniques as paired-end mapping even detect balanced translocations and inversions and SNP arrays can detect copy number neutral alterations as uniparental disomies.

These new tools are needed to unravel the causal defects in patients with idiopathic developmental delay with or without congenital abnormalities. It was already known that the causes for developmental delay could be very diverse, from large chromosomal or numerical alterations to single gene deletions, disruptions or mutations and even to environmental factors or metabolic disease. Nevertheless, the development of array-CGH proved to be of significant value in the diagnostics of mental retardation in terms of resolution and accuracy of the detection of the abnormality. An example for this is shown in chapter 2, where several cell lines of patients with chromosomal alterations were investigated. In one case, G-banding revealed an aberrant chromosome 8 that was judged to be an isochromosome 8q. After array-CGH analysis the marker chromosome was found to have a partial 8p deletion together with a partial 8q duplication, instead of an isochromosome 8q. Subsequently, the mother was found to be carrier of a pericentric inversion of chromosome 8. A second case was identified with a ring chromosome X, while G-banding was unable to resolve the origin of the ring chromosome. In a third case array-CGH showed the capacity to detect different sub-lineages in a ring chromosome 13 cell line, showing the instability of this particular ring chromosome. This instability was proven with FISH.

A separate example of the power and additional value of array-CGH is given in chapter 5. Here, a patient with a complex rearrangement as detected with G-banding, was thought to originate from a balanced three way translocation and a separate insertion. Detailed investigation using array-CGH and FISH revealed eight breakpoints with an additional deletion in one breakpoint region. This and other publications [1-4] proved that a significant portion of complex

rearrangements that appear balanced with G-banding have additional rearrangements or deletions not detected using classical cytogenetics.

Several cases of ring chromosomes have also shown to be more complex when detailed array analysis was performed after conventional G-banding. Chapter 6 describes a ring chromosome 14 which next to a terminal deletion also has an inverted duplication and a triplication. These additional alterations were not seen initially nor retrospectively using cytogenetic G-banding investigation. Based on this chapter and the publication of Rossi et al. [5], one can conclude that screening with high resolution molecular techniques is needed for correct interpretation of acrocentric ring chromosomes.

When patients with idiopathic mental retardation are studied using array-CGH, providing a 10-fold better resolution than G-banding, 10-15% more causal genetic alterations are detected, as shown in this thesis in chapter 3 and other published studies [6]. These figures match the percentages that were estimated at the start of the project to establish array-CGH within our laboratory. The results also emphasized that the alterations found by array-CGH provide superior resolution and are mostly heterogeneous, which limits the possibilities for targeted screening for unknown genetic factors [7].

Nevertheless, many recurrent deletions and duplications are detected by array-CGH. They are often found to be flanked by repetitive sequences, by which the formation of these alterations is mediated. Non-allelic homologous recombination is described as the main responsible mechanism. During meiosis misalignment takes place between the two repeats and subsequent recombination causes deletion or duplication of the unique sequence between the repeats. The finding of such recurrent deletions and duplications resulted in the identification of the underlying genetic cause of several different syndromes. This is sometimes called “genotype-first” type of research, whereby contrary to the phenotype-first studies the genetic alteration of a patient with idiopathic mental retardation is elucidated first, after which the patient can be categorized in relation to other patients with similar genetic alterations. Often the phenotypic characteristics of these patients are retrospectively comparable, but for example because of heterogeneity in the phenotype, it proves to be a difficult task for clinical geneticists to diagnose the right syndrome, without *a priori* whole genome screening results [8].

In chapter 7 an example of a recurrent duplication is described, which is mediated by the recombination of flanking duplicons. This report describes a genetic alteration with a rather variable phenotype. The fact that this recurrent duplication is not found in unrelated normal individuals supports the conclusion that it represents the causal alteration. The variation seen in the phenotype may possibly be explained by other unknown genetic or environmental factors.

