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High-resolution karyotyping by oligonucleotide microarrays : the next revolution in cytogenetics

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High-resolution karyotyping by oligonucleotide microarrays: the next revolution in cytogenetics

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Chapter 1

Introduction

1. Clinical Cytogenetics

1.1 Cytogenetics

Cytogenetics is a branch of genetics concerned with the study of the structure of chromosomes and their role in heredity. Conventional chromosome analysis using G-banding is widely used for clinical diagnostics and genomic research. However, over the past 30 years the development of new techniques with increasingly higher resolution has led to the new field of molecular cytogenetics.

1.2 History of Cytogenetics

The field of human cytogenetics emerged in 1879 when Walther Flemming described the first human chromosomes and coined the term mitosis (Flemming, 1879). In 1888, Waldeyer introduced the word chromosome which comes from the Greek words chroma for colour and soma for body (Waldeyer, 1888). The term cytogenetics (from cytology and genetics) was formulated by Sutton in 1903 (Sutton, 1903). In the years that followed there were several publications on the human chromosome number (Von Winiwarter, 1912; Painter, 1923; Hsu, 1952). For a long time the chromosome number of humans was considered to be 48. Finally, in 1956 using improved tissue culture techniques, Tjio and Levan showed the correct human chromosome number in lung fibroblasts to be 46 (Tjio and Levan, 1956). This was soon confirmed on meiotic chromosomes from human testicular tissue (Ford and Hamerton, 1956). Three years later the first chromosomal aberration was discovered by Lejeune and his colleagues (1959) who identified an extra small chromosome in fibroblast cultures from patients with Down syndrome. Soon thereafter more chromosomal abnormalities were described involving both sex chromosomes and the autosomes: 45,X in Turner syndrome (Ford et al., 1959), 47,XXY in Klinefelter syndrome (Jacobs and Strong, 1959), 47,XXX (Jacobs et al., 1959), trisomy 13 in Patau syndrome (Patau et al., 1960), trisomy 18 in Edwards syndrome (Edwards et al., 1960), and deletion of the short arm of chromosome 5 in Cri-du-chat syndrome (Lejeune et al., 1963).

1.3 Chromosome banding and nomenclature

At first the chromosome pairs were classified into seven different groups (A-G), based on morphology (Patau, 1960). A system of nomenclature was proposed at a conference in Denver (Denver Conference, 1960). This classification was officially approved at a conference in London (London Conference, 1963). However, although certain chromosomes could be identified by their size and centromere position, it was not possible to identify individual chromosomes as we know them today. Furthermore, many structural abnormalities, such as inversions, that were suspected could not be proven.

In 1968, it was demonstrated that plant chromosomes, stained with fluorescent quinacrine compounds (Q-banding), showed a distinct staining whereby each chromosome could be identified by its unique banding pattern (Caspersson et al., 1968). In 1970 the first human banded chromosomes were produced by this method (Caspersson et al., 1970). Q-banding proved to be very useful in identifying many chromosome abnormalities. However, as it requires a relatively expensive fluorescence microscope, and the fluorescence fades rapidly, Q-banding was largely replaced by G-banding.

For G-banding, which gives permanent staining, the chromosomes are treated with trypsin followed by staining with a Giemsa dye (Seabright, 1971). This method

is now the most commonly used banding technique in most laboratories worldwide, except in France. In France and many French speaking countries R-banding, which gives a banding pattern that is reverse to that of G-banding, is used as a standard technique (Dutrillaux and Lejeune, 1971). G-banding, a simple and an inexpensive technique, enabled rapid identification of many new chromosomal abnormalities, including deletions, duplications, translocations, inversions and insertions. In 1971, at the Paris Conference, a new system for the classification of chromosomes, based on Q, G, and R banding patterns, was introduced to identify individual chromosomes and chromosome regions (Paris Conference, 1971). This was followed in 1978 by a new document entitled "An International System for Human Cytogenetic Nomenclature" which included the major decisions of the Denver, London and Paris Conferences (ISCN 1978). This nomenclature for human chromosomes became widely used and is regularly updated (most recently in 2009, ISCN 2009). G-banding of long pro-metaphase chromosomes gave high resolution banding (Yunis et al, 1980) and enabled the identification of subtle chromosome alterations.

2. Chromosome morphology

2.1 Chromosome variants

Chromosomes consist of chromatin which is a combination of DNA and proteins. There are two types of chromatin, euchromatin and heterochromatin, which show different degrees of condensation. Euchromatin is the less condensed form, is generally rich in genes and is actively transcribed whereas heterochromatin is normally more condensed, poor in gene content, and rich in repetitive DNA. Even before the advent of banding it was known that the size and position of the heterochromatic segments could vary between individuals without a phenotypic effect. They were called heterochromatic variants. In recent years, variants of certain euchromatic chromosome segments have also been described.

2.2 Heterochromatic variants

Already in 1960 it was evident that the Y chromosome could vary considerably between individuals (Patau, 1960). At the London Conference on 'The normal human karyotype' in 1963, it became apparent that also the secondary constrictions near the centromeres of chromosomes 1, 9 and 16 could vary in size. Although these so-called heteromorphisms had been widely reported, it was unclear whether or not they were associated with clinical abnormalities (Cooper and Hernits, 1963; Yunis and Gorlin, 1963; Palmer and Schroder, 1971; Lobitz et al., 1972). It was only after large studies on consecutive newborns that it became clear that most of the heterochromatic variants were not disease-related (Sergovich et al., 1969; Lubs and Ruddle, 1970; Friedrich and Nielsen, 1973; Jacobs et al., 1974; Nielsen and Sillesen, 1975; Hamerton et al., 1975). Eventually, large scale population studies based on banded chromosomes revealed heteromorphisms on chromosomes 1, 3, 4, 6, 9, 13-16, 21, 22 and the Y (Geraedts and Pearson, 1974; Madan and Bobrow, 1974; Müller et al., 1975; McKenzie and Lubs, 1975; Madan and Bruinsma, 1979) and demonstrated that these were heritable (reviewed by Wyandt, 2004). The heritability of heteromorphisms was used in various ways, such as to demonstrate maternal contamination in prenatal samples (Olson et al., 1987), to determine paternity (Olson et al., 1983; Olson et al., 1986) and to determine the parental origin of chromosome abnormalities (Magenis et al., 1977; Mikkelsen et al., 1980; Jacobs et al., 1982; Juberg and Mowrey, 1983).

2.3 Euchromatic variants

Deletions and duplications of euchromatic segments are usually pathogenic. However, several microscopically visible euchromatic deletions and duplications without any phenotypic consequences have been described. These euchromatic variants reflect copy number variation of chromosomal segments containing genes and pseudogenes. They can be polymorphic in the normal population and only reach a cytogenetically detectable level when the multiple copies are long enough to be observed under a microscope as constitutional cytogenetic amplifications. Euchromatic variants segregate in families without apparent phenotypic consequences. The first euchromatic variant was reported on the short arm of chromosome 9 in a G-banded chromosome study of live-born infants (Buckton et al., 1980). Other examples of regions with known euchromatic variants are 8p23.1, 9p12, 9q12, 9q13, 15q11.2 and 16p11.2 (reviewed by Wyandt, 2004; Barber et al., 2005; Hansson et al., 2007). In many cases it is difficult to decide whether a euchromatic deletion or a duplication is pathogenic or benign. Since most of the aberrations are initially detected in phenotypically abnormal individuals, their benign or pathogenic status can be established only after family studies.

3. Molecular Cytogenetics

3.1 Overview

Although G-banding has improved substantially since its initial discovery in 1971, it enables the detection of deletions or duplications only in the order of 5-10 Mb. For the identification of smaller aberrations new techniques are needed. Fluorescence *in Situ* Hybridization (FISH) was the first of a series of methods that led to the emergence of the field of molecular cytogenetics, a combined application of cytogenetics and molecular biology. Some of the new methods are used only for the confirmation or further characterization of previously identified chromosomal aberrations (FISH, Multiplex Ligation-dependent Probe Amplification, MLPA and Quantitative Fluorescence-Polymerase Chain Reaction, QF-PCR) whereas others are used for whole genome screening with different resolutions (array Comparative Genome Hybridization, aCGH and Single Nucleotide Polymorphism array, SNP array). Table 1.1 gives an overview of the (molecular) cytogenetic techniques and their possible resolutions. The techniques most commonly used in cytogenetic diagnostics are described below.

3.2 Fluorescence *in Situ* Hybridization

In Situ Hybridization (ISH) was developed to detect specific DNA sequences on chromosomes and was initially based on radioactively labelled probes (Pardue and Gall, 1969). This technique was used for the localization of genes to chromosomes (Harper et al., 1981; Gerhard et al., 1981). FISH, a locus-specific technique, was introduced in the 1980s (Prooijen-Knegt et al., 1982; Landegent et al., 1986). It allows the detection of chromosomal abnormalities directly on metaphase chromosomes and in interphase nuclei. A fluorescent labelled DNA probe hybridizes to its complementary sequence in chromosomes and is visualized with a fluorescent microscope. Probes used for FISH can be made from Bacterial Artificial Chromosome (BAC), P1 Artificial Chromosome (PAC), cosmid or fosmid clones, or from PCR products. The resolution depends on the size of the probe (>50 kb - 2 Mb). This method can be used to examine those regions of chromosomes that are suspected of carrying a specific abnormality based on the clinical picture of the patient, for instance microdeletion syndromes (Dauwerse et al., 1990). FISH is a powerful technique that can be used to detect specific structural

Table 1.1 Overview of cytogenetic techniques

	Conventional karyotyping	Locus specific metaphase FISH	Locus specific interphase FISH	Fibre FISH	SKY/M-FISH	MLPA	QF-PCR	arrayCGH (BAC)	arrayCGH (oligo's)	SNP array
Whole genome screening	+	-	-	-	+	-	-	+	+	+
Resolution	5-10Mb	50 kb-2 Mb*	50 kb-2 Mb*	5 kb -500 kb	3-5 Mb	50-70 bp	50-70 bp	100-300 kb*	10 kb⁺	10 kb⁺
Deletion	+	+	+	+	±	+	+	+	+	+
Duplication	+	-	±	+	±	+	+	+	+	+
Triplication	+	-	±	+	±	±	+	+	+	+
Trisomy	+	+	+	-	+	+	+	+	+	+
Triploidy	+	+	+	-	+	-	+	-	-	±
Monosomy	+	+	+	-	+	+	+	+	+	+
Inversion	+	++	-	+	-	-	-	-	-	-
Insertion	+	++	-	+	++	-	-	-	-	-
Balanced translocation	+	++	-	-	++	-	-	-	-	-
Unbalanced translocation	+	++	-	-	++	±	-	+	+	+
Complex rearrangement	+	++	-	+	++	±	-	±	±	±
Identification marker chromosomes	+	+	+	-	++	-	-	±	±	±
UPD	-	-	-	-	-	-	+	-	-	+
LOH	-	-	-	-	-	-	+	-	-	+
Genotyping	-	-	-	-	-	-	-	-	-	+

Table is obtained and adapted from Schoumans and Ruivenkamp (2010) *Resolution depending on the size of the probes; ⁺ resolution depending on the number of probes on the array

chromosome abnormalities. However, it is relatively labour intensive and its use in diagnostics is limited because of its low resolution.

Fiber FISH allows the detection of deletions and duplications of even just a few kilobases (Heng et al., 1992; Wiegant et al., 1992). This technique uses extended chromatin fibers and is able to resolve complex chromosomal rearrangements (Florijn et al., 1995; Giles et al., 1997). The preparation of fiber FISH samples, however, requires a highly skilled technician, and the technique is used only in specialized laboratories. Furthermore it is only used for a more detailed characterization of complex chromosomal aberrations that have been identified already.

Other FISH applications use telomere-specific probes and whole-chromosome painting probes. Spectral karyotyping (SKY) and multicolour-FISH (M-FISH) are methods whereby in one experiment a whole karyotype can be produced in which each chromosome has a different fluorescent colour (Schröck et al., 1996; Speicher et al., 1996). These techniques allow the detection of interchromosomal aberrations such as translocations, insertions, complex chromosome rearrangements and the origin of marker chromosomes. The major disadvantage of SKY and M-FISH is that inversions, deletions and small duplications are not detectable.

While using different types of FISH for increasingly detailed analysis of chromosomes, it became apparent that techniques used for DNA analysis essentially also give information on chromosome morphology, albeit at another level of resolution. It was soon realized that these high throughput DNA techniques could also be used for studying chromosome morphology.

3.3 Array-CGH using BAC clones

Array comparative genome hybridization (aCGH) is a technique which compares DNA of a test sample with DNA of a reference sample and is generally seen as the first cytogenetic application of genomic (DNA-based) array technology. These arrays contained BAC and PAC clones covering the whole genome (BAC arrays) and were mostly in-house made by spotting (Solinas-Toldo et al., 1997; Pinkel et al., 1998). aCGH using BAC clones allows the detection of copy number variants (deletions and duplications) that are approximately 100 times smaller than those identifiable with conventional karyotyping. The resolution of these arrays depends on the distance between the probes as well as on the sizes of the probes. Initially, the BAC arrays contained approximately one clone per Mb (i.e. about 3500 BAC clones for the coverage of the full human genome) followed by a tiling path array with a resolution that is 10 fold higher (including 33000 BACs). The technique is based on competitive hybridization of test and reference DNA labelled with different fluorochromes (e.g. red for test and green for reference) on the spotted BAC clones. The array is imaged by a scanner and the relative fluorescence intensities are calculated. The chromosomal regions that are equally represented in the test and reference samples appear yellow, but those that are deleted in the test sample appear more green and those that are amplified appear more red. This results in intensity ratios for each mapped clone and reflects the copy number difference which can be visualized by a number of software packages.

3.4 Array-CGH using oligonucleotides

The procedure for oligonucleotide CGH arrays is similar to that for BAC arrays, using differentially labelled test and reference DNA. However, these arrays are commercially manufactured by lithography, and consist of 45- to 85- mer oligonucleotide probes.

The major manufacturers are Agilent and Nimblegen. Their first arrays contained 44.000 and 72.000 oligonucleotides respectively, but the latest released oligonucleotide CGH arrays contain 1 and 2.1 million probes respectively.

3.5 Single Nucleotide Polymorphism (SNP) array

A different type of oligonucleotide array is the SNP array which was initially developed for genome-wide linkage and association studies. This method allows the detection of SNP-genotype as well as the presence of deletions and amplifications. The major manufacturers of SNP arrays are Affymetrix and Illumina. These two companies use different technologies for the discrimination of alleles and their sensitivity for the detection of CNVs is different. A major advantage of this type of array is that it can be used both for genotyping studies and for copy number screening.

Affymetrix

The first SNP array produced by Affymetrix contained 10.000 (10K) SNP probes on a single array slide. This was followed by a 100K and 500K, the probes being distributed in the latter case over two array slides of 250K each. Later the 5.0 (500K) array combined the 500.000 probes on a single slide and the newer arrays 6.0 and 2.7M contain 1.8 and 2.7 million probes respectively on a single slide. These arrays include SNP probes and additional probes specific for the detection of copy number variation.

The Affymetrix method is a single color assay and is composed of multiple overlapping allele-specific hybridization probes that are complementary to SNP regions present in the reduced fraction of the genome amplified in the assay. Since the 500K assay is now used for routine cytogenetic diagnostics in a number of laboratories, the method (adapted from www.affymetrix.com) is explained here in detail. The probes are 25-mer oligonucleotides with the variable SNP located at the 13th nucleotide. The probes present are for both possible alleles of each SNP, so-called 'perfect match' (PM). Besides these PM probes, mismatch (MM) probes are included for each allele to allow discrimination between signal and noise. Four additional probe quartets are present for each SNP where the probe sequences are different at four other positions. Additionally, all the quartets are present in both the forward and the reverse orientations. As a consequence, each SNP is represented by 40 distinct probe sequences. The probes are scattered over the array to diminish any effects of array variation and each probe has a fixed location on the array.

Approximately 250 ng of genomic DNA is digested with restriction enzymes and ligated to adaptors recognizing the overhangs (Figure 1.1). A universal primer, which recognizes the adaptor sequence, is used to amplify ligated DNA fragments and PCR conditions are optimized to preferentially amplify fragments in the 200-1000 bp size range. These products are purified and fragmented to 50-200 bp products. The amplified and fragmented DNA is incorporated with biotine labeled nucleotides and hybridized to GeneChip arrays. After hybridization of 16-18 hours, the arrays are washed, fluorochromes are labeled to the biotine on the GeneChip fluidics station, and scanned on a GeneChip Scanner 3000. Signals from the allele-specific probes are analyzed to determine whether a SNP is AA, AB, or BB. The signal intensity is quantified and compared to signal intensities of normal individuals to determine SNP copy number. Several software packages have been developed to analyze SNP genotypes and to determine copy number.

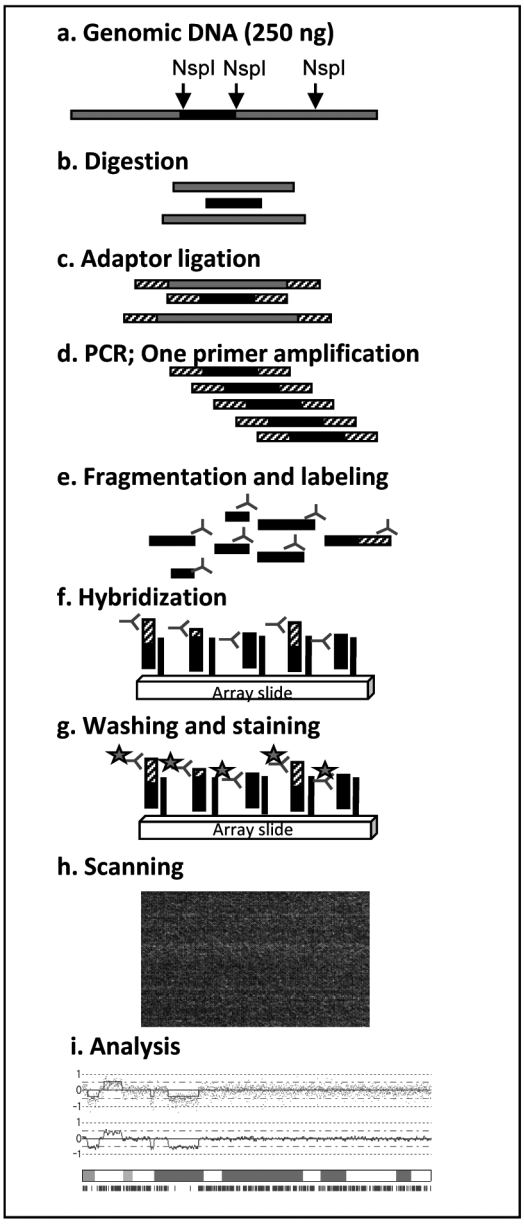


Figure 1.1 Schematic overview of the Affymetrix platform procedure

Illumina

The first SNP arrays from Illumina contained 317.000 probes (300K) on a single slide. The next generation contained twice the same 317.000 probes on a single slide, thus enabling hybridization of DNA from two individuals (317K-duo). This was followed by a 370K-duo and a 550K-duo. The latest SNP arrays from Illumina contain 650.000 and 1 million probes.

The concept of the Illumina assay is based on direct hybridization of whole genome-amplified genomic DNA to a bead array of 50-mer locus-specific probes. These probes end one nucleotide before the SNP. After hybridization each SNP is scored by a single base extension assay using different labeled nucleotides. These labels are visualized by staining with an immunohistochemistry assay. The A and T nucleotides are stained in one color and C and G in another. The signal intensity is used to determine copy number. On each array the beads are randomly assembled, therefore every array is provided with a file with the exact probe locations. A brief description of

the protocol (adapted from www.illumina.com) is described here. The first step in the Illumina assay is a whole genome amplification of 750 ng of genomic DNA (Figure 1.2). The amplified genomic DNA is fragmented to an average size of approximately 300 bp using an endpoint enzymatic fragmentation protocol. These fragments are precipitated and re-suspended in a hybridization buffer. The precipitated DNA is hybridized to a BeadChip in a humidified chamber. After 16-18 hours the hybridized arrays are washed and the next step is a single base extension with differentially labeled nucleotides. The BeadChips are scanned with a two-color confocal Illumina BeadArrayTM Reader. Image intensities are extracted and genotypes and copy number are determined using Illumina's BeadStudio software. Several other software packages have been developed to analyze Illumina data.

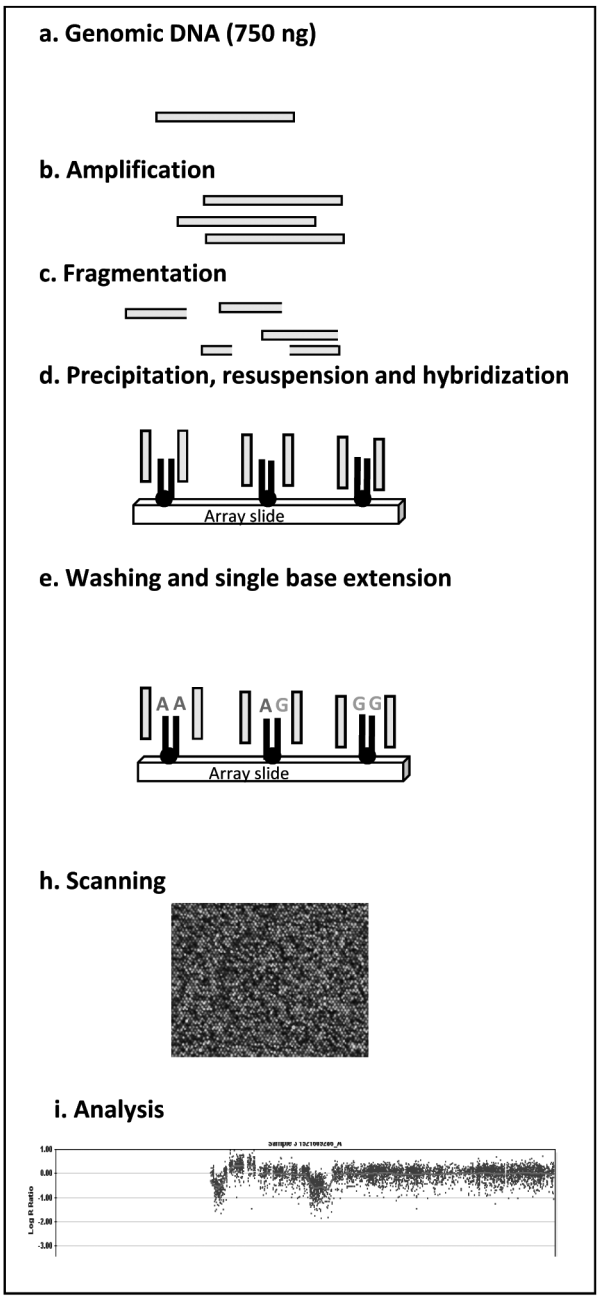


Figure 1.2 Schematic overview of the Illumina platform procedure

4. Copy number variants

As mentioned earlier, variation in chromosome morphology has been known from the earliest days of cytogenetics. It has now become possible to study variation in the composition of chromosomes right down to the nucleotide base level. Variation in the human genome takes many forms, ranging from the heterochromatic and euchromatic variants that have been described above to SNPs. With the advent of high-resolution whole-genome array technologies it has become evident that many submicroscopic copy number variants (CNVs), varying in size from kilobases to megabases, are present in all humans (Iafrate et al., 2004; Sebat et al., 2004; Sharp et al., 2005; Tuzun et al., 2005; Hinds et al., 2006; McCarroll et al., 2006; Locke et al., 2006; Feuk et al., 2006; Friedman et al., 2006; Redon et al., 2006; Conrad and Hurles, 2006). The identification of disease causing CNVs is hampered by our inability to distinguish between normal and causative variants.

New recurrent deletions and duplications for which the clinical significance is not directly evident have been reported. In 2007, a deletion of approximately 600 kb on 16p11.2 was reported in 1% of individuals with autistic features (Weiss et al., 2008). This was supported by two additional studies (Kumar et al., 2008; Marshall et al., 2008). However, in one of our studies we identified the same CNV in patients with mental retardation (MR) as well as in healthy individuals, both groups without autism (**Chapter 3.1**). This indicates that the recurrent deletion of 16p11.2 gives rise to a broad and highly variable phenotype, including a normal phenotype. Another aberration which is detected in MR patients as well as in healthy individuals is a 253 kb deletion in 15q11.2 (Murthy et al., 2007; Doornbos et al., 2009). This deletion was first reported in a boy with MR and dysmorphic features. He inherited it from his father, who had a history of developmental delay (Murthy et al., 2007). Doornbos and colleagues studied nine patients with the same deletion. In seven cases it was inherited from one of the parents. Only one of these parents showed an affected phenotype (the same developmental and behavioural problems as in the child). They concluded that a deletion in this region was associated with variable phenotypes, some of which could be explained by other genetic or environmental modifiers.

One of the consequences of the continuous increase in the resolution of the whole-genome arrays is that also the number of detected pathogenic, benign and potentially pathogenic CNVs has increased. Estimates indicate that approximately 12% of the human genome may involve CNVs (Redon et al., 2006). The detected CNVs may be difficult to interpret in both clinical and research settings. Different workflows have been developed and are used to categorize CNVs into the different groups (Lee et al., 2007; Koolen et al., 2009; **Chapter 2**). In general, CNVs that overlap with known microdeletion or microduplication syndromes are likely to be pathogenic. Benign CNVs are mostly determined by comparing them with healthy or normal reference sets from one's own laboratory or with the data in the Database of Genomic Variants (DGV). The DGV documents structural variants of 1 kb or more that were detected in apparently healthy individuals (http://projects.tcag.ca/variation). This database is still growing and laboratories that use high-resolution whole-genome arrays use it to screen for genomic deletions and amplifications to exclude the benign CNVs. Caution should be exercised when using this database as DGV is not always reliable. This is because many CNVs have been reported in single individuals, different platforms have been used in determining the CNVs and data submitted to this database is not subject to curation or an editorial screening. It is possible that 'normal' healthy individuals reported in the

DGV have phenotypic abnormalities which would have been noticed if the person had been seen by a clinical geneticist. Another example illustrating the need for caution is a case where a heterozygous deletion of 15q15.3 reported in the DGV turned out to be pathogenic in the homozygous state (Knijnenburg et al., 2009). Some heterozygous deletions could be pathogenic if there is a mutation on the other allele. Furthermore, there are CNVs such as 22q11.2 deletion that may show incomplete penetrance.

CNVs that do not overlap with known microdeletion or microduplication syndromes and are not reported as benign variants are categorized as potentially pathogenic. Databases have been developed to collect cases of potentially pathogenic CNVs found in individuals seen in genetic clinics. DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER) collects clinical information about patients with microdeletions, microduplications, insertions, translocations and inversions (https://decipher.sanger.ac.uk). Another database with similar aims is the European Cytogenetics Association Register of Unbalanced Chromosome Aberrations (ECARUCA, http://www.ecaruca.net). CNVs that are not found in the DGV, should be compared with and added to one of these databases to create a platform of molecular cytogenetic and clinical data. This would enable collaboration between clinical genetic centres that have found (approximately) the same CNV in phenotypically similar patients and should make it possible to unravel new microdeletion and microduplication syndromes. The gene content, the size, and the inheritance pattern of potentially pathogenic CNVs are important factors that influence pathogenicity.

The implementation of high-resolution whole-genome arrays in cohorts with unexplained MR patients has resulted in the identification of new microdeletion and microduplication syndromes. These new syndromes were determined by screening large groups of patients with a similar clinical phenotype, mostly unexplained MR (reviewed by Slavotinek 2008; Vissers et al., 2009).

Chapter 2 describes the implementation of the SNP arrays in the diagnosis of MR. An increase from approximately 5% chromosomal aberrations found by conventional karyotyping to 22.6% aberrations by SNP array was noticed. However, most of the detected CNVs are neither described in the DGV nor are they known to be associated with microdeletion or microduplication syndromes. At present we do not know their clinical significance, so most of them have been classified as potentially pathogenic for the time being. More patients or healthy individuals with the same CNV are needed before we can understand their clinical significance.

We are seeing history repeat itself. Similar problems arose with the heterochromatic variants when karyotyping and banding were first introduced. The same was true for euchromatic variants identified by high resolution banding. In a few years one can expect that most of the potentially pathogenic CNVs will have been classified into either pathogenic or benign CNVs. We will then be faced with the same challenge at the base pair level with the introduction of the next-generation whole genome sequencing.

5. Clinical cytogenetic diagnostics

Patients are referred for chromosome analysis for various reasons which can be divided in three groups. 1) Postnatal diagnosis: this includes patients with multiple congenital abnormalities with or without mental retardation (MCA/MR), individuals with infertility and abnormal sexual development and couples with recurrent miscarriages.

2) Prenatal diagnosis: increased maternal age, ultrasound abnormalities in the foetus and chromosome abnormalities in the family. 3) Tumorcytogenetics: acquired chromosome abnormalities in tumor cells.

Identification of chromosomal abnormalities is particularly important for a child with MCA/MR, for diagnosis and prognosis and for estimating the recurrence risk for the parents and other family members. Mental retardation is a highly diverse neurologic disorder with an incidence of 1-3% in the general population (Roeleveld et al., 1997; Leonard and Wen 2002). It is a lifelong disability characterized by impairment of cognitive and adaptive skills, with or without dysmorphic features. MR presents most often during infancy or in the first years of school. The cause of MR can be identified in only about 50% of all patients and is therefore one of the major unsolved problems in modern medicine (Battaglia and Carey, 2003).

The underlying causes of MR are extremely heterogeneous. There are non-genetic factors that can act prenatally, perinatally or during early infancy to cause brain injury. These include infectious diseases (such as rubella, toxoplasmosis and cytomegalovirus during pregnancy and postnatal meningitis), oxygen deprivation (perinatal event), very premature birth and fetal alcohol syndrome, which is caused by excessive maternal alcohol consumption during pregnancy. Genetic factors include (1) single-gene disorders, such as Rubinstein-Taybi syndrome (*CREBBP*-gene) (Petrij et al., 1995) and Pitt-Hopkins syndrome (*TCF4*-gene) (Amiel et al., 2007), (2) chromosomal abnormalities, including presence of an extra chromosome, as in Down syndrome (Lejeune et al., 1959), or a deletion or a duplication of a chromosome segment, for example 5p- in Cri-du-chat syndrome (Lejeune et al., 1963) and microdeletion in 22q11.2 in DiGeorge syndrome (de la Chapelle et al., 1981; Kelley et al., 1982), (3) multifactorial disorders, due to a combination of multiple genetic as well as environmental causes and (4) mitochondrial disorders, caused by alterations in the small cytoplasmic mitochondrial chromosome (e.g. Leigh syndrome OMIM # 256000).

Patients with unexplained MR/MCA, who are referred to genetic laboratories, were screened initially with conventional karyotyping and if necessary with targeted FISH or MLPA analysis. The combined diagnostic yield of these analyses is approximately 5-10% (de Vries et al., 2005). Consequently, a clinical diagnosis is lacking in the majority of these patients and this impedes development of treatment strategies and adequate genetic counseling. Therefore, new high-resolution whole-genome technologies facilitating an increased detection rate of subtle chromosome imbalances have been developed to improve diagnosis of MR/MCA patients.

6. Scope of this thesis

The main aim of this thesis is to detect genome-wide submicroscopic CNVs at high resolution in a cohort of idiopathic MR patients to identify genomic regions or loci involved in developmental disorders. SNP arrays, from two manufacturers (Affymetrix and Illumina) were used to explore the possibility of identifying these abnormalities. The different arrays were directly compared for detection rate, accuracy, software, and costs. By applying these arrays, new regions involved in the etiology of MR were identified, resulting in an increase in the number of patients with a known cause for their developmental disorder. These methods have a significantly improved sensitivity as compared to the conventional karyotyping and FISH analyses. The Affymetrix array was finally chosen and implemented as a first-tier routine diagnostic analysis for MR patients.

Chapter 2 describes the platform comparison and implementation of the SNP array in the diagnosis of MR. **Chapter 3** demonstrates the difficulty in differentiating between CNVs that are possibly implicated in the etiology of MR and those that are not. **Chapter 4** presents two case reports. The first shows that the SNP arrays can be used to further characterize a complex chromosomal abnormality detected with conventional karyotyping. The second shows that not every CNV, in this case a large CNV, is pathogenic. **Chapter 5** is comprised of two studies using patients with phenotypes other than MR. The first report is about patients with a BPES-like phenotype and the second is about patients with a SOTOS-like phenotype. Both patient groups were screened for CNVs. **Chapter 6** includes the conclusions and a discussion on the future applications of the SNP array technology.

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Chapter 2

SNP array and mental retardation diagnostics

Chapter 2.1

A new diagnostic workflow for patients with mental retardation and/or multiple congenital abnormalities: test arrays first

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Abstract
High-density Single Nucleotide Polymorphism (SNP) genotyping technology enables extensive genotyping as well as the detection of increasingly smaller chromosomal aberrations. In this study we assess molecular karyotyping as first round analysis of patients with mental retardation and/or multiple congenital abnormalities (MR/MCA).

We used different commercially available SNP array platforms, the Affymetrix GeneChip 262K *NspI*, the Genechip 238K *StyI*, the Illumina HumanHap 300 and Human CNV 370 BeadChip, to detect copy number variants (CNVs) in 318 with unexplained MR/MCA. We found abnormalities in 22.6 % of the patients; including six CNVs which overlap known microdeletion/duplication syndromes, eight CNVs which overlap recently described syndromes, 63 potentially pathogenic CNVs (in 52 patients), four large segments of homozygosity, and two mosaic trisomies for an entire chromosome.

This study demonstrates that high density SNP array analysis reveals a much higher diagnostic yield as that of conventional karyotyping. SNP arrays have the potential to detect CNVs, mosaics, uniparental disomies (UPD) and loss-of-heterozygosity (LOH) in one experiment. Furthermore, this study shows that two distinct SNP array platforms from different commercial suppliers can be readily used in a diagnostic setting. We therefore propose a novel diagnostic approach to all MR/MCA patients by first analyzing every patient with a SNP array instead of conventional karyotyping.

Introduction
Mental retardation (MR) is a life-long disability with a major impact on the lives of the patients and their families. The prevalence of MR is 2-3% and the underlying cause remains unknown in 65-80% of patients (Flint and Knight, 2003; Rauch et al., 2006; Hoyer et al., 2007). Diagnosing is a challenge due to the broad spectrum of potentially underlying disorders and the wide range of available tests. Knowing the cause is necessary in order to assess recurrence risk, short and long term prognosis and to decide on treatment options.

Changes in genetic dosage of one or more genes are common causes of MR (Rauch et al., 2006). Routine microscopic analysis of chromosomes isolated from peripheral blood lymphocytes has been used successfully to identify such genetic imbalances over the past 50 years. This conventional karyotyping has the advantage of surveying the entire genome for chromosome abnormalities in a single experiment, but it cannot detect imbalances smaller than approximately 5 Mb. Smaller chromosomal aberrations can be identified with Fluorescent *In Situ* Hybridization (FISH) and Multiplex Ligation-dependent Probe Amplification (MLPA) analysis. These techniques are used either to confirm a clinical suspicion by screening for well-known microdeletion syndromes associated with MR or for the analysis of all subtelomeric regions of the genome. The subtelomeric regions are known to be frequently affected in MR (Flint and Knight, 2003). The use of FISH and MLPA analysis is limited because only a few genomic regions can be screened in a single experiment and it can therefore not be applied genome wide.

Patients with unexplained MR with or without multiple congenital abnormalities (MR/MCA), whom are referred to genetic laboratories, are initially screened with conventional karyotyping and if required targeted FISH or MLPA analysis. The combined diagnostic yield of these analyses is approximately 5-10% (de Vries et al., 2005). Consequently, a clinical diagnosis is lacking in the majority of these patients,

which impedes development of treatment strategies and adequate genetic counseling. Therefore, new high resolution whole genome technologies facilitating an increased detection rate of subtle chromosome imbalances are needed to improve diagnosis of MR/MCA patients.

Recent developments in array technology allow whole genome analysis for copy number variation (CNV) at a resolution 10 to 10 000 times higher than that of routine chromosome analysis by conventional karyotyping. Comparative genome hybridization (CGH) studies using arrays with large insert clones (usually Bacterial Artificial Clones (BACs)) have demonstrated the potential of array technology to identify diagnostic CNVs in generally 16.7% of the unexplained MR/MCA patients (Vissers et al., 2003; Shaw-Smith et al., 2004; Schoumans et al., 2005; Tyson et al., 2005; Menten et al., 2006; Rosenberg et al., 2006; Fan et al., 2007). The pathogenic CNVs detected in CGH studies range in size from 0.25 Mb to 15 Mb (Kirov et al., 2008). Resolution is limited by the size of the probes and the distance between the clones, i.e. 100 kb-1 Mb. Therefore, the ideal technique would identify abnormalities with an even higher resolution. The single nucleotide polymorphism (SNP) arrays have been widely used for genotyping and can identify submicroscopic CNVs as well as low-level chromosomal mosaicisms and uniparental disomies (UPDs) (Altug-Teber et al., 2005; Friedman et al., 2006; Hoyer et al., 2007; Bruno et al., 2008).

We performed SNP array analysis on DNA from 318 patients with unexplained MR/MCA and an apparently balanced karyotype to search for potentially pathogenic submicroscopic CNVs with two different commercially available SNP array platforms. In this study, we demonstrate the importance of implementing the SNP array analysis in a diagnostic setting and we advocate a whole-genome copy number screening using a SNP array as a new diagnostic tool for every MR/MCA patient rather than conventional karyotyping.

Materials and methods

Patients

A total of 318 patients referred for MR/MCA were recruited without further selection. Previously performed conventional karyotyping, targeted FISH or molecular tests revealed no etiological diagnosis. Detailed phenotypic information on all patients found to have a pathogenic or potentially pathogenic CNV is provided in Table 2.1. DNA was extracted from whole blood using a Gentra Puregene DNA purification Kit (Gentra Systems, Minneapolis, USA), following the manufacturer's instructions. The study was approved by the Leiden University Medical Center Clinical Research Ethics Board, conforming to Dutch law and the World Medical association Declaration of Helsinki.

SNP Arrays

The Affymetrix GeneChip Human Mapping 262K *Nsp I* and 238K *Sty I* arrays (Affymetrix, California, USA) contain 262 262 and 238 304 25-mer oligonucleotides respectively with an average spacing of approximately 12 kb per array. An amount of 250 ng DNA was processed according to the manufacturer's instruction. SNP copy number was assessed using the software program CNAG Version 2.0 (Nannya et al., 2005). The Illumina HumanHap300 BeadChip (Illumina, Inc., San Diego, USA) contains 317 000 TagSNPs with an average spacing of approximately 9 kb. The Illumina HumanCNV370 BeadChip (Illumina, Inc., San Diego, USA) contains 317 000 TagSNPs and 52 000 non-polymorphic

markers to specifically target nearly 14 000 known CNVs. This array has an average spacing of approximately 7.7 kb. A total of 750 ng DNA was processed according to the manufacturer's instruction. SNP copy number (log R ratio) and B allele frequency were assessed using the software programs BeadStudio Version 3.2 (Illumina, Inc., San Diego, USA) and Partek Genomics Suite Version 6.3 (Partek, Inc. St. Louis, USA).

Evaluation of CNVs

Deletions of at least five adjacent SNPs or of a minimum region of 150 kb and duplications of at least seven adjacent SNPs or of a minimum region of 200 kb were analyzed (Hehir-Kwa et al., 2007). This approach was adopted to minimise the number of false positive findings. The detected CNVs were classified in three different groups: I, known pathogenic CNVs (known microdeletion or microduplication syndrome); II, potentially pathogenic CNVs, not described in the Database of Genomic Variants (DGV; http://projects.tcag.ca/variation/); and III, known polymorphic CNVs described in the DGV, or observed in our in-house reference set (60 controls), whereby at least three individuals must be reported with the same rearrangement. All type III CNVs were further excluded from this study. All type II CNVs were assessed with Ensembl (http://www.ensembl.org: Ensembl release 52 - dec 2008) and DECIPHER for gene content and similar cases respectively. All patients with a type II CNV were added to DECIPHER when consent was obtained.

Validation of CNVs

The known and potentially pathogenic CNVs were confirmed with MLPA, FISH or another type of SNP array on a second independent sample. If parents were available segregation analysis was performed by MLPA, FISH or SNP array.

MLPA experiments were performed as described (White et al., 2004). At least two synthetic MLPA probes were designed within the CNV and probes were commercially obtained from Biolegio (Malden, the Netherlands). Amplification products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Fragment analysis was performed with the GeneMarker Software V1.51 (SoftGenetics, State College, USA). Thresholds for deletions and duplications were set at 0.75 and 1.25 respectively.

FISH analysis was carried out by standard procedures as described (Dauwerse et al., 1990). BAC clones mapping to the CNVs were selected based on their physical location within the affected region (http://www.ensembl.org: Ensembl release 49 - mar 2008).

Results

A total of 318 patients were screened for submicroscopic CNVs. All patients had an apparently normal balanced karyotype and, if performed, targeted FISH or molecular tests revealed no rearrangements. The Affymetrix GeneChip was applied to 132 patients and the Illumina BeadChip platform was applied to 186 patients. Eight (5.71 %) Affymetrix and two (1.06 %) Illumina experiments failed. On average two CNVs per patient were obtained (Affymetrix 3 and Illumina 1.7). All polymorphic CNVs were excluded from further research. Supplementary Table 2.1 shows a summary of all detected CNVs. Six patients showed a CNV that has a clear clinical significance since it overlaps a known microdeletion/duplication syndrome. In eight patients we detected a CNV that was recently described as a new microdeletion/duplication syndrome (Rauch

et al., 2005; Ballif et al., 2008; Courtens et al., 2008; Kumar et al., 2008; Weiss et al., 2008; Bijlsma et al., 2009; Hannes et al., 2009). 63 Potentially pathogenic CNVs were observed in 52 patients (16.4%). Four patients showed striking regions of loss-of-heterozygosity (LOH) (Table 2.1 and Figure 2.1). Regions of homozygosity, ranging in size from 200 kb to 15 Mb are common in healthy individuals (Lencz et al., 2007). Here four patients showed regions of LOH extending more than 15 Mb. Two patients showed a single segment of LOH (BC227 and BC318), one patient a single segment, however in mosaic form (BC302) and one patient two segments (BC308). The parents of the patients were not related.

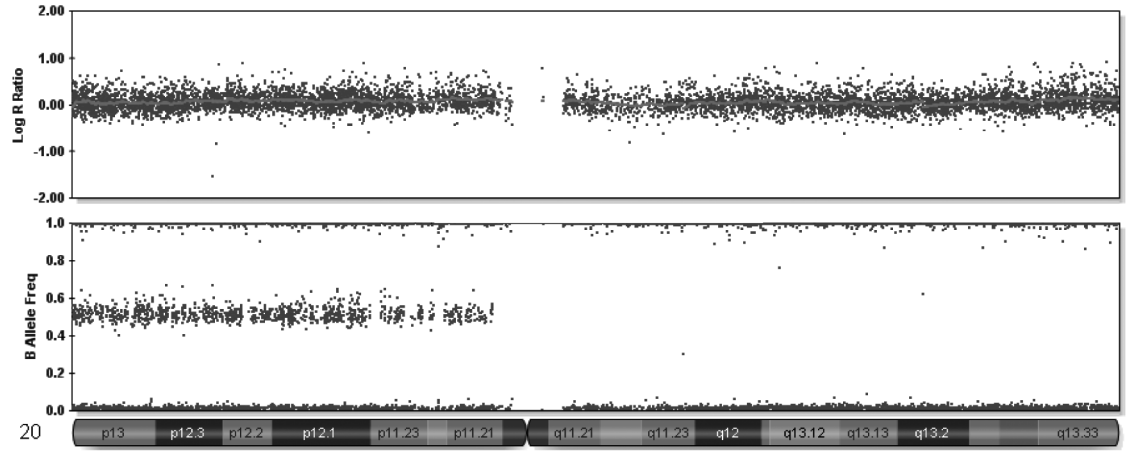


Figure 2.1 37.26 Mb region of LOH on chromosome 20q in case BC311 detected with the Illumina 317K BeadChip. Beadstudio logRratio estimate for each individual SNP in the first plot and genotype call for every SNP in the second plot. The X-axis shows the position on the chromosome.

Table 2.1 Regions of LOH detected in patients with no consanguine parents

ID	Chromosome	Starting SNP	Ending SNP	Size in Mb
BC227	12q15q23.2	rs3905279	rs7312593	33.78
BC302*	16q11.2qter	rs166161	rs7498985	43.31
BC311	13q14.2q22.1	rs12875203rs	rs9318228	27.16
	20p11.21qter	6138531	rs10485816	37.26
BC318	17q21.32q23.2	rs6503455	rs758466	15.17

* LOH was found in mosaic

Two patients showed a low-level chromosomal mosaicism. Patient CR355 was a girl diagnosed with microcephaly, ventricular septum defect, diaphragmatic hernia, umbilical hernia and postaxial polydactyly of the left hand (Figure 2.2g and h). Pregnancy was conceived by in vitro fertilization and the girl was born at a gestational age of 36 5/7 weeks with a birthweight of 2475 grams. Her psychomotor development was delayed and she failed to thrive. She developed severe respiratory insufficiency and died at the age of 7 months. Initial conventional karyotyping of five metaphases did not show rearrangements. SNP array analysis demonstrated a subtle increase in copy number for chromosome 13, suggesting an extra copy of chromosome 13 in 14% of the cells (Figure 2.2a). FISH experiments confirmed the presence of trisomy 13 in 18% of cultured lymphocytes (Figure 2.2c and d) and supplementary karyotyping detected in seven of the 50 (13%) metaphases an extra chromosome 13.

Patient CR377 was a boy referred at the age of 2 years and 9 months because of short stature, speech delay and motor delay (Figure 2.2i). Pregnancy had been uneventful and the boy was born at a gestational age of 40 5/7 weeks after vacuum extraction, with a birthweight of 3610 grams. In early childhood, he suffered from recurrent respiratory infections and recurrent otitis media. At referral height was 84 cm (-3,4 SDS). He had a broad thorax, pectus excavatum, a right-sided simian crease and short 2nd phalanges of both digiti V. On follow up at the age of 3 years and 7 months height was even more compromised (-4.2 SDS). At the age of 4 years and 8 months a marked discrepancy in leg length was noted, the right being shorter. At that time the skin around both wrists and ankles showed an apparent reticular pattern of hypo- and hyperpigmentation. The body asymmetry combined with an abnormal skin pigmentation pointed in the direction of a mosaic condition. Conventional karyotyping on 31 metaphases had shown one cell with trisomy 14 which, confirming to professional guidelines, was interpreted as an

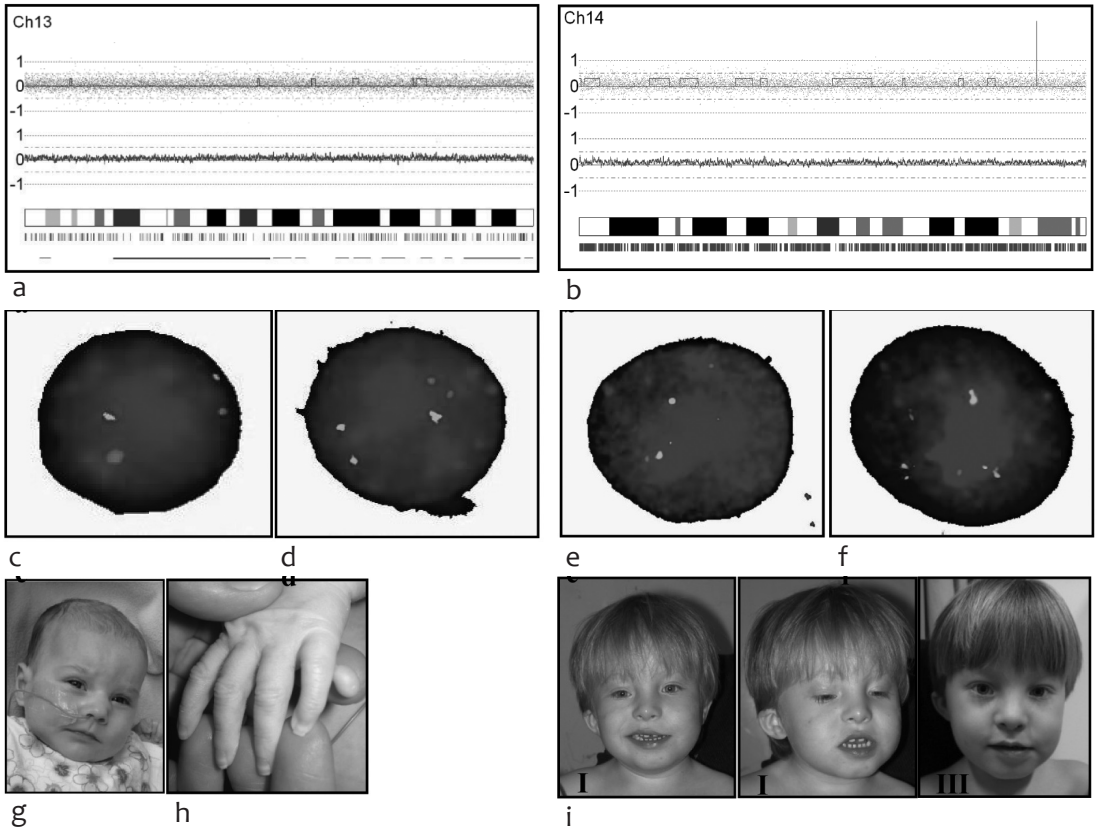


Figure 2.2 (a) CNAG copy number analysis for patient CR355 using the Affymetrix 262K GeneChip. LogRratio estimate for each individual SNP in the first plot and for an average of 10 SNPs in the second plot. Both plots show a slight increase in logRratio for whole chromosome 13. Blue line in first plot: copy-number estimate calculated with the Hidden Markov Model. The X-axis shows the position on the chromosome. Green stripes: heterozygous SNP calls. (b) CNAG copy number analysis output for patient CR377 using the Affymetrix 262K GeneChip. Both plots show a slight increase in logRratio for chromosome 14. (c) FISH experiment (probes LSI13 (green) and LSI21 (red), Vysis) showing a normal cell. (d) FISH experiment showing the presence of a mosaic trisomy 13 in 18% of the 200 cells analyzed. (e) FISH experiment (probes LSI CCNDI, 11q13 (red) and LSI IGH, 14q32 (green), Vysis) showing a normal cell. (f) FISH experiment showing the presence of a mosaic trisomy 14 in 9% of the 200 cells analyzed. (g) Facial picture of patient CR355. Facial dysmorphisms included upslant of palpebral fissures, a broad nasal bridge and uplifted earlobes. (h) Picture of postaxial polydactyly of the left hand of CR355. (i) Facial pictures of case CR377, 3 years and 7 months (I, II), and 4 years and 8 months (III). Note marked asymmetry when smiling, asymmetric upslanted palpebral fissures, left sided epicanthus, hypertelorism, low-set and small right ear.

artifact. SNP array results displayed a subtle increase in copy number for chromosome 14, suggesting an extra copy of chromosome 14 in 19% of the cells and mosaicism was confirmed with FISH experiments on cultured lymphocytes (9%) (Figure 2.2b, e and f). UPD of chromosome 14 for the normal cells was excluded (results not shown).

Discussion

In this study SNP arrays were used to search for pathogenic CNVs in patients with unexplained MR/MCA. The detected CNVs can be divided into the following groups: clearly pathogenic CNVs which overlap known microdeletion/duplication syndromes, CNVs that overlap recently described syndromes, potentially pathogenic CNVs, and polymorphic CNVs (Supplementary Table 2.1). In total we detected known syndromes in six patients, recently described CNVs in eight patients and 63 potentially pathogenic CNVs in 52 patients (in total 20.7%). The polymorphic CNVs were excluded from further research. Six CNVs were considered pathogenic as they are associated with well-established microdeletion syndromes. These syndromes were recognized afterwards by a clinical geneticist, which underlines the difficulty of establishing a diagnosis by clinical observation. Eight patients showed CNVs that were recently identified in other studies. For these new syndromes no obvious phenotype has been established yet and more patients with the same abnormalities are needed to unravel the associated phenotype. The discovery of these 'known' CNVs highlights the advantage of the whole genome screening methods to detect a known deletion or duplication syndrome in one single experiment.

Unraveling the clinical relevance for the potentially pathogenic CNVs is a new challenge. Regions containing coding genes can be present in variable copy number without obvious clinical manifestations, which makes it very hard to determine whether a subtle CNV has a clinical significance. Recent papers have already presented flow schemes for the interpretation of these CNVs (Lee et al., 2007; Koolen et al., 2008). In this study, first all polymorphic CNVs were excluded by comparing against the DGV and our in-house reference set. Secondly, for all CNVs containing coding genes annotated by Ensembl (release 52, Dec 2008), the inheritance was determined by checking both parents (if available).

For 27 potentially pathogenic CNVs we could establish that the rearrangement was inherited from one of the unaffected parents. Several studies have shown that some CNVs are indeed polymorphisms contributing to common variation in healthy individuals (Iafrate et al., 2004; Sebat et al., 2004). A large number of small rearrangements, detected in patients with MR and inherited from phenotypically normal parents have been reported, whereby it was speculated that some of these imbalances may indeed be benign variations and others are likely to represent susceptibility loci for disease (Barber, 2005; de Ravel et al., 2006). A particularly intriguing example is the submicroscopic 1q21 deletion characteristic for thrombocytopenia absent radius (TAR) syndrome, which is found in all patients with the syndrome, but is inherited from a phenotypically normal parent in a subset of cases (Klopocki et al., 2007). It is becoming increasingly clear that many CNVs come with a highly variable phenotype, including what is considered as 'normal'. Examples among many are the 22q11 deletion and duplication (Courtens et al., 2008), the 16p11.2 deletion (Kumar et al., 2008; Weiss et al., 2008, Bijlsma et al., 2009), and the Xp deletions involving the neuroligin and *VCX* genes (Mochel et al., 2008). Mechanisms which can explain why some inherited CNVs occasionally result in abnormal

development have been postulated (Barber, 2005; Lesnik Oberstein et al., 2006). These mechanisms include: a mutation in the same region on the other chromosome; a mutation in one or more unlinked modifying genes; imprinting; mosaicism in one of the parents; or any other unidentified genetic, epigenetic or environmental factor (Barber, 2005; Hannes et al., 2008). Furthermore, it is frequently assumed that parents are phenotypically normal although closer inspection by a clinical geneticist might reveal subtle anomalies (Barber, 2005).

Twenty-two *de novo* potentially pathogenic CNVs are detected and are likely to be relevant for the phenotype of the patient. For 14 potentially pathogenic CNVs the inheritance could not be determined. Interpretation of these CNVs is even more difficult. Attempts should be made to receive DNA from the parents or alternatively other relatives. However, for all potentially pathogenic CNVs phenotypically concordant patients with the same abnormality need to be found to be sure of their pathogenicity. Therefore, databases like DECIPHER (https://decipher.sanger.ac.uk/) have been created in order to compile molecular cytogenetic data from clinical studies all over the world to provide the basis for understanding the role of different CNVs in genetic diseases. For the 63 potentially pathogenic CNVs detected in this study no complete overlapping cases were described in DECIPHER. More array data on MR patients and healthy controls will be needed to determine the clinical relevance of these CNVs.

In 9 of the 26 *de novo* CNVs (pathogenic and potentially pathogenic), DNA from the parents was tested on SNP array, enabling us to determine the parental origin. Seven CNVs occurred in the paternally derived chromosome. Only two CNVs occurred in maternally derived chromosomes, giving a paternal maternal ratio of 6:2. Parental origins of microdeletions and duplications have been investigated in several genomic disorders. Deletions in Williams and DiGeorge syndrome were of paternal and maternal origin equally (Baumer et al., 1998). Deletions in Neurofibromatosis type 1 and in 1p36 syndrome were predominant on the maternally derived chromosome (Lazaro et al., 1996; Wu et al., 1999). In contrast, duplications in Charcot-Marie-Tooth disease type I, deletions in Wolf-Hirschhorn syndrome and Cri Du Chat syndrome occur more frequently in the paternally derived chromosome (Lopes et al., 1997; Wieczorek et al., 2000; Mainardi et al., 2001). Much more parent-of-origin data are needed to document the possible existence of regional parental bias.

ArrayCGH (aCGH) screenings performed on mentally retarded patients were found to be a powerful tool for the detection of CNVs (Vissers et al., 2003; Shaw-Smith et al., 2004; Schoumans et al., 2005; Tyson et al., 2005; de Vries et al., 2005; Menten et al., 2006; Rosenberg et al., 2006; Fan et al., 2007). These arrays consist of large-insert clones and the smallest pathogenic CNVs detected are approximately 0.25 Mb. The high-density whole-genome SNP arrays, which were initially developed for genotyping, are now widely used to search for smaller CNVs (Hoyer et al., 2007; Altug-Teber et al., 2005; Friedman et al., 2006; Bruno et al., 2008). In approximately 25% of patients with unexplained MR/MCA CNVs are detected by aCGH and SNP array studies. The array technology is the most effective method resulting in the most clinical diagnoses compared to conventional karyotyping, FISH analysis and mutation screening. Although cytogeneticists suspected that array analysis would not be able to detect mosaicisms, the aCGH and SNP array techniques actually appear to be more sensitive in detecting low-level mosaicism than conventional karyotyping (Ballif et al., 2006; Powis et al., 2007). If mosaicism is not suspected, the number of cells counted with conventional karyotyping may not be sufficient to detect the aberrant subset of cells,

and a single abnormal cell might be interpreted as an artifact of cell culture (Cheung et al., 2007). Two such cases of low-level mosaicism were reported in this study. Our patients with mosaic trisomy 13 and 14 have phenotypical characteristics which resemble the reported phenotypes of mosaic trisomy 13 and 14 (Figure 2.2).

A major advantage of SNP array analysis is the extra SNP genotyping information, which enables the detection of copy-number neutral chromosomal aberrations such as UPD and LOH (Zhang et al., 2008). UPD, which arises when an individual inherits two copies of a chromosome pair from one parent and no copy of the other parent, can result in rare recessive disorders, or developmental problems due to the effects of imprinting (Casidy et al., 2000). Examples of genetic diseases linked to UPD are Prader-Willi syndrome (MIM 176270), Angelman syndrome (MIM 105830), Beckwith-Wiedemann syndrome (MIM 130650) and Silver-Russell syndrome (MIM 180860). SNP array analysis is able to detect uniparental isodisomy and uniparental heterodisomy (when both parents are included in the experiment), but the interpretation of new UPD regions is difficult and further research is required to confirm the clinical consequences. Recessive and normally non-penetrant alleles in isodisomic form (two copies of the same parental chromosome) may cause recessive diseases. Gene defects underlying autosomal recessive disorders can be localized and identified by homozygosity mapping. Furthermore, patients with consanguineous parents display many regions of homozygosity (LOH) that might result in a recessive disorder. In this study we identified an extended segment of LOH in four patients, with no consanguineous parents. To identify the responsible gene or genes is a challenge now, but may become a realistic possibility with next generation high throughput DNA sequencing technology. Finally, the information on the SNP genotype could be used to verify biological parentage and in cases of suspected incest.

Conversely, a disadvantage of using arrays instead of conventional karyotyping is the inability to detect balanced rearrangements. Around 6% of antenatal cases with balanced reciprocal translocations and inversions are associated with abnormal phenotypes (Warburton, 1991). In these cases the breakpoints of the rearrangement probably disrupt a gene, or small duplications or deletions beyond microscopic resolution are present. The SNP array analysis will (depending on the resolution) detect the small abnormalities, but the disruption of genes will remain unknown. A Dutch retrospective study showed that only approximately 0.78% potentially pathogenic balanced rearrangements of all referrals will be undetectable by array analysis without conventional karyotyping (Hochstenbach et al., 2009).

The absence of an aberration or the presence of only polymorphic CNVs after SNP array analysis does not exclude a syndrome caused by a mutation at gene level. Therefore we emphasize that MR/MCA patients with normal array results should always be referred to a clinical geneticist to exclude such known syndromes. Furthermore, genomic data obtained from the SNP array analysis can be used in future research for association between genetic markers and specific phenotypes to hopefully diagnose even more patients.

In 2006, Rauch et al. compared the diagnostic yield of various techniques in MR/MCA patients (Rauch et al., 2006). These authors suggested targeted analysis in patients with a clear diagnosis and in the remaining patients conventional karyotyping and molecular screening (Rauch et al., 2006). Kriek et al. proposed another diagnostic approach to MR/MCA patients, suggesting a screening with MLPA first and based on the outcome additional aCGH or karyotyping (Kriek et al., 2007). However, more recent

studies already mentioned the partial replacement of conventional karyotyping by molecular karyotyping (Zhang et al., 2008). In addition, Koolen et al. described a workflow for the clinical interpretation of CNVs in individuals with MR (Koolen et al., 2008). Our results demonstrate that high-density SNP arrays can be successfully used as tool for the detection of CNVs, low-level mosaicism and copy-number neutral abnormalities. Their high resolution and commercial availability make them attractive to implement into a routine diagnostic setting.

Here, we combine the flowcharts designed by Kriek et al. and Koolen et al. in a novel approach to the patient with MR/MCA (Figure 2.3) (Kriek et al., 2007; Koolen et al., 2008). We recommend testing every patient first with a SNP array instead of conventional karyotyping. The results will be classified in patients with polymorphic CNVs or no CNV ('normal'), patients with CNVs which overlap known syndromes and patients with potentially pathogenic CNVs. The 'normal' patients could be screened for gene mutation in targeted genes or in the future with whole genome next generation high throughput DNA sequencing technology. The patients with CNVs overlapping known syndromes are diagnosed and family members could be checked for inheritance and recurrence risk. The inheritance of the potentially pathogenic CNVs should be tested and the patients should be reported in a database like DECIPHER. The clinical relevance for these CNVs can be determined when the specific CNV is reported in adequate numbers of healthy individuals or phenotypically concordant patients.

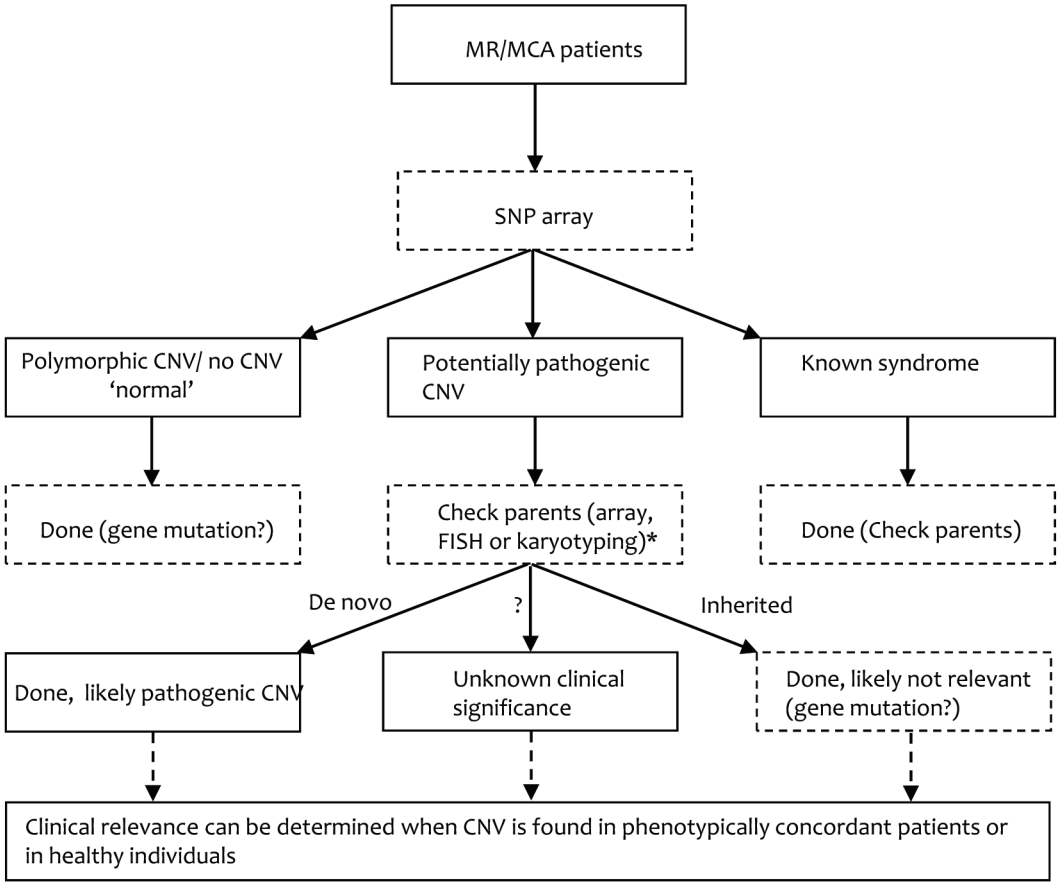


Figure 2.3 Flow chart for the new diagnostic approach to patients with mental retardation. * If the CNV exceeds 200 kb we recommend additional FISH analysis to confirm the CNV in the patient and screen for balanced translocations or insertions in the parents. If the CNV is smaller than 200 kb we recommend a second array analysis on the patient to confirm the CNV and the parents to test heritability.

This new approach will diagnose a larger proportion of CNVs in the first round, however the interpretation of the CNVs will be the major challenge. Eventually, more families will be informed on the cause of the disease of their family member. This will improve medical care and genetic counseling. Furthermore, since the SNP array approach will make targeted FISH and MLPA analysis redundant, less laboratory tests will be needed which leads to a substantial reduction of cost.

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Supplementary Table 2.1 Pathogenic and potentially pathogenic CNVs detected by Affymetrix 262K, Illumina 317K and 370K arrays

ID	Platform	Gain/ Loss	Chr	Starting SNP	Ending SNP	Size in bp	Inheritance	OMIM genes	
Known microdeletion syndromes									**Syndrome**
CR30	262K	loss	2q22.3	rs1517914	rs2381683	1339008	*de novo*	1	Mowat-Wilson syndrome
BC373	370K	loss	2q37.3	rs3935423	rs7577243	1530000	n/a	0	2q37.2 monosomy
BC43	317K	loss	17p11.2	rs9905272	rs9909852	3530000	*de novo*	7	Smith-Magenis syndrome
CR295	262K	loss	18q21.1-q21.31	rs1383531	rs502430	6300000	n/a	3	Pitt-Hopkins syndrome
BC300	317K	loss	22q11.2	rs2543958	rs140392	2530000	*de novo*	7	DiGeorge syndrome
CR353	262K	loss	Xp22.31	rs6654821	rs17300660	1550000	maternal	1	X-linked ichthyosis
Recently described CNVs									**Syndrome (Phenotype present case)**
CR103	262K	gain	3q29	rs9883087	rs1431501	1429695	paternal	0	3q29 microduplication syndrome
BC65	317K	loss	16p11.2	rs8054172	rs7202714	522140	n/a	1	16p11.2 microdeletion syndrome (Mild mental retardation, speech delay. Short palpebral fissures, dysplastic ears, retrognathia, unilateral simian crease, broad neck, sloping shoulders)
BC93	317K	loss	16p11.2	rs8054172	rs7202714	522140	*de novo*	1	16p11.2 microdeletion syndrome (Mild mental retardation. Short palpebral fissures, mild malaar hypoplasia, simple external ears, retrognathia, broad neck, sloping shoulders)
BC235	370K	loss	16p11.2	rs28455095	rs7202714	554000	paternal	1	16p11.2 microdeletion syndrome (Mild mental retardation, speech delay. Mild malar hypoplasia)
BC245	370K	gain	16p13.11	rs7404843	rs402522	820000	paternal	2	16p13.11 recurrent microduplication syndrome
BC260	370K	gain	16p13.11	rs2941256	rs6498606	1330000	paternal	2	16p13.11recurrent microduplication syndrome (Prominent parietal bones, narrow bitemporal, small and short chin, simple helices, narrow external ears, short upper

Supplementary Table 2.1 continued

									arms and legs, severe lissencephaly, corpus callosum agnesia)
BC259	370K	gain	22q11.2	rs2543958	rs140391	2530000	maternal	7	22q11 duplication syndrome (Bilateral ptosis, brachycephaly, low set ears, hypertelorism, downslant, bulbous nasal tip, broad nasal bridge, overriding toes, syndactyly, hypospadia, cryptorchism.
BC104	317K	loss	22q11.22-q11.23	rs4481090	rs131690	383630	n/a	1	22q11.2 distal deletion syndrome
Potentially pathogenic CNVs									**Phenotype**
CR19	262K	Gain	1p32.3	rs3013747	rs2793669	2666889	*de novo*	3	Arched and thick eyebrows, long eyelashes, strabismus, auricular pits, prominent ear helix, cleft palate, cleft upper lip, fetal finger pads, segmentation defects of spine
CR550	262K	loss	1p21.1-p13.3	rs6695016	rs2797520	5160000	n/a	2	Mental retardation, psychotic behavior, sleep disorders, seizures. Blepharophimosis, bulbous nasal tip, thick upper and lower lip, narrow palate, low-pitched voice, deafness, loose skin in neck, joint stiffness,
CR304	262K	loss	1q32.3	rs3768547	rs320391	854000	n/a	0	Mental retardation, autism. Congenital cataract.
BC20	317K	gain	1q44	rs4564164	rs7548751	187220	n/a	0	Mental retardation
BC9	317K	gain	2p25.3	rs11211657	rs4853841	434730	paternal	1	Bitemporal narrowing, small palpebral fissures, broad nasal bridge, small ears, narrow inner ear, small hands, short arms.
BC324	317K	loss	2q11.2-q13	rs1110947	rs832665	6610000	*de novo* (pat)	0	Mental retardation
BC322	317K	loss	2q24.2	rs1896270	rs7423892	670910	*de novo* (pat)	0	Mental retardation, behavioral problems. Almond shaped palpebral

Supplementary Table 2.1 continued

									fissures, slant down palpebral fissures, hypotelorism, simple ears, bound tongue, long philtrum, full cheeks, abnormal palmar creases, clinodactlyly, short phalanges, tapering fingers, joint laxity, over-riding toes, sandal gap of toes, short toes, tall stature, generalized obesity.
BC249	370K	loss	2q31.1	rs2676145	rs10497422	3060000	*de novo* (pat)	1	Mental retardation. Ear pits, ptosis, mild downslant, long eyelashes, small mouth, abnormal inner ear on right side, mild hypospadia.
CR20	262K	gain	2q35	rs1979028	rs1992235	823000	maternal	3	Mental retardation
BC30	317K	loss	3p25.1-p24.3	rs4685442	rs2638134	5050000	*de novo*	0	Mental retardation. Large nose, long nasal septum, arachnodactyly, clinodactyly, over-riding toes, short toes, pectus excavatum.
BC103	317K	gain loss	3p26.3-p26.1 3p26.1	rs9681213 rs11712496	rs2570006 rs7621754	5080000 90080	*de novo* *de novo*	2 0	Mild mental retardation, autism. Low weight
BC17	317K	gain	3q13.13	rs12637387	rs9861358	400060	n/a	0	Mental retardation
CR296	262K	loss	5p12	rs2548343	rs12655990	2250000	paternal	1	Large fontanelles, prominent ear helix, small and absent nails, lung hypoplasia.
BC24	317K	gain	5p12-p11	rs11743392	rs10941803	733510	n/a	0	Mental retardation
BC332	317K	loss loss loss loss loss loss	5q14.3 5q14.3 5q14.3 5q14.3 5q33.3 5q33.3	rs4920853 rs16902356 rs7715840 rs357509 rs6556381 rs6884239	rs1862233 rs10051603 rs1062035 rs304151 rs9637861 rs3846687	59000 83000 320000 638000 129940 158600	*de novo* *de novo* *de novo* *de novo* *de novo* *de novo*	0 0 1 0 0 0	Mental retardation, sleep disorder, grand-mal seizure. Sensorineural deafness, epicanthic folds, severe visual loss, strabismus, VSD, trachea/laryngeal malacie, general hypotonia.
CR507	262K	gain	6q14.1	rs3828761	rs17783795	989760	maternal	2	Mild mental retardation. Temporal narrowing, plagiocephaly, small ears, earpit, high and narrow arched

Supplementary Table 2.1 continued

									palate, sacral dimple, café au lait spot.
CR78	262K	loss	6q16.3	rs9376514	rs7754315	333350	paternal	0	Mental retardation
BC313	317K	loss	6q25.3	rs2181068	rs9365947	2660000	n/a	0	Mental retardation. Plagiocephaly, deep-set eyes, up-slant palpebral fissures, small ears, deep palmar creases, clubbed nails, wide-spaced nipples, ASD, lung cysts.
CR14	262K	loss	7q21.11-q21.3	rs2373207	rs17166393	8291932	*de novo* (pat)	7	Mental retardation, anxiety disorder, autism. Small palpebral fissures, simply formed low set ears, abnormal palmar creases, scoliosis, long halluxes, proximally implanted thumbs.
BC333	370K	gain	8q24.22	rs4736413	rs6599566	12870000	maternal	10	Mental retardation, behavioral problems.
CR11	262K	gain	9p21.3-p21.3	rs4618784	rs7389761	164000	paternal	0	Mental retardation.
CR66	262K	loss	10p12.31-p12.33	rs11254467	rs11012683	4396349	*de novo* (pat)	0	Blepharophimosis, ptosis, epicanthus, telecanthus, VSD.
BC78	317K	loss	10p12.1-p11.23	rs2998285	rs754865	990454	n/a	0	Mental retardation, autism. Flat occiput, spasticity, strabismus, small ears, coarse hair, short metacarpals, patent ductus arteriosus, scoliosis.
BC63&64	317K	loss	11centr	rs11227836	rs688740	157440	paternal	0	Mild mental retardation. Microcephaly, oligodontia, velopharyngeal insufficiency, abnormal corpus callosum.
BC232	370K	loss	13q12.2	rs9512755	rs881084	188000	n/a	0	Mental retardation. Treacher-collins like.
BC180	317K	loss	13q12.3	rs1023166	rs7332693	1250000	n/a	0	Mental retardation, hyperactivity. Microcephaly, hypotelorism, large ears, columella below alae nasi, thin nasal bridge, coarse hair, syndactyly

Supplementary Table 2.1 continued

									of 2-3 toes, patchy depigmentation of skin, pectus excavatum.
BC37	317K	loss	13q34	rs4293272	rs3813131	2590027	n/a	3	Mental retardation.
CR566	262K	loss	15q13.1-q13.2	rs4966231	rs8028345	1340000	maternal	0	Mental retardation, febrile convulsion. Flat occiput, hypotonia, heterotopia, hypertelorism, down-slant palpebral fissures, ptosis, large ears and era lobule, micrognathia, loose skin in neck, short neck, deep palmar creases, abnormal plantar creases, cavernous haemangioma, eczema, dry skin, constipation, feeding problems, pectus excavatum, spina bifida occulta, short stature, thin.
CR506	262K	gain	16p13.2	rs1273350	rs30781	203250	n/a	2	Percepto-motor delay without mental retardation, hypotonia. Down-slant palpebral fissures, flat nasal bridge, large ear lobules, prominent forehead, long toes, syndactyly of toes, patchy pigment of skin, feeding problems, pectus excavatum, recurrent infections.
BC302	317K	gain	16p13.2	rs7186634	rs10500367	236720	paternal	0	Mental retardation.
CR569	262K	loss	16q22.2-q22.3	rs9302640	rs3743977	379140	*de novo* (pat)	1	Mental retardation.
BC335	370K	gain	16q23.3	rs1056629	rs4558403	1480000	maternal	0	Mild mental retardation. Congenital nystagmus, VSD, frontal bossing, high frontal hairline, sparse hair, simple ears, deep-set eyes upslant, high and narrow palate, clinodactyly V left, fetal finger pads of fingers and toes.
CR93	262K	gain	17q12	rs17836884	rs4795952	989950	paternal	0	Mental retardation. Blepharophimosis, deep-set eyes,

Supplementary Table 2.1 continued

									hypertelorism, short palpebral fissures, strabismus, hypoplastic ear lobule, posteriorly rotated ears, prominent anti-helix, choanal atresia, overhanging nasal tip, short nasal septum, high and narrow palate, microstomia, short neck, micrognathia, cavernous haemangioma, flexion deformity of hip, post-axial polydactyly of fingers, short stature.
BC101	317K	gain	17q25.3	rs7219745	rs6502043	2203063	*de novo*	3	Severe mental retardation. Prominent forehead, small eyes, broad nasal tip, clinodactyly V, abnormal palmar crease right.
CR39	262K	loss	18p11.31-p11.23	rs625106	rs12454998	1226479	maternal	0	Mental retardation. Assymetric ears, plagiocephaly.
CR295	262K	loss	18q21.2-q21.31	rs1383531	rs502430	6300000	n/a	3	Mental retardation, tonic seizures, speech delay. Hypertelorism, myopia, up-slant palpebral fissures, deafness, flat nasal bridge, flat nose, macrostomia, wide-spaced teeth, prominent mandible, round face, clinodactyly, small hands, over-riding toes including clinodactyly, small feet, constipation, scoliosis, short stature, primary amenorrhea, quadriplegia.
BC336	370K	loss loss	18q21.31 18q21.32-q21.33	rs4940582 rs1877055	rs4940754 rs588677	877000 2600000	n/a	1 2	Mental retardation. Obesity, valvular pulmonary stenosis.
BC320	317K	loss	18q22.1	rs4334403	rs9964774	1320000	maternal	0	Mental retardation.
BC362	370K	gain	19q13.41-q13.42	rs3810115	rs8111919	728000	maternal	0	Mental retardation, seizures, hypotonia. Deep-set eyes, flat nasal bridge, gum hypertrophy, cleft and

Supplementary Table 2.1 continued

									high palate, high frontal hairline, dolichocephaly, abnormal palmar creases, cryptorchid testes, hypospadias, diastasis recti, joint laxity.
CR273	262K	gain	20p11.21	rs2387578	rs446649	370870	maternal	1	Mental retardation.
BC247	370K	loss	21q22.3	rs2839110	rs2839193	159000	paternal	0	Mental retardation.
CR63	262K	gain	22q11.2	rs361689	rs5762401	718540	paternal	2	Mental retardation, seizures. Oligodontia, posteriorly rotated ears.
CR69	262K	loss	22q13.1	rs3021274	rs11089991	355205	*de novo* (pat)	1	Mental retardation, autism. Long philtrum, thick lower lip, wide-spaced teeth, diffuse depigmentation of skin, dolichocephaly, tall stature.
CR100	262K	gain	Xp22.12	rs5955562	rs16981283	349540	maternal	0	Mental retardation.
BC258	370K	loss	Xp22.1	rs5925675	rs5970987	223000	maternal	0	Mental retardation.
CR533	262K	gain	Xp22.31	rs6654821	rs17300660	1550000	maternal	1	Mental retardation. Up-slant palpebral fissures, non-specific vision impairment, large tongue, open mouth appearance, wide-spaced teeth, nasal speech, coarse facial features.
CR306	262K	gain	Xq21.2	rs2887392	rs2369242	325410	*de novo* (mat)	0	Mental retardation.
CR177	262K	gain	Xq28	rs732572	rs5983907	606191	maternal	1	Telecanthus, epicanthic folds, hypotelorism, infra-orbital creases, up-slant and short palpebral fissures, small nose, wide nasal bridge, flat face, metopic ridge, narrow and sloping forehead, abnormal palmar creases, fetal finger pads, proximal placement of thumbs, single palmar crease, early

Supplementary Table 2.1 continued

									closure of fontanelles, trigonocephaly, wide-spaced nipples.
BC319	317K	loss gain	1p31.1-p22.2 5q11.2	rs1926256 rs169382	rs2991701 rs4865815	1858000 167000	*de novo* (mat) maternal	3 0	Severe mental retardation. Assymetric face, microtia, macrostomia, downslant, high palate, croede teeth, pectus excavatum, scoliosis, fetal finger pads, extreme hyperlaxity of joints, deformaties of feet.
CR416	262K	gain gain	13q12.11 20p13-p12.1	rs4400902 rs17685809	rs9506958 rs6043648	4410000 15890000	*de novo* *de novo*	4 7	Mild mental retardation, psychiatric disorder.
BC153	317K	gain loss	15q26.3 21q22.3	rs8030950 rs2839102	rs7183226 rs2839378	348590 583730	paternal maternal	1 2	Mental retardation. Hypertelorism, strabismus, cleft upper lip, cleft palate, prominent forehead.
CR174	262K	loss gain	Xp22.32-p22.31 Xq27.3-q28	rs5916033 rs728395	rs16984818 rs6641352	2696475 6306898	maternal maternal	2 3	Mental retardation.

n/a, DNA of parents not available; pat, CNV in paternal chromosome; mat, CNV in maternal chromosome

Chapter 3

Interpretation of copy number variants

Chapter 3.1

Extending the phenotype of recurrent rearrangements of 16p11.2: deletions in mentally retarded patients without autism and in normal individuals

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Abstract
Array CGH (comparative genomic hybridization) screening of large patient cohorts with mental retardation and/or multiple congenital anomalies (MR/MCA) has led to the identification of a number of new microdeletion and microduplication syndromes. Recently, a recurrent copy number variant (CNV) at chromosome 16p11.2 was reported to occur in up to 1% of autistic patients in three large autism studies.