In the latter case, as well as in many other individuals with mental retardation, epigenetic factors may also play a role in the origin of the retardation. For instance imprinting is known to play a role in the causality of Prader-Willi

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syndrome, Angelman syndrome and Beckwith-Wiedemann syndrome, in which in some cases the expression of a gene is influenced by uniparental disomy of the involved chromosome. In Angelman syndrome a fraction of patients was found with a paternal imprinting pattern on the maternal allele affecting the expression of the involved gene [9]. Probably more syndromes of mental retardation are caused by differences or defects in hypo- or hypermethylation of promoter regions or genes involved in development or of aberrant methylation or acetylation of histones, thereby influencing underlying gene expression.

High resolution whole genome molecular screening such as array-CGH results in the detection of many sub-microscopic copy number variations. Chapter 8 shows clearly that in a homozygous state normal copy number variations may have phenotypic impact and it is rather likely that more regions currently reported as normal variation may have a similar influence. Also one can expect that some regions of variation can be tolerated in a person without consequence, while in another individual the same region causes a phenotype because of a different genetic background. With more detailed investigations and further documentations it is expected that so-called polygenic causes for disease will become a more frequent finding.

To maximally benefit from data on copy number variation it is very important that international arrangements are made for the documentation of the data. The Hospital of Sick Children in Toronto, Canada, started to host a database called the Database of Genomic Variants (DGV) [10,11]. This database contains data of structural variation in normal individuals or cell lines, generated with microarray platforms and mass sequencing techniques. Free accessibility for the whole community is offered allowing everybody to profit from the wealth of information that arises from individual experiments. Nevertheless care must be taken to only implement data of a high quality standard, so that the information collected from the database can be interpreted correctly. The database should also be maintained properly, according to the latest results and insights, since new data could shed different light on the influence of structural variation to disease. Two other databases, a European one named the “European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations” or ECARUCA [12], and an international database called the “Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources” or DECIPHER [13], register chromosomal imbalances that are presumably linked to a phenotype. Registered geneticists and cytogeneticists are able to upload molecular data on reported copy number variations, combined with the phenotypic patient information. These databases are by necessity partly restricted to the registered professionals to protect private patient information. Worldwide submitted data can serve as a reference in explaining found copy number data in a single patient. Also for these databases control and quality assurance of submitted data is a prerequisite. The collaboration that resulted in

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the publication of chapter 7 was based on contacts initiated through the DECIPHER platform.

Molecular genetic techniques for whole genome screening have proven to be highly valuable in research settings. In chapter 4 a different strategy for routine screening of developmentally delayed patients is proposed, in which routine screening with MLPA precedes array-CGH and conventional G-banding. This approach may result in a more cost effective screening of patients.

The use of array-CGH in the studies presented in this thesis has been successful. In chapter 3 an increase of 16% of the genetic alterations found in a patient group with mental retardation with or without congenital anomalies was found using array-CGH. This percentage was comparable to percentages found in other studies of array-CGH screening in mental retardation patients. Moreover, the introduction of the technique created a number of possibilities for follow-up research and interesting research questions, resulting in detailed molecular characterization of several genetic alterations as presented in this thesis.

The field of genetics is developing very rapidly, with techniques such as paired-end mapping and mass-sequencing being introduced recently. Therefore, it is far from clear what the gold standard will be for cytogenetic laboratories in the near future. However, as this thesis and many other publications prove, it is certain that the field of cytogenetic screening with mainly G-banding and FISH needs to change dramatically. The latest developments make new diagnostic approaches possible to maximize the yield of genomic information in a cost-efficient way. On the other hand, the era of classical chromosome investigation is not over yet. Correct interpretation and confirmation of the molecular genetic data as well as the detection of Robertsonian and reciprocal translocations and inheritance patterns continue to require chromosome analysis by microscopy.

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## Summary

In the past decades it gradually became clear that genetics play a significant role in idiopathic mental retardation. The search for genetic alterations responsible for idiopathic mental retardation has been dominated by cytogenetic screening methods as chromosome banding. Many genetic alterations, such as trisomies, partial trisomies or monosomies and balanced or unbalanced translocations were detected. However, as the detection limit of classical cytogenetic screening is about 5-10 megabases, this limitation has precluded the finding of a positive genetic cause in a large portion of cases.