In the screening of 4284 patients with MR/MCA with various array platforms, we detected 22 individuals (14 index patients and 8 family members) with deletions in 16p11.2, which are genomically identical to those identified in the autism studies. Though some patients shared a facial resemblance and a tendency to overweight, there was no evidence for a recognizable phenotype. Autism was not the presenting feature in our series.

The assembled evidence indicates that recurrent 16p11.2 deletions are associated with variable clinical outcome, most likely arising from haploinsufficiency of one or more genes. The phenotypical spectrum ranges from MR and/or MCA, autism, learning and speech problems, to a normal phenotype.

Introduction
Array CGH (comparative genomic hybridization) screening of large patient cohorts with mental retardation (MR) and/or multiple congenital anomalies (MCA) has lead to the identification of a number of new microdeletion and microduplication syndromes (for recent review see [20]). An additional and important outcome of this testing has been the discovery that several recurrent microdeletion and microduplication syndromes are caused by non-allelic homologous recombination (NAHR) between paired segmental duplications. As the short arm of chromosome 16 is rich in intrachromosomal segmental duplications (also known as low copy repeats, LCRs), it has previously been suggested that this region may harbour novel genomic disorders [19]. Indeed, a number of recent reports have provided evidence for this. Ballif et al. identified a microdeletion syndrome in 16p11.2-p12.2 involving a 7-8 Mb deletion [1]. Ullman et al. reported reciprocal 16p13.1 deletions and duplications which predispose to MR and/or autism [21], while Hannes et al. found that this deletion was significantly associated with MR/MCA, and that the reciprocal duplication was a common variant in the general population [8]. Finally, copy number variants (CNVs) in the region of 16p11.2 have been identified in up to 1% of autistic individuals [10, 13, 22], representing a substantial susceptibility risk to development of autism. The phenotypic spectrum of rearrangements in this genomic region remains to be fully characterized, especially in regard to their association with autism.

By screening 4284 patients with MR/MCA, we detected 14 patients with deletions in 16p11.2, which are genomically identical to those identified in the autism studies [10, 13, 22]. Of these, six deletions were de novo and six were inherited from parents with a milder or normal phenotype; in one index case the inheritance could not be assessed, in another case segregation analysis is pending. We also detected an inherited smaller deletion of an adjacent region on 16p11.2.

Here we present clinical and molecular data on our patients with a 16p11.2 deletion and compare them with previously reported cases. As autism was not the presenting symptom in our series of patients, our data indicate that the recurrent deletion of 16p11.2 gives rise to a broader phenotype than autism alone.

Methods

Selection of patients tested by various array platforms.

We studied 4284 patients with MR/MCA in several genetic centres. Patients were ascertained by clinical geneticists in Leiden, the Netherlands (n = 318), and through a collaborative effort with cytogenetic laboratories of Groningen, the Netherlands (n = 600), Nijmegen, the Netherlands (n = 1525), Stockholm, Sweden (n = 560), Melbourne, Australia (n = 325), Madrid, Spain (n = 60), and Ghent, Belgium (n = 896).

Array platforms

Each of the genetic centres used one of the following array platforms to analyse their group of patients.

The Affymetrix GeneChip Human Mapping 262K *NspI* and 238K *StyI* arrays (together 500K) (Affymetrix, California, USA) contain 262,262 and 238,304 25-mer oligonucleotides respectively, with an average spacing of approximately 12 kb per array. An amount of 250 ng DNA was processed according to the manufacturer's instruction (http://www.affymetrix.com). Single nucleotide polymorphism (SNP) copy numbers were assessed using the software program CNAG Version 3.0 [15].

The Affymetrix Genome-Wide Human SNP Array 6.0 features 1.8 million genetic markers, including more than 906,600 SNPs and more than 946,000 probes for the detection of copy number variation. DNA was processed according to the manufacturer's instruction (http://www.affymetrix.com). SNP copy numbers were assessed using Genotyping Console™ version 3.0.2.

The Illumina HumanHap300 BeadChip contains 317,000 TagSNPs with a mean resolution of approximately 9 kb. The Illumina HumanCNV370 BeadChip contains 317,000 TagSNPs and 52,000 non-polymorphic markers to specifically target nearly 14,000 known CNVs. This array has a mean resolution of approximately 7.7 kb. A total of 750 ng DNA was processed according to the manufacturer's instruction (http://www.illumina.com). SNP copy numbers (logRratio) and B allele frequencies were assessed using the software programs BeadStudio Version 3.2 (Illumina, Inc.) and Partek Genomics Suite Version 6.3 (Partek, Inc.).

The 38K high-resolution CGH array contains 41,760 bacterial artificial chromosome (BAC) clones produced by the Swegene DNA Microarray Resource Centre, Department of Oncology, Lund University, Sweden (http://swegene.onk.lu.se) as previously described [17].

The Agilent Human Genome CGH Microarray Kit 44K contains 42,433 probes and the assay was performed according to the manufacturer's instructions with minor modifications. In brief, 400 ng of genomic DNA was labeled with Cy3 (patient) or Cy5 (control) (BioPrime Array CGH Genomic Labeling System, Invitrogen). After precipitation, patient and control samples were pooled together with Cot-1 DNA, Agilent 10X Blocking Agent and Agilent 2X Hybridization Buffer. This hybridization mixture was hybridized on the microarrays for 24 hours at 65°C. After washing, the slides were scanned with an Agilent DNA microarray scanner. The scan images were processed with Agilent Feature extraction software version 9 and further analysed with an in-house developed and freely available software tool arrayCGHbase (http://medgen.ugent.be/arraycghbase/) [14]. Profiles were also evaluated by circular binary segmentation (CBS) to detect regions with aberrant copy number.

The Agilent Human Genome CGH Microarray Kits 105K and 244K (Santa Clara, CA) contain respectively ~99,000 and ~237,000 probes, and the assays were performed

following the protocols provided by the manufacturer. The slides were scanned on an Agilent microarray scanner. Data analysis was performed using the Agilent Feature extraction software version 9 and Agilent CGH analytics version 3.5.14 software.

Multiplex Ligation-dependent Probe Amplification (MLPA)
MLPA experiments were performed as described previously [23]. MLPA probes were designed within the 16p11.2 region (located in the genes *MVP*, *SPN*, *CORO1A* (kit1); *SEZ6L2*, *MVP*, *FAM57B* (kit2); *SPN*, *ALDOA* (kit 3)). Amplification products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Fragment analysis was performed with the GeneMarker Software V1.51 (SoftGenetics, USA). Thresholds for deletions and duplications were set at 0.75 and 1.25 respectively.

Fluorescent In Situ Hybridisation (FISH)
FISH analysis was carried out by standard procedures as described previously [4]. BAC clones mapping to the 16p11.2 deletion region were used (RP11-114A14 and RP11-301D18).

Gene prioritisation and sequencing
The software tool Anni 2.0 (http://www.biosemantics.org/Anni) was used to search for candidate genes in the 16p11.2 region. For each gene a profile of related concepts is constructed that summarizes the context in which the gene is mentioned in the literature. Genes associated with similar topics are identified by hierarchical clustering of the corresponding gene concept profiles [9].The software was used according to the software's manual.

Direct sequencing of genomic PCR products covering the coding regions of *ALDOA*, *TBX6* and *SPN* was performed. Primers were selected using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequencing was performed as described previously [12].

Case reports
Patients carrying a ~600 kb 16p11.2 deletion
Case 1 is a mildly retarded, 44-year old male with normal height (1.72 m, +0.2 SDS) and overweight (BMI 28.7 kg/m2). He does not have autistic behaviour, but has significant speech problems. He has some mild dysmorphic features: short palpebral fissures, dysplastic ears, retrognathia, a broad neck with sloping shoulders, and a unilateral simian crease. Parents were not available for testing, but were reported to be of normal intelligence.

Case 2 is a mildly retarded 18-year old male (Fig. 3.1.1a), born to a 30-year old mother and a 39-year old father. He was born at term with a birth weight of 4 kg. His motor development was normal (started walking at 13 months of age), however speech development was delayed (first words at 2.5 years of age). A formal test for autism in childhood showed no autism spectrum disorder (ASD).

When first assessed at the age of 7 years and 3 months, his height was 1.36 m (+2.3 SDS), and his head circumference 54.5 cm (+1.3 SDS). At the age of 17 years and 2 months his height was 1.80 m (-0.2 SDS), his weight was 130.5 kg (+4.2 SDS, BMI 40.1 kg/m2) and his head circumference 60 cm (+1.8 SDS). He does not show autistic

behaviour. He has mild dysmorphic features: short and down-slanted palpebral fissures, mild malar hypoplasia, anteverted nares, simple external ears, retrognathia, a broad neck, and sloping shoulders.

An MRI at the age of 8 years showed an arachnoidal cyst with a diameter of 5 cm, and partial agenesis of the left temporal lobe.

His family history is negative for obesity or mental retardation. MLPA analysis of the 16p11.2 region (kit 1) showed normal copy numbers in both parents.

Case 3 is a mildly retarded, 10-year old girl, born to a 38-year old mother and a 31-year old father (Fig. 3.1.1b). She was born at term with a birth weight of 3300 g. In infancy she was treated for epilepsy. Her motor milestones were reached late and speech development was delayed (first words at the age of 20 months). A formal test for autism in childhood showed no ASD.

On examination at the age of 8 years and 2 months her height was 1.30 m (-0.4 SDS), her weight 45 kg, (+3 SDS for height, BMI 26.6 kg/m2) and her head circumference 54.5 cm (+1.7 SDS). She did not have autistic features. She speaks with a lisp. Apart from mild malar hypoplasia, she has no apparent facial dysmorphisms. She has a unilateral single palmar crease and mild syndactyly of the 2nd and 3rd toes.

A brain MRI was reported normal.

The paternal family history is positive for dyslexia and mild mental retardation. Only her parents and a paternal uncle could be examined with MLPA-analysis (kit 1). Other family members were not accessible.

Her father carried the same microdeletion. He had speech retardation (first words at the age of 4 years) and is dyslectic. Because of this, he went to a special school. Apart from bilateral 4/5 syndactyly of his toes, he has no apparent dysmorphic features.

The deletion was also found in the the paternal uncle. In infancy he was treated for pyloric stenosis. He is reported to have mental retardation and dyslexia. Dysmorphic features were not recorded. He lives in a sheltered home.

Case 4 is a 13-year old boy. He is the only child of healthy, non-consanguineous parents. He was born at term after a normal pregnancy and delivery. His birth weight was 4.3 kg. In early childhood, he suffered from obstructive bronchitis. Enuresis was present till the age of 10 years. His motor and speech development were slightly delayed; he started to speak single words at 2 years of age. He was diagnosed with Attention Deficit Disorder (ADD) with motor immaturity and muscular hypotonia. His cognitive level was in the low normal range. On examination he was rather tall (+2 SDS), and overweight (+3 SDS). He had no apparent dysmorphic features.

FISH analysis for the 16p11.2 region showed normal copy numbers in both parents.

Case 5 is a 3-year old boy, the second in a sibship of three. His parents are first cousins. Pregnancy, delivery and neonatal period were reported as normal. His growth parameters are within normal limits (0 SDS for height and weight, -0.8 SDS for head circumference). His development (motor function, speech and cognitive function) was severely delayed. He walked independently at the age of 24 months. He has a disturbed sleeping pattern, with difficulties falling asleep as well as waking up in the middle of the night.

At the age of 18 months he scored positive in the CHecklist for Autism in Toddlers (CHAT)-screening. Evaluation at the age of 20 months showed lack of eye contact, stereotypic behaviour and no speech. He had no dysmorphic features. Since the age of 18 months, he has had frequent periods with diarrhea.

Psychological evaluation (Griffith and Merril Palmer R) revealed a cognitive level corresponding to the 4-10 months of age interval at a chronological age of 20 months, in addition to autistic symptoms. A brain MRI at 17 months of age was normal.

His family history is positive for speech retardation. MLPA analysis of the 16p11.2 region (kit 2) in his parents and brother showed a normal copy number in his mother, and a deletion in his father and brother. As a child, his father had delayed speech development. At present he is working full time as a sailor. He has no apparent dysmorphic features.

His 5-year old brother was born at term with good Apgar scores. At birth, his weight was 3055 g, his length 49 cm. His motor development was unremarkable (crawling at 8-9 months, walking independently at 19 months). He had a severe speech delay; at the age of 2 years he only spoke a few single words. Psychological evaluation at the age of 4 years and 6 months revealed a speech disorder. His cognitive function is in the low normal range. His growth parameters are at the median for weight and height. He has no apparent dysmorphic features.

Case 6 is a 7-year old mentally retarded girl (IQ 65), without autistic behaviour (Fig. 3.1.1c). On examination, her height was 122 cm (-0.7 SDS), her weight 25 kg (0 SDS), and her head circumference 52.5 cm (+0.8 SDS). She has a nasal speech.

She has several dysmorphic features: low frontal hairline, hypertelorism, bilateral epicanthic folds, short palpebral fissures, mild ptosis, a broad nasal bridge, a broad based nose with upturned nares, a long philtrum, a tented mouth with thin upper lip, a high and narrow palate, a pointed chin, low set ears, a broad neck, widely spaced nipples, short fingers, broad and proximally implanted thumbs, and mild clinodactyly of both fifth fingers.

Testing of the 16p11.2 region in the parents, using the 44K Agilent Human Genome CGH Microarray Kit detected the same deletion in the mother. She is a normal functioning female, with an IQ within normal limits, without speech problems or major health problems. The further family history is negative for mental retardation or autism.

Case 7 is a 1 year and 8 month old boy, born after an uneventful pregnancy with a birth weight of 2770 g, a birth length of 46 cm and a head circumference of 34 cm. His neonatal period was uncomplicated. His neuromotor development was slow, with sitting independently at 13 months of age. At the age of 18 months he was able to crawl, roll over, and pull himself up to an upright position, but he could not yet stand unsuppported. He could speak two words. Formal developmental testing revealed a developmental level of 12 months at the age of 17 months. He had no major health problems. At the age of 18 months his height was 72 cm (-4 SDS), his weight 8.2 kg (-1.5 SDS for height), and his head circumference 47.5 cm (-0.7 SDS). Physical examination revealed mild facial dysmorphism with sparse blond hair, anteverted nares, low-set ears, a broad mouth and a narrow nasal bridge. He has mild hypospadias with bilateral descended testis. He has small hands. Neurological examination showed symmetrical reflexes, axial hypotonia, and joint hyperlaxity.

His family history is positive for developmental delay: his mother, father

and brother are all developmentally delayed. The probands brother had normal antropometric parameters at birth, but suffered from asphyxia and has convulsions and developmental delay. He has not been tested for the deletion.

Analysis of the 16p11.2 region in the parents, using the 44K Agilent Human Genome CGH Microarray Kit detected the same deletion in the mother. She has short stature (height 1.50 m, -3.2 SDS). She attended a school for children with learning disabilities and works in a sheltered workshop. There is no further family history of developmental delay, other family members were not available for testing.

Case 8 is a 11-year old, mildly mentally retarded girl. She was born as the third child of healthy, non-consanguineous parents. Pregnancy and delivery were uneventful. At the age of 2 years speech retardation was evident. At the age of 2.5 years, she used about 20 words and at the age of 3 years she spoke 2-word sentences. At the age of 3 years, she suffered from complex partial epilepsy, which was successfully treated.

Psychological testing revealed mild mental retardation (total IQ (TIQ) 62) and a severe expressive language disorder. Apart from some hand stereotypies, she had no behavioural problems or autistic features. On examination, she had a normal height (146 cm, 0 SDS) and weight (42.8 kg, +0.7 SDS). Apart from mild truncal obesitas no abnormalities were noted, especially no dysmorphic features. A brain MRI showed no abnormalities.

Despite intensive therapy, she did not achieve scholarly skills such as reading, writing and calculating. At the age of 11 years she still has poor expressive verbal skills.

Her family history is positive for mental retardation. Her oldest sister is equally affected with mild mental retardation (TIQ 66), expressive language disorder and epilepsy. Their father had learning problems. Analysis of the 16p11.2 region in the parents, using the 44K Agilent Human Genome CGH Microarray Kit detected the same deletion in the mother, who is of normal intelligence and without major health problems. Array analysis in the sister is pending.

Case 9 is a 34-year old mentally retarded male, reported as case 7 in a previously described series [2]. He was born at term after normal pregnancy and delivery. He was reported to be small for gestational age (2.8 kg). His developmental milestones were reached late. In childhood he had intensive speech therapy because of speech delay. On examination at the age of 34 years, he had slow speech and was overweight. Apart from mild malar hypoplasia, he had no apparent dysmorphisms. He had no autistic features.

Formal neuropsychological assessment indicated moderate mental retardation, with major difficulties with working memory, attention and self-monitoring. He does not live independently, but has been able to do simple (cleaning) jobs.

FISH analysis in his parents confirmed a *de novo* deletion.

Case 10 is an 8-year old girl with significant intellectual disability, born to a 30-year old mother and a 31-year old father. She has no speech, but she is socially interactive. She is able to walk and has a happy disposition. She is not toilet trained and has major sleeping problems. Apart from pyloric stenosis at the age of 5 weeks, she had no major health problems, especially no seizures.

On examination she had normal height (128 cm, –0.5 SDS), weight (24 kg, –0.7 SDS), and head circumference (53 cm, +1 SDS). She has subtle dysmorphic features

(Fig. 3.1.1d): a relatively flat nose and maxilla, prominent infra-orbital skin creases, small ears, an unusual hairline which extends over the lateral forehead, small hands with abnormal palmar creases and small feet.

Her family history is negative for mental retardation. FISH analysis in the parents is ongoing.

Case 11 is a 4.5-year old boy, born to a 33-year old father and a 28-year old mother. He was born at term in poor condition, but quickly recovered from hypotonia and cyanosis using an oxygen mask (Apgar scores 5 and 9 at 1 and 5 minutes, respectively).

At birth, his weight was 3 kg, his length 49 cm, and his head circumference 35 cm. In infancy and childhood he had failure to thrive. He has no autistic features. He loves water and music. He has normal hearing and vision.

His gross and fine motor development were delayed; he walked independently at the age of 2.5 years. At the age of 3 years and 8 months he had no comprehensible language, but only varied utterances mimicking sentences. Language comprehension was clearly present. At the age of 4 years and 4 months he was able to speak a few single words, with poor articulation.

IQ-testing at the age of 3 years and 8 months showed scores in the slightly retarded range (non verbal IQ (tested with SON-R 2½-7) 74, language comprehension quotient (Reynell): 73, and expressive language quotients words/sentences (Schlichting): 58/55).

Physical examination at the age of 4 years showed a skinny boy with a height of 101.5 cm (–0.5 SDS), a weight of 15 kg (–0.9 SDS for height), and a head circumference of 51.5 cm (+0.1 SDS). He had mild facial dysmorphism: retrognathia, small teeth, and posteriorly rotated ears with a slightly larger ear on the left (Fig. 3.1.1e). Neurological examination showed clumsy walking, but was otherwise unremarkable. There was no indication of a specific motor deficit affecting articulation.

A brain MRI was reported normal.

The maternal family history is positive for mental retardation: two maternal uncles are mentally retarded. Analysis of the 16p11.2 region using the 105K Agilent Human Genome CGH Microarray Kit showed normal copy numbers in both parents.

Case 12 is a 3.5-year old girl, born as the third child of a 32-year old mother and the second child of a 31-year old father (Fig. 3.1.1f). She presented prenatally with intrauterine growth retardation. In pregnancy, her mother had thrombosis and hypertension. Delivery was induced at 37 weeks of gestation. Birth weight was 2.5 kg (-1.3 SDS), and head circumference 31.5 cm (-2 SDS). In the first year, she had muscular hypertonia. Her development is within normal limits and she has no autistic features.

Physical examination at the age of 11 months showed a length of 77.5 cm (+1.5 SDS), a weight of 8.4 kg (-0.9 SDS), and a head circumference of 45.5 cm (0 SDS). She had minor facial dysmorphisms: slightly deep-set eyes, a thin upper lip, a smooth philtrum, long and slender fingers, and camptodacyly of both fifth fingers.

At the age of 2 years and 9 months she was diagnosed with a Wilms' tumor with liver metastases. At the age of 3 years and 4 months she was fully recovered after standard treatment.

The family history is positive for mental retardation. Her older brother is mentally retarded, with an estimated IQ of 60. He also had muscular hypertonia in infancy. He was not tested for the deletion. Her maternal half-sib is mentally retarded

and has attention deficit hyperactivity disorder (ADHD).

The Wilms' tumor, combined with the family history and hypertonia in the neonatal period, were reason to perform array analysis.

Analysis of the 16p11.2 region in her parents, using the 105K Agilent Human Genome CGH Microarray Kit, detected the same deletion in the father. He is of normal intelligence and has no apparent dysmorphic features (Fig. 3.1.1g). In childhood, he had neither learning problems nor speech problems. He had two episodes of meningitis, at the ages of 9 months and 31 years, respectively. During the latter episode he developed seizures due to post-viral cerebral damage. He has a full time job.

He is the only child of healthy parents. Further family studies have not been performed.

Case 13 is a 4 years and 10 months old boy (Fig. 3.1.1h). He is the second child of a 31-year old mother and a 33-year old father. He was born at term after a normal pregnancy, with a birth weight of 2790 g. At the age of 7 weeks he was diagnosed with short segment (~5 cm) Hirschsprung's disease, for which he underwent surgery. At the age of 2 years and 6 months he started to have seizures, for which he was successfully treated. He suffers from frequent infections, particularly otitis media and upper respiratory tract infections.

His psychomotor development was delayed: he walked independently at the age of 21 months and spoke his first words at the age of 3 years and 9 months. At the age of 4 years and 6 months his IQ score was 83. A formal test for autism showed no ASD.

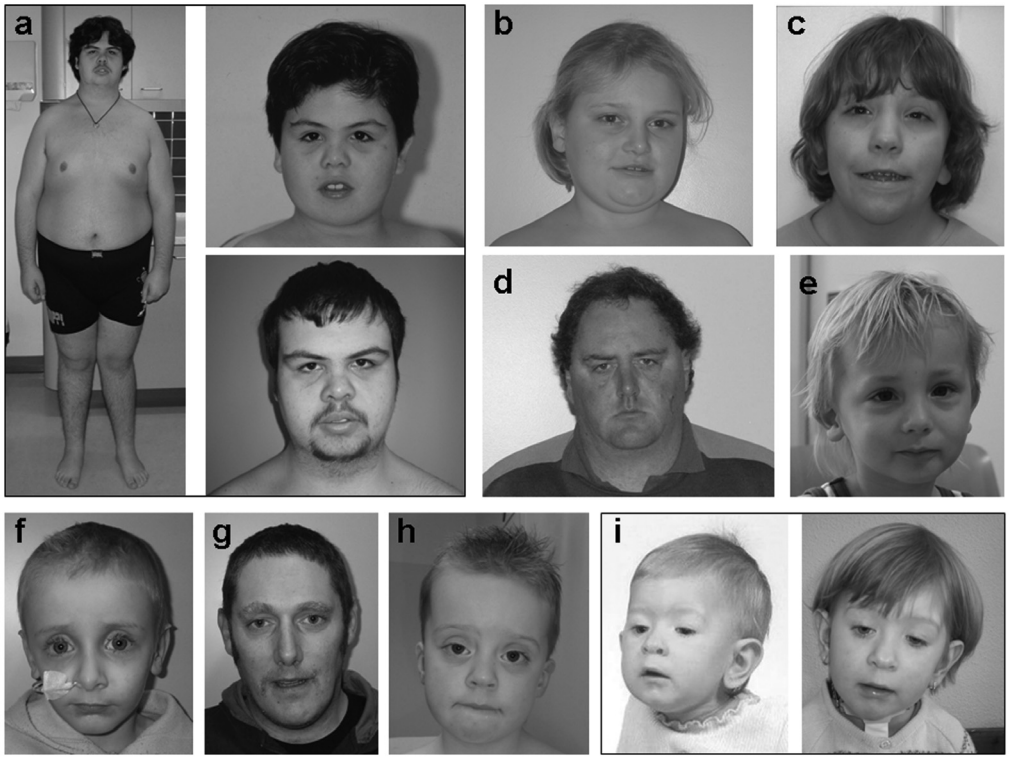


Figure 3.1.1 Phenotypical characteristics of cases with a 16p11.2 deletion. (a) case 2 (overview aged 16 years (left), face aged 7 years (top) and 17 years, (b) case 3, (c) case 6, (d) case 10, (e) case 11, (f) case 12, (g) father of case 12, (h) case 13, (i) case 14 (at the age of 9 months (left) and 4 years). Some of the cases share facial characteristics (long nose in cases 6, 12 and, father of case 12) (c, f, g); narrow palpebral fissures in cases 6 and 14(c, i); periorbital fulness in cases 2, 3, and 11(a, b, e); ptosis in cases 13 and 14 (h, i). Overall however, patients show no common facial features.

On examination he had normal height (112 cm, 0 SDS) and weight (21 kg, +1 SDS). He has a mild ptosis, a unilateral simean crease, and a pectus excavatum. He has a high forehead, but this is also observed in his unaffected brother and father.

Analysis of the 16p11.2 region using the 500K Affymetrix GeneChip Human Mapping array, showed normal copy numbers in both parents.

Case 14 is a 4-year old girl with psychomotor and growth retardation. She is microcephalic with facial dysmorphisms: blepharophimosis, ptosis, epicanthus inversus, telecanthus, a flattened and broad nose with bifid tip, and large ears (Fig. 3.1.1i). She also has scoliosis and clinodactyly of her fingers. Autistic features are not reported.

MLPA analysis of the 16p11.2 region (kit 3) showed normal copy numbers in both parents.

Patients carrying atypical 16p11.2 deletions

Case 15 is a 5-year old mentally retarded boy. He was born at term after an uncomplicated pregnancy. Birth weight and length were 3750 g and 53 cm, respectively. Both his motor and speech development were delayed; he started to walk at the age of 2 years, and at times speech is barely comprehensible. Because of behavioural problems he is on Risperdal. He has a normal sleeping pattern.

On examination at the age of 5 years and 3 months he was hypotonic and had dysmorphic features: a long narrow face, a prominent forehead, downslanted and narrow palpebral fissures, an open mouth with down turned corners, and fleshy earlobes (Fig. 3.1.2a).

A brain MRI was reported normal.

Analysis of the 16p11.2 region in the parents, using the 44K Agilent Human Genome CGH Microarray Kit detected the same deletion in the father. As a child, his father had learning difficulties. He works as a truck driver. He has the same facial appearance as his son (Fig. 3.1.2b). Further family members were not accessible. The family history is negative for learning problems.

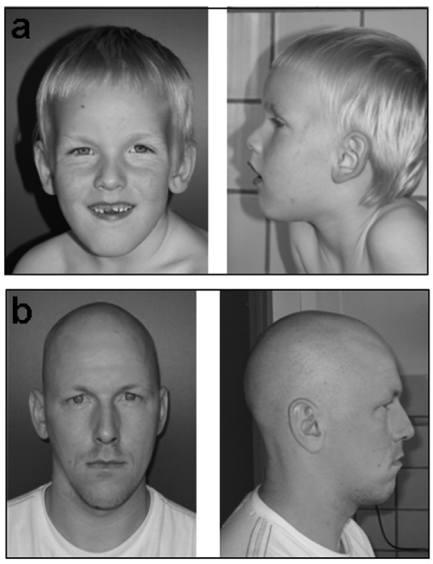


Figure 3.1.2 Facial characteristics of cases with an atypical 16p11.2 deletion. (a) case 15, (b) father of case 15. Note long narrow face, prominent forehead, downslanted and narrow palpebral fissures, and down turned corners of the mouth in both.

Results

Recurrent microdeletion of 16p11.2

Array analysis was performed on 4284 patients with MR/MCA. We detected 14 cases (0.3%) with a microdeletion of approximately 600 kb in the same area of 16p11.2, from genomic location 29.5 to 30.1 Mb (Ensembl release 52, Dec 2008) (Fig. 3.1.3). In case 14 a de novo deletion of 16p11.2 was found in a mosaic state (Fig. 3.1.4). Table 3.1.1

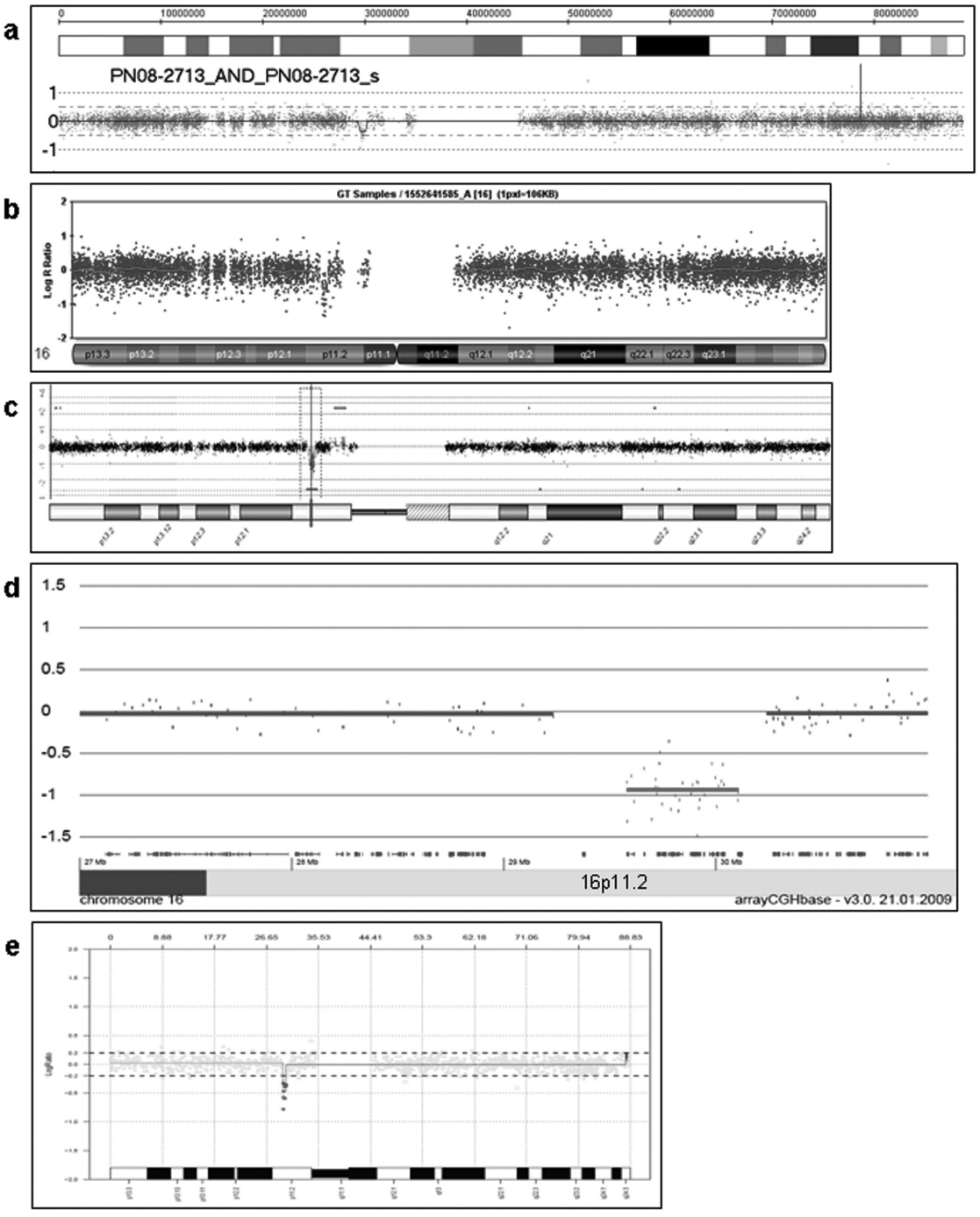


Figure 3.1.3 Examples of array results showing a 16p11.2 deletion using various array platforms. (a) LogR-ratio profile of a 500K Affymetrix array, showing a minimal deletion of ~525 kb in case 13. (b) LogRratio profile of a 317K Illumina array, showing a minimal deletion of ~522 kb in case 1. (c) LogRratio profile of a 244K Agilent array, showing a minimal deletion of ~505 kb kb in case 5. (d) LogRratio profile of a 44K Agilent array, showing a minimal deletion of ~526 kb kb in case 6. (e) LogRratio profile of a 38K BAC array, showing a minimal deletion of ~612 kb kb in case 4.

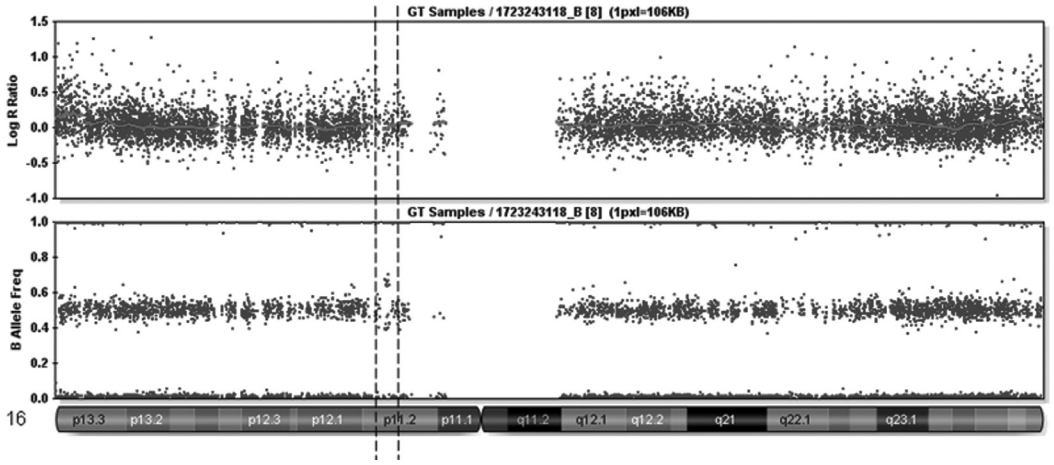


Figure 3.1.4 Mosaic 16p11.2 deletion. LogRratio profile (upper panel) showing a slight decrease in copy number and B-allele frequency plot (lower panel) showing ABB and AAB genotypes (317K Illumina array).

summarizes all detected 16p11.2 deletions in the index cases (n =14). Of these, six occurred *de novo*, six were inherited (three paternal and three maternal), one could not be assessed, and one is still under study.

Table 3.1.2 provides a summary of the phenotypic characteristics of the 14 index cases with a 16p11.2 deletion. Twelve out of 14 had developmental delay, ranging from motor retardation to severe mental retardation. Ten were recorded to have speech problems. Autism was formally diagnosed in one index patient (case 5). In nine index cases dysmorphic features were noted. Five index cases had overweight or obesity. Major congenital malformations were not a frequent symptom, however pyloric stenosis was reported twice (uncle of case 3, case 10), but this co-occurence is most likely coincidental. The intracerebral cyst in case 2 is regarded a chance finding, as this anomaly is not known to be associated with (speech) retardation. Among the index cases, one patient had a malignancy (case 12, Wilms' tumor).

In the six familial cases, three of the transmitting parents (two males, one female) had developmental problems of a varying degree (parents of cases 3, 5, and 7). Other family members carrying the deletion were also reported to have developmental problems (uncle and sib of cases 3 and 5, respectively). Three apparently normal transmitting parents were identified (in cases 6, 8, and 12).

In the common 600 kb recurrent microdeletion more than 25 genes are located. To determine whether the remaining intact 16p11.2 region harbored recessive mutations, which might be contributing to the phenotype in these individuals, we sought to identify gene candidates for sequence analysis. After analysing this region with the Anni tool, the genes *ALDOA*, *TBX6* and *SPN* seemed good candidates to test for a mutation on the remaining allele. However, sequencing of genomic PCR products covering the coding regions of *ALDOA*, *TBX6* and *SPN* in four of the deletion patients (cases 1-3, and the paternal uncle of case 3) showed no mutations.

Atypical deletion of 16p11.2

In addition to the common recurrent microdeletion of 16p11.2 observed in 22 individuals (14 patients and 8 family members), an atypical rearrangement was detected in two related patients. In case 15, a 205 kb deletion in 16p11.2 was detected (28,74 Mb - 28,95 Mb) (Fig. 3.1.5). The same deletion was detected in his father. The 205 kb deletion in 16p11.2 is flanking the common deleted region.

Table 3.1.1 Index patients with 16p11.2 deletion

Case ID (DECIPHER code)	Platform	Starting probe	Ending probe	Start position	End position	Status of inheritance
1 (LEI00248634)	317K Illumina	rs8054172	rs7202714	29563365	30085308	n/a
2 (LEI00248635)	317K Illumina	rs8054172	rs7202714	29563365	30085308	*de novo*
3 (LEI00248635)	370K Illumina	rs28455095	rs7202714	29531748	30085308	paternal
4	38K BAC array	RP11-753J6	RP11-787F23	29536903	30149118	*de novo*
5	244K Agilent	A_16_P03134009	A_14_P129228	29500284	30240082	paternal
6 (GHE00002722)	44K Agilent	A_14_P200817	A_14_P124319	29581455	30106054	maternal
7 (GHE00248753)	44K Agilent	A_14_P200817	A_14_P124319	29581455	30106054	maternal
8 (GHE00248754)	44K Agilent	A_14_P200817	A_14_P124319	29581455	30106054	maternal
9	262K *NspI* Affymetrix	SNP_A-2236020	SNP_A-2272043	29580704	30085308	*de novo*
10	Affymetrix SNP 6.0	SNP_A-8559376	CN_728581	29488122	30134457	pending
11	105K Agilent	A_16_P20442189	A_14_P124319	29563984	30106253	*de novo*
12	105K Agilent	A_16_P20442189	A_14_P124319	29563984	30106253	paternal
13	500K Affymetrix	SNP_A-2117335	SNP_A-2272043	29559989	30085308	*de novo*
14*	317K Illumina	rs8054172	rs7202714	29563365	30085308	*de novo*
15 (atypical)	44K Agilent	A_14_P134515	A_14_P106155	28744951	28949560	paternal

n/a, DNA of parents not available; *, deletion found in mosaic state

Table 3.1.2 Phenotypic features of index patients with a 16p11.2 deletion

Index patients	Gender	Developmental delay	Speech retardation	Autism	Dysmorphism	Overweight	Seizures [a]
This series							
case 1	m	+	+	–	+	+	–
case 2	m	+	+	–	+	+	–
case 3	f	+	+	–	–	+	+
case 4	m	–	+	–	–	+	–
case 5	m	+	+	+	–	–	–
case 6	f	+	?	–	+	–	–
case 7	m	+	?	–	+	–	–
case 8	f	+	+	–	–	–	+
case 9	m	+	+	–	–	+	–
case 10	f	+	+	–	+	–	–
case 11	m	+	+	–	+	–	–
case 12	f	–	–	–	+	–	–
case 13	m	+	+	–	+	–	+
case 14 [b]	f	+	?	?	+	–	–
Marshall et al. [13]							
MM 0088-003	f	–	+	+	–	?	?
SK 0019-004	m	–	+	+	+	+	?
Weiss et al. [22]							
pt 1	m	+	+	+	–	–	–
pt 2	m	+	+	+	–	?	–
pt 3	m	+	+	+	–	–	–
pt 4 [c]	m	+	+	+	–	+	–
pt 5 [c]	m	+	+	+	–	+	–
Aut 1	f	+	+	+	?	–	–
Aut 2	m	–	+	+	?	+	+
Aut 3	f	+	?	+	?	?	+
Total (index cases)	15m/9f	19/24	19/20	11/23	10/21	9/21	5/22
Combined carrier family members (this series; n = 8)	5m/3f	2/8	3/6	0/8	0/6	0/3	0/6
Total (all carriers)	20m/12f	21/32	22/26	11/31	10/27	9/24	5/28

+, present; –, absent; ?, unknown; a, excluding seizures secondary to known causes; b, mosaic deletion; c, monozygous twins

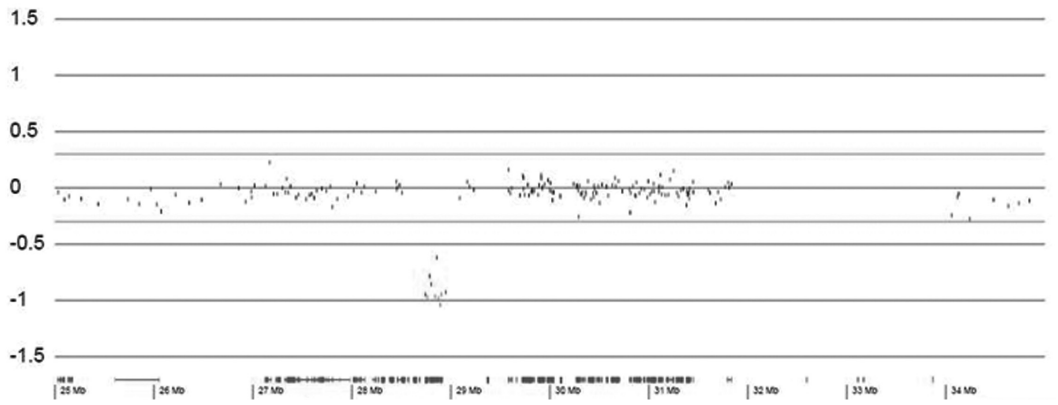


Figure 3.1.5 Atypical 16p11.2 deletion. Array analysis with 44K Agilent array revealing a 205 kb atypical 16p11.2 deletion from 28,74 Mb to 28,95 Mb.

Discussion

We describe 22 individuals (14 index patients and 8 family members) carrying a common deletion in 16p11.2 (from genomic location 29.5 Mb to 30.1 Mb), one of them in mosaic form. In addition, two related patients showed a smaller deletion of an adjoining region, presumably representing a rearrangement of adjacent LCRs.

Previous reports have shown that the same (~ 600 kb) 16p11.2 deletion recurrently occurs in patients diagnosed with autism [10, 13, 22]. Weiss et al. reported a recurrent microdeletion on chromosome 16p11.2 in five of 751 families with one or more cases with ASD, in three of 299 ASD patients, in five of 512 children referred for MR and/or autism, and in two of 18,834 Icelandic controls who had not been screened for psychiatric or language disorders [22]. The reciprocal duplication was found in 11 patients and in five controls. In another study, the same deletion was detected in four of 712 autistic patients and none of 837 controls [10]. This study identified the reciprocal duplication in one autism case and two controls. Similarly, Marshall et al. detected two *de novo* 16p11.2 deletions in 427 families with autism [13]. In this series, the reciprocal duplication was also found twice. The authors stated that deletions and duplications of 16p11.2 carry substantial susceptibility to autism, and that the deletions appear to account for approximately 1% of cases. In contrast, in our series autism was not a frequent symptom, only one of the cases had formally been diagnosed with autism. Although the other patients were not extensively assessed for autistic features, their behaviour and social interaction were not suggestive of autism.

Additional, single reports of individuals with 16p11.2 microdeletions have been documented, mainly without autism. Rosenberg et al. reported a deletion in a patient with mild mental retardation, severe speech delay, and facial dysmorphism [16]. A ~600 kb 16p11.2 microdeletion was reported in a pair of monozygotic twins with mild mental retardation, mild dysmorphism, a seizure disorder and aortic valve disease. Autistic features were not reported [7]. A similar *de novo* deletion was identified in a female with Asperger syndrome, without further details regarding her phenotype [18].
Comparison of the phenotypes is hampered by limited clinical data in previous reported series (Table 3.1.2). Dysmorphic features were not reported in the deletion cases in the series of Kumar et al. [10]. As further phenotypical data are not provided, these patients were not included in Table 3.1.2. Regarding behaviour, there was a trend towards aggression and overactivity in patients carrying the deletion [10]. This was not observed in our series.

Phenotypic data are available on two autism patients [13], five children referred for MR and/or autism (including a pair of monozygous twins), and three autism patients in the series of Weiss et al. [22]. Seven out of ten cases had developmental delay, and speech development was delayed in each case, when it was recorded (n = 9). Only one patient was reported with facial dysmorphisms, which were not further specified [13]. In our series, dysmorphic features were reported in nine of 14 index cases. Some patients have facial features in common (Fig. 3.1.1), however no specific pattern of dysmorphic features could be distinguished.

Five of our 14 index cases were overweight, as were four out of ten autism cases [13, 22]. However, patients with a weight on the other side of the spectrum are also reported (case 12, [22]). Three of our index cases and four previously reported patients (including monozygous twins) had seizures [7, 22].

In summary, though some of our patients show a facial resemblance and 16p11.2 deletion patients share a tendency to overweight and obesity, there is no evidence in our group of index cases to suggest a recognizable phenotype.

In the previous autism studies almost all microdeletions were *de novo* [10, 13, 22]. Among a total of 20 cases (including monozygous twins), 18 were *de novo* and only one familial case was reported: an index patient with autism inherited the deletion from his father with ADHD [22]. In contrast, we identified six familial cases among 14 index patients. In half of familial cases, the transmitting parent (and other family members that carried the deletion) had developmental problems, which were frequently speech related. Interestingly, in a study of Icelandic subjects with a psychiatric or language disorder, the 16p11.2 deletion was found in a higher frequency than in the control population, 0,1% vs. 0,01%. For instance in patients with dyslexia, 1 in about 750 carried the 16p11.2 deletion, suggesting an association between the deletion and this specific phenotype [22].

In two families, the transmitting fathers were less affected than their children (cases 3 and 5). In one family with maternal transmission, the mother was mentally retarded and probably as affected as her child (case 7). As the region of chromosome 16 is not known to be imprinted, it is unlikely that imprinting explains this phenotypic variability.

Noticeably, in case 5 and his family clinical expression seems to include both ends of the phenotypic spectrum: case 5 is severely mentally retarded and the only patient in our series with autism; his father and brother are of normal intelligence but do have speech problems. It is possible that the consanguinity in this family plays a role in this variability. As his parents are first cousins, an additional effect of an autosomal recessive trait may be present. Homozygosity for this hypothetical trait in case 5, but not in his brother and father, would then explain the difference in expression in this family. Segregation studies however, point to considerable intrafamilial variability in expression in our non consanguineous families as well.

Unexpectedly, the mosaic 16p11.2 deletion was identified in a patient with a severe phenotype (case 14), not observed in any other known carrier. Without knowledge about the full spectrum of the 16p11.2 deletion phenotype, it is difficult to presume a causal relationship between the mosaic deletion and the severe phenotype in this patient. As this patient seems to have dysmorphic features suggestive of BPES (Blepharophimosis, Ptosis, Epicanthus inversus Syndrome), this may well be caused by another genetic defect (*FOXL2* gene mutations were excluded).

Likewise, it is difficult to speculate on the significance of the atypical deletion found in case 15 and his father. There are no previous reports about patients with this deletion. The deletion may be causal, since the father has a comparable phenotype and had learning problems. More evidence is needed however, and further family studies would have to be performed to gain more insight in the segregation of the deletion and the phenotype. The identification and characterization of additional deletions like the two described here are needed.

In a previous paper, the question was raised whether the 16p11.2 microdeletion might be non-pathogenic, or a coincidental finding [10]. Given the negative results in their control group, the authors suggest that chance finding is unlikely. Further evidence to support pathogenicity for this microdeletion has come from several sources: in the autism studies, the deletion was almost always *de novo* [10, 13, 18, 22], and the deletion was found in only 2 of almost 18,900 non-characterized Icelandic controls [22]. In our series, six out of 14 deletion cases were familial, half of the transmitting parents however had a (mild) phenotype. Hence, it seems plausible that the 16p11.2 deletion is pathogenic.

Kumar et al. suggested that the 16p11.2 microdeletion is not associated with MR, and is more likely to cause autism [10]. Our series proves that the deletion does not necessarily cause autism, but is associated with other developmental and speech disorders as well, and may even be found in normal individuals. Finally, the marked phenotypic variation in our series of deletion cases proves that the 16p11.2 deletion does not by itself cause ASD, as has been suggested previously [10].

The assembled evidence indicates that recurrent 16p11.2 deletions are associated with variable clinical outcome, most likely arising from haploinsufficiency of one or more genes located between the two paired LCRs. Several well known microdeletion syndromes, such as the 22q11 microdeletion syndrome, show a wide range in phenotypic expression with non-obligatory MR, congenital anomalies, dysmorphisms and psychiatric disorders, including autism. The reciprocal 22q11 duplication appears to be even more variable, including more 'normal' individuals with mild, but characteristic facial features [3, 5, 6].

As an alternative explanation for the variability in phenotype in new microdeletion cases, it has been hypothesized that the deletion may unmask a mutation in a recessive gene on the homologous allele, and thus cause a more severe phenotype [11]. However, sequencing of three selected candidate genes in four patients described here, failed to detect any sequence alterations. As there are more than 25 genes in this region, one explanation for the negative findings could be the fact that we chose the wrong genes. However, the cellular functions of *TBX6* (a transcription factor) and *ALDOA* (a glycolytic enzyme) make them strong candidate genes, and it may be worthwhile to explore them in more detail. We may have missed mutations in the promotor regions, the untranslated regions or introns. Alternatively, we may have selected an inappropriate set of patients. Better still would be to screen patients with a more severe phenotype than their transmitting parent. Further studies of additional genes and patients are needed.

In summary, a ~ 600 kb deletion in 16p11.2 is described in autistic patients without apparent dysmorphic features [10, 13, 18, 22], in mentally retarded patients with minor dysmorphic features ([7, 16], this series), and in individuals with normal intelligence (this series) who may have had isolated developmental problems such as speech retardation and dyslexia. We therefore conclude that this deletion at 16p11.2

is associated with a variable phenotype. With our series of 16p11.2 deletion carriers we have extended the spectrum of the associated phenotype. The phenotype is not restricted to autism, and the deletion does not always result in autism. The deletions are most likely pathogenic and are associated with a variable clinical outcome, including a normal phenotype. However, further studies of more patients and normal individuals with 16p11.2 deletions (and duplications) are needed to gain better insight in the potential pathology associated with rearrangements in this area.

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Chapter 3.2

Additional cryptic CNVs in mentally retarded patients with apparently balanced karyotypes

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Abstract
Apparently balanced chromosome abnormalities are occasionally associated with mental retardation (MR). These balanced rearrangements may disrupt genes. However, the phenotype may also be caused by small abnormalities present at the breakpoints or elsewhere in the genome. Conventional karyotyping is not instrumental for detecting small abnormalities because it only identifies genomic imbalances larger than 5-10 Mb. In contrast, high-resolution whole-genome arrays enable the detection of submicroscopic abnormalities in patients with apparently balanced rearrangements.

Here, we report on the whole-genome analysis of 13 MR patients with previously detected balanced chromosomal abnormalities, five *de novo*, four inherited, and four of unknown inheritance, using Single Nucleotide Polymorphism (SNP) arrays. In all the cases, the patient had an abnormal phenotype. In one familial case and one unknown inheritance case, one of the parents had a phenotype which appeared identical to the patient's phenotype. Additional copy number variants (CNVs) were identified in eight patients. Three patients contained CNVs adjacent to one or either breakpoints. One of these patients showed four and two deletions near the breakpoints of a *de novo* pericentric inversion. In five patients we identified CNVs on chromosomes unrelated to the previously observed genomic imbalance.

These data demonstrate that high-resolution array screening and conventional karyotyping is necessary to tie complex karyotypes to phenotypes of MR patients.

Introduction
Recent advances in molecular cytogenetic technologies provide a resolution that exceeds that of conventional karyotyping and increased the detection of aberrations from 5% to approximately 17% in patients with mental retardation (MR) [13,16,17,19,23,28,31,32,34-36].

A disadvantage of the array technique is the incapability to detect balanced structural abnormalities such as translocations and inversions. Balanced rearrangements have a prevalence of at least 1:500 and in approximately 6% of antenatal patients with a balanced rearrangement an abnormal phenotype is found [20,37]. The abnormal phenotype of these patients can be explained by (1) breakpoint regions directly disrupting genes or transcription regulatory regions [21], (2) indirectly by submicroscopic copy number variants (CNVs) near one or both of the breakpoints [4], (3) the rearrangement hosts 'cryptic' complex chromosomal rearrangements (CCRs) [24], (4) submicroscopic CNVs unrelated to the translocation or inversion [3,6,18], or (5) another unidentified genetic or environmental factor.

Reports of single patients or small series of patients with apparently balanced aberrations have identified unexpected complexity and instability of the human genome [3,6,18,24]. Some studies investigated the difference between additional CNVs in carriers of *de novo* 'balanced' reciprocal translocations and CCRs with normal and abnormal phenotypes [1,11]. In approximately 35% of the phenotypically abnormal patients additional candidate disease-causing CNVs were identified, mostly occurring around the breakpoints of the translocations. In the phenotypically normal cohort no additional genomic CNVs were identified. Sismani and colleagues studied 12 MR patients both with *de novo* and familial apparently balanced translocations for the presence of cryptic CNVs [33]. Two *de novo* and one familial case had additional abnormalities. Recently, Schluth-Bolard and colleagues analysed 47 MR patients with *de novo* and familial apparently balanced chromosomal rearrangements [30]. All familial

rearrangements were inherited from phenotypically normal parents. Imbalances were detected in 16 *de novo* cases (48.5%) and in 4 inherited cases (28.6%).

We report on 13 patients carrying an apparently balanced translocation or inversion detected with conventional karyotyping. High-resolution Single Nucleotide Polymorphism (SNP) array analysis was performed to search for cryptic CNVs. In eight patients additional CNVs were detected. Herein we focus on the interpretation of the detected CNV in relation to the phenotypes of the patients.

Materials and methods

Patients

This study included 13 patients with MR, with or without multiple congenital malformations, and an apparently balanced translocation or inversion observed with conventional karyotyping (five *de novo*, four inherited, and four of unknown inheritance). A summary of the clinical and cytogenetic data of all patients is shown in Table 3.2.1. Karyotyping on GTG-banded chromosomes from cultured lymphocytes of the patient was performed according to standard techniques. The study was approved by the Leiden University Medical Center Clinical Research Ethics Board, conforming to Dutch law and the World Medical association Declaration of Helsinki.

SNP arrays

DNA was extracted from whole blood by a Gentra Puregene DNA purification Kit (Gentra Systems, Minneapolis, USA), following the manufacturer's instructions. The Affymetrix GeneChip Human Mapping 262K *NspI*, 238K *StyI* arrays (Affymetrix, Santa Clara, CA, USA) and Illumina HumanHap300, Human CNV370 BeadChips (Illumina Inc., San Diego, CA, USA) were performed following the manufacturers' instructions and data was analyzed as described previously [17]. Table 3.2.1 shows which SNP array platform was used for each patient.

Evaluation and validation of CNVs

Detected CNVs were evaluated as described previously [17]. The potentially pathogenic CNVs were confirmed with Fluorescence *In Situ* Hybridization (FISH) analysis or another type of SNP array using an independent DNA sample. If parents were available, segregation analysis was performed by FISH or SNP array analysis. FISH analysis was carried out by standard procedures as described previously [9]. BAC clones mapping to the unbalanced chromosome regions were selected based on their physical location within the affected region (http//: www.ensembl.org, Ensembl release 54 - May 2009, Genome build NCBI36).

All potentially pathogenic CNVs were assessed with Ensembl (Ensembl release 54 - May 2009, Genome build NCBI36) and DECIPHER (https://decipher.sanger.ac.uk) for gene content and patients with similar CNVs respectively. Finally, data of all patients with (potentially) pathogenic CNVs was added to the DECIPHER database.

Results

SNP array analysis demonstrated 16 additional submicroscopic CNVs in eight of the 13 patients (61.5%); five out of the five *de novo*, one out of the four familial and two out of the four unknown inheritance cases. In the remaining five patients no additional CNVs were detected. The 16 CNVs consisted of 15 interstitial deletions ranging in size from 59 kb to 10.11 Mb and one interstitial duplication of 2.78 Mb. Results are described in detail in Table 3.2.1.

Table 3.2.1 Cytogenetic, clinical and SNP array data of all patients

ID	Structural aberration	Phenotype	SNP array	Chromosome	Starting SNP	Ending SNP	Size
	CNV near / at breakpoint(s)						
1	inv(5)(q22q31.3) *dn*	Mental retardation *	*NspI* and 317K	4x del 5q14.3, *dn*	rs4920853	rs1862233	59 kb
					rs16902356	rs10051603	83 kb
					rs7715840	rs1062035	320 kb
					rs357509	rs304151	638 kb
				2x del 5q33.3, *dn*	rs6556381	rs9637861	130 kb
					rs6884239	rs3846687	159 kb
2	t(11;12)(q13.3;p12.3), inv(12)(p12.3p13.1) n/a	Mental retardation, psychiatric problems	*NspI*	del 12p12.3p12.3, n/a	rs7306438	rs1865936	2.51 Mb
3	inv(7)(q31.3q34) *dn*	Mental retardation, anxiety disorder, autistic behavior	*NspI*	del 7q21.11q21.3, *dn*	rs2373207	rs17166393	8.29 Mb
	CNV on unrelated chromosome(s)						
4	inv(6)(p21.3q15) n/a	Mental retardation, obesity	*NspI* and 370K	del 18q21.31q21.31, n/a	rs4940582	rs4940754	877 kb
				del 18q21.32q21.33, n/a	rs1877055	rs588677	2.6 Mb
5	t(3;18)(p14.2;q23) mat	Mental retardation *	317K	del 22q11.22q11.23, n/a	rs38114997	rs6003620	630 kb
6	t(2;6)(q37.1;q13) *dn*	Mental retardation *	317K	del 13q12.3q12.3, *dn*	rs1023166	rs7332696	1.24 Mb
7	t(2;6)(q24.1;p24.3) *dn*	Developmental delay *	*NspI*	dup 1p32.3p32.2, *dn*	rs563403	rs6670302	2.78 Mb
	CNV at or near the breakpoint plus additional, unrelated CNV						
8	t(12;14)(q21.3;q32.1) *dn*	Dysmorhpic features*	*NspI*	del 3p12.3p12.3, *dn*	rs7622824	rs11920974	1.15 Mb
				del 4q28.3q31.23, *dn*	rs1519335	rs6838916	10.11 Mb
				del 12q21.31q21.33, *dn*	rs1994104	rs7133204	6.88 Mb
	No additional CNV						
9	t(1:4), pat	Psychomotor retardation	*NspI*	balanced			
10	t(2;9)(q14.2;q32), mat	Mild developmental delay	*NspI*	balanced			
11	t(12;13)(p11.2;q31), n/a	Severe speech delay, mild developmental delay, pectus carinatum, high and thin palatum	317K	balanced			
12	t(9;13)(p22;q14.3), pat	'psuedo'achondroplasia	317K	balanced			
13	t(4;10)(q21.1;q25.2), n/a	Short stature	*StyI*	balanced			

dn, de novo; n/a, (one of the) parents not available; mat, maternally inherited; pat, paternally inherited; *, extended clinical information provided in results

The parental origin of the known *de novo* deletions could be determined in three patients (1, 3, and 8). In all these patients the deletions occurred on the paternal chromosome. In patients 4, 5, and 6 the breakpoints of the deletions were flanked by segmental duplications (according to the Database of Genomic Variants, DGV; http://projects.tcag.ca/variation).

CNVs at or near the breakpoint regions

Eight CNVs in three patients were located at or near one of the breakpoints of the apparently balanced chromosome abnormalities (Table 3.2.1). Two of these patients (1 and 3) had more complex chromosome abnormalities.

Patient 1

The patient was a 1-year old boy with MR, sleeping problems, grand-mal seizures, sensorineural deafness, severe loss of vision, epicanthic folds, strabismus, ventricular septal defect (VSD) and general hypotonia. He was initially diagnosed with a *de novo* paracentric inversion of region 5q22q31.3 (Fig. 3.2.1a). Additional SNP array screening showed four *de novo* interstitial deletions on band 5q14.3 (Fig. 3.2.1b and c) and two *de novo* interstitial deletions on band 5q33.3 (Fig. 3.2.1b and d). The presence of the six deletions was confirmed by a different SNP array platform (NspI, Affymetrix). Based on the SNP array data the inversion breakpoints were revised to 5q14.3 and 5q33.3. These breakpoints were confirmed by high resolution G- banding. The 6 deletions contain eight known coding genes (*COX7C*, *RASA1*, *CCNH*, *TMEM161B*, *MEF2C*, *EBF1*, *RNF145* and *UBLCP1*).

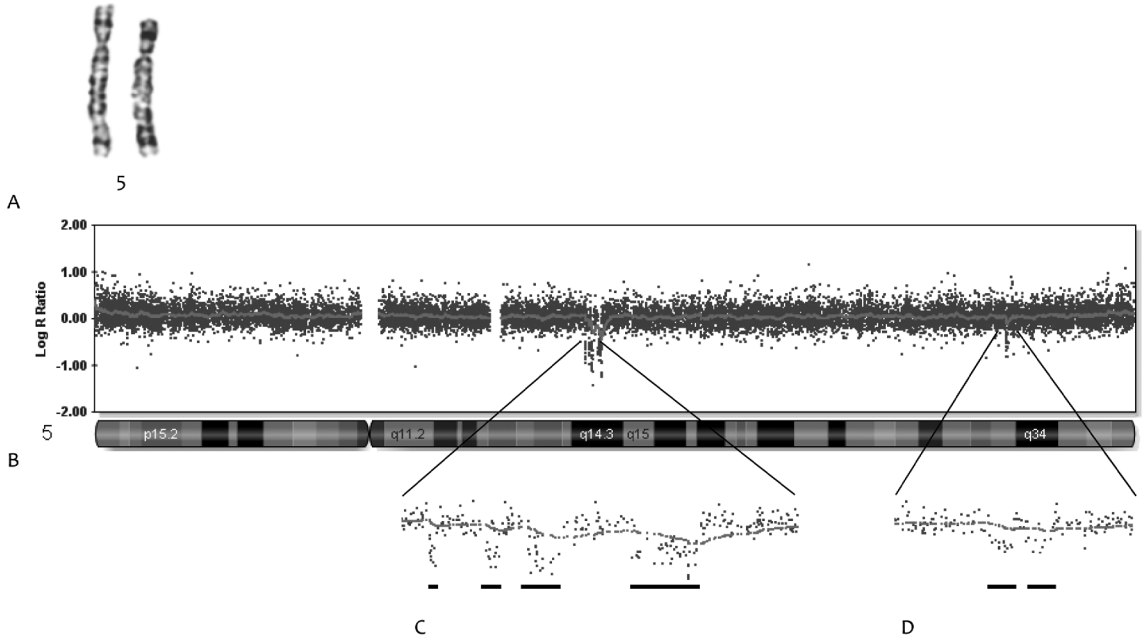


Figure 3.2.1 Cytogenetic and molecular results for patient 1. (a) Partial karyotype showing chromosome 5. Right: abnormal chromosome 5 with inversion 5q14.3q33.3. (b) SNP array copy number plot (Illumina HumanHap 300 BeadChip) for chromosome 5. (c) Four deletions at 5q14.3 and (d) two deletions at 5q33.3

Patient 2

The patient was a 61-year old male with MR and psychiatric problems. Conventional karyotyping showed a translocation between the long arm of chromosome 11 and the short arm of chromosome 12. The breakpoints were determined at 11q13.3 and 12p12.3. In addition, a pericentric inversion of region 12p12.3q13.1 was observed in the derivative chromosome 12. Subsequent SNP array analysis identified an interstitial

deletion on band 12p12.3. The parents were not available for testing. The 2.51 Mb deleted region contains eight known coding genes (*RERG*, *PTPRO*, *EPS8*, *STRAP*, *DERA*, *SLC15A5*, *MGST1* and *LMO3*).

Patient 3

The patient was a 15-year old mentally retarded girl. She had a severe anxiety disorder and autistic features. The patient is described in detail by Dauwerse and colleagues [10]. Conventional karyotyping showed a *de novo* paracentric inversion of region 7q31.3q34. However, FISH analysis in order to map the exact breakpoints identified an insertion of region 7q31.31q35 within band 7q21.3. SNP array screening demonstrated a *de novo* interstitial deletion at the insertion site from chromosome bands 7q21.11 to 7q21.3. The deletion contains approximately 40 known coding genes.

CNVs on unrelated chromosomes

Six CNVs in four patients were detected on chromosomes not related to the chromosomes involved in the balanced inversions or translocation (Table 3.2.1).

Patient 4

The patient was a 42-year old male, diagnosed with MR and obesity. Conventional karyotyping showed a pericentric inversion of chromosome region 6p21.3q15 (Fig. 3.2.2a). Additional SNP array analysis identified two interstitial deletions on the long arm of chromosome 18 in chromosome bands q21.31 and q21.32 (Fig. 3.2.2b and c). The patient's mother and brother showed a normal karyotype and normal SNP array results. His father was not available for testing. The two deletions contain 13 known coding genes (*NEDD4L*, *ALPK2*, *MALT1*, *ZNF532*, *SEC11C*, *GRP*, *RAX*, *CPLX4*, *LMAN1*, *CCBE1*, *PMAIP1*, *MC4R*, *CDH20*).

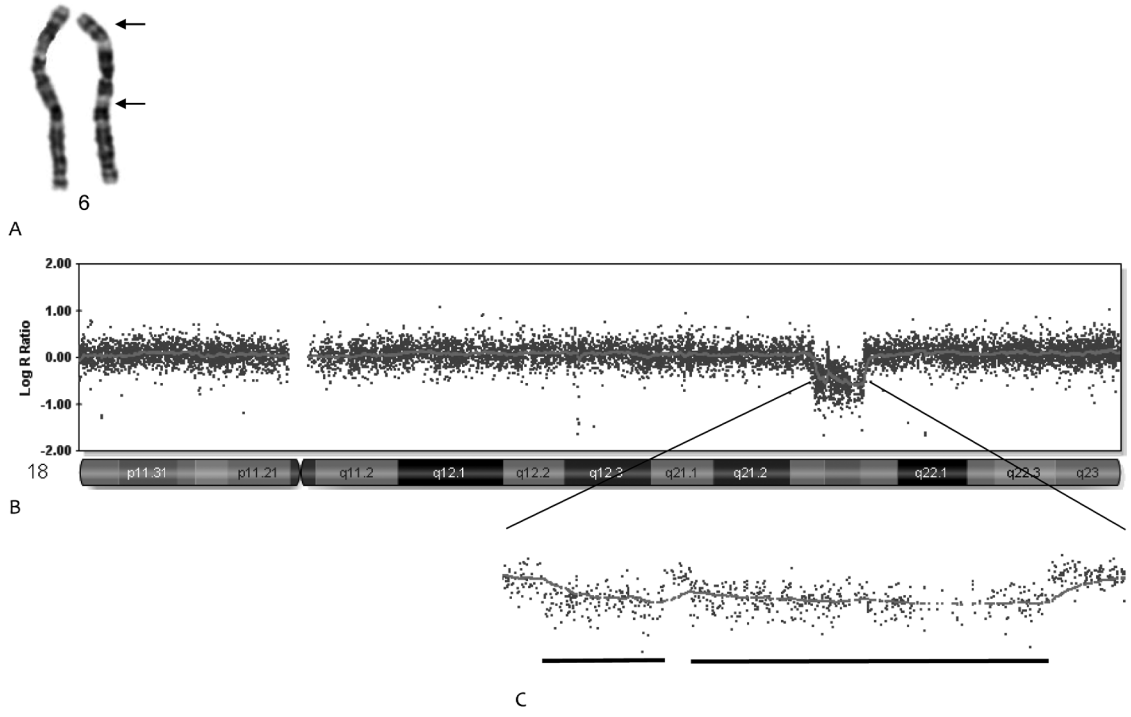


Figure 3.2.2 Cytogenetic and molecular results for patient 4. (a) Partial karyotype showing chromosome 6. Right: abnormal chromosome 6 with inversion 6p21.3q15. (b) SNP array copy number plot (Illumina HumanCNV370 BeadChip) for chromosome 18. (c) Two deletions at 18q21.31q21.31 and 18q21.32q21.33.

Patient 5
The patient was a 46-year old female with MR, deafness, heterochromia of the iris, a depigmented forelock, hypertension and hypothyroidism. Mutations in the coding region of *MITF* (Waardenburg syndrome) were excluded by sequencing. Conventional karyotyping showed a balanced translocation t(3;18)(p14.2;q23). SNP array analysis identified one additional deletion on the long arm of chromosome 22 between bands 22q11.22 and q11.23. Conventional karyotyping and SNP array results of the father were normal. The mother was not available for testing. However a healthy sister of the proband showed the same t(3;18)(p14.2;q23). The 22q deletion contains four known coding genes (*RTDR1*, *GNAZ*, *RAB36* and *BCR*).

Patient 6
The patient was a 5-year old boy with mild MR and hyperactivity. He had several dysmorphic features, including microcephaly, coarse hair, hypotelorism, a narrow nasal bridge, a long columella, large ears, pectus excavatum, syndactyly of 2-3 toes, and patchy depigmentation of the skin. Conventional karyotyping identified a *de novo* balanced translocation t(2;6)(q37.1;q13). SNP array results revealed an interstitial deletion on the long arm of chromosome 13 band q12.3. In the 1.24 Mb deletion five known coding genes (*KIAA0774*, *SLC7A1*, *UBL3*, *KATNAL1*, *HMGB1*) are located. FISH analysis on the parents showed that the deletion occurred *de novo*. Parental DNA for SNP array analysis was not available.

Patient 7
The patient was a 1-year old boy with mild developmental delay, a unilateral cleft palate and dysmorphic features, including protruding ears and a unilateral preauricular earpit, long eyelashes, prominent arched eyebrows and strabismus. On his philtrum he had a dimple. X-ray of the spine showed posterior fusion defects of several thoracic vertebrae. Conventional karyotyping showed a *de novo* balanced translocation t(2;6)(q24.1;p24.3). SNP array results revealed a *de novo* interstitial duplication on chromosome 1p32.3p32.2 that contains 23 known coding genes. The parental origin of this duplication could not be determined with SNP array analysis.

CNV at or near the breakpoint plus additional, unrelated CNV
Patient 8
The patient was a new born girl with low-set ears, a prominent forehead, pulmonary stenosis and a VSD. Conventional karyotyping showed a *de novo* translocation t(12;14)(q21.3;q32.1). Subsequent SNP array analysis detected three *de novo* deletions on chromosome bands 3p12.3, 4q28.3q31.23, and 12q21.31q21.33, the latter at the breakpoint of the translocation. The deletions contain in total 52 known coding genes.

Discussion
The development of high-resolution array platforms allows the detection of CNVs in carriers of apparently balanced chromosome aberrations. In this study we have analyzed 13 MR patients with previously detected apparently balanced chromosomal rearrangements. Three of the patients had a breakpoint-associated imbalance, four had an imbalance on an unrelated chromosome and one patient had both an additional imbalance near the breakpoint of a translocation as well as cryptic deletions on unrelated chromosomes.

CNVs at or near the breakpoint regions

Three of the 13 patients showed additional CNVs at the breakpoints (patients 1-3). The deletions in patients 1 and 3 are highly likely contributing to the patients' phenotypes, since both are *de novo* and other patients have been reported with deletions in the same regions.

The 5q14.3 deletion region of patient 1 is recently described, five patients showed a 5q14.3 microdeletion and phenotypic similarities, including severe MR with absent speech, epilepsy, hypotonia and stereotypic movements [22]. The minimal overlapping region in their study encompassed the *MEF2C* gene. The phenotype of patient 1 is therefore most probably caused by haploinsufficiency of the *MEF2C* gene. None of the 12 breakpoint regions of the 6 *de novo* deletions in patient 1 contained low copy repeats (LCRs). It is therefore not likely that non-allelic homologous recombination (NAHR) underlies this complex single chromosome rearrangement. Poot and colleagues proposed that such a complex single chromosome rearrangement may be the result of mismatched repair of multiple double-strand breaks that co-localize in a chromosome at the time of DNA-damage induction [25].

None of the genes in the 12p12.3 deletion of patient 2 could be directly related to the phenotype. To our knowledge, a deletion of the same region has not been reported yet. Since we were not able to investigate the parents, pathogenicity of the deletion remains uncertain.

The phenotype of patient 3 overlaps with patients reported with *de novo* 7q21.1q21.3 deletions [8]. As explained previously disruption of the C7orf58 gene in band 7q31.31, one of the insertion breakpoints, may explain anxiety disorder and/or autistic features [10].

CNVs on unrelated chromosomes

In four patients the translocation or inversion appeared balanced, but SNP array analysis detected cryptic CNVs on unrelated chromosomes (patients 4-7). Haploinsufficiency of one or more genes in the deletions of patients 4, 5, and 6 may have contributed to the patients' phenotypes.

Partial deletions of the long arm of chromosome 18 lead to variable phenotypes. The region 18q12.1q21.33 could be associated with mild to severe MR [12,14], explaining the phenotype of patient 4. Furthermore, *MC4R* (melanocortin 4 receptor) may have contributed to the obesity in this patient. The *MC4R* gene is a member of the melanocortin receptor family and represents a G-protein coupled seven transmembrane receptor. Genetic studies related melanocortin receptors to genetically determined obesity [5,7].

The deletion detected in patient 5 overlaps partly with a known microdeletion syndrome. The recurrent 22q11.2 distal deletions are either approximately 1.4 Mb or 2.1 Mb in size with a common proximal breakpoint flanked by LCR22-4 [2]. They differ at the distal breakpoints flanked by either LCR22-5 for the smaller deletion or LCR22-6 for the larger deletion [2]. The breakpoints for the deletion of patient 5 are LCR22-5 and LCR22-6. The same deletion has been reported before in one patient and her healthy father [26]. This patient had a congenital heart defect, normal appearance and psychomotor development, and minimal dysmorphic features. The clinical features of patient 5 do not resemble the previously reported patient.

Patients with larger 13q deletions than patient 6 have been reported, but presented no recognizable phenotype [29]. One patient, with a deletion of 1.43 Mb on

13q12.3 (DECIPHER ref. 2154), is partly overlapping with the deletion in our patient. The common clinical features are MR, microcephaly, and large ears. The smallest region of overlap contains two genes, *KATNAL1* and *HMGB1*, which might be responsible for the overlap in phenotype.

The clinical relevance of the *de novo* interstitial 1p duplication in patient 7 is not clear. Interstitial duplications of chromosome 1p are rare and are associated with a variable phenotype [15]. One patient has been described with a similar duplication, however this patient also carried a deletion within 1p36.32 [15].

CNVs at or near the breakpoint plus additional, unrelated CNV

All three deletions in patient 8 occurred *de novo* and it is highly likely that haploinsufficiency of one or more genes have contributed to the patient's phenotype. Each deletion partly overlaps with patients described in the DECIPHER database (refs. 2059, 790 and 1020), although no similar phenotypes have been described.

General discussion and conclusion

Since there is no applicable technique available yet to detect balanced chromosome rearrangements and cryptic imbalances in one experiment, both high-resolution array screening and conventional karyotyping were necessary to unravel the complex karyotypes described in this paper. In 61.5% of our patients with an apparently balanced aberration, we detected an additional cryptic CNV.

In all five *de novo* cases an additional cryptic CNV was identified. Previous published data have shown cryptic imbalances in approximately 30-50% of MR patients with *de novo* apparently balanced chromosome rearrangements [11,33]. The high occurrence in our study is probably due to the small sample size. In only two of the four patients with an inherited translocation or inversion, one of the parents showed a similar phenotype as the proband. No additional CNVs were detected in these two cases. In one of the other familial cases an additional CNV not related to the translocation was detected (patient 4). Since, DNA of the mother was not available the inheritance of this CNV could not be determined.

By SNP haplotype analysis we could determine for three of the patients that the deletions had occurred in the paternal allele. This observation is consistent with previous studies suggesting that male gametogenesis is more susceptible to this type of chromosome abnormalities [11].

In the remaining patients where no additional abnormalities were detected the presence of cryptic imbalances explaining the phenotype obviously cannot be excluded. It is possible that higher resolution arrays may reveal smaller aberrations that could have been missed in our analysis. Alternatively, the breakpoints of the apparently balanced rearrangements might disrupt putative disease genes or cause a position effect giving rise to the abnormal phenotype.

With the advent and application of high-resolution array screening it was demonstrated that man is more genetically variable than previously considered [27]. Each individual (healthy or not) presents multiple CNVs in its genome. Yet, unless reported in healthy individuals or patients with similar phenotypes, the pathogenicity of a substantial number of CNVs remains uncertain. The clinical interpretation of CNVs is even more difficult in patients with multiple chromosomal aberrations like the ones described in this paper, as the phenotypes might be the result of a combination of two or more chromosome aberrations [3,6,18].

The findings described here once more illustrate that genomic CNVs are an important cause of phenotypic abnormalities in carriers of apparently balanced chromosome aberrations [1,3,6,11,18,24,30,33]. We recommend re-evaluating MR patients with an apparently balanced karyotype (translocation, inversion or CCR) by whole-genome high-resolution array analysis. Furthermore, we advise to first investigate MR patients with a SNP array analysis and if more complex abnormalities are suspected a follow-up by conventional karyotyping [17].

Acknowledgements
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Chapter 3.3

X-chromosome duplications in males with mental retardation: pathogenic or benign variants?