In order to detect smaller genetic alterations genome-wide a new technique was developed, called array comparative genomic hybridization (array-CGH). This molecular genetic technique is based on the comparison of two differentially fluorescently labeled genomic DNA samples (typically a test or patient sample and a reference sample of a normal individual), hybridized to an array of immobilized DNA fragments representing specific locations on the genome. The resolution of this array-CGH technique depends on the spacing of spotted DNA fragments and the length of each spotted probe.

In chapter 1 array-CGH is introduced and placed into the spectrum of other cytogenetic and molecular screening techniques currently used. Chapter 2 to 8 of this thesis describe the development and use of an array-CGH platform in the field of molecular and cytogenetics, built up using large insert clones with a spatial resolution of about 1 Megabase throughout the whole euchromatic genome. Added functionality of array-CGH compared to conventional banding techniques is shown in chapter 2. Chromosomal imbalances could be detected more accurately and even be revised in primary cell lines from patients with known aberrations.

In a consecutive study array-CGH was compared to current techniques with respect to detection of potential pathogenic genetic alterations. Array-CGH detected 16% more confirmed potential pathogenic genetic alterations in idiopathic mental retardation as shown in chapter 3. This percentage is in concordance with other published studies.

The advantages described above may change the approach that is currently used for postnatal genetic testing. While conventional chromosome banding has been the method of choice for screening for years, in chapter 4 a fundamental new approach is proposed that used a panel of molecular techniques as MLPA and array-CGH as the first step in screening for genetic alterations. This robust, cost-effective and high-throughput procedure may lead to a faster and more precise elucidation of chromosomal imbalances.

The following chapters then explain in a detailed way that array-CGH including high resolution oligo array-CGH, are useful tools to elucidate chromosome imbalances in detail and a convenient step towards characterize alterations down to a single base. This type of studies not only detects genetic alterations, but

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also contributes to the common knowledge of the understanding of how chromosome rearrangements take place. They may result in better genotype to phenotype correlation of patients and can pinpoint the influence of a given gene or genetic region to a specific disease. These chapters also show that it is highly important to establish and maintain international collaborations within the field of genetics to share data on copy number variation, in order to distinguish disease related changes and normal variation.

Chapter 5 and chapter 6 describe examples of complex chromosomal imbalances that are elucidated in detail using array-CGH. In chapter 5 a seemingly balanced complex translocation involving three chromosomes and eight breakpoints was additionally found to contain a cryptic deletion. Chapter 6 shows a complex ring chromosome with an additional duplication and triplication, which initially was concluded to only have a simple subtelomeric deletion with standard microscopical evaluation. This chapter proves that in order to obtain a correct genotype to phenotype correlation detailed screening using G-banding analysis in combination with high resolution molecular techniques is necessary.

In chapter 7 a microduplication of chromosome 3q29 found in four different families is described. It is shown that this specific chromosomal rearrangement has a reduced penetrance and a variable phenotype. To conclude causality of a copy number variation like this duplication it is important to document case reports and families with uncommon variation in broadly accessible databases. The phenotypic influence of a copy number variation can then be recognized earlier. On the other hand it remains a challenge to correctly interpret the data on copy number variation that is recorded in these databases, as is shown in chapter 8. Here, a deletion of chromosome 15q15.3 which is described as a normal copy number variant is proven to be causing hearing loss in a syndromic patient in a homozygous state.

Now or in the near future array-CGH techniques will generally be implemented in diagnostic laboratories to support or partially replace the classical cytogenetics. They have proven to be a very valuable addition to the diagnostics of clinical genetics, but in order to correctly interpret structural alterations metaphase chromosome analysis remains an important technique. Nevertheless, the field of genetics proves to be highly innovative as next generation sequencing techniques are already on the doorstep to take over array comparative genomics. These next generation sequence techniques will be able to sequence the whole genome of a patient in search of causal alterations and will result in superior resolution over the previous techniques. It proves that the development within the field of diagnostics has certainly not come to an end.