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Clin Genet, in press

Abstract
Studies to identify copy number variants (CNVs) on the X-chromosome have revealed novel genes important in the causation of X-linked mental retardation (XLMR). Still, for many CNVs it is unclear whether they are associated with disease or are benign variants. We describe six different CNVs on the X-chromosome in five male patients with mental retardation (MR) that were identified by conventional karyotyping and Single Nucleotide Polymorphism (SNP) array analysis. One deletion and five duplications ranging in size from 325 kb to 12.5 Mb were observed. Five CNVs were maternally inherited and one occurred *de novo*. We discuss the involvement of potential candidate genes and focus on the complexity of X-chromosomal duplications in males inherited from healthy mothers with different X-inactivation patterns. Based on size and/or the presence of XLMR genes we were able to classify CNVs as pathogenic in two patients. However, it remains difficult to decide if the CNVs in the other three patients are pathogenic or benign.

Introduction

The prevalence of mental retardation (MR) in the general population is 2-3% (1, 2). As there are more males than females diagnosed with MR, the search for candidate genes or loci has been largely focused on the X-chromosome (3, 4). To date, there are 82 genes for which mutations have been demonstrated in X-linked MR (XLMR) (5).

However, in many XLMR families and sporadic cases the cause of MR remains unknown. Copy number variants (CNVs) involving one or more genes on the X-chromosome have been reported in patients with MR (6 - 8). Decrease in gene dosage due to deletions can be related to the phenotype in many cases. Although partial duplications in either arm of the X-chromosome are rare and difficult to interpret, it appears that an increase in gene dosage may be the cause of MR more often than expected (6 - 9).

In this report we describe the use of whole-genome Single Nucleotide Polymorphism (SNP) arrays to precisely map one previously detected large X-chromosomal aberration, and to identify novel X-chromosome abnormalities in patients with MR. In addition, we discuss the difficulty in determining whether an X-chromosomal aberration is pathogenic or a benign variant.

Materials and methods

Patients

Patients were selected from a series of idiopathic MR patients with or without multiple congenital abnormalities, which had been investigated with SNP array analysis. Some were previously found in a cohort of 318 idiopathic MR patients (10). For this study we selected only those patients that had an aberration on the X-chromosome.

Conventional karyotyping and Fluorescence in Situ Hybridisation (FISH)

Conventional cytogenetic analysis on GTG-banded chromosomes from cultured lymphocytes of the patients was performed according to standard techniques. FISH analysis was carried out by standard procedures as described previously (10). FISH analysis was performed on metaphase chromosomes of all patients to confirm whether the duplicated regions were located on the X-chromosome or on another location of the genome.

SNP Arrays

The Affymetrix GeneChip Human Mapping 262K *NspI* (used for patients 2, 3 and 5), 238K *StyI* (used for patient 4) and Affymetrix Genome-Wide Human SNP Array 6.0 arrays (used for patient 1) (Affymetrix, Santa Clara, CA, USA) were performed following the manufacturer's instructions. The 262K and 238K arrays have an average probe spacing of 12 kb and were analyzed as described previously (10). The average probe spacing of the 6.0 array is 0.7 kb. SNP copy number was assessed in the patient using Affymetrix Genotyping Console 2.1 software. Aberrations of at least 100 kb were analyzed.

All CNVs identified in this study were evaluated as described earlier (10). All patients with a (potentially) pathogenic CNV were added to the DECIPHER database.

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis

MLPA experiments were performed as described previously to narrow down the breakpoints of patient 2 (10).

X-inactivation

X-inactivation was investigated with Southern blot analysis with a probe for the M27B (DXS255) locus for the mothers of patients 1 and 4. The intensity of the unmethylated (2.8 kb) and the methylated (5.2 kb) bands were compared to determine random or non-random X-inactivation. The ratio of active to inactive X could not be calculated by this method. X-inactivation was investigated with a methylation-specific PCR for family members of patients 2 and 3 (11). Inactivation was considered to be random if the ratio was less than 75:25 (12).

Results and discussion

We present six different CNVs, five interstitial duplications and one interstitial deletion, on the X-chromosome in five male MR patients (Table 3.3.1). Five CNVs were inherited from the mother (patients 1-4) and one had occurred *de novo* (patient 5). Clinical features of these patients are shown in Table 3.3.2 and an extended clinical description is provided as supplementary material.

The array-based technology for the detection of CNVs in MR patients has proven to be a powerful tool. However, the main challenge is ranking these CNVs as pathogenic, potentially pathogenic or polymorphic variants. Here, CNVs were classified into these groups based on various factors, including size, gene content, and whether they were inherited or occurred *de novo* (13).

Pathogenic CNVs

Conventional karyotyping of patient 1 (Fig 3.3.1a-c) and his brother (Fig 3.3.1d-f) showed an interstitial duplication of Xp21.3p22.2, which was inherited from their healthy mother. SNP array analysis revealed a duplication of 12.5 Mb (Fig 3.3.1g). FISH analysis demonstrated that the duplication was present in tandem on the X-chromosome. Duplications of the individual XLMR genes in this region (*AP1S2*, *CDKL5*, *SCML1*, *PDHA1*, *RPS6KA3*, *SMS* and *ARX*) have so far not been associated with MR. One family with a partly overlapping duplication of Xp22.11p22.2 (from 15.0 Mb to 23.5 Mb) has been reported and the most striking similarities between the patients of that family and patient 1 and his brother are MR, long face and large ears (14). The large size of the duplication in patient 1 and his brother is expected to be associated with the abnormal phenotype due to a two-fold increase in dosage of the included

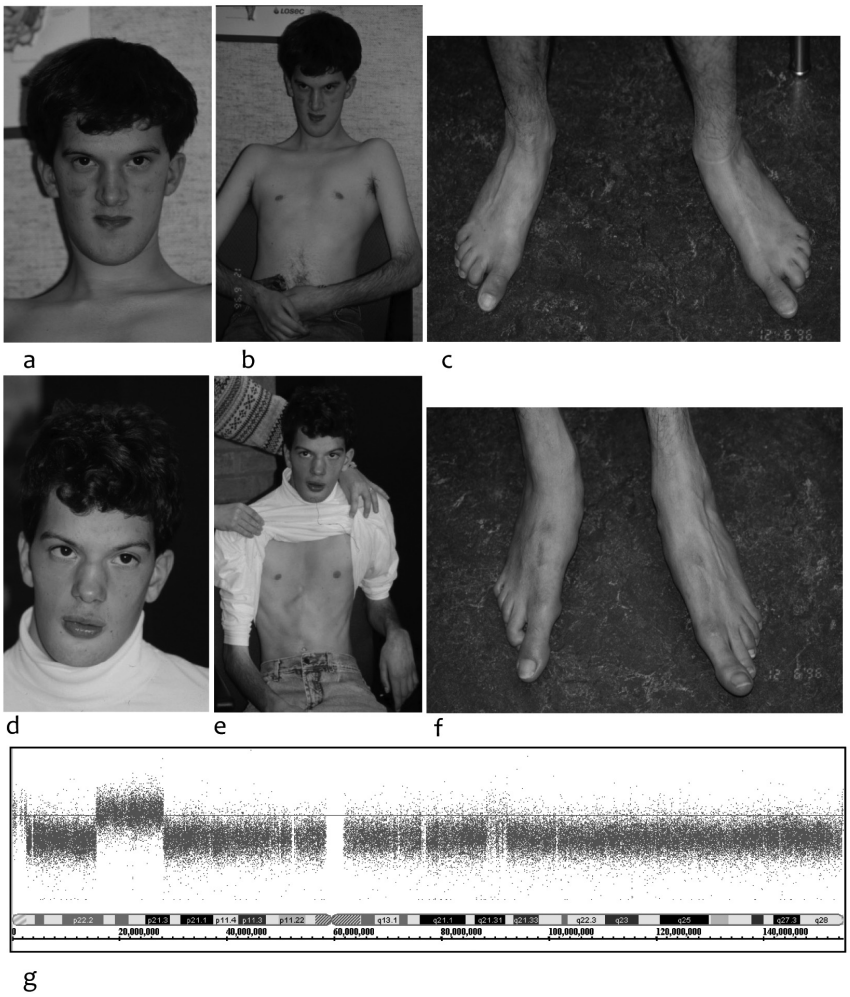


Figure 3.3.1 Patient 1 and his brother. (a) Patient 1 with elongated face and flat midface. (b) Scoliosis of patient 1. (c) Flat, thin and long feet of patient 1. (d) Brother of patient 1 with similar facial appearance. (e) Scoliosis and pectus excavatum of the brother. (f) Flat, thin and long feet with long first digits of the brother. (g) SNP array (Affymetrix Genome-Wide Human SNP Array 6.0) results of patient 1 showing a 12.5 Mb duplication. Log2ratios for each probe are plotted against the position on the X-chromosome. Intensity values are compared with normal males and females resulting in Log2ratios below zero for one copy of the X-chromosome for males. Duplicated regions have a Log2ratio of 0, including the pseudoautosomal regions 1 and 2 (PAR 1 and 2) at Xp22.3 and Xq28.

genes (7). Furthermore, pathogenicity of the CNV is supported by the fact that the phenotypically normal carrier mother showed a skewed X-inactivation pattern in which the X-chromosome with the aberration is most probably the one that is inactivated (12, 15).

SNP array analysis of patient 3 showed a deletion of approximately 2.7 Mb in chromosome band Xp22.32p22.31 and a duplication of at least 6.3 Mb in chromosome band Xq27.3q28 (Fig 3.3.2b). SNP array analysis on the mother, who was slightly retarded, demonstrated that both aberrations had been inherited. The 2.7 Mb deleted region contains *STS* (steroid sulfatase), which is associated with X-linked ichthyosis, and can explain the very dry skin of the boy. Patients with X-linked ichthyosis often suffer from MR and *VCX3A* has been proposed as candidate gene for the MR, although this has not yet been confirmed (16 - 18). A 757 kb deletion within *NLGNX4* has been

Table 3.3.1. Overview of the X-chromosomal CNVS detected in males

Pt	Decipher ID	Gain/Loss, inheritance	Band	Size	Start position (bp)	End position (bp)	FISH clones	Genes	Known XLMR genes	X-inactivation (in mother)
1	LEI001573	gain, mat	Xp22.11p22.13	12.5 Mb	15682525	28207699	RP11-431J24 RP11-489K4	52	*AP1S2*, *CDKL5*, *SCML1*, *PDHA1*, *RPS6KA3*, *SMS*, *ARX*	skewed
2	LEI249293	gain, mat	Xp22.12	349 kb	19425768	19775308	RP11-191B09 RP11-393C11	2	-	skewed (90:10)
3	LEI251249	loss, mat	Xp22.3	2.7 Mb	5195649	7892124	RP11-359O20 RP11-142M16	6	*NLGN4X*, *VCX3A*,	random (70:30)
		gain, mat	Xq27.3q28	6.3 Mb	142274697	148581595	RP11-375C13 RP11-142M16	11	*FMR*	
4	LEI004684	gain, mat	Xq28	606 kb	148147654	148753845	RP11-378O8 RP11-202E19	7	-	random
5	LEI251250	gain, *dn*	Xq21.2	325 kb	85525834	85851245	RP11-507L11	1	-	n/a

Pt, patient number; Mat, maternally inherited; *dn*, *de novo*; n/a, not applicable

Table 3.3.2 Clinical description of the patients with an X-chromosomal aberration

	Patient 1	Younger brother of patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Weeks of gestation	43	34	40	41	41	42
Birth weight (g)	2460	1610	3520	3350	3930	3050
Growth retardation	-	-	+	-	-	-
Neurologic features	Moderate	Moderate	Severe	Severe	-	Moderate
-Mental retardation	+	-	-	+	-	+
-Hypotonia	+	+	+	+	-	++
-Absent or delayed speech	Myoclonic jerks	Myoclonic jerks, spastic gait	Stereotypic movement of head and hands	Palilalia	-	+
-Other						
Facial dysmorphism	Submucous cleft of the palate, bifid uvula, abnormal dental development, absent maxillary lateral incisors, long and slender habitus, elongated and small face, flat midface	Absent maxillary lateral incisors, asymmetric face	Bitemporal narrowing, narrow palpebral fissures, deep-set eyes, large mouth, widely spaced teeth	Oval eyes, short nose, small chin	Early closure of the fontanelles, trigonocephaly with narrow and sloping forehead and metopic ridge, flat face, upslant and short palpebral fissures with telecanthus and epicanthic folds, hypotelorism, infra-orbital creases, wide nasal bridge	Deep set small eyes, hypotelorism narrow nose, and small mouth
Other	Bradycardia, scoliosis, long and thin feet, bilateral simian creases, long fingers	Open ductus Botalli, spastic gait, scoliosis, pectus excavatum, long and thin feet	-	Very dry skin, inverted nipples, gynaecomastia, unusual subcutaneous fat distribution.	Proximal placement of the thumbs, single palmar creases, fetal finger pads, widely space nipples	-

reported in a boy with autism and mental retardation (19). The 6.3 Mb duplicated region contains one known XLMR gene, *FMR1* which is associated with the fragile X-syndrome. Recently, a familial interstitial Xq27.3q28 duplication has been reported partly overlapping the duplication of patient 3 (20). The phenotype of patient 3 shows no striking similarities with affected members of this family. The combination of the loss of these genes and the relative large size of the duplication in our patient most probably accounts for his cognitive phenotype as well as his other clinical features. Additionally, his slightly retarded mother, who carries the same X-chromosomal aberrations, showed a random X-inactivation (70:30) which might support the causative role of the aberrations.

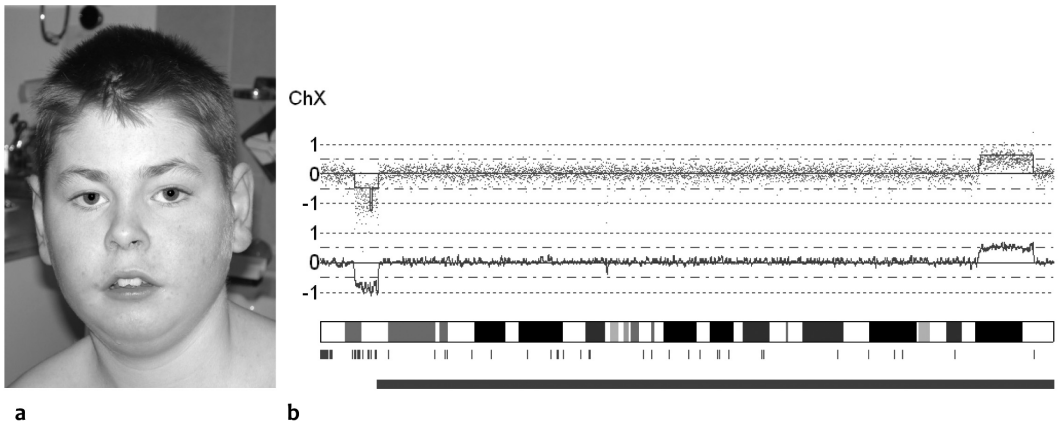


Figure 3.3.2 Patient 3. (a) Facial appearance showing oval eyes, short nose and a relative small chin. (b) SNP array (Affymetrix GeneChip Human Mapping 262K NspI and 238K StyI) results showing the 2.71 Mb deletion and 6.22 Mb duplication. Intensity values are compared with normal males resulting in Log2ratios of zero for one copy of the X-chromosome. Log2ratios for each probe are plotted against the position on the X-chromosome.

Potentially pathogenic CNVs

High-resolution whole-genome screening arrays have enabled the detection of small abnormalities. Yet, the discovery of small CNVs in healthy individuals has revealed that many CNVs are not pathogenic (21-23). We found three CNVs of relatively small sizes (325 kb – 606 kb) in patients 2, 4 and 5. As these CNVs are neither described in the DGV nor do they contain known XLMR genes they could be potentially pathogenic.

SNP array analysis of patient 2 presented an interstitial duplication of at least 349 kb in chromosome band Xp22.12. The healthy mother and grandmother carried the same Xp22.12 duplication. The small duplication was located in the same duplicated region as observed in patient 1. However, the aberration contains only two genes, *SH3KBP1* and *MAP3K15*. The SH3-domain kinase-binding protein, a multifunctional protein which is expressed in the nervous system (24), plays key roles in endocytic down-regulation of receptor tyrosine kinases, in apoptosis, in cell adhesion and in the rearrangement of the cytoskeleton. The *MAP3K15* gene is a member of the Mitogen activated protein (MAP) kinase family. Although a mutation has been recently described in this gene in a patient with MR, the clinical significance of the mutation is not known (25). So far no MR patients with a similar duplication of these genes have been described. The unaffected carrier mother and grandmother of the patient showed skewed X-inactivation patterns (both ratios were 90:10), which could be an indicator for the functional effect of the aberration.

SNP array analysis on patient 4 showed a duplication of approximately 606 kb in chromosome band Xq28. SNP array analysis on the mother showed that the abnormality had been inherited. The duplication partially overlaps the duplication of patient 3, but contains no known XLMR genes. All seven duplicated genes located in this region overlap with patient 3. According to the DGV there are some copy number polymorphisms described in this region but the whole duplication in patient 4 is not covered by the variants. Only four of the seven genes were not located in one of these polymorphic regions. One gene belongs to the MAGEA-family (*MAGEA9B*), and members of this family may play a role in embryonic development, although they are better known for their expression in melanomas and other cancers (26). Mutations and deletions of *IDS* cause sex-linked mucopolysaccharidosis type II, which is a lysosomal storage disorder. No cases have been reported with a duplication of this gene. The other genes, *HSFX1* (heat shock transcription factor family) and *TMEM185A*, are not known to be disease-related. The healthy carrier mother showed a random X-inactivation pattern indicating that the duplication might not be the cause of the phenotype. However, a CNV associated with an abnormal phenotype with random X-inactivation in a phenotypically normal carrier mother has been described (case 5 in reference 7).

SNP array analysis on patient 5 revealed a *de novo* duplication of 325 kb in chromosome band Xq21.1, comprising almost the entire *DACH2* gene, dachshund homolog 2 (Drosophila). The dachshund/Dach gene family is conserved in insects and vertebrates and encodes putative transcriptional cofactors (27). Dachshund is expressed in the developing eyes, brain, limbs and genital disc of the fly, however *Dach1/2* double homozygous newborn mice have intact eyes and limbs (28 -30). Mutations in *DACH2* have been documented in patients with premature ovarian failure, but an association between *DACH2* mutations and infertility has not yet been established (27, 31). Since the duplication is intragenic it might result in gene disruption, leading to either absence of protein or a non-functional protein (32). Functional studies should be performed to check mRNA levels of this gene. Although the duplication is *de novo* it remains difficult to determine whether it is pathogenic or not.

General discussion

In two of the five patients the CNVs could explain their phenotypes (1 and 3) based on size and/or the presence of XLMR genes. For patients 2, 4 and 5 it remains unclear whether the increase in gene dosage has contributed to the phenotype. Besides, establishing the pathogenicity of the duplication in patient 4 is difficult because of the random X-inactivation pattern in the healthy carrier mother. The X-inactivation pattern in females with an abnormal X-chromosome is usually skewed with the abnormal X being preferentially inactivated. In this way they are protected from genetic imbalance. Here, the X-inactivation pattern was measured only in peripheral blood which may not reflect the actual situation in the brain. In any case, the gene dosage in males with a duplication of a segment of the X will be higher than in females with random X-inactivation and therefore could explain the pathogenicity in our patient.

To gain more insight in the pathogenicity of the CNVs in patient 2, 4 and 5 we checked our in-house reference set (including 118 healthy males and 82 healthy females) for the presence of these CNVs. None of the CNVs were found in this set. Additionally, family studies for the patients with the inherited potentially pathogenic CNVs could give a better indication of the pathogenicity if there are healthy or affected

male carriers. Furthermore, the DGV should be regularly checked for these CNVs, since the data in this database is increasing rapidly. More patients or normal individuals with overlapping X chromosomal duplications will be needed to elucidate whether the CNVs that we have reported are pathogenic or benign variants

In conclusion, this study highlights the difficulty in pinpointing the underlying causes of disease. Nevertheless knowing whether a CNV is pathogenic or benign is of major importance for counselling and diagnosis.

Acknowledgements
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Supplementary material

Patient descriptions

Patient 1 was a 33-year old male. He was born by caesarian section at 43 weeks of gestation following a normal pregnancy. The parents were healthy and non-consanguineous. Birth weight was 2460 g. He had bradycardia and hypotonia and assisted ventilation had been necessary for a short period. A submucous cleft of the palate was closed before the age of 1 year. As an infant he developed myoclonic jerks which stopped spontaneously after a few years. His psychomotor development was delayed, he was able to sit without support at 15 months. His dental development was abnormal: at the age of 11 he had only 5 teeth, several incisors and molars had never appeared.

Examination at the age of 20 showed: length 182 cm, span 170 cm and head circumference 56 cm. He had a friendly deposition and was able to speak a few single words. He had a long and slender habitus. The face was elongated with a relatively small mouth and a flat midface (Fig. 3.3.1a). The uvula was bifid. The maxillary lateral incisors were absent. His hands showed bilateral simian creases and long fingers. He had scoliosis (Fig. 3.3.1b). His feet were long and thin (Fig. 3.3.1c).

The 31-year old younger brother of patient 1 had a similar phenotype. He was born by caesarian section at 34 weeks. Birth weight was 1610 g. Assisted ventilation was necessary in the neonatal period because of respiratory distress syndrome. An open ductus Botalli closed after medication. Like his brother he developed myoclonic jerks, which ceased spontaneously before the age of four. Also as in his brother the psychomotor development was delayed, he walked independently at the age of four. Neurological examination at the age of 16 years showed a spastic gait.

On examination at the age of 18 years his length was 190 cm, span 187 cm and head circumference 54 cm. He spoke a few words and communicated with signing. The face was somewhat asymmetric (Fig. 3.3.1d). The maxillary lateral incisors were absent. He had pectus excavatum and scoliosis (Fig. 3.3.1e). The hand creases were normal. The feet were flat, thin and long with long first digits (Fig 3.3.1f).

Patient 2 was a boy, born at term after an uncomplicated pregnancy. He had a normal birth weight. His development has been delayed form infancy, he was able to walk at the age of 4 years and he has no speech. He has a high threshold for pain. He has no sleeping problems.

When first seen at the age of 4 years, he had a happy disposition and made stereotypic movements with his head and hands. Height, weight and head

circumference were within normal limits. He had several dysmorphic features, including bitemporal narrowing, narrow palpebral fissures, deep set eyes, large mouth, and widely spaced teeth. He had a broad-based gait.

Patient 3 was born at 41 weeks of gestation with a normal birth weight (3350 g). The pregnancy had been complicated by pre-eclampsia. He was admitted to the department of pediatrics for observation because of hypotonia. His skin was very dry. There were no feeding problems during infancy. He did not speak any words at the age of three.

At 13 years of age he was found to be severely retarded. He displayed palilalia and was not toilet-trained. His height was normal. His facial features were slightly dysmorphic with oval eyes, a short nose and a relatively small chin (Fig. 3.3.2a). He had inverted nipples, gynaecomastia, and an unusual subcutaneous fat distribution with large pads of fat above the buttocks.

Patient 4 was born at 41 weeks of gestation with a normal birth weigth (3930 g). He had an early closure of the fontanelles and some facial dysmorphic features, including trigonocephaly with narrow and sloping forehead and metopic ridge, flat face, upslant and short palpebral fissures with telecanthus and epicanthic folds, hypotelorism, infra-orbital creases, wide nasal bridge. He had proximal placement of thumbs, single palmar creases, fetal finger pads, and widely-spaced nipples.

Patient 5 was born at 40 weeks of gestation with a normal birth weight (3050 g). He had hypotonia and moderate MR. His facial features were very similar to those of his father with deep set small eyes, hypotelorism, narrow nose and small mouth.

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Chapter 3.4

Mosaicism with a normal cell line and an unbalanced autosomal reciprocal translocation; three new cases and review of the literature

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Abstract
Mosaicism involving a normal cell line and an unbalanced autosomal translocation are rare. In this study we present three new cases detected by Single Nucleotide Polymorphism (SNP) array analysis in our routine diagnostic setting. These cases were further characterized using Fluorescence *in situ* Hybridisation (FISH) analysis and conventional karyotyping.

The first case is a mentally retarded male who carries an unbalanced translocation in 87% of his cells. Remarkably, the phenotypically normal mother carries the balanced form of the translocation in all her cells. The second case is a phenotypically normal female who has an unbalanced translocation in 52% of her cells. She passed the unbalanced translocation to her daughter who has mild mental retardation and serious behavior disturbance. The third case is a female referred for Rubinstein-Taybi syndrome who carries a complex unbalanced translocation in 60% of her cells. Her mother showed a normal karyotype.

The mechanisms that might be responsible for these mosaic karyotypes are discussed. Furthermore, we demonstrate that high-resolution whole-genome array is a powerful tool to reveal cryptic unbalanced translocations and mosaicisms, including the more rare cases.

Introduction
Mosaicism is the presence of genetically different cell lines in one individual derived from a single zygote. Mosaicism can be caused by several mechanisms, including chromosomal abnormalities and DNA mutations (reviewed by Youssoufian and Pyeritz, 2002). Whether a mosaicism is disease-causing depends upon the abnormality, on which tissue is abnormal and on how much of a tissue is affected. If only a fraction of the soma is abnormal (somatic mosaicism), the phenotype is likely to be normal and will probably never be recognized (Gardner et al., 1994). If it involves a substantial part of the soma, it can cause dysmorphisms and malformation, and if the brain is included, mental retardation (MR). Abnormality involving only a part of the gonad (gonadal mosaicism) is not associated with an abnormal phenotype of the carrier and will usually only be recognized after two siblings are born with the same '*de novo*' abnormality (Youssoufian and Pyeritz, 2002).

Chimerism is the presence of genetically different cell lines in one individual derived from two or more different zygotes. If both cell lines contain the same sex chromosomes, conventional karyotyping is generally not able to distinguish between mosaicism and chimerism. Support for the mechanism causing two or more different cell lines as a result of chimerism can be obtained by comparison of DNA microsatellite markers (Cotter and Hirschhorn, 1998).

Somatic mosaicism with an abnormal cell line can be missed by conventional karyotyping if masked by a high percentage of normal cells or even dismissed as a culture artefact (Ballif et al., 2006). Additionally, healthy individuals with a mosaicism will in most cases not be investigated. The prevalence of mosaicisms is therefore difficult to establish. Lebbar et al. (2008) reviewed the literature for mosaicism with a normal cell line and a balanced rearrangement and reported 35 cases. Most of these carriers have a normal phenotype and were referred due to recurrent miscarriages, infertility or the birth of an abnormal child. Furthermore, they described the first two cases of mosaicism for a normal cell line and a complex chromosome rearrangement (CCR) in patients ascertained through infertility. Zaslav and colleagues (1999) reported

23 cases of mosaicism for a normal cell line and an unbalanced autosomal structural rearrangement (N/UASR). These UASRs included duplications, deletions, insertions, isochromosomes, and derivative chromosomes. In total they reported only 4 cases with a mosaicism for a normal cell line and an autosomal derivative chromosome (Zaslav et al., 1999). To our knowledge, only 3 additional mosaicism cases with a normal cell line and an unbalanced autosomal translocation have been described (Stallings et al., 1997; Kulharya et al, 2002; Petkovic et al., 2003).

The application of high-resolution whole-genome array technology to cytogenetic testing has improved the ability to detect smaller abnormalities and mosaicisms (Ballif et al., 2006; Gijsbers et al., 2009). We report on three unrelated cases with a mosaicism for a cryptic unbalanced reciprocal translocation detected by Single Nucleotide Polymorphism (SNP) array analysis and Fluorescence *in situ* hybridisation (FISH). Our results illustrate that array technology is a sensitive method for the detection of mosaicisms, unbalanced translocations and, as described here, a combination of these two.

Material and methods

Patients

This study is based on results obtained from patients with MR submitted to our laboratory for SNP array screening. In this report we include three cases (two patients with MR and one healthy parent) in which a mosaicism with a normal cell line and an unbalanced autosomal translocation was detected. Pedigrees are shown in Figure 3.4.1.

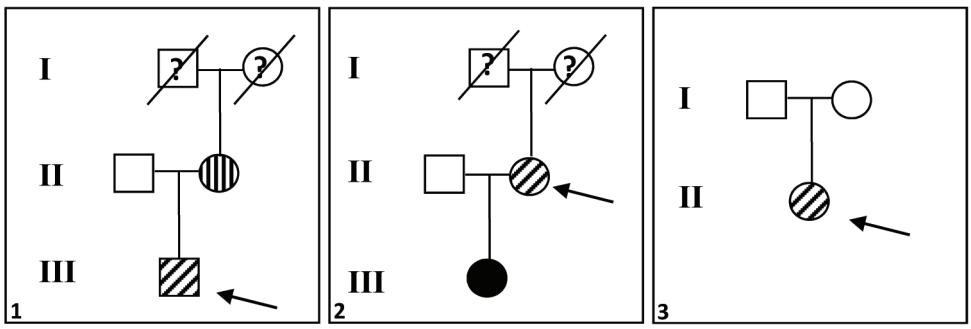


Figure 3.4.1 Pedigrees of the three cases with a normal cell line and an unbalanced autosomal translocation. Arrow indicates case with the mosaicism.

Conventional karyotyping and FISH

Conventional cytogenetic analysis on GTG-banded chromosomes from cultured lymphocytes was performed according to standard techniques. FISH analysis was carried out by standard procedures as described (Dauwerse et al., 1990). Bacterial artificial clones (BAC) mapping to the affected chromosome regions were selected based on their physical location within the affected region (http//: www.ensembl.org, Ensembl release 54 - May 2009).

SNP arrays

DNA was extracted from whole blood by a Gentra Puregene DNA purification Kit (Gentra Systems, Minneapolis, USA), following the manufacturer's instructions. The Affymetrix GeneChip Human Mapping 262K *Nsp*I array (Affymetrix, Santa Clara, CA, USA) was performed following the manufacturers' instructions and data was analyzed

as described previously (Gijsbers et al., 2009). The Affymetrix Genome-Wide Human SNP Array 6.0 contains more than 1.8 million markers for genetic variation, including more than 906,600 SNP probes and more than 946,000 probes specific for the detection of copy number variation (Affymetrix). 500 ng of genomic DNA was processed according to the instruction provided in the Affymetrix Cytogenetics Copy Number Assay user guide. SNP copy number was assessed in the patient using Affymetrix Genotyping Console 2.1 software. Aberrations of at least five consecutive probes were considered as significant.

Results

Case 1

The patient was a 42-year old male with MR and dysmorphic features including coarse hair, small dysplastic low-set ears, deep-set eyes, hypotelorism and short 4th metatarsal of the left foot. He also had scoliosis, aortic stenosis, coarctation of the aorta, hypertension, anal stenosis, sensineural deafness and distal symphalangism of toes.

SNP array analysis with the Affymetrix Genome-Wide Human SNP Array 6.0 detected a terminal duplication of 10.11 Mb on the long arm of chromosome 10 and a 7.51 Mb deletion on the long arm of chromosome 11 (Fig. 3.4.2a and b). Additional FISH analysis showed in 87 of the 100 metaphases an unbalanced translocation between the long arm of chromosome 10 and the long arm of chromosome 11 (Fig 3.4.2c. The karyotype of this patient was 46,XY,der(11)t(10;11)(q26.13;q24.2) [87]/46,XY [13]. DNA microsatellite markers showed no extra alleles.

FISH analysis of the parents identified that the mother was a carrier of the balanced translocation between chromosomes 10 and 11.

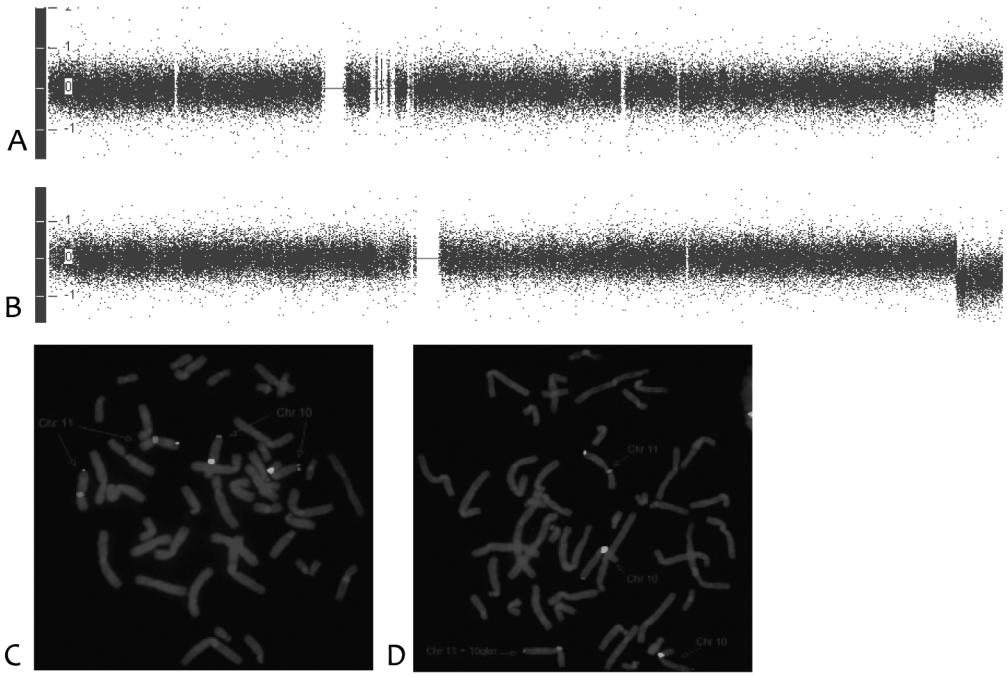


Figure 3.4.2 Case 1. (a) SNP array analysis revealed a duplication of 10.11 Mb on chromosome 10 and (b) a 7.51 Mb deletion on chromosome 11. (c) FISH analysis results showing a normal cell line and (d) a cell line with the der(11)t(10;11)(q26.13;q24.2).

Case 2

Case 2 was a 51-year old healthy female. The daughter of case 1 was referred because of developmental delay and serious behavioral problems.

SNP array analysis with the Affymetrix GeneChip Human Mapping 262K *Nsp*I array on the daughter of case 2 revealed a terminal duplication of 12.87 Mb on the long arm of chromosome 8. FISH analysis identified an unbalanced translocation between the long arm of chromosome 8 and the short arm of chromosome 22. Additional SNP array analysis for case 2 showed the same duplication, however with a lower intensity and FISH analysis confirmed this was due to a mosaic unbalanced t(8;22) resulting in a karyotype of 46,XX,der(22)t(8;22)(q24.2;p10) [15]/46,XX [14]. DNA microsatellite markers showed no extra alleles.

The parents of case 2 were not available for testing

Case 3

The patient was a female referred for Rubinstein-Taybi syndrome. FISH and sequence analysis for the *CREBBP* gene excluded the presence of a deletion or a mutation.

SNP array analysis with the Affymetrix GeneChip Human Mapping 262K *Nsp*I revealed a terminal duplication of 6.8 Mb on the long arm of chromosome 6 and a terminal deletion of 11.2 Mb on the long arm of chromosome 11. Additional FISH analysis in the patient confirmed the presence of an unbalanced translocation between the long arm of chromosome 6 and the long arm of chromosome 11 in 6 of the 10 cells. The karyotype of this patient was 46,XX,der(11)t(6;11)(q26;q24.1) [6]/46,XX [4]. DNA microsatellite markers showed no extra alleles. In addition, the SNP analysis showed large stretches of homozygosity, suggesting consanguinity of the parents.

FISH results for the parents were normal. The parents are first cousins once removed.

Discussion

Mosaicism involving a normal cell line and an unbalanced autosomal translocation is rare. To our knowledge, only 7 cases have been reported (Table 3.4.1) (Stallings et al., 1997; Kulharya et al, 2002; Zaslav et al., 1999; Petkovic et al., 2003). Zaslav et al. (1999) reported a mosaicism for three cell lines, present in fibroblasts and lymphocytes and a patient with mosaicism for two cell lines in both lymphocytes and fibroblasts. Two cases are described with normal cell line in lymphocytes and mosaicism in fibroblasts (Stallings et al., 1997; Kulharya et al, 2002). The last three cases are mentioned with mosaicism in their lymphocytes only (Zaslav et al., 1999; Petkovic et al., 2003). The inheritance was unknown for three cases (Zaslav et al., 1999; Petkovic et al., 2003), three were *de novo* (Tsien et al., 1991; Stallings et al., 1997; Zaslav et al., 1999), and for one case the mother was carrier of the balanced translocation (Kulharya et al, 2002). In this study we reported three new cases with a normal cell line and an unbalanced autosomal translocation identified in lymphocytes. Other tissues were not analyzed.

The presence of a normal cell line and an unbalanced translocation can arise in several ways. 1) A mitotic exchange of nonhomologous chromatids followed by the loss of one of the translocated chromatids, subsequent segregation would result in a normal and an unbalanced cell line (Zaslav et al., 1999). 2) An unbalanced zygote followed by loss of the abnormal chromosome, a subsequent monosomy rescue event and a duplication of the normal chromosome. This would result in an isodisomy for this chromosome (Cotter and Hirschhorn, 1998). 3) 3:1 segregation with the derivative

Table 3.4.1 Mosaicism with a normal cell line and an unbalanced autosomal reciprocal translocation

Reference	Karyotype	Parents
Stallings et al., 1997	46,XX (blood)-46,XX/46,XX,der(15)t(3;15)(q11;p11) (fibro)	normal
Zaslav et al., 1999	46,XX,der(4)t(4;15)(q35;q22)/46,XX	normal
	45,XX,-15,-18,+der(18)t(15;18)(q13;q23)/46,XX	n/a
	47,XX,+mar/47,XX,+der(9)t(9:9)(p13;q34) (blood) - 47,XX,+der(9)t(9:9)(p13;q34)/46,XX (fibro)	normal
	45,XY,-7,-15,+der(15)t(7;15)(q34;q13)/46,XY	n/a
Kulharya et al., 2002	46,XY,der(22)t(11;22)(q23.3;q11.2)/46,XY (fibro)- 46,XY (blood)	mother carrier balanced
Petkovic et al., 2003	46,XX,der(10)t(6;10)(p11;q11)/46,XX	n/a
Present cases	46,XX,der(22)t(8;22)(q24.2;p10) [15]/46,XX [14]	n/a
	46,XY,der(11)t(10;11)(q26.13;q24.2) [87]/46,XY [13]	mother carrier balanced
	46,XX,der(11)t(6;11)(q26;q24.1) [6]/46,XX[4]	normal

chromosome and two normal associated chromosomes. Loss of a normal chromosome in one cell and loss of the derivative chromosome in the other cell will result in two different cell lines (Kulharya et al, 2002). Depending on the stage of development the abnormal cell line involves a greater or lesser fraction of the embryo for these three mechanisms. 4) Chimerism (Nyberg et al, 1992).

Here, we report on three unrelated cases of mosaic unbalanced translocations detected by SNP array and FISH analysis. Case 1 inherited the unbalanced translocation from his mother who carried the balanced translocation. To our knowledge this is only reported once (Kulharya et al., 2002). This mosaicism could only be explained by events 2, 3 or 4. No extra alleles were detected with DNA microsatellite marker study and the genotype information was not suggestive for isodisomy. Since there was approximately in only 10% of the cells a normal karyotype this might be under the detection level of both techniques, and therefore mechanism 2 and 4 could not be excluded. For case 2, we were not able to determine the inheritance of the unbalanced translocation since the parents were not available for testing. Case 3 showed a *de novo* mosaic unbalanced translocation. The origin of these mosaicisms is most likely postzygotic. Chimerism was excluded for both cases and genotype information derived from SNP array analyses showed no allele differences suggestive for isodisomy. The first mechanism seems to be most likely in these cases.

To detect low-level mosaicisms with conventional karyotyping, large cell numbers need to be examined. Therefore, mosaicism screening is time consuming and expensive (Ballif et al., 2006). The application of high-resolution whole-genome arrays in a diagnostic setting has the potential to improve the identification of mosaicisms (Ballif et al., 2006; Gijsbers et al., 2009). Furthermore, the increased resolution of arrays enables the detection of smaller deletions and duplications. The gains and losses detected in this study were all larger than 5 Mb, which should be identifiable with conventional karyotyping. This highlights that the subtelomeric regions are more difficult to characterize by conventional karyotyping due to their G-negative staining. Therefore, unbalanced translocations could be easily masked and missed by routine cytogenetic testing.

This study reports three new cases of a mosaicism with a normal cell line and an unbalanced autosomal translocation, which is a rare phenomenon. The exact mechanism responsible for these karyotypes is not clear. We demonstrate that high-resolution whole-genome arrays will reveal more mosaicisms, unbalanced translocations and a combination of both.

Acknowledgements

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Chapter 4

Case reports

Chapter 4.1

A 400 kb duplication, 2.4 Mb triplication and 130 kb duplication of 9q34.3 in a patient with severe mental retardation

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Abstract
The presence of a duplication as well as a triplication in one chromosome is a rare rearrangement and not easy to distinguish with routine chromosomal analysis. Recent developments in array technologies, however, not only allow screening of the whole genome at a higher resolution, but also make it possible to characterize complex chromosomal rearrangements in more detail. Here we report a molecular cytogenetic analysis of a 16-year old female with severe mental retardation and an abnormality on the end of the long arm of chromosome 9. Subtelomeric multiplex ligation-dependent probe amplification (MLPA) analysis revealed that the extra material originated from the telomeric end of chromosome 9q. Fine mapping using a high-resolution single nucleotide polymorphism (SNP) array detected a duplication of ~400 kb upstream of a ~2.4 Mb triplication followed by a duplication of ~130 kb of chromosome 9q34.3. This study underscores the value of combining conventional karyotyping with novel array technologies to unravel complex chromosomal alterations in order to study their phenotypic impact.

1. Methods of detection

1.1 Cytogenetic analysis

Conventional cytogenetic analysis on GTG-banded chromosomes from cultured lymphocytes of the patient was performed according to standard techniques. All metaphases studied demonstrated a 46,XX, add(9)(q34) karyotype (Fig. 4.1.1a).

1.2 MLPA

Two specifically designed sets of probes for testing subtelomeric chromosomal imbalances, SALSA P036B and P070 Human Telomere Test Kit (MRC Holland, Amsterdam, The Netherlands), were used for subtelomere screening of the patient and the parents. MLPA experiments were performed as described by MRC Holland (http://www.mlpa.com/pages/indexpag.html) with slight modifications. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Peak analysis was performed with the GeneMarker Software V1.51 (SoftGenetics, USA).

1.3 SNP- array

The Affymetrix GeneChip Human Mapping 238K *StyI* array was used. This SNP array contains ~238.000 25-mer oligonucleotides with a ~12 kb resolution. A sample of 250 ng DNA was processed according to the instruction provided in the Affymetrix GeneChip Human Mapping 500K Manual (http://www.affymetrix.com). SNP copy number was assessed in the patient using DNA-Chip Analyzer (dChip) software (version release 02-16-06) [8]. Regions of copy number gain and loss were detected using the hidden Markov model output of dChip.

1.4 Chromosomal anomaly

To further characterize the extent of the aberration found by karyotyping, subtelomeric MLPA was performed. A duplication of both 9q probes, located in the *MRPL41* gene and *EHMT1* gene, was observed (Fig. 4.1.1b).

Fine mapping of the duplication was performed with a 238K SNP array. This array illustrated a terminal duplication of ~2.93 Mb (Fig. 4.1.1c). Due to exceptional high intensity values from both, the MLPA and the SNP array analyses, a 9q34.3

triplication was suggested. Detailed analysis of the SNP array data revealed a ~400 kb duplication followed by a ~2.4 Mb triplication and a ~130 kb duplication (Fig. 4.1.1d). The proximal breakpoint is mapped in 9q34.3 with the last SNP (rs11103754) normal located in 137.24 Mb and the first SNP (rs7043655) duplicated in 137.26 Mb. The second breakpoint is mapped between the duplicated SNP (rs3849220) located in 137.62 Mb and the triplicated SNP (rs7873626) in 137.63Mb. The most distal breakpoint in 9q34.3 is mapped with the last SNP (rs7848769) triplicated in 140.075 Mb and the first SNP (rs7860423) duplicated in 140.076 Mb. Based on this result, the patient's karyotype was refined as:

46,XX,add(9)(q34).arr SNP 9q34.3q34.3(rs7043655->rs3849220)x3,
9q34.3q34.3(rs7873626->rs7848769)x4,9q34.3q34.3(rs7860423->rs1820789)x3 dn

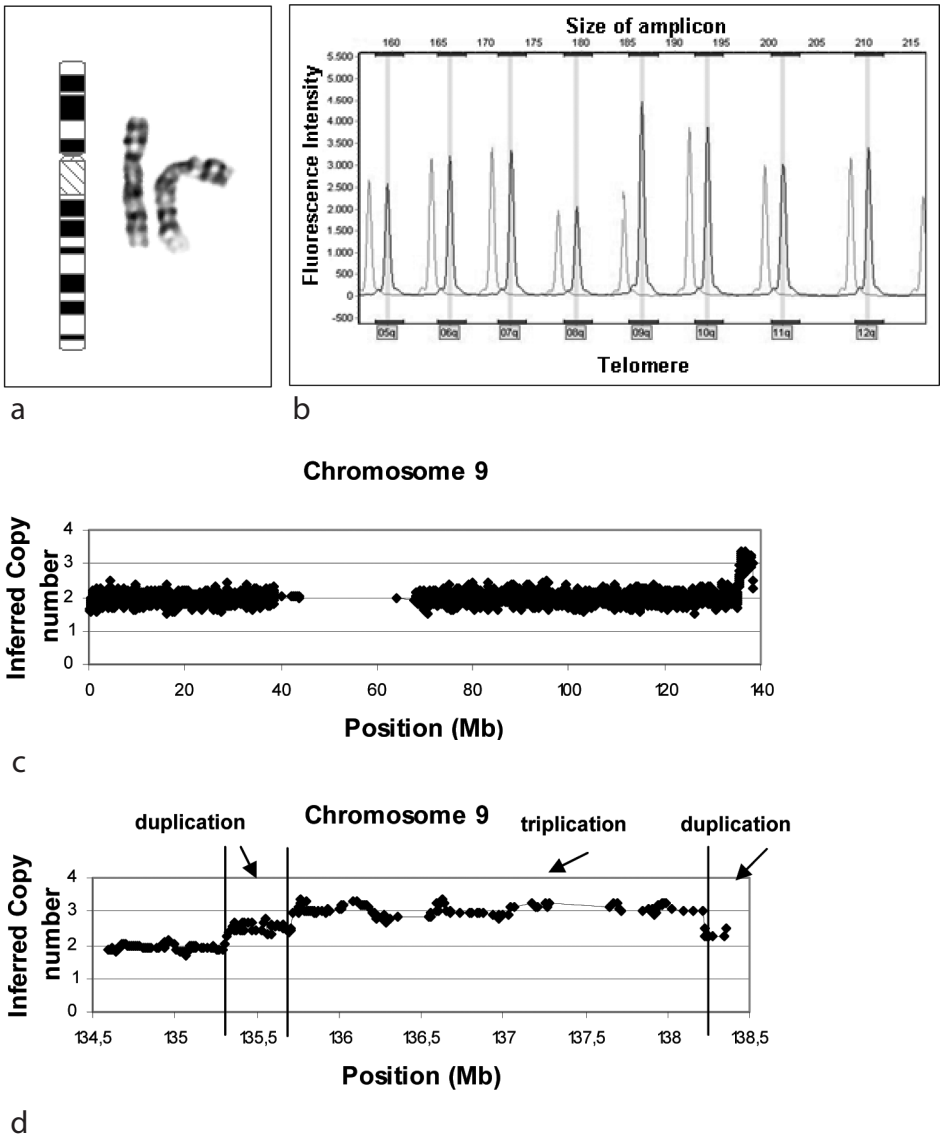


Figure 4.1.1 Cytogenetic and molecular results. (a) Partial karyotype showing both chromosomes 9. Right: abnormal chromosome 9. (b) MLPA analysis revealing an aberration of the 9q probe. Red peaks: control panel; blue peaks: patient. (c) SNP array analysis of the patient revealed an aberration on the long arm of chromosome 9 of approximately 2.93 Mb. The threshold for deletions and duplications is, respectively, 1.6 and 2.4. (d) SNP array copy number plot focused on the duplicated region of chromosome 9, showing a ~400 kb duplication, a ~2.4 Mb triplication and a ~130 kb duplication.

1.5 Methods of confirmation

To verify these results additional MLPA probes were developed within the 2.93 Mb critical interval for 9q. MLPA probes for the duplicated and triplicated region of chromosome 9q34.3 (located in genes *MRPS2*, *CAMSAP1*, *LHX3*, *PTGDS*, *ANAPC2*, *COL5A1*, *OLFM1*, *CACNA1B*) and control probes (genes *GPR64* and *FAM46D* located on chromosome X) were designed at our laboratory and commercially obtained from Biolegio (Malden, The Netherlands). The MLPA experiments were performed as described [15].

Furthermore BAC clones specific for human chromosome 9q were selected based on their physical location within the affected 9q region (http://www.ensembl.org). Fluorescent *in situ* hybridization (FISH) experiments were performed by standard procedures [2]. BAC clones RP11-270D17 and GS-135I17 were used to confirm both duplications (Fig. 4.1.2a). To determine the precise orientation of the fragments involved in the triplication, BAC clones RP11-413M3 and RP11-48E05 were used and labeled with different fluorochromes (Fig. 4.1.2b, c).

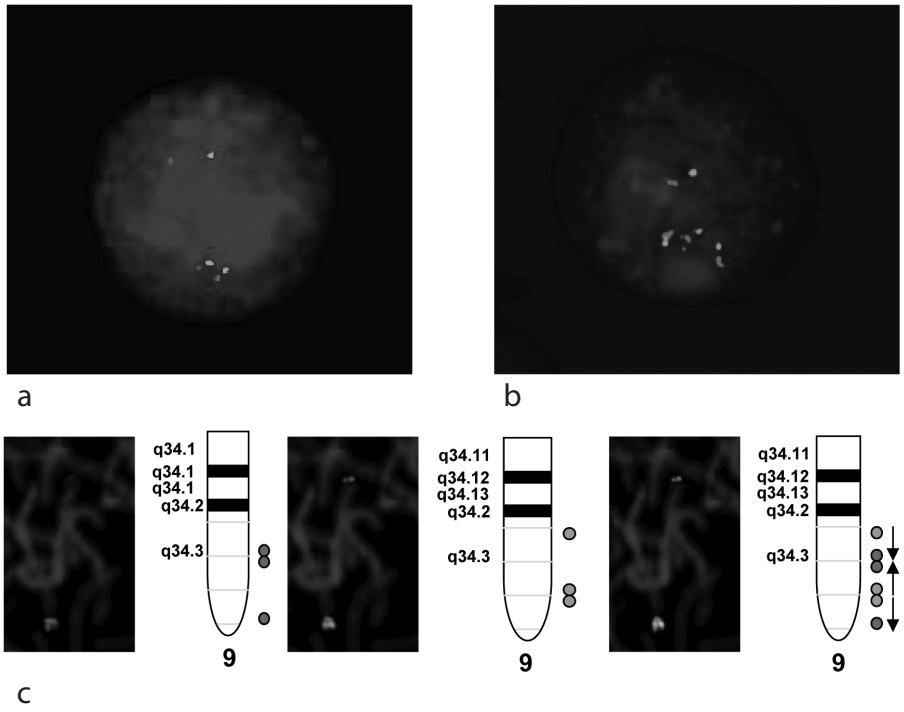


Figure 4.1.2 (a) FISH analysis with RP11-270D17 (green) and GS-135I17 (red) observed a duplication for both the probes confirming the 400 kb and 130 kb duplication, respectively. (b) Hybridization with RP11-413M3 (green, centromeric side of chromosome 9q) and RP11-48E05 (red, telomeric side of chromosome 9q) revealed the presence of four red and four green signals. The signals for the chromosome with the triplicated region showed that the middle repeat is inverted (green-red-red-green-green-red). (c) For probe RP11-48E05 the centromeric signal was of double intensity as compared to the telomeric signal. For probe RP11-413M3 (green) the telomeric signal was stronger than the centromeric signal. This indicates that the repeats of the triplication are normal in the proximal and distal region and inverted in the central region.

1.6 Causative of the phenotype

Conventional karyotyping of the parents presented a normal karyotype. Furthermore the parents showed normal subtelomeric MLPA, FISH and SNP array results. These results indicate that the aberration was *de novo*.

2. Clinical description

The female patient was born after an uncomplicated pregnancy, weighing 2750 g. She is the youngest child in a sibship of three. Parents are healthy and non-consanguineous. A previous pregnancy had resulted in unexplained stillbirth around 38 weeks gestation. She has one healthy older sister.

In early infancy, psychomotor retardation was noted (walking after 2.5 years of age, no speech development). At physical examination at the age of 2.5 years height was 89 cm (10th centile), head circumference 47.5 cm (25th centile). She was reported to have no apparent facial dysmorphism, and apart from tapering fingers and a sacral dimple no phenotypic anomalies were present. Despite extensive investigations at the time, no reason for the developmental delay was found. At the age of 6 years she underwent surgery because of convergent strabismus.

At the age of 16 years she was referred to our department for re-evaluation (Fig. 4.1.3). She was severely mentally retarded. She had limited speech; she only used a few words and was dysarthric. There was constant drooling. She had hearing loss. She was able to communicate using signs and pictures. She had a high threshold for pain. There was no history of sleep disturbance, seizures or constipation. Recently, she developed aggressive behaviour and was found to have more difficulties with walking.

At physical examination her growth parameters were within normal limits. Her facial features showed narrow palpebral fissures and a wide/large mouth. Both earlobes were upturned. Apart from slender fingers with clinodactyly of both fifth fingers and bilateral pes cavus, no dysmorphic features were present.

DNA analysis for Angelman syndrome and Mowat-Wilson syndrome showed a normal methylation pattern and no mutation in the *ZFHX1B* gene, respectively. MRI and metabolic screening showed no abnormalities.

We obtained samples from the patient and family members after acquiring informed consent approved by the Institutional Review Board at the Leiden University Medical Center, Leiden.



Figure 4.1.3 Picture of the female patient at the age of 16 years. Note mild facial asymmetry and narrow palpebral fissures.

3. Discussion

Cytogenetic karyotyping was performed on a female patient with unexplained severe mental retardation and showed an abnormal karyotype, 46,XX,add(9)(q34). To determine the extent of the aberration on chromosome 9, subtelomeric MLPA analysis was performed showing an abnormality for both probes on chromosome 9q. Subsequent SNP array analysis revealed a ~400 kb duplication followed by a ~2.4 Mb triplication and a duplication of 130 kb of chromosome 9q34.3 demonstrating the added value of this technique to conventional karyotyping.

To the best of our knowledge, this is the first report on a case with a partial duplication and partial triplication of chromosome 9q34.3. In the literature a few cases of duplication 9q are described. In 1975 the first association between clinical features and a partial duplication of chromosome 9q in two patients was reported [12]. Later four families with seven affected children, presumably descending from a common ancestor, in which an inverted insertion (9)(q22.1q34.3q34.1) chromosome was segregating were described [1]. Meiotic recombination led to the duplication of the 9q34 region, and to a phenotype they called the duplication 9q34 syndrome. Recently a new case with a duplication of chromosome 9q34 was described [4]. Furthermore, patients with a duplication of the 9q34 region and an additional aberration elsewhere in the genome were reported [6,9,10]. It may be expected that in those cases the phenotype is also affected by the other observed deletions and duplications in the genome. Nevertheless, the authors recognized a clinical syndrome similar to the duplication 9q34 syndrome [1]. Phenotypic similarities are observed between patients with a duplication of chromosome 9q34 and patients with larger duplications of chromosome 9q [10]. Larger duplications of chromosome 9q appear to be associated with a more severe developmental delay.

Although there are no cases described of partial triplication of this region before, it would be logical to compare the clinical phenotype of our patient with cases with duplication of chromosome 9q34 (Table 4.1.1). Only one study described patients with a pure 9q34 duplication overlapping the 9q34.3 abnormality in our patient [1]. In their patients common clinical features were dolichocephaly, facial asymmetry, deep-set eyes, narrow horizontal palpebral fissures, prominent nasal bridge, beaked nose, small mouth, retrognathia, arachnodactyly, camptodactyly, joint contractures, and scoliosis. The facial phenotype seemed to change with age. Reported medical problems were feeding difficulties and failure to thrive in infancy, strabismus and ptosis. All patients had psychomotor retardation and speech development was frequently impaired. Several patients were reported to have behavioral problems (hyperactivity, tantrums). Our patient seems to have some of the reported facial features. Furthermore, her arachnodactyly, limited speech and behavioral problems may be explained by the chromosome aberration.

It has been described that a microdeletion syndrome can show overlapping features with a microduplication syndrome of the same region, e.g. 22q11 [3]. Patients with a deletion 9q34 however, show a distinct phenotype that does not bear a resemblance to our patient [5]. As yet there are no arguments for overlapping clinical features in microduplication and microdeletion in the 9q34 region.

In the 9q34.3 deletion syndrome, haploinsufficiency of *EHMT1* (MIM 607001) is recognized as the causative gene [7]. The *EHMT1* gene belongs to the histone-lysine methyltransferase family and plays a role in the central nervous system development and function through epigenetic histon modification (http://www.dsi.univ-paris5.fr/

Table 4.1.1 Comparison of clinical features with other reported cases with duplication 9q34

Phenotypic features	Allderdice et al. 9q34.1q34.3	Gawlik-Kuklinska et al. 9q33.3q34.1	Present case 9q34.3
Facial asymmetry	+	+	±
Deep-set eyes	±	+	-
Narrow horizontal palpebral fissures	+	+	+
Abnormally shaped ears	+	-	+
Prominent chin	+	+	-
Beaked nose	+	-	-
Microstomia	+	+	-
Retrognathia	+	+	-
Abnormal position of digits	+	+	-
Arachnodactyly	+	+	+
Camptodactyly	+	+	-
Scoliosis	±	+	-
Overweight/obesity	-	+	-
Tongue/palate abnormalities	-	+	-
Cardiac anomalies	-	-	-
Low birth weight	+	-	-
Hypotonia	+	+	-
Cyanosis	+	+	-
Failure to thrive	+	+	-

genatlas/). It is unknown whether duplication or triplication of this gene results in an equal disturbance of function.

In our case the most distal breakpoint disrupts the *CACNA1B* gene. *CACNA1B* is a calcium channel, voltage dependent, N-type, alpha subunit gene (MIM 601012). It encodes an N-type calcium channel, which controls neurotransmitter release from neurons. It has been suggested that a heterozygous deletion of *CACNA1B* may result in the reduction of N-type channel activity [5]. In this way, in addition to the triplication of *EHMT1*, haploinsufficiency of *CACNA1B* may be a contributing factor for mental retardation and/or epilepsy. However, FISH analysis revealed the orientation of the triplication and supports an inverted orientation of the middle fragment (Fig. 4.1.3b, c). This results in two normal functioning copies of the *CACNA1B* gene and three

interrupted copies.

In short, copy number variations of the *EHMT1* gene may probably explain some features of the phenotype of this patient. However, according to the Ensembl Genome Browser (http://www.ensembl.org/index.html) release 44 based on the NCBI 36 assembly of the human genome (November 2005), the 2.93 Mb duplicated and triplicated regions of our patient contains approximately 100 genes. It is therefore unlikely that the clinical features in our patient are the result of a copy number change in just one gene. We expect that the phenotype associated with this rearrangement will be clarified when other cases with comparable rearrangements are disclosed.

According to the Database of Genomic Variants (http://projects.tcag.ca/variation/) the 9q34.3 region corresponds to a copy number variation region (CNVR), which is an artificial grouping of CNVs overlapping or in close proximity of each other [16]. This CNVR consists of 3 individual CNVs (gains) detected in 1190 controls (0.3%). Since this aberration is repeatedly described as disease causing [1,4,12], it is likely that the complex rearrangement described here causes the phenotype.

The three breakpoint regions of the 9q aberration present no architectural features that might be involved in the mediation of duplications and triplications. We speculate that first a 2.93 Mb duplication occurred and subsequently, an interstitial triplication in the duplicated region. Intrachromosomal triplications are rare and most of them concern the proximal 15q region [13]. Most of the reported triplications seem to result from a common mechanism as indicated by the inverted orientation of the middle repeat [14]. However, it is not clear which mechanism can explain the genesis of these triplications.

In conclusion, using conventional karyotyping characterization of complex rearrangements and identification of the origin of extra chromosomal material is often difficult. The introduction of new molecular cytogenetic methods such as MLPA analysis and SNP arrays provides cytogenetics with new approaches to define these abnormal chromosomes. The resolution of chromosome studies is markedly improved by the availability of SNP arrays that enable high-resolution genome analysis. This technique proves to be a more accurate method for the identification and delineation of chromosomal rearrangements. As a result, precise definition of complex rearrangements can be better established. It is, however, noteworthy that current array technologies are incapable of detecting balanced alterations such as inversions, and only give information regarding genomic gains and losses. In this study we underline the added value of array technologies in defining chromosomal aberrations observed with conventional chromosome analysis.

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Chapter 4.2

A subtle familial translocation t(3;21)(p26.3;q22.3): apparently healthy boy with a 3p deletion and 21q duplication

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Abstract
Here we report on the clinical and cytogenetic results in a family carrying a cryptic translocation involving chromosome 3pter and 21qter detected by Single Nucleotide Polymorphism (SNP) array and subtelomeric Fluorescent *In Situ* Hybridisation (FISH) analysis. The index patient, with mild mental retardation (MR) in combination with minor dysmorphic features, inherited the derivative chromosome 21 resulting in a partial trisomy of the short arm of chromosome 3 and a partial monosomy of the long arm of chromosome 21. Her apparently healthy brother inherited the derivative chromosome 3 resulting in a terminal deletion of the short arm of chromosome 3 and a terminal duplication of the long arm of chromosome 21. We discuss the different phenotypes for the two genotypes and argue for the importance of reporting these imbalances to achieve accurate genetic counseling in prenatal and postnatal diagnosis.

Introduction
Several patients with chromosome aberrations involving chromosomes 3pter and 21qter have been reported. The first case with the 3p deletion syndrome was described in 1978 (Verjaal and De Nef, 1978), and is characterized by low birth weight, developmental delay, growth retardation and dysmorphic facial features (Malmgren et al., 2007; Barber et al., 2008; Fernandez et al., 2008). The smallest region of overlap for all reported 3p deletion patients is 1.5 Mb on chromosome band 3p26, including the candidate genes *CHL1* (MIM# 607416), *CNTN4* (MIM# 607280) and *CRBN* (MIM# 609262) (Cargile et al., 2002; Dijkhuizen et al., 2006). The clinical manifestations of the trisomy 3p syndrome are quite variable depending on the size of the duplication (for review see Schinzel, 2001). The syndrome includes psychomotor retardation, mental retardation (MR) and minor dysmorphic features.

Genotype-to-phenotype correlations for partial monosomy and partial trisomy of chromosome 21 have recently been reviewed (Lyle et al., 2009). Deletions of the terminal 21q22.2q22.3 region produce a mild phenotype including MR and holoprosencephaly (Lyle et al., 2009). Cases with terminal duplications of 21q22 are rare. Most patients have a Down syndrome phenotype including cognitive impairment, congenital heart disease and characteristic facial and physical appearance (Lyle et al., 2009).

Conventional karyotyping can detect chromosomal abnormalities larger than approximately 5 Mb. For detection of smaller abnormalities Fluorescent *In Situ* Hybridisation (FISH)-, Multiplex Ligation-dependent Probe Amplification (MLPA)-,and whole genome high resolution array - analyses are necessary (Knight and Flint, 2000; de Vries et al., 2005; Rauch et al., 2006; Hoyer et al., 2007; Kriek et al., 2007; Gijsbers et al., 2009). In 5% of patients with MR with or without multiple congenital abnormalities subtelomeric abnormalities are identified (Flint et al., 1995; Knight et al., 1999; de Vries et al., 2003). Nevertheless, there are subtelomeric rearrangements reported in unaffected normal individuals and therefore considered to be not pathogenic (Hengstschläger et al., 2005; Balikova et al., 2007). Hence it is of great importance to report these polymorphisms for genetic counseling in prenatal and postnatal screening.

Here we report on the segregation of a submicroscopic familial reciprocal translocation between the subtelomeric regions of the short arm of chromosome 3 and the long arm of chromosome 21, and describe the different phenotypes in two siblings who inherited the different unbalanced products.

Materials and methods

Index patient

The index patient in this family was a 5-year old girl (Fig. 4.2.1a and b), the first child of healthy, unrelated parents. She was born after an uneventful pregnancy after 42 weeks of gestation with a birth weight of 2500 g and birth length of 43 cm. She spoke her first words before her first year and walked unsupported at the age of 2 years. At 2 years and 8 months delayed motor and speech development was noted. She had mild MR (IQ 65) and clinical examination showed frontal bossing, hypotelorism, thin and long face, hoarse voice and laxity of all joints.

Williams syndrome was excluded by FISH analysis with the DNA probe LSI ELN/ LSI D7S486, D7S522 (Vysis, Il, USA).

Brother of index patient

The 2.5-year old, younger brother (Fig. 4.2.1c) of the index patient was born after an uneventful pregnancy after 40 weeks of gestation. His birth weight was 3350 g. He walked unsupported at 14 months. His motor and speech development was normal. Clinical examination showed no dysmorphic features. At 14 months mild strabismus was noted. Before the age of 2 years he was able to count and to recognize figures. He will be kept under review in case later onset health problems emerge.



Figure 4.2.1 Pictures of the index patient and her apparently healthy brother. (a) (b) Facial picture of the index patient at the age of 3 (a) and 4 (b) years. Note frontal bossing, hypotelorism and thin and long face. (c) Facial picture of her brother at the age of 2 years.

Conventional karyotyping and FISH analysis
Conventional cytogenetic analysis on GTG-banded chromosomes from cultured lymphocytes and FISH analysis were performed according to standard techniques (Dauwerse et al., 1990).

SNP arrays
DNA was extracted from whole blood by a Gentra Puregene DNA purification Kit (Gentra Systems, Minneapolis, USA), following the manufacturer's instructions. The Affymetrix GeneChip Human Mapping 262K *NspI* array contains 262.262 25-mer oligonucleotides, with an average spacing of approximately 12 kb (Affymetrix, Santa Clara, CA, USA). An amount of 250 ng DNA was processed according to the manufacturer's instruction. SNP copy number was assessed using the software program CNAG Version 2.0 (Nannya et al., 2005).

The result of the index patient was added to the DECIPHER database (Wellcome Trust Genome Campus, Hinxton, Cambridge, UK) when consent was obtained (ID LEI248616).

Results
Conventional karyotyping showed no abnormalities in the index patient (Fig 4.2.2a). SNP array screening, however, revealed a terminal duplication on the short arm of chromosome 3 and a terminal deletion on the long arm of chromosome 21 (Fig 4.2.2b). The size of the aberrations was 3 Mb (from probe SNP_A-1971271 to SNP_A-2081957, respectively at 73.603 bp and 3.085.004 bp according to http//:www.ensembl.org, Ensembl release 56 – Sept 2009) and 5 Mb (from probe SNP_A-2019989 to SNP_A-2020813, respectively at 43.020.221 bp and 48.069.930 bp). FISH analysis confirmed the presence of a derivative chromosome 21, originating from a translocation between the short arm of chromosome 3 and the long arm of chromosome 21 (Fig 4.2.2c).

FISH analysis revealed a normal karyotype for the father, while the mother was carrier of a *de novo* balanced t(3;21)(p26.3;q22.3) (Fig 4.2.2d). The brother of the index patient inherited the derivative chromosome 3 resulting in a 3 Mb deletion of the terminal part of the short arm of chromosome 3 and a 5 Mb terminal duplication of the long arm of chromosome 21 (Fig. 4.2.2e).

Discussion
The presence of a double chromosome imbalance complicates genotype-phenotype correlations in patients. Our index patient carrying the derivative chromosome 21, resulting in a partial trisomy of 3p and a partial monosomy of 21q, presented mild MR, frontal bossing, hypotelorism, thin and long face, hoarse voice and laxity of all joints. The phenotype of trisomy 3p patients is quite variable and depends on the size of the duplication (Schinzel, 2001). The duplication of 3p in the index patient is 3 Mb and contains three genes: *CHL1*, *CNTN6* and *CNTN4*. In the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation/), all these genes are reported as gains in normal individuals. Therefore we suggest that the 3p duplication in our patient is probably not contributing to the phenotype. The 5 Mb deletion on chromosome 21, however, contains approximately 88 genes and is not described in normal individuals according to the DGV. Despite the phenotypic variability in partial monosomy 21, there are some common features among the reported cases including craniofacial, skeletal and cardiac effects, genital malformations and severe MR (Lyle et al., 2009). Yet, most

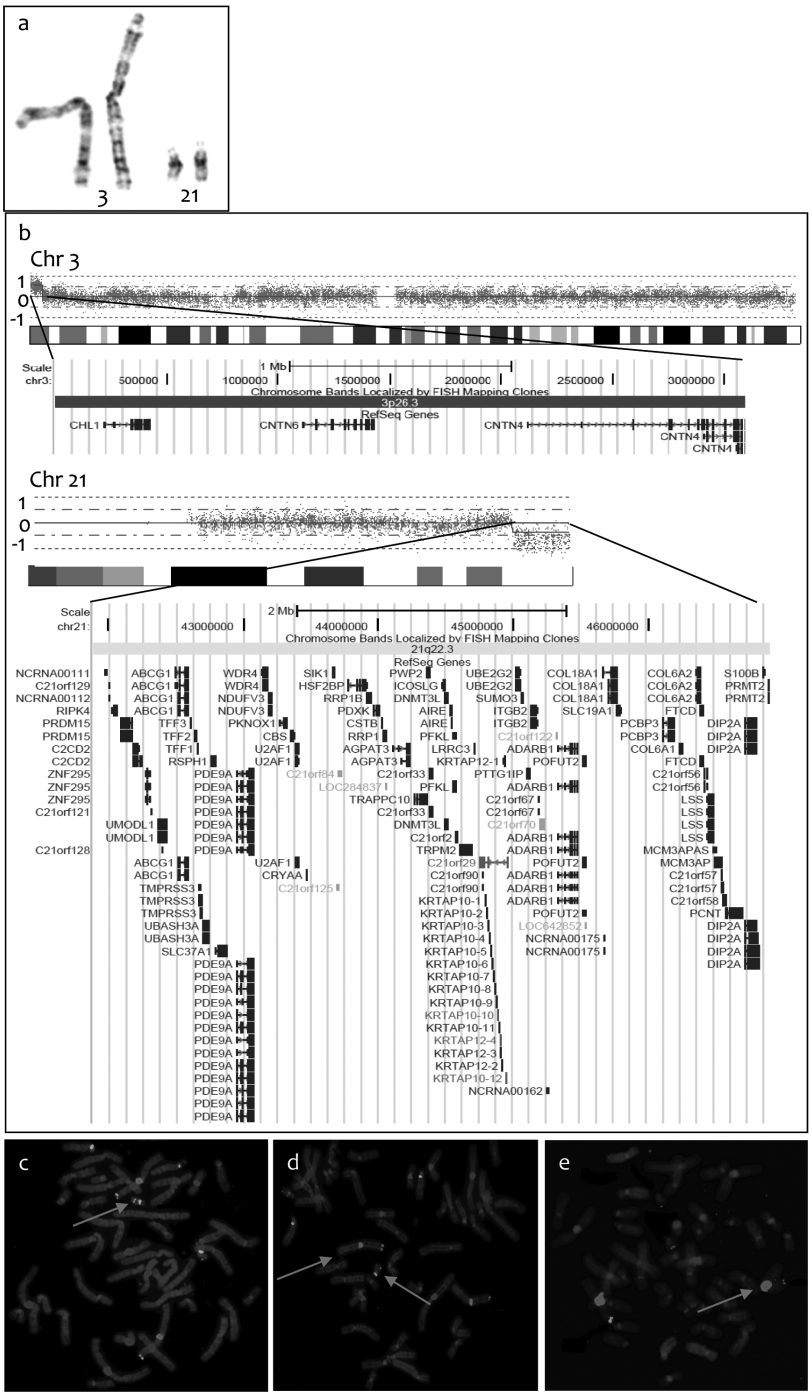


Figure 4.2.2 Cytogenetic and molecular analysis of the familial t(3;21)(p26.3;q22.3). (a) Conventional karyotyping revealed no abnormalities for chromosomes 3 and 21 in the index patient. The arrow indicates the aberrant chromosome 21. (b) SNP array analysis (Affymetrix GeneChip Human Mapping 262K NspI) results for the index patient demonstrating a 3 Mb duplication on the short arm of chromosome 3 and a 5 Mb deletion on the long arm of chromosome 21. Genes involved on both regions, UCSC Human Genome Browser (http://genome.ucsc.edu), Mar 2006 build (hg18). (c) FISH analysis confirmed the presence of the derivative chromosome 21 (black arrow), originating from a translocation between 3p (green; GS-1186B18; Flint) and 21q (red; GS-63H24; Flint). Control probes used for centromere chromosome 3 (red; CEP3; Vysis) and satellites of chromosome 13 and 21 (green; a-sat 13/21; Cytocell). (d) FISH analysis for the mother of the index patient demonstrated that she is carrier of the balanced t(3;21)(p26.3;q22.3) (black arrows) (probes: GS-1186B18, GS-63H24, CEP3 and a-sat 13/21). (e) FISH analysis for the brother of the index patient showing the derivative chromosome 3 (black arrow) (probes: GS-1186B18, GS-63H24, CEP3).

of these patients have a larger deletion than our patient. The most terminal 10 Mb of chromosome 21 contains approximately 130 genes and monosomy of this region contributes to a milder phenotype (Lyle et al., 2009). Accordingly the relative mild phenotype of our index patient is most likely explained by the even smaller distal 21q deletion. The phenotype might be the result of a gene-dosage effect of a combination of genes. Moreover, five of the 88 genes have been associated with MR; *CBS* (MIM# 236200), *COL6A1* (MIM# 120220), *CSTB* (MIM# 601145), *S100B* (MIM# 176990) and *SLC19A1* (MIM# 600424) (http://www.ncbi.nlm.nih.gov/sites/entrez).

The brother of the index patient, carrying the derivative chromosome 3, resulting in a terminal deletion of the short arm of chromosome 3 and a terminal duplication of the long arm of chromosome 21, shows an apparently healthy phenotype. The distal 3p deletion syndrome is reported with a recognizable phenotype, including developmental delay, low birth weight, growth retardation and several dysmorphic features (Malmgren et al., 2007; Barber et al., 2008; Fernandez et al., 2008). The critical region was defined as a 1.5 Mb region on chromosome 3p26, containing the candidate genes *CHL1*, *CNTN4* and *CRBN* (Cargile et al., 2002; Dijkhuizen et al., 2006). The genes *CHL1* and *CNTN4* map within the brother's deletion excluding haploinsufficiency of these genes as a cause for MR. Nevertheless, cases have been reported with a deletion of the distal 3p region without any phenotypic effects (Knight et al., 1995; Shrimpton et al., 2006; Takagishi et al., 2006; Hoo et al., 2008). Shrimpton et al (2006) even postulated that the distal 3p26 deletion is probably associated with normal intelligence and normal physical features. However, it is possible that mutations in one of the candidate genes on the normal chromosome 3 are responsible for an abnormal phenotype. This could also be suggested for the candidate gene *CRBN*, which was not deleted in our patient, but in both phenotypically abnormal and normal previously reported cases (Knight et al., 1995; Dijkhuizen et al., 2006). Another explanation might be that the distal 3p region is a susceptibility locus for MR, and therefore the deletion is not presenting MR in all 3p- cases. A further possible explanation might be the involvement of epigenetic and environmental factors.

Since terminal deletions of the 3p26 may be associated with a normal phenotype we suggest that prenatally detected de novo distal 3p deletions may warrant further molecular analysis to accurately size the deletion.

Patients with partial trisomy 21 are rare and the Down syndrome phenotype in most of these cases has been associated with duplication of 21q22 (Horn et al., 2003). A 250 kb trisomy of the distal subtelomeric region of 21q22.3 was reported as a benign variant (Bonaglia et al., 2007). Here we show that a terminal duplication of at least 5 Mb on distal 21q22.3q22.3 is not related with an abnormal phenotype. While a deletion of this region is associated with an abnormal phenotype in our index patient.

In conclusion, a genotype-phenotype correlation is difficult to make in regions with large number of genes and in regions in which deletions as well as duplications have been reported in healthy individuals. Yet, reporting these pathogenic and polymorphic rearrangements is of great importance for genetic counseling and prenatal screening. The detailed clinical description of our cases, along with a precise cytogenetic designation of chromosome breakpoints, allows further refinement of genotype-phenotype correlation for terminal imbalances in 3p and 21q.

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Chapter 5

Other patient groups

Chapter 5.1

Identification of copy number variants associated with BPES-like phenotypes

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Abstract
Blepharophimosis-Ptosis-Epicanthus inversus Syndrome (BPES) is a well characterized rare syndrome that includes an eyelid malformation associated with (type I) or without premature ovarian failure (type II). Patients with typical BPES have four major characteristics: blepharophimosis, ptosis, epicanthus inversus and telecanthus. Mutations in the *FOXL2* gene, encoding a forkhead transcription factor, are responsible for the majority of both types of BPES. However, many patients with BPES-like features, i.e. having at least 2 major characteristics of BPES, have an unidentified cause. Here, we report on a group of 27 patients with BPES-like features, but without an identified genetic defect in the *FOXL2* gene or flanking region. These patients were analyzed with whole-genome high-density arrays in order to identify copy number variants (CNVs) that might explain the BPES-like phenotype. In 9 out of 27 patients (33%) CNVs not previously described as polymorphisms were detected. Four of these patients displayed psychomotor retardation as an additional clinical characteristic. In conclusion, we demonstrate that BPES-like phenotypes are frequently caused by CNVs, and we emphasize the importance of whole-genome copy number screening to identify the underlying genetic causes of these phenotypes.

Introduction
Blepharophimosis and ptosis are a reduction in the horizontal and vertical dimensions of the palpebral fissures respectively (Guercio et al. 2007). From the 135 syndromes in the London Dysmorphology Database with blepharophimosis and ptosis, 6 are known to be associated with a chromosomal microdeletion (Winter et al. 2008). In addition, a recent literature review illustrates that chromosomal aberrations are probably the most important underlying cause in patients with blepharophimosis and mental retardation phenotypes (Bartholdi et al. 2008). Only a few blepharophimosis syndromes have known causative genes: the Schwartz-Jampel syndrome (OMIM 255800), Freeman Sheldon syndrome (OMIM 193700), Hereditary neuralgic amyotrophy syndrome (HNA; OMIM 162100) (Laccone et al, 2008) and Blepharophimosis-Ptosis-Epicanthus inversus syndrome (BPES) (OMIM 110100). BPES is a rare autosomal dominant disorder characterized by a dysplasia of the eyelids. There are two types of clinical presentation: type I is associated with premature ovarian failure, whereas type II has no associated symptoms (Zlotogora et al. 1983). The typical BPES phenotype is characterized by four major features blepharophimosis, ptosis, epicanthus inversus (a small skin fold which arises from the lower eyelid and runs inwards and upwards) and telecanthus (increased distance between the medial lid margins). Haploinsufficiency of the *FOXL2* gene, encoding a putative forkhead transcription factor located at chromosome 3q23, is responsible for both types of BPES (Crisponi et al. 2001; De Baere et al. 2001; De Baere et al. 2003). Using a combined mutation detection approach, it is possible to reveal the causal genetic defect in 88% of typical BPES patients. Molecular defects include chromosomal rearrangements (2%), intragenic mutations (81%) and genomic rearrangements comprising both deletions encompassing *FOXL2* (12%) and deletions located outside its transcription unit (5%) (Beysen et al. 2005; Beysen et al. 2008). The lack of a detectable genetic *FOXL2* defect might be explained by several grounds, including subtle extragenic rearrangements, epigenetic changes and referral bias by the different clinicians (Beysen et al. 2008). Here, we focus on series of 27 patients with BPES-like features (i.e. having at least two main characteristics of typical BPES), who tested negative for *FOXL2* mutations and deletions of the *FOXL2* region, and in whom

the causal defect remains to be elucidated (Beysen et al. 2008).