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## Samenvatting

Sinds de jaren vijftig van de afgelopen eeuw is het duidelijk geworden dat genetica een belangrijke rol speelt bij mentale retardatie. De zoektocht naar genetische afwijkingen die mentale retardatie veroorzaken, is gedomineerd door cytogenetische technieken als chromosoombandering. Hoewel vele genetische afwijkingen zoals trisomieën, gedeeltelijke trisomieën of monosomieën en (on)gebalanceerde translocaties zijn gevonden, is bij een significant deel van de gevallen geen genetische afwijking gedetecteerd, omdat de ondergrens van detectie bij chromosoombandering rond 5 tot 10 megabasen ligt.

Om kleinere genetische afwijkingen op te sporen is een nieuwe techniek ontwikkeld genaamd “array comparative genomic hybridization” (array-CGH). Deze moleculair-genetische techniek is gebaseerd op het vergelijken van twee genomische DNA monsters, die een verschillend fluorescerend label bevatten (meestal een patiëntmonster en een referentiemonster van een normaal persoon). Beide gelabelde monsters worden tegelijkertijd gehybridiseerd op een array van geïmmobiliseerde DNA fragmenten die specifieke stukken van het genoom vertegenwoordigen. De resolutie van deze array-CGH techniek hangt af van de mate van representatie van het hele genoom en de lengte van de geïmmobiliseerde DNA fragmenten.

In hoofdstuk 1 van dit proefschrift wordt array-CGH geïntroduceerd en besproken in de context van de algemeen gangbare cytogenetische en moleculaire technieken. Hoofdstukken 2 tot en met 8 beschrijven de ontwikkeling en het gebruik van een array-CGH platform in de moleculaire en cytogenetica. De desbetreffende array bestond uit DNA fragmenten van gemiddeld 100 kilobasen en een spatiële resolutie van ongeveer 1 megabase. De toegevoegde waarde van array-CGH ten opzichte van de conventionele banderingstechnieken wordt getoond in hoofdstuk 2. Chromosomale afwijkingen in primaire cellijnen van patiënten met bekende afwijkingen konden nauwkeuriger worden vastgesteld en zelfs worden verbeterd.

In een daaropvolgende studie is array-CGH vergeleken met technieken die op dit moment gebruikt worden voor de detectie van potentiële pathogene genetische afwijkingen. Met array-CGH konden 16% meer afwijkingen aangetoond worden in patiënten met mentale retardatie, welke ook bevestigd zijn met andere technieken. Deze resultaten zijn weergegeven in hoofdstuk 3 en het gevonden percentage is in overeenstemming met andere gepubliceerde studies.

De voordelen van array-CGH kunnen de huidige werkwijze in de postnatale diagnostiek volledig veranderen. Terwijl conventionele chromosoombandering de standaard is sinds vele jaren, wordt er in hoofdstuk 4 een nieuwe aanpak binnen de postnatale cytogenetica voorgesteld waarbij moleculair-genetische technieken als MLPA en array-CGH worden gebruikt als eerste stap in het onderzoek naar genetische afwijkingen. Deze robuuste, relatief goedkope en snelle procedure kan leiden tot een preciezere en snellere detectie van chromosoomafwijkingen.

De volgende hoofdstukken zetten op een gedetailleerde manier uiteen dat array-CGH een nuttige techniek is om chromosoomafwijkingen in detail aan te tonen en het nauwkeurig karakteriseren van afwijkingen mogelijk maakt. Dit type onderzoek toont genetische afwijkingen niet alleen aan, maar draagt ook bij tot de algemene kennis over hoe chromosoomafwijkingen ontstaan. Mogelijk resulteren deze studies in een beter inzicht tussen de correlatie tussen het genotype en het fenotype van patiënten en kunnen ze de invloed van een gen of genetisch gebied binnen een bepaald ziektebeeld vaststellen. Deze hoofdstukken laten ook zien dat het van wezenlijk belang is dat internationale samenwerkingsverbanden gecreëerd en onderhouden worden waarbinnen data over genetische variatie gedeeld worden. Immers alleen op deze manier kan beoordeeld worden of genetische variaties ziekteveroorzakend dan wel normaal zijn.