Novel whole-genome array-based technologies can detect copy number variants (CNVs) at much higher resolution than conventional cytogenetic methods, and hence might reveal CNVs that were previously unidentified. These techniques have been used with considerable success to reveal *de novo* CNVs in several phenotypes, including mental retardation and dysmorphism (Stankiewicz and Beaudet 2007; Slavotinek 2008).Yet, the association between CNVs and BPES-like phenotypes has not been investigated. We performed whole-genome high-density arrays in the group of 27 *FOXL2* mutation-negative patients with a BPES-like phenotype in order to identify the underlying genetic cause explaining the phenotypes.

Methods

Patients

Genomic DNA was obtained from 27 patients with a BPES-like phenotype, being patients for whom at least two of the four diagnostic criteria of BPES (i.e. blepharophimosis, ptosis, epicanthus inversus and telecanthus), were mentioned on a clinical questionnaire. In 11 of the patients psychomotor retardation was identified as an additional clinical feature. Furthermore, all patients were clinically assessed by different clinicians. The patients were recruited in three genetic centers: (1) 22 patients from the Center for Medical Genetics, Ghent University Hospital, Belgium. Genomic patient DNA was used that was available from previously approved mutation studies (De Baere et al. 2001; De Beare et al. 2003; Beysen et al. 2005; Beysen et al. 2008); (2) 3 families from the Department of Clinical Genetics, Amsterdam Medical Centre, The Netherlands; (3) 2 patients from the Department of Clinical Genetics, Leiden University Medical Center, The Netherlands.

All patients were initially referred for *FOXL2* screening. Intragenic *FOXL2* mutations and copy number variants of the *FOXL2* gene and surrounding region were excluded in all patients by sequencing of the coding region and multiplex ligation-dependent probe amplification (MLPA) (P054, MRC Holland). In addition, conventional karyotyping was performed in 13 patients and considered normal.

Single Nucleotide Polymorphism arrays

Affymetrix GeneChip Human Mapping 262K *NspI*. This array was used for one patient and his parents. This SNP array contains ~262,000 25-mer oligonucleotides with an average spatial resolution of ~12 kb. An amount of 250 ng DNA was processed according to the manufacturer's instruction (http://www.affymetrix.com). SNP copy number was assessed in the patient using DNA-Chip Analyzer (dChip) software (version release 02-16-06) (Li and Wong, 2001).

Illumina's Sentrix HumanHap300 Genotyping Beadchip. This platform was used for 23 patients. This array contains ~317,000 TagSNPs with a mean spatial resolution of approximately 9 kb. A total of 750 ng DNA was processed according to the manufacturer's instruction (http://www.illumina.com). SNP copy number (logRratio) and B allele frequency were assessed in the patients using BeadStudio Version 3.2 (Illumina, Inc.).

Illumina's HumanCNV370-Duo BeadChip. For three patients this platform was used. This array contains ~317,000 TagSNPs and 52,000 non-polymorphic markers to specifically

target nearly 14,000 known copy number variants with a mean spatial resolution of approximately 7.7 kb. The experiment was performed as described above. SNP copy number and B allele frequency were assessed in BeadStudio, Version 3.2 (Illumina, Inc.).

Evaluation of CNVs
Based on in-house validation studies deletions of at least 5 adjacent SNPs or with a minimum size of 150 kb and duplications of at least 7 adjacent SNPs or with a minimum size of 200 kb were considered significant. All CNVs identified in this study were assessed by screening them against the Database of Genomic Variants (DGV) (http://projects.tcag.ca/variation/) and against our in-house available reference set of approximately 1000 individuals. All regions that significantly overlapped with known polymorphic CNVs were excluded from further research.

MLPA
MLPA experiments were carried out to validate the presence of deletions and duplications identified by the arrays. When an aberration was confirmed by MLPA, the same probe set was used to perform segregation analysis in the parents (if available). At least two synthetic MLPA probes were designed within the aberration and MLPA experiments were performed as described (White et al. 2004). Probes were commercially obtained from Biolegio (Malden, The Netherlands). Amplification products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Fragment analysis was performed with the GeneMarker Software V1.51 (SoftGenetics, USA). Thresholds for deletions and duplications were set at 0.75 and 1.25 respectively (White et al. 2004).

Fluorescent In Situ Hybridisation Analysis (FISH)
FISH analysis was carried out by standard procedures as described (Dauwerse et al. 1990). FISH analysis was performed to verify the imbalances found with the array experiments and to exclude more complex rearrangements. BAC clones mapping to the unbalanced chromosome regions were selected based on their physical location within the affected region (http://www.ensembl.org: Ensembl release 48 - dec 2007).

Gene prioritisation
The software tool Anni 2.0 (http://www.biosemantics.org/Anni) was used to search for potential candidate genes for BPES-like features in the CNVs found. For each gene a profile of related concepts is constructed that summarizes the context in which the gene is mentioned in the literature. Genes associated with similar topics are identified by hierarchical clustering of the corresponding gene concept profiles. The software was used according to the software's manual (Jelier et al. 2007).

Results and discussion
Identification and confirmation of 12 significant CNVs
In this study we included 27 patients based on the following criteria: (1) BPES-like phenotype (i.e. 2 or more major features of BPES, with or without additional dysmorphic features and psychomotor retardation); (2) exclusion of a *FOXL2* mutation or deletion of the *FOXL2* region. These patients were analyzed with whole-genome high-density arrays with an average spatial resolution of approximately 10 kb, in

an attempt to identify the underlying genetic cause and to reveal potential novel candidate loci and genes for BPES-like phenotypes.

In total we identified 12 CNVs not previously described in phenotypically normal individuals according to the DGV and our in-house reference set, in 9 out of 27 patients (33%). An overview of these changes is provided in Table 5.1.1. We found 6 duplications and 6 deletions. In 3 patients (cases 7, 8 and 9) a deletion as well as a duplication was identified, suggesting the presence of an unbalanced translocation. The identified CNVs vary in size from 485 kb to 21.9 Mb. Table 5.1.2 represents the available clinical features from 8 out of the 9 patients carrying chromosome imbalances. Four of these patients display psychomotor retardation as an additional clinical feature.

Each of the 12 CNVs was confirmed by a second independent technique, either MLPA (patients 1-3, 5, 7-8), FISH (patients 4, 9) or another SNP array platform (patient 6). In 8 of 9 cases parental DNA was available for segregation analysis. MLPA and FISH analysis in these cases determined that chromosomal aberrations arose *de novo* in 5 out of 8 individuals (patients 1-4, 8).

De novo single CNVs

The CNVs identified in cases 1 and 2 were recently described in patients with BPES-like features, developmental delay and other dysmorphic features (Schinzel et al. 1991; Tinkle et al. 2003; Shaw-Smith et al. 2006; Koolen et al 2006; Koolen et al 2008).

Patient 1 had an interstitial deletion of 18q12.2q21.1 (14.3 Mb), and displayed blepharophimosis, ptosis, epicanthus inversus, and mild dysmorphic features (abnormal nose and ears). The patient died at the age of 4. Several cases have been described with del(18)(q12.2q21.1) showing a consistent clinical pattern of mild dysmorphic features, blepharophimosis, obesity, mental retardation, seizures, behavioral problems, and lack of major congenital anomalies (Schinzel et al. 1991; Tinkle et al. 2003).

In patient 2 an interstitial microdeletion of 17q21.31 (485 kb) was found (Fig. 5.1.1), overlapping with the region that was recently described in the new microdeletion syndrome 17q21.31 (Shaw-Smith et al. 2006; Koolen et al 2006). This new microdeletion syndrome was originally identified by high-resolution genome analyses in patients with unexplained mental retardation, and was recently further characterized clinically and molecularly (Koolen et al. 2008). The deletions are associated with a common inversion polymorphism. Individuals carrying this deletion display a recognizable phenotype of blepharophimosis, a characteristic long face with pear shaped nose, large ears, hypotonia and mental retardation. Interestingly, the phenotype of patient 2, characterized by blepharophimosis, bilateral ptosis, epicanthus inversus, telecanthus, broad pear shaped nose, large ears, central and peripheral hypotonia, and psychomotor retardation, is in agreement with this recognizable 17q21.31 microdeletion syndrome.

Patient 3 had a small interstitial duplication of 16p13.3 (1.1 Mb). To our knowledge, two patients have been reported with a partially overlapping duplication of this region, including a patient with an interstitial duplication of 345 kb - 480 kb on 16p13.3, encompassing the *TRAP1* and *CREBBP* genes that are also duplicated in patient 3 (Thienpont et al. 2007). The second patient was reported to have a duplication of 16p13.3 with a minimal and maximal size of 4.5 Mb and 7 Mb respectively (de Ravel et al. 2005). The duplicated region of patient 3 overlaps over at least 620 kb with the reported duplications. However, the two reported patients display no BPES-like features, suggesting that the region of approximately 600 kb that does not overlap is

Table 5.1.1 CNVs detected by whole-genome high-density SNP arrays

ID	Gain/ Loss	Chromosome	Starting probe[1]	Ending probe[2]	Size of CNV[3]	Array	Confirmation	Status of inheritance	Number of genes
1	Loss	18q12.2q21.1	rs1693336	rs2576050	14.3 Mb	II	MLPA	*de novo*	18
2	Loss	17q21.31q21.31	rs393152	rs2016730	485 kb	II	MLPA	*de novo*	3
3	Gain	16p13.3p13.3	rs11077336	rs2239316	1.1 Mb	II	MLPA	*de novo*	21
4	Loss	10p12.33p12.31	rs17140331	rs11012683	4.5 Mb	I	FISH	*de novo*	7
5	Gain	11p15.4p15.4	rs7117223	rs4237772	545 kb	II	MLPA	maternal	5
6	Gain	9q34.1q34.2	rs3012755	rs2027963	1.4 Mb	II	I	maternal	23
7	Gain	10q25.2	rs10885243	rs9630042	21.9 Mb	II	MLPA	n/a	61
	Loss	11q24.3	rs1940363	rs4508216	5 Mb	II	MLPA	n/a	18
8	Gain	3q29	rs1021662	rs12639242	4 Mb	II	MLPA	*de novo*	43
	Loss	13q33.3	rs7321084	rs7316983	5 Mb	II	MLPA	*de novo*	36
9	Loss	3p25.3	rs9681213	rs3774210	8.6 Mb	II	FISH	paternal[4]	12
	Gain	6p24.3	rs6596796	rs927402	7.7 Mb	II	FISH	paternal[4]	23

I, Affymetrix GeneChip Human Mapping 262K *NspI*; II, Illumina's Sentrix HumanHap300 Genotyping Beadchip; n/a, parental DNA not available; [1], first aberrant probe; [2], last aberrant probe; [3], minimal size of aberration; [4], paternal inheritance can be concluded from the pedigree (see Fig. 3c)

Table 5.1.2 Clinical characteristics of BPES-like patients with significant CNVs

	1	2	3*	4	5	6	7	8	9
BPES features									
Blepharophimosis	+	+	n/a	+	+	-	+	+	+
Ptosis	+	+	n/a	+	+	+	+	+	+
Epicanthus inversus	+	+	n/a	-	-	+	+	+	-
Telecanthus	-	+	n/a	+	-	-	+	+	-
Additional features									
Menstrual abnormalities	male	male	male	male	-, age?	-, 15y	+, 19y	-, 7y	-,43y
Psychomotor retardation	-	+	n/a	-	-	-	+	+	+
Growth retardation	-	-	n/a	-	-	-	+	-	-
Microcephaly	-	-	n/a	-	-	-	+	+	-
Abnormal ears	+	+	n/a	-	+	-	+	+	-
Abnormal nose	+	+	n/a	-	+	-	+	-	-
Flattened and broad nose	+	+	n/a	-	-	+	+	-	+
Skeletal anomaly	-	+	n/a	-	-	-	-	+	-
Syndactyly	-	-	n/a	-	-	-	-	+	-
Clinodactyly	-	-	n/a	+	-	-	+	+	-
Other	-	central and peripheral hypotonia, pectus excavatum,	n/a	Atrial septal defect type II	reduced iris pigmentation, mild Brushfield spots	-	midface hypoplasia, round face, narrow palate	brachycephaly, diastasis recti	-

n/a, not available; *, patient shows at least two characteristics of typical BPES and other features are unknown

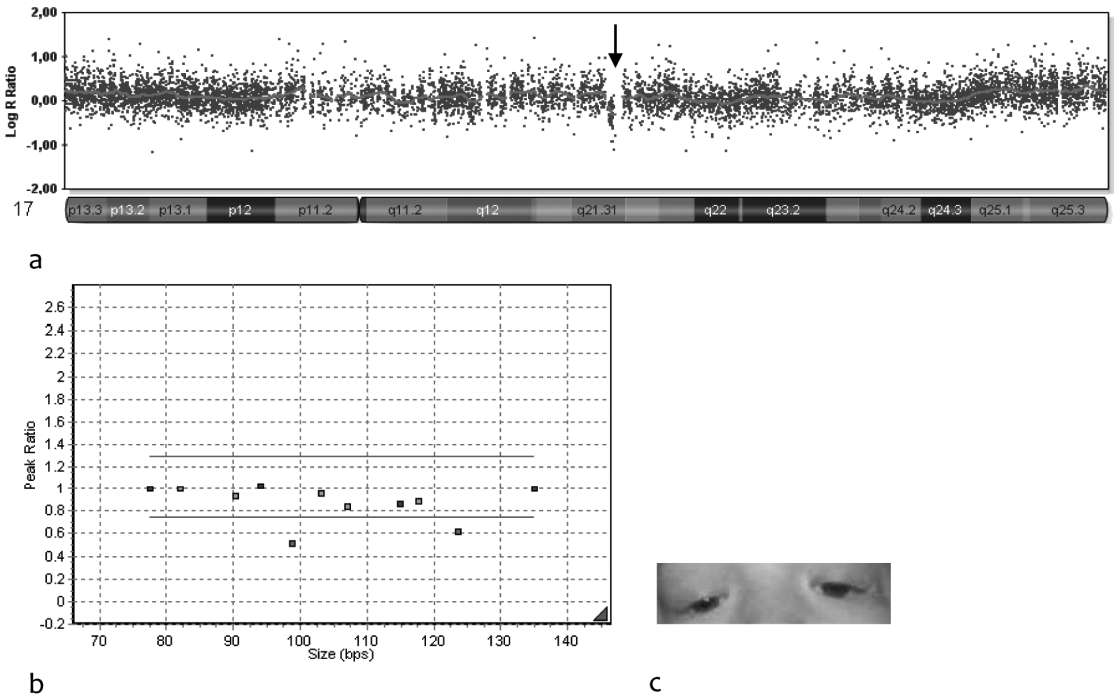


Figure 5.1.1 Case 2 (a) Deletion of chromosome 17q21.31 (485 kb) found with the Illumina's Sentrix HumanHap300 Genotyping Beadchip. (b) MLPA analysis confirmed the deletion. Deleted probes: MAPT and KIAA1267. (c) Picture of the eyes of case 2, characterized by blepharophimosis, bilateral upper lid ptosis, epicanthus inversus and telecanthus.

responsible for the BPES-like features in patient 3.

In patient 4 we detected a novel rearrangement, being an interstitial deletion of chromosome 10p12.33p12.31 (4.5 Mb) (Fig. 5.1.2). It was shown to occur *de novo* and it is therefore likely to be causative. The clinical features of this patient include blepharophimosis, ptosis, epicanthal folds, ectropion of the lower eyelid, S-shaped upper eyelid, synophrys, high palate, broad hands and feet, and an atrial septal defect type II. This patient has a normal psychomotor development.

Inherited single CNVs

In patient 5 a small interstitial duplication of 545 kb on chromosome 11p15.4 was detected. This gain was not reported as a variant in the DGV, but was found in the phenotypically normal mother of this patient, probably suggesting absence of a causal relation between the duplication and the BPES-like phenotype in the child.

Furthermore, in case 6 an interstitial duplication of chromosome 9q34.1q34.2 (1.4 Mb) was found that was not described in the DGV. The mother of this patient was shown to carry the same duplication. The phenotype of patient 6 is characterized by bilateral ptosis, mild epicanthus inversus, horizontal nystagmus and high hypermetropia, while her mother displayed a mild ptosis and strabismus for which she underwent surgery. The occurrence of a phenotype in mother and child might suggest a causal relationship of the novel duplication and the phenotype.

Complex CNVs

In three cases more complex rearrangements were identified (cases 7, 8 and 9). In these patients both a duplication and a deletion were found on different chromosomes, suggesting the occurrence of an unbalanced translocation (Table 5.1.1).

Patient 7 was found to carry a terminal duplication of chromosome 10q (21.9 Mb) and a terminal deletion of chromosome 11q (5 Mb). Recently, a patient was described with approximately the same duplication of chromosome 10q and a deletion located on chromosome 4q (Bartholdi et al. 2008). This patient displayed the following clinical features: blepharophimosis, ptosis, epicanthus inversus, downslanting palpebral fissures, generalized hypotonia and developmental delay. The phenotype was ascribed to the duplication of chromosome 10q (Bartholdi et al. 2008). Our patient 7 showed a similar phenotype, therefore we hypothesize that the duplication of chromosome 10q might contribute to the BPES-like phenotype in this patient. For patient 7 it could not be confirmed whether this complex rearrangement arose from a balanced translocation in one of the parents.

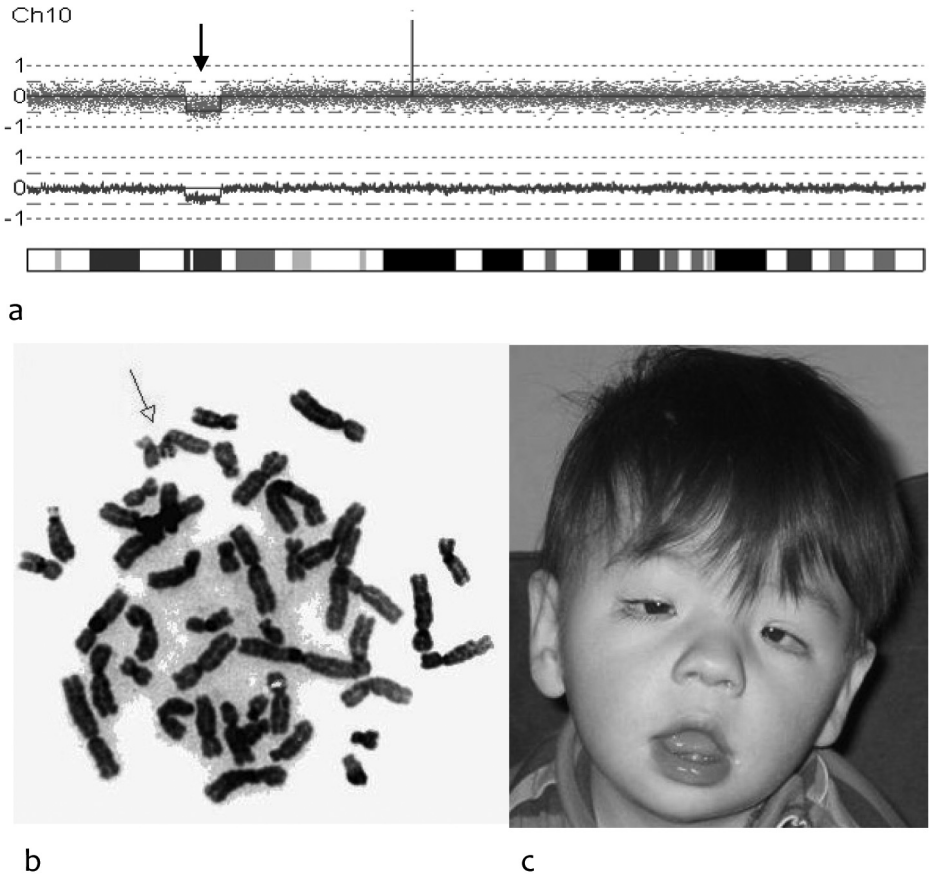


Figure 5.1.2 Case 4 (a) Deletion of chromosome 10p12.33-p12.31 (4.5 Mb) detected with Affymetrix GeneChip Human Mapping 262K NspI .(b) FISH analysis confirmed the deletion. Control probe: RP11-390B4 (red), deleted probe 10p12.31: RP11-177H22 (green). (c) Facial picture of case 4, showing blepharophimosis, ptosis, epicanthal folds, ectropion of the lower eyelid, S-shaped upper eyelid, synophrys.

Patient 8 showed a terminal duplication of chromosome 3q (4 Mb) and a terminal deletion of chromosome 13q (5 Mb). FISH analysis revealed the presence of an unbalanced translocation in the patient and showed no abnormalities in the parents. This patient showed a BPES-like phenotype and some additional dysmorphic features. In the ECARUCA database 28 cases were listed with a del(13)(q33.3q34). In 4/28 blepharophimosis was described as a clinical feature, in 3/28 ptosis, and in 2/28 telecanthus. No association between BPES-like features and 3q29 could be found in literature.

In patient 9 a terminal deletion of chromosome 3p (8.6 Mb) and a terminal duplication of chromosome 6p were observed (7.7 Mb) (Fig. 5.1.3a). Her facial appearance is characterized by blepharophimosis, ptosis, flattened and broad nose, long philtrum (IV-2) (Fig. 5.1.3d). In addition she displays psychomotor retardation. Patients with a terminal deletion of the short arm of chromosome 3 have been described to display blepharophimosis, epicanthus, upturned nose, long philtrum, microcephaly and polydactyly (Malmgren et al. 2007). Both the terminal deletion of 3p and terminal duplication 6p are associated with BPES-like features according to literature (Schinzel 2001). With standard G-banding the deletion and duplication were not visible. FISH with DNA probes specific for the subtelomeric region of chromosome 3p and 6p revealed that this rearrangement arose by an unbalanced translocation between chromosome 3p and 6p (Fig. 5.1.3b). Furthermore, another patient of this family with similar clinical features was found to have the same unbalanced rearrangement using SNP array analysis (III-5) (Fig. 5.1.3d). Subsequently, segregation analysis of the 3;6 translocation was performed in other members by FISH analysis (Fig. 5.1.3c), and revealed a familial 3;6 translocation. An unbalanced translocation, resulting in a terminal duplication of chromosome 3p and a terminal deletion of chromosome 6p, was observed in one family member (V-2). This patient showed no blepharophimosis, but displayed macrocephaly, Dandy-Walker malformation, hypoplasia of the cerebellum, hypertelorism, divergent strabismus, broad and flat nasal bridge and low set ears (Fig. 5.1.3d).

Previously performed conventional karyotyping revealed no chromosome abnormalities in patients 7, 8 and 9. These findings demonstrate that even large chromosomal imbalances are not always easily detected with standard G-banding, emphasizing the importance of a whole-genome screen with a high resolution array technique.

Candidate gene analysis

Assuming that the identified CNVs are causative for BPES-like phenotypes, it can be postulated that the corresponding chromosomal regions might reveal some relevant candidate genes contributing to some specific features. Most of these rearrangements, however, are too large to allow pinpointing of major candidate genes. Despite this, we used the software tool Anni in order to find functional associations between large numbers of genes contained in the identified CNVs and biomedical information from literature (Jelier et al. 2007). However, for all these regions no significant candidate genes were found. The availability of more BPES-like patients and more subtle rearrangements may be useful in the search for candidate genes involved in BPES-like phenotypes.

General conclusion

BPES-like features are associated with many syndromes and chromosomal disorders. In this study novel rearrangements were identified in 33% of patients with BPES-like phenotypes. These changes are presumed to contribute to the phenotype in most cases. Overall, we demonstrated that whole-genome high-density array screening is a powerful strategy to reveal the underlying genetic defect in BPES-like phenotypes. We conclude that patients with all 4 main features of typical BPES should be tested first for genetic defects in *FOXL2*. However, patients with a BPES-like phenotype that can be distinguished from typical BPES, should be tested with a genome wide array prior to

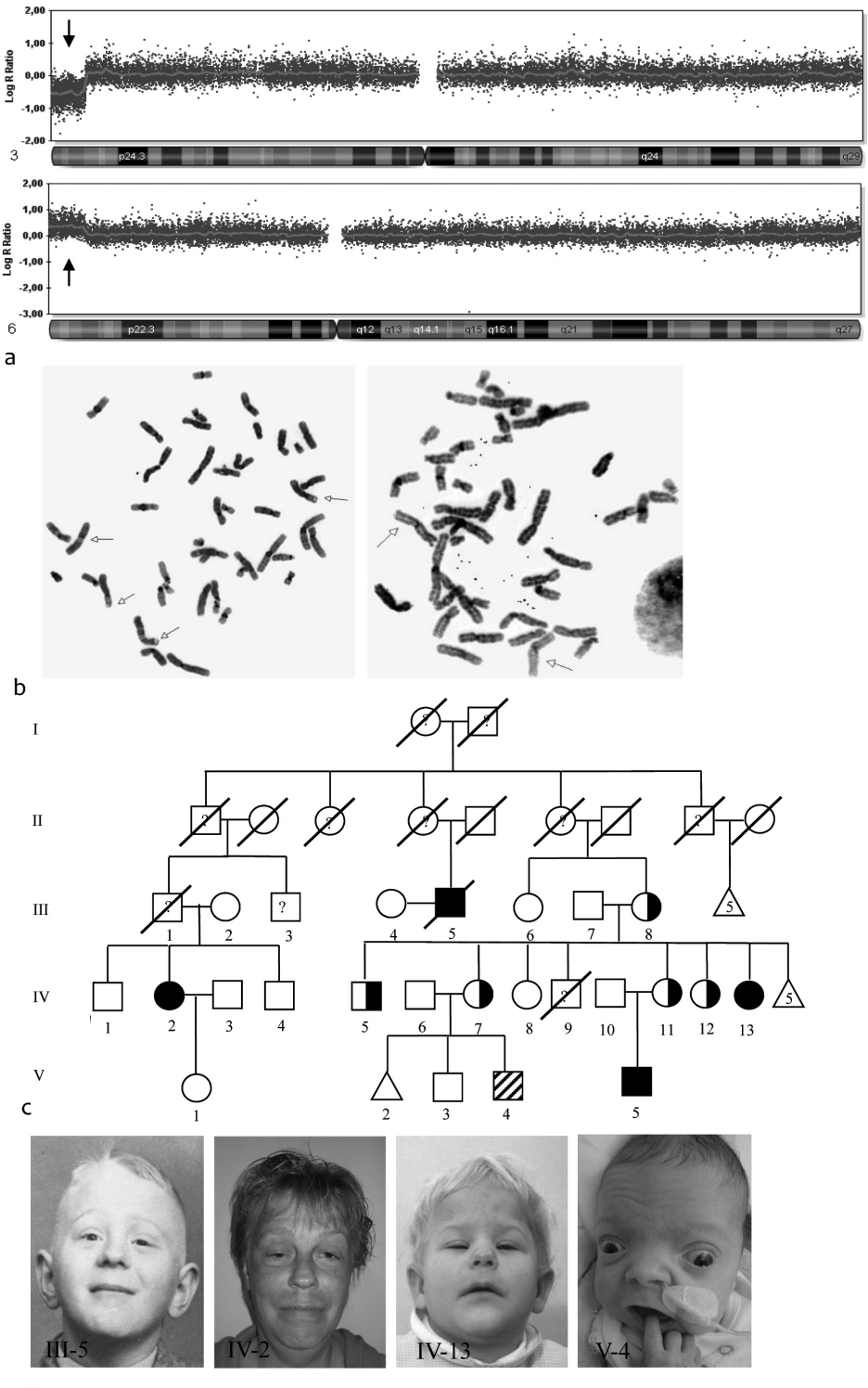


Figure 5.1.3 Case 9 (a) Terminal deletion of chromosome 3p25.3 (8.6 Mb) and terminal duplication of 6p24.3 (7.7 Mb) detected with the Illumina's Sentrix HumanHap300 Genotyping Beadchip. The derivative chromosome 3 was not observed by conventional G-banding. (b) FISH analysis revealed the presence of an unbalanced 3;6 translocation. Left panel, control probe: CEP3 (red) (Vysis), 6pter probe: GS-196I5 (green); right panel, control probe: CEP3 (red) (Vysis), 3pter probe; GS-186B18 (green). (c) Pedigree of the family with the segregating t(3;6) analyzed with FISH experiments. ◧ : balanced t(3;6); ■ : 3p25.3 deletion and 6p24.3 duplication; ▨: 3p25.3 duplication and 6p24.3 deletion. (d) Facial picture of case 9 (IV-2), characterized by blepharophimosis, ptosis, flattened and broad nose. Facial pictures of two relatives (III-5, IV-13) with the same unbalanced translocation as case 9 are presented. These members show similar clinical features. In addition, a facial picture of a relative (V-2) with the opposite unbalanced translocation is shown. No BPES-like features are seen in this child.

FOXL2 genetic testing.

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Chapter 5.2

Genome-wide SNP array analysis in patients with features of Sotos syndrome

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Abstract

Background Sotos syndrome is characterized by overgrowth, facial dysmorphism and learning impairment. Haploinsufficiency of *NSD1* accounts for approximately 60%-90% of the patients. Consequently, a considerable number of patients with features of Sotos syndrome remain without a molecular diagnosis. To date, target-gene approaches in these patients have not been successful.

Methods Twenty-six Sotos syndrome-like patients were analyzed with a high resolution whole genome SNP array and segregation was studied in the parents.

Results Four possible pathogenic copy-number variants including deletions of 10p12.32-p12.31, 14q13.1, Xq21.1-q21.31 and a duplication of 15q11.2-q13.1 were detected. They varied in size from 155 kb to 13.36 Mb. The 10p12.32-p12.31 deletion revealed a candidate gene (*PLXDC2*) for overgrowth. The 14q13.1 deletion affected only the *NPAS3* gene and the patient carrying this deletion displayed mental retardation as the main feature. The Xq21.1-q21.31 deletion and the 15q11.2-q13.1 duplication encompassed multiple genes of which several could be associated with phenotypic expression.

Conclusion The high resolution genome-wide SNP array approach resulted in a detection percentage of 15% of novel abnormalities and is therefore a powerful method to attain a molecular diagnosis in Sotos syndrome-like patients. Identified candidate genes provide directions for future screening of larger patient cohorts.

Introduction

Sotos syndrome (SoS; MIM #117550]) is an autosomal dominant overgrowth disorder characterized by three cardinal features: height and/or head circumference > +2.0 SDS, facial dysmorphism and learning disability [1]. The typical craniofacial features include macrodolichocephaly, a broad forehead with a receding hairline, a prominent chin and downslanting of the palpebral fissures [2]. SoS is caused by haploinsufficiency of the *NSD1* gene at 5q35.2-35.3. The detection percentage varies but overall *NSD1* abnormalities are detected in ~60% - 90% of the cases [3]. Therefore, there are a considerable number of patients suspected of SoS but without a molecular explanation. The phenotypic spectrum of these patients is usually broad, varying from a classical SoS phenotype to patients exhibiting only a few Sotos features. The latter group is sometimes referred to as "Sotos-like" [4;5].

In order to identify genetic alterations in SoS patients without *NSD1* abnormalities, several gene-targeted approaches have been performed [6-8]. In 78 overgrowth syndrome patients in whom *NSD1* abnormalities were excluded, the *NSD*-gene-family members *NSD2* and *NSD3* were screened but no aberrations were detected [6]. Furthermore, screening of the *NSD1* interacting protein 1 gene (*NIZP1*) did not reveal any sequence abnormalities in 97 patients referred for *NSD1* analysis (Visser et al, unpublished data) and no *NIZP1* deletions were detected in 12 typical non-*NSD1* SoS patients [7]. In 38 patients with SoS features, a duplication of *IGF1R* was found in a single patient [8]. Recently, *RNF135* (MIM #611358) abnormalities were identified as the cause of a new overgrowth syndrome in combination with learning disability [9]. However, analysis of *RNF135* in 160 patients referred for *NSD1* screening, did not reveal any alterations [10]. These results render the gene-targeted approach rather unsuccessful in detecting a molecular diagnosis in these patients. Therefore, in this study we have chosen a high resolution genome-wide approach using a Single Nucleotide Polymorphism array (SNP array) with the objective to detect pathogenic copy-number variants (CNVs) in 26 patients with certain features of SoS.

Subjects and methods

Patients

The present study includes 26 patients described previously by de Boer et al. [11] in whom *NSD1* mutations or microdeletions were excluded. From family B of the original study only the proband (nr. 41) was included. From 3 patients (nr. 21, 29 and 52), DNA was no longer available. In patient 17 a duplication of *IGF1R* had been previously found and this patient was therefore excluded from the present analysis [8]. According to the clinical scoring system previously used [11], this study included 3 "typical" SoS patients, 15 from the "dubious" SoS group, and 8 from the "atypical" SoS group. Phenotypic details and results of the clinical scoring system are shown in Table 5.2.1. Approval was obtained from the Medical Ethical Committee of the Leiden University Medical Center and consent was given by the patients and/or their parents or legal guardians. Standard deviation scores (SDS) for growth are expressed in reference to the Dutch population [12] using the Growth Analyser version 3.5 software (http://www.growthanalyser.org/). Weight SDS refers to the SDS for weight corrected for height. Target height was calculated with a correction for the secular trend as described previously [13]. Growth parameters at birth were corrected for gestational age using Swedish references [14].

SNP arrays

Genome-wide high density SNP array screening was performed with HumanCNV370-Duo Genotyping BeadChips (Illumina Inc., Eindhoven, The Netherlands). These chips contain ~318.000 SNP probes and ~52.000 non-polymorphic probes per chip, which results in a median spacing of 1 probe per 5 kb. Procedures were performed according to manufacturer's protocol (http://www.illumina.com). Fluorescence intensities were read with the BeadArray Reader (Illumina) and data-files were analyzed with the Beadstudio Data Analysis Software Version 3.2.

CNV validation

Copy-number polymorphisms were excluded with the Database of Genomic Variants (DGV; http://projects.tcag.ca/variation/). Detected CNVs were mapped to the human genome assembly (NCBI build 36.1) using the UCSC-genome browser (http://genome.ucsc.edu/). They were confirmed using a SNP-array from a different manufacturer, i.e. the GeneChip Human Mapping 500k array Set (Affymetrix Inc., Santa Clara, CA). These chips contain another set of SNP probes, either using the restriction enzyme *NspI* (~262.000 SNPs) or *StyI* (~238.000 SNPs), and are prepared and analyzed following different practical procedures and analysis methods. If available, parental DNA was analyzed as well using the Affymetrix-platform. Practical procedures were conducted following manufacturer's guidelines (http://www.affymetrix.com). SNP copy-number was assessed using CNAG v2 software [15].

X-inactivation study

X-inactivation was investigated at the Androgen Receptor locus as described previously [16].

Results

Genome-wide SNP array was performed in 26 patients with SoS features. In total we identified five deletions (patients 6, 39, 45, 54, 58) and one duplication (patient 49) which were not found in the DGV. The CNV plots are shown in Figure 5.2.1 and are

Table 5.2.1 Clinical scores for 26 patients diagnosed as having Sotos syndrome and the results of the molecular studies. Adapted from De Boer et al. [11] with permission from Karger AG, Basel.

	Patient[a]	Sex	Age (years)	Fac*	1	2	3	4	5	6	Gr[b]	Ba[b]	Hc[b]	Dev[b]	Sum[b]	NSD1	SNP array
Group 1	6	M	4.6	5	+	+	-	+	+	+	1	2	1	1	10	-	-
	7	M	5.8	5	+	+	+	+	+	+	1	2	1	1	10	-	-
	15	M	15.3	3	-		+	+	-	-	2	2	1	1	9	-	-
Group 2	24	M	3.8	3	+	-	+	-	+	+	0	2	1	1	7	-	-
	25	M	4.1	3	+	-	+	-	+	+	1	2	0	1	7	-	-
	30	M	5.6	3	-	+	-	+	+	-	0	2	1	1	7	-	-
	31	F	7.2	3	-	+	+	-	+	+	0	2	1	1	7	-	-
	32	F	9.2	3	-	+	-	+	-	-	0	2	1	1	7	-	-
	33	M	20.6	3	+	+	-	-	+	+	0	2	1	1	7	-	-
	37	M	1.8	3	-	-	-	+	+	+	0	2	0	1	6	-	-
	39	F	4.8	3	-	+	-	+	+	+	1	0	1	1	6	-	del(X)(q21.1q21.31)
	40	F	6.3	0	-		-	-	-	-	2	2	1	1	6	-	-
	41	M	10.7	3	-	+	-	+	-	-	0	2	0	1	6	-	-
	42	F	14.0	3	-	+	+	-	+	+	0	1	1	1	6	-	-
	43	M	36.3	3	-	+	-	-	+	+	0	1	1	1	6	-	-
	45	M	6.2	3	-	+	-	-	+	+	1	0	0	1	5	-	del(10)(p12.32p12.31)
	46	F	10.2	0	-	-	-	-	+	-	1	2	1	1	5	-	-
	49	M	48.4	3	-	+	+	-	-	-	0	1	0	1	5	-	dup(15)(q11.2q13.1)
Group 3	50	M	7.6	3	-	-	+	-	+	-	0	0	1	0	4	-	-
	51	M	10.1	0	-	-	-	-	-	-	2	1	0	1	4	-	-
	53	M	12.8	0	-	+	-	-	-	-	2	0	1	0	3	-	-
	54	F	5.7	0	-	+	-	-	-	-	2	0	0	0	2	-	-
	56	M	8.6	0	-	-	-	-	-	-	0	0	1	1	2	-	-
	57	M	8.8	0	-	-	-	-	-	-	0	0	1	1	2	-	-
	58	M	7.3	0	-	-	-	-	-	-	0	0	0	1	1	-	del(14)(q13.1)
	59	M	9.6	0	-	-	-	-	-	-	0	0	0	1	1	-	-

Group 1 = Typical Sotos syndrome; Group 2 = Dubious Sotos syndrome; Group 3 = Atypical Sotos syndrome; Fac = Facial characteristics (1 = down-slant palpebral fissures, 2 = high arched palate, 3 = prominent jaw, 4 = dolichocephaly, 5 = frontal bossing, 6 = high hairline); Gr = growth; Ba = Bone age; Hc = Head circumference; Dev = Development; Mut = mutation; Del = Deletion; Dup = Duplication; [a] Numbering of patients is according the original article of De Boer et al. [11]; [b] For the details about the marks used in the clinical scoring system we refer to De Boer et al. [11]

Table 5.2.2 Identified CNVs by genome-wide SNP arrray screening

Patient	Chromosome	SNP-start	SNP-end	Start-position	End-position	Size	Type	Genes	Inheritance
Possible pathogenic CNVs									
39	Xq21.1-q21.31	rs5912576	rs3126874	77400259	90759007	13.36Mb	Deletion	25	*de novo*
45	10p12.32-p12.31	rs10827906	rs11012086	20262098	20812000	550kb	Deletion	1	N.D.[a]
49	15q11.2-q13.1	rs34742996	rs8040604	20315855	26849239	6.53Mb	Duplication	20	N.D.
58	14q13.1	rs10872871	rs6571577	32456510	32611701	155kb	Deletion	1	mother carrier[b]
Likely non-pathogenic CNVs									
6	11p14.2	rs12275693	rs1442927	26971983	27197527	225kb	Deletion	2	maternal
54	1p22.1-p21.3	rs2747038	rs1041282	94007455	94705022	698kb	Deletion	5	father, paternal uncle and aunt

[a] N.D. Not Determined because parental DNA was not available; [b] Only maternal DNA available

Table 5.2.3 List of Refseq genes involved in the deleted or duplicated regions

Patient	Refseq Genes
Possible pathogenic CNVs	
39	CYSLTR1, ZCCHC5, LPAR4, P2RY10, GPR174, ITM2A, TBX22, FAM46D, BRWD3, NSBP1, SH3BGRL, POU3F4, CYLC1, RPS6KA6, HDX, APOOL, SATL1, ZNF711, POF1B, CHM, DACH2, KLHL4, CPXCR1, TGIF2LX, PABPC5
45	PLXDC2
49	TUBGCP5, CYFIP1, NIPA2, NIPA1, GOLGA8E, MKRN3, MAGEL2, NDN, C15orf2, SNRPN, SNURF, UBE3A, ATP10A, GABRB3, GABRA5, GABRG3, OCA2, HERC2, GOLGA8G, FLJ32679
58	NPAS3
Likely non-pathogenic CNVs	
6	FIBIN, BBOX1
54	DNTTIP2, GCLM, ABCA4, ARHGAP29, ABCD3

N.B. Genes are ordered according to their genomic position.

summarized in Table 5.2.2. Table 5.2.3 lists the genes involved per CNV. Based on the size, the *de novo* character and/or the genes involved, the chromosomal alterations identified in patients 39, 45, 49 and 58 were categorized as possible pathogenic.