Hoofdstuk 5 en 6 beschrijven complexe chromosoomafwijkingen die tot in detail geanalyseerd zijn met array-CGH. In hoofdstuk 5 is bij een schijnbaar gebalanceerde complexe driewegtranslocatie met extra insertie een submicroscopische deletie vastgesteld. Hoofdstuk 6 beschrijft een complex ringchromosoom met een deletie, duplicatie en triplicatie, waarbij met G-banding en FISH in eerste instantie alleen de ringstructuur met een terminale deletie gevonden werd. Dit hoofdstuk laat zien dat voor een correcte correlatie tussen genotype en fenotype G-banding gecombineerd moet worden met een moleculair-genetische techniek met hoge resolutie.

In hoofdstuk 7 is een microduplicatie 3q29 beschreven die gevonden werd in vier verschillende families. Dit hoofdstuk toont aan dat deze specifieke chromosoomafwijking een causale maar een variabele en soms verminderde invloed heeft op het fenotype. Om te kunnen bewijzen dat een genetische afwijking een causale invloed heeft, is het van belang te komen tot een algemeen toegankelijke databank, waarin “case reports” en families met ongewone variatie staan geregistreerd. De interpretatie van dit soort data blijft een uitdaging, zoals blijkt uit hoofdstuk 8. Een deletie die herhaaldelijk gerapporteerd werd als een normale variatie bleek in homozygote vorm toch een causaal effect te hebben in een patiënt met syndroomaal gehoorsverlies.

Op dit moment worden in diagnostische laboratoria array-CGH technieken geïmplementeerd als ondersteuning of deels ter vervanging van klassieke cytogenetische technieken. Er is immers aangetoond dat deze een waardevolle aanvulling op de huidige diagnostiek zijn. Echter, om structurele chromosoomafwijkingen correct te kunnen typeren blijft chromosoombandering een belangrijke techniek.

De genetica bewijst een uiterst innovatief vakgebied te zijn, want nieuwe generatie sequencingtechnieken staan op het punt array-CGH te vervangen. Deze nieuwste technieken zullen het mogelijk maken het genoom van een patiënt volledig te sequencen om genetische afwijkingen op te sporen en hebben zodoende een superieure resolutie in vergelijking tot voorgaande technieken. Het is dus evident dat de ontwikkeling van de diagnostiek allerminst ten einde is met de introductie van de array-CGH.

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## Curriculum vitae

Jeroen Knijnenburg (27 mei 1977, Borculo) behaalde het VWO diploma in 1995 aan het Staring College te Lochem en het HBO diploma (HLO, Hogere Laboratorium Opleiding, richting Biochemie) in 1999 aan de Rijkshogeschool IJselland te Deventer. In het laatste jaar van de opleiding heeft hij zijn stageperiode vervuld op de afdeling Moleculaire Celbiologie van Pharming Technologies B.V. te Leiden, onder de leiding van drs. G. Platenburg en dr. J.J. Heus. Vanaf juni 1999 is hij ruim twee jaar werkzaam geweest als analist op dezelfde afdeling van Pharming Technologies B.V. te Leiden.

Vanaf oktober 2001 was hij werkzaam bij het Leids Universitair Medisch Centrum op de afdeling Moleculaire Celbiologie van prof. dr. H.J. Tanke, in eerste instantie onder de leiding van dr. C. Rosenberg, later onder de leiding van dr. K. Szuhai. In maart 2005 is hij begonnen als promovendus op dezelfde afdeling onder de leiding van dr. K. Szuhai, met als onderzoeksdoel structurele genetische afwijkingen op te sporen en te karakteriseren bij patiënten met mentale retardatie met het eerder opgezette array-CGH platform.

Sinds januari 2009 is Jeroen Knijnenburg werkzaam bij de afdeling Klinische Genetica (prof. dr. F.G. Grosveld) van het Erasmus Medisch Centrum te Rotterdam, alwaar hij een opleiding volgt tot Klinisch Cytogeneticus.



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