The deletion in patient 6 was inherited from a phenotypically normal mother suggesting absence of a causal correlation between the deletion and the phenotype in the child. Furthermore, in patient 54 the father (possibly affected) and two of his siblings (normal phenotype) were carrier of the deletion detected. Therefore, the chromosomal imbalances detected in patients 6 and 54 were considered to be likely non-pathogenic and these two patients will not be described here.

In patient 39 a 13.36 Mb deletion was found encompassing Xq21.1-q21.31. The deletion involved 25 Refseq genes and occurred in the paternally derived chromosome. X-inactivation study showed bi-allelic activity, with the paternal chromosome being more active (71% versus 29%). Although chromosome analysis at the age of three had been described as a normal 46,XX karyotype, with the current techniques the deletion was visible in the repeated karyogram (data not shown).

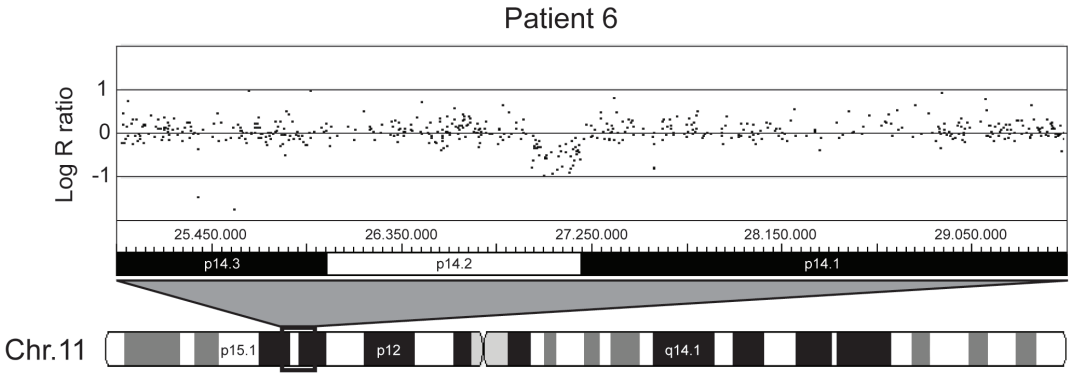
In patient 45 a 550 kb deletion was detected at 10p12.32-p12.31 deleting the last 12 exons, out of a total of 14 exons, of the Plexin Domain Containing 2 (*PLXDC2*) gene. *PLXDC2* encodes a transmembrane protein and is expressed in mouse brain, limbs, spinal cord, dorsal root ganglia, lung buds and heart [17]. Unfortunately parental DNA was not available to test inheritance. In the DGV, variants were reported in four individuals, including loss of exon 1 (variant 9140), loss of exon 2 (variant 2855), loss of exon 2 and 3 (variant 9141) and gain of exon 2 (variant 2855) [18;19].

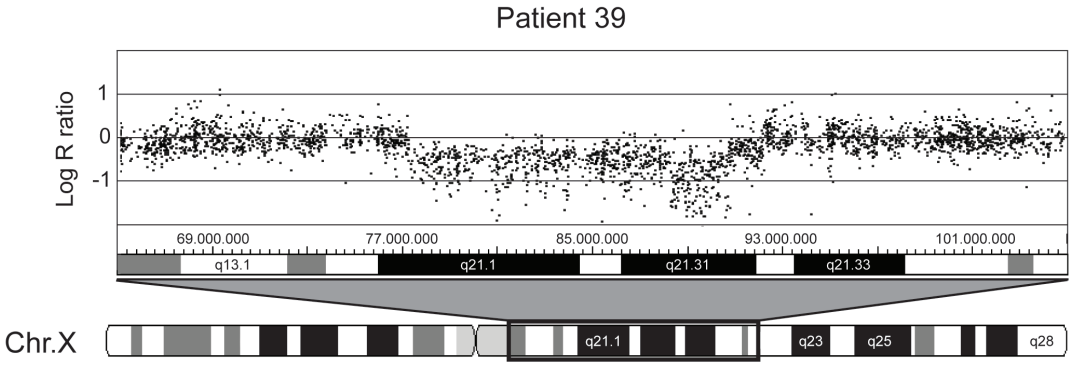
A 6.53 Mb-sized duplication was found in patient 49 encompassing 15q11.2-q13.1 and harboring 20 Refseq genes. The proximal and distal breakpoints were mapped within two known low copy repeats (LCRs) [20]. Non-allelic homologous recombination between LCRs at 15q11-q13 cause interstitial deletions which account for approximately 70% of the patients with Prader-Willi syndrome or Angelman syndrome [21]. Reciprocal duplications of 15q11-q13 occur less frequent and breakpoints are clustered in the same LCRs as Prader-Willi and Angelman syndromes: BP1 or BP2 for proximal and BP3 for distal breakpoints [22]. The same breakpoints, BP1 and BP3 respectively, were found in our patient

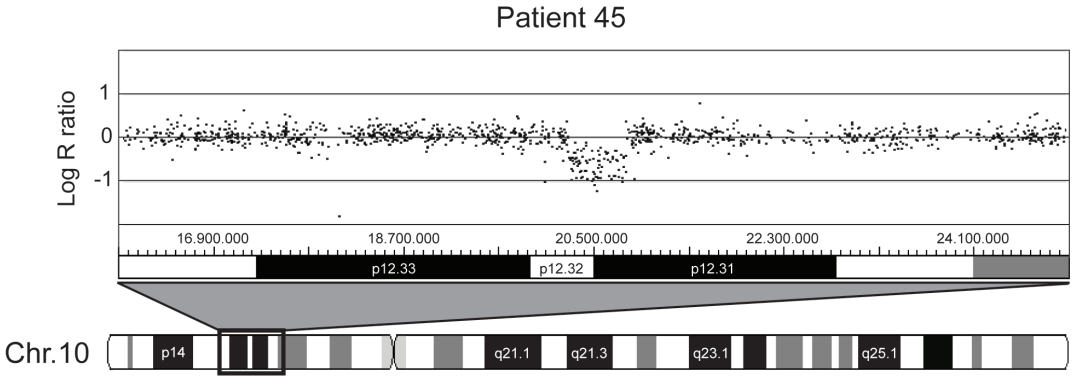
In patient 58 a 155 kb deletion of 14q13.1 was identified comprising the first exon of the Neural Pas Domain Protein 3 (*NPAS3*) gene, which was also detected in his mother. Two of his brothers were tested and both were confirmed to be non-carriers. To exclude compound heterozygosity, sequence analysis of *NPAS3* was performed in our patient but no pathogenic changes were detected (data not shown). *NPAS3* has two transcript isoforms (NM_022123 and NM_173159) which both include exon 1 and encodes for a neuronal transcription factor [23].

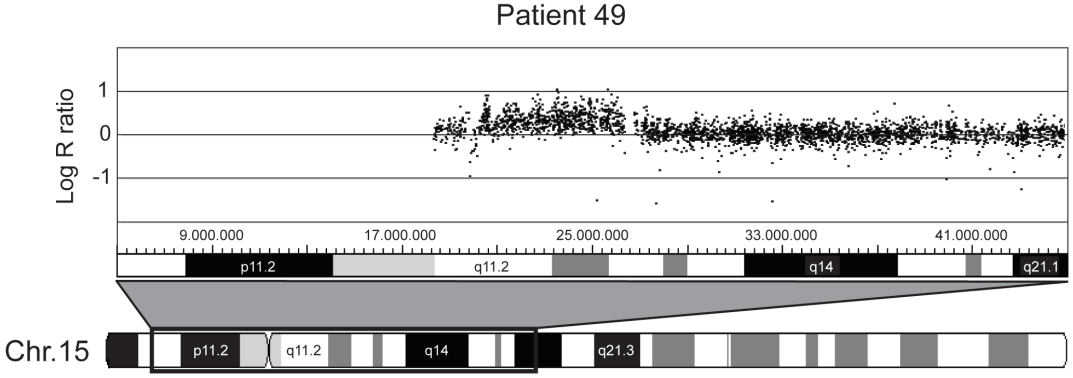
Discussion

Studies examining *NSD1* abnormalities in SoS patients have refined the clinical diagnostic criteria which has resulted in high detection rates for *NSD1* abnormalities in classical SoS patients. The diagnostic challenge nowadays lays therefore in the heterogeneous group of patients with a few phenotypic features of SoS, but without *NSD1* abnormalities. With a genome-wide approach four possible pathogenic CNVs were detected in patients previously diagnosed as “dubious” SoS (patients 39, 45, 49) and “atypical” SoS (patient 58) [11]. Phenotypic details and possible phenotypic expression of the genes involved are discussed per patient below.


Patient 6
Log R ratio
1
0
-1
25.450.000
26.350.000
27.250.000
28.150.000
29.050.000
p14.3
p14.2
p14.1
Chr.11
p15.1
p12
q14.1


Patient 39
Log R ratio
1
0
-1
69.000.000
77.000.000
85.000.000
93.000.000
101.000.000
q13.1
q21.1
q21.31
q21.33
Chr.X
q21.1
q23
q25
q28


Patient 45
Log R ratio
1
0
-1
16.900.000
18.700.000
20.500.000
22.300.000
24.100.000
p12.33
p12.32
p12.31
Chr.10
p14
q21.1
q21.3
q23.1
q25.1


Patient 49
Log R ratio
1
0
-1
9.000.000
17.000.000
25.000.000
33.000.000
41.000.000
p11.2
q11.2
q14
q21.1
Chr.15
p11.2
q11.2
q14
q21.3

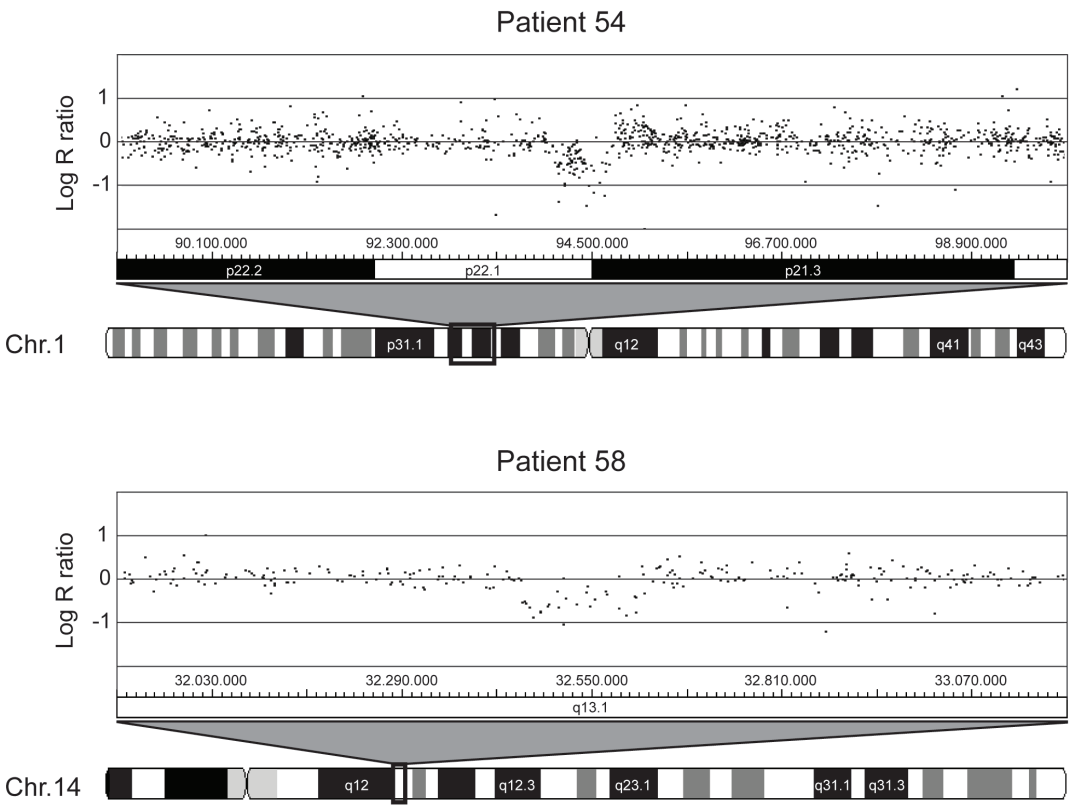


Figure 5.2.1 Copy-number analysis plots of the CNVs detected. The log R ratio's (Y-axis) of the SNP probes for the detected CNVs and their normal flanking regions are shown. A log R ratio of >0.3 or <-0.3 indicates a duplication or deletion respectively. Corresponding chromosomes, chromosomal bands and positions (in base pairs) are depicted below each CNV and are according the NCBI Build 36.1 genome assembly in the UCSC genome browser.

Patient 39

This female patient was born as the first child of healthy, non-consanguineous white Dutch parents at 35^{+2} weeks of gestation after premature rupture of membranes and gestational diabetes. At birth, weight was 3240 grams (+1.9 SDS) and length 50 cm (+1.5 SDS). Parental heights were 192.2 cm (+1.2 SDS) and 168.3 cm (-0.4 SDS) for the father and mother, respectively, which results in a target height of 178.2 cm (+1.2 SDS). Her motor milestones were within the normal range. Audiologic examination at 2.2 years of age was normal. At the age of 2.9 years, her height was 107 cm (+3.2 SDS) and occipital frontal circumference (OFC) was 54.4 cm (+3.4 SDS). She showed dysmorphic features including dolichocephaly, frontal bossing, parietal balding, a high, narrow palate (Figure 5.2.2A-C) and there was an advanced osseous maturation of 6 months. Two IQ-tests at around three years of age showed an IQ of 57 and 74. Concerning her family history, her father's growth pattern and head circumference were reportedly similar, although his OFC measured 60 cm (+1.4 SDS). Two younger siblings of the proband showed normal development. Our patient was last seen at the age of 9.4 years, with a height of 152.4 cm (+2.0 SDS), weight 49.5 kg (+1.2 SDS) and OFC 57.5 cm (+3.0 SDS). She receives special education. During a recent routine check-up for amblyopia, abnormalities of the retina were detected and retinitis pigmentosa was suspected.

An Xq21.1-q21.31 deletion with a size of 13.36 Mb was detected encompassing 25 genes. In literature several genes in this region have been associated with phenotypic expression. Firstly, deletions of Xq21 in male patients were reported to cause a contiguous gene syndrome including choroideremia (*CHM* gene), mental retardation (possibly *RPS6KA6*) and X-linked deafness type 3 (*POU3F4* gene) [24]. In our patient audiologic testing at the age of two was normal and presently she had no apparent hearing loss. However, recently retinal abnormalities were found which might be the first signs of choroideremia. The variable phenotypic expression of deleted genes in our patient could be explained by the incomplete skewed X-inactivation.

Secondly, *BRWD3* is another gene possibly related to our patient's phenotype, because loss of function mutations were identified in four male patients with X-linked mental retardation, macrocephaly and dysmorphic features [25]. Interestingly, one of them showed also tall stature (> +1.9 SDS) and his affected uncle was tall with a final height of +1.3 SDS. Thirdly, a gene which might also be associated with tall stature, is ITM2A. This gene encodes a transmembrane protein which has been shown to be involved in chondrogenesis and a significant association with height was recently found in a genome-wide association study [26;27].

Thus, although the implication of multiple genes complicates linking individual genes to the phenotypic features in our patient, the deleted region contains likely candidate genes explaining the retinal alterations, overgrowth and mental retardation.

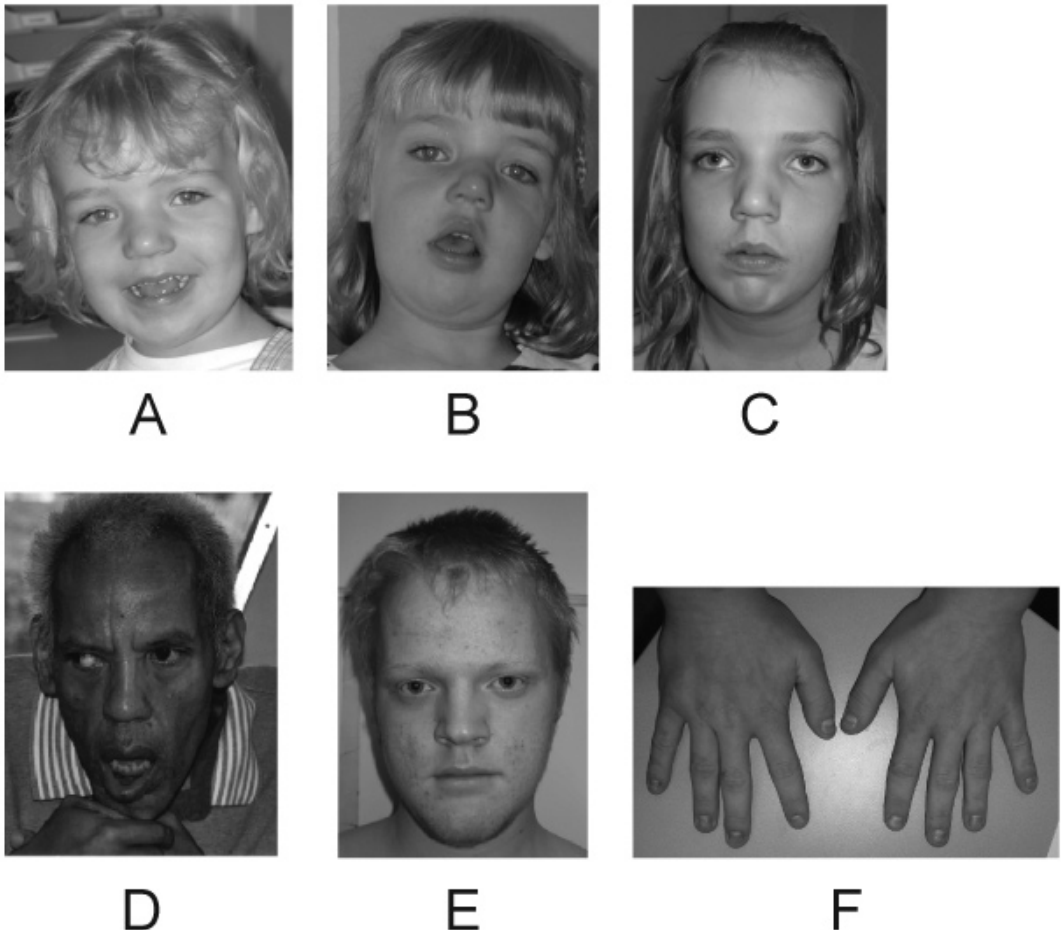


Figure 5.2.2 Facial features of the patients carrying a possible pathogenic CNV. Written permission for publication of the photographs was obtained. For patient 45 photographs were not available. (a-c) Patient 39 at the age of 2.9, 4 and 9.4 years respectively. (d) Patient 49 at the age of 51 years. (e) Patient 58 at the age of 15.8 years. (f) Hands and fingers of patient 58.

Patient 45

This male patient was born as the first child of healthy, non-consanguineous white Dutch parents at a gestational age of 35^{+3} weeks with a birth weight of 3500 grams (+2.2 SDS). Mother was hospitalized for preecclampsia, but the delivery was uneventful. Parental heights were 185.0 cm (+0.2 SDS) and 179.7 cm (+1.4 SDS) for the father and mother, respectively which results in a target height of 193.4 cm (+1.3 SDS). Although his motor development milestones were normal, his gross and fine motor skills were assessed as delayed during childhood. He spoke his first words at the age of 30 months and talked complete sentences at the age of 60 months. For this language and speech development delay he attended special education, after which he proceeded to a mainstream elementary school. During childhood he had an increased statural growth near the +2.5 SDS curve. Bone age at the age of 2.5 was conform calendar age. At the age of 6.2 years his height was 133.1 cm (+2.4 SDS), weight 29.9 kg (+0.6 SDS), OFC 55 cm (+1.9 SDS) and arm span 131.5 cm (+2.0 SDS). His facial features included frontal bossing, a receding hairline and a high arched palate. A simian crease was found in his right hand. His IQ was tested to be 84. Chromosome analysis showed a normal male 46,XY karyotype.

A small deletion was found, affecting the last 12 exons out of 14 exons of *PLXDC2*. A patient (patient KK) with a 4.3 Mb-interstitial deletion of 10p12.1-p12.31 was described, including amongst others *PLXDC2* in the deleted region [28]. This patient showed also overgrowth with height, weight and OCF > +1.6 SDS, mild dysmorphic features and a delay in language development [28]. Patients with larger interstitial deletions or 10pter deletions have been reported but they showed postnatal growth retardation [29]. In addition to the much larger size of the deletion, involvement of *PLXDC2* in these deletions could not always be confirmed (Figure 5.2.3).

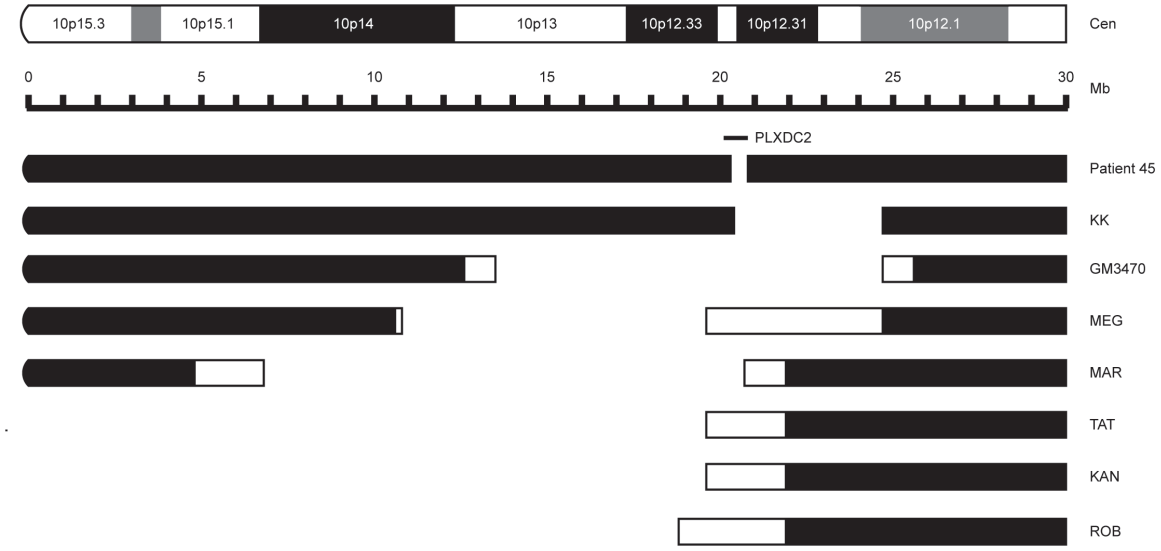


Figure 5.2.3 Schematic representation of reported patients with deletions of the short arm of chromosome 10. On top, the chromosomal bands of 10p are shown in scale with the physical distance in Mb according the NCBI Build 36.1 genome assembly in the UCSC genome browser (below). The deletions of patient 45, KK, GM3470 and MAR comprise *PLXDC2* (horizontal black line), while the deletions of patients MEG, TAT, KAN and ROB might include *PLXDC2*. For each patient, horizontal black bars depict non-deleted chromosomal regions. White bars represent the undetermined breakpoint regions and the blank spaces in between the deleted region. Mapping data for patient KK was obtained from [26] and for patients GM3470, MAR, MEG, TAT, KAN and ROB from [27] and references therein.

Due to the missing parental DNA for inheritance studies and the detection of variants within *PLXDC2* in four control individuals [18;19], the contribution of haploinsufficiency of *PLXDC2* to a clinical phenotype remains equivocal. However, all the more since growth and height data of the four control individuals is lacking, phenotypic expression should be considered as well. The findings in our patient suggest then that *PLXDC2* is a candidate gene to be involved in growth regulation and/or intellectual development.

Patient 49

This male patient of Indonesian descent was first seen at the age of 45.2 years. His height was 1.78 cm (-0.8 SDS), weight 78.5 kg (+0.3 SDS) and OFC 60 cm (+1.4 SDS). The patient presented with a long face, a prominent jaw, a high arched palate and strabismus divergens (Figure 5.2.2D). He had a thoracic kyphosis and scoliosis. He was institutionalized for his moderate to severe mental retardation. In his forties he developed periods of depression followed by severe behavioural problems such as screaming, restlessness and aggression. His medical history showed hypertension and Parkinsonism and his family history was normal. Chromosome analysis revealed a normal 46,XY karyotype. The patient died at the age of 52, due to complications after surgery for a femoral fracture.

A duplication of 15q11-q13 was identified, which is a region containing imprinted and non-imprinted genes. The clinical phenotype of the 15q11-q13 duplication is variable even within families and is associated with varying levels of mental retardation and problems in motor coordination [30]. On physical examination, hypotonia, decreased deep tendon reflexes and joint laxity are frequently found. Distinct dysmorphic features are not present, although an antimongoloid-slant of the eyes and thick or pouting lips have been described [30]. An association with autism spectrum disorders has been postulated, but this is not a common feature [30;31]. Paternal inherited duplications have been reported to associate with a normal or less severe phenotype [30;32], but affected patients have been reported as well [22;33;34]. Considering the phenotypic features associated with 15q11-q13 duplications, it is likely that the chromosomal imbalance in our patient is the cause of his mental retardation and behavioural problems.

Patient 58

This male patient was born as the 7th child of healthy non-consanguineous white Dutch parents at 42 weeks of gestational age after an uneventful pregnancy. At birth, weight was 3550 grams (-0.5 SDS) and length 54 cm (+1.2 SDS). His target height was 186 cm (+0.3 SDS). Although he was hypotonic for which he received physiotherapy from 7 till 14 months of age, his motor development milestones were within normal range. He followed two years of mainstream elementary school, but continued with special education. His IQ was tested to be 76 at the age of 7.3 years. He was last seen at the age of 15.8 with a height of 180.3 cm (+0.3 SDS), weight 84 kg (+1.9 SDS) and OFC 57.4 cm (+0.6 SDS). He did not show any major dysmorphic features, although he had short fingers and a simian crease in his left hand. His father died at the age of 40 years, due to a ruptured abdominal aneurysm. Both his father and mother attended normal elementary schools and followed lower secondary education. He had nine siblings who were healthy, but learning problems were prevalent. There was no history of psychiatric problems.

A small deletion of the first exon of *NPAS3* was identified and his mother was confirmed to be a carrier. Previously, a mother and daughter have been described who carried a balanced reciprocal translocation t(9;14)(q34;q13) disrupting *NPAS3* on chromosome 14 without disrupting genes on chromosome 9 [35]. The mother showed mild learning disability and was diagnosed with schizophrenia [36]. The daughter had severe learning impairment and suffered from schizophreniform psychosis [35]. Additionally, a recent study revealed four *NPAS3* related haplotypes which were associated with bipolar disorder and schizophrenia [37]. The association of *NPAS3* with these psychiatric disorders remains subject for discussion since our patient and mother did not show signs of psychiatric illness. Yet, there is still a possibility that our patient will develop schizophrenia since the onset of schizophrenia in males is usually in their late teens and early twenties [38]. Another possibility is that due to the translocation, the disruption of *NPAS3* resulted in a dominant negative effect and therefore caused a more severely affected phenotype [36]. More pronounced behavioural abnormalities were also seen in homozygous knock-out mice, while only a non-significant trend was observed in the heterozygous mutants [39]. Compound heterozygosity in our patient was excluded.

The findings in our patient support the postulated association of *NPAS3* with intellectual development, although admittedly the intelligence of the mother of our patient was borderline normal. In contrast, they are not in support of a causative relation of *NPAS3* with psychiatric disorders and more patients with *NPAS3* abnormalities are necessary to elucidate this association.

Conclusion

A genome-wide SNP array analysis detected four possible pathogenic CNVs in 26 (15%) patients with features of SoS. This approach is therefore a powerful method to provide a molecular explanation in patients with features of SoS but without *NSD1* alterations, although still molecular validation is required to establish a definite causal relationship. A candidate gene for overgrowth (*PLXDC2*) was identified and a deletion of *NPAS3* in two carriers without psychiatric illness warrants caution of linking this gene to schizophrenia. Screening of these genes in larger patient populations is necessary in order to further delineate the associated phenotype.

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Chapter 6

Discussion

Conventional karyotyping by G-banding has been in use since the 1970s as the standard technique in many laboratories to detect chromosomal aberrations. With this technique it is possible to identify chromosomal rearrangements of at least 5-10 Mb. Other techniques have been developed to detect smaller aberrations. These methods, such as Fluorescence *in situ* Hybridization (FISH), Multiplex Ligation-dependent Probe Amplification (MLPA) and Quantitative Fluorescence-Polymerase Chain Reaction (QF-PCR), are useful only for identifying microdeletions and subtelomeric regions or for confirming and further characterizing previously identified chromosomal aberrations. In recent years a technique to screen the whole human genome in a single experiment, the array Comparative Genome Hybridization (aCGH), has been developed. It enables the detection of copy number variants (CNVs) (deletions and duplications) that are approximately 100 times smaller than those that can be identified by conventional karyotyping. The first genome-wide CGH arrays were based on large DNA fragments such as bacterial artificial chromosomes (BACs) (Solinas-Toldo et al., 1997; Pinkel et al., 1998). The resolution of these arrays depends on the size of the probes. Other CGH arrays were developed with small oligonucleotide probes (45-85 - mer) (Agilent and Nimblegen) that allowed the detection of smaller CNVs. More recently, the Single Nucleotide Polymorphism (SNP) array, which was originally developed for genome wide association studies, turned out to be ideal also for the detection of CNVs. These oligonucleotide SNP arrays (25-50 - mer) (Affymetrix and Illumina) are based on probes containing a SNP. With this oligonucleotide array technique (both aCGH and SNP) the resolution has increased to approximately 10 - 100 kb and depends on the genomic spacing between the probes.

Mental retardation (MR) occurs in 2-3% of the population and in approximately 5% of these patients a chromosomal aberration can be detected by conventional karyotyping (de Vries et al., 2005). In contrast, the overall yield of CNVs detected by high-resolution array (aCGH and SNP) in MR patients is approximately 17% (Vissers et al., 2003; Shaw-Smith et al., 2004; Schoumans et al., 2005; Tyson et al., 2005; Friedman et al., 2006; Menten et al., 2006; Rosenberg et al., 2006; Fan et al., 2007; Hoyer et al., 2007). We studied 318 MR patients using high-resolution SNP arrays in order to establish the frequency of submicroscopic CNVs in patients with idiopathic MR (**chapter 2**). In this cohort we identified different chromosomal aberrations in 22.6% of the MR patients. It should be noted, however, that most of the above mentioned reports, including our study, represent highly selected patient cohorts. In all these patients conventional karyotyping had been performed already and in a smaller group of patients also locus-specific FISH and subtelomeric MLPA had been done, however no aberration was detected. Testing every MR patient with a SNP array instead of conventional karyotyping would further increase the diagnostic power of this method and we have, therefore, recommended it as a new diagnostic tool to be included in the workflow for MR patients (**chapter 2**). At present, high-resolution array is being successfully implemented in routine clinical diagnostic laboratories. It allows the screening for all known microdeletion and microduplication syndromes as well as novel CNVs in a single experiment. As a consequence, fewer diagnostic tests are needed to facilitate a rapid diagnosis in many patients.

A major advantage of the SNP array is that it provides information on the genotype and enables the detection of regions of loss of heterozygosity (LOH) (**chapter 2**). LOH can be the result of a deletion or of uniparental disomy (UPD), which is known to cause genomic disorders such as Prader-Willi syndrome and Silver-Russel syndrome.

With a SNP array it is possible to distinguish between heterodisomy and isodisomy if both parents are hybridized on an array. Furthermore the genotype information can be used to identify homozygous regions in offspring from consanguineous parents, to resolve questions concerning mix-up of samples and paternity, and to determine the parental origin of a deletion. An additional advantage of the genotype information is that it serves as an extra control step to confirm a deletion or a duplication. In case of a deletion all SNPs appear homozygous because of loss of one allele; duplications show four possible genotypes, including AAA, AAB, ABB and BBB.

Although it was originally suspected that array analysis would not be able to detect mosaicism, it appears that the aCGH and SNP array techniques are actually more sensitive in detecting low-level mosaicism than conventional karyotyping (**chapter 2** and **3.4**). Usually, an insufficient number of cells is counted unless mosaicism is suspected. Also, a single abnormal cell might be interpreted as an artifact of cell culture. So, one can easily fail to detect an aberrant subset of cells with conventional karyotyping. Furthermore, as shown in **chapter 2**, **4.2** and **5.1**, the array technique is more sensitive in detecting unbalanced translocations. Relatively large aberrations (3 – 21 Mb) are missed by conventional karyotyping because telomeric bands of many chromosomes are similar in appearance.

In **chapter 3.2** and **4.1** we have shown that the SNP array is useful in characterizing previously detected microscopically visible chromosomal rearrangements. In **chapter 3.2** we have described apparently balanced translocations and inversions where the SNP array detected additional CNVs. These CNVs were either at the breakpoints of the rearrangement or were on chromosomes unrelated to the previously detected aberration. In **chapter 4.1** we have presented a patient where conventional karyotyping detected extra material of unknown origin on the long arm of chromosome 9. SNP array analysis detected a duplication of 400 kb, a triplication of 2.4 Mb and a duplication of 130 kb of chromosome band 9q34.3. In this case, the SNP array proved to be an accurate method for the identification and delineation of the chromosomal rearrangement. As there is no technique available at present that can detect complex chromosome rearrangements as well as cryptic CNVs in one experiment, conventional karyotyping, FISH and high-resolution array screening remain essential for unravelling complex karyotypes.

The disadvantage of using arrays instead of conventional karyotyping is that arrays cannot detect balanced rearrangements. A large prenatal study has shown that approximately 0.5% and 0.1% of the antenatal cases carry an apparently balanced reciprocal translocation or an inversion respectively (Warburton, 1991). Only 6% of these cases are associated with abnormal phenotypes. The abnormal phenotype can be caused by the disruption of a gene at the breakpoint or by a small duplication or a deletion that is beyond the resolution of the microscope. The SNP array analysis would (depending on the resolution) detect the small abnormalities, though the disruption of genes would remain undetected. A Dutch retrospective study has shown that of all referrals only about 0.78% potentially pathogenic balanced rearrangements would remain undetected by array analysis without conventional karyotyping (Hochstenbach et al., 2009). Conventional karyotyping, on the other hand, will miss a much higher percentage of unbalanced rearrangements if no array analysis is performed.

An ideal technique would combine whole-genome high-resolution screening for CNVs and the detection of inversions, insertions and translocations. This is possible with paired-end mapping, a new sequencing method, whereby aberrations from

approximately 3 kb can be identified (Korbel et al., 2007). It involves fragmentation of genomic DNA to 3 kb fragments; these fragments are circularized and randomly sheared. These products are sequenced and mapped back to the reference sequence. Paired-end mapping has already been used successfully in breakpoint mapping of balanced chromosome rearrangements (Chen et al., 2009). However, experience with this technique in whole-genome screening is limited. More importantly, the costs are high and the process is very laborious. A rapid and routine implementation of this technique is therefore not to be expected soon.

Finally, another example of the power of high-resolution arrays is given in **chapter 5**. Two studies in which the SNP arrays were used to detect CNVs in patients with phenotypes other than MR are presented. In **chapter 5.1** we have reported the detection of CNVs in 33% of patients with a BPES-like phenotype. In **chapter 5.2** we have used high-resolution SNP arrays to detect CNVs in patients with features of the SOTOS syndrome. We found chromosomal aberrations in 15% of these SOTOS-like patients.

The major recurring theme in this thesis is that the clinical consequence of novel CNVs is not always immediately evident in many cases (**chapter 2**, **3.1**, **3.2**, **3.3**, **5.1** and **5.2**). High-resolution array studies have revealed many new microdeletion and microduplication syndromes (reviewed by Slavotinek, 2008). There are other recurrent deletions and duplications for which the clinical significance is not immediately clear. A good example of the difficulty in assigning clinical significance is discussed in **chapter 3.1**. A ~600 kb 16p11.2 deletion was initially detected in patients with autism, but later it was seen also in patients with MR, in healthy individuals, and finally in obese patients (Walters et al., 2010). This suggests that the recurrent 16p11.2 deletion is associated with a variable outcome and it is even uncertain whether it is pathogenic. There are other well known microdeletion syndromes with a wide phenotypic spectrum (e.g. 22q11.2) (Edelmann et al., 1999). Since geneticists often assume that only *de novo* CNVs are pathogenic, one of the first steps in a routine diagnostic workflow is to check the parents for transmission of a CNV. Besides, it has been shown already that patients with known syndromes can inherit this deletion from one of their healthy parents (Edelmann et al., 1999). The cause for this phenotypic variability is unknown. Several hypotheses have been proposed to explain the variable effect of deletions: stochastic variation of gene expression at a lower level (Cook et al., 1998), presence of modifying genes on the other undeleted homologue as was demonstrated recently at the locus for adult polycystic kidney disease, *PKD1* (Rossetti et al., 2009), influence of unlinked genes or epigenetic factors that may play a role. Recently, Girirajan and colleagues suggested a two-hit model for a recurrent 16p12.1 microdeletion (Girirajan et al., 2010). They found that 30% of the affected individuals with a 16p12.1 microdeletion carried a second CNV. These results show for the first time two independent chromosomal changes in a mentally retarded patient, confirming the multifactorial model proposed decades ago. With the increasing resolution of genome analysis similar examples are likely to follow soon. The big challenge, however, is to prove the pathogenicity of each CNV or genomic variant, which is becoming more difficult with the increasing complexity. To gain better insight in the pathogenicity of these CNVs large numbers of patients and their families need to be analyzed in great detail.

Even before the introduction of G-banding it was clear that two chromosomes of a pair were not always alike. As most variants were found in individuals with a clinical abnormality, it was not until large newborn studies that it became evident that

these variants were benign (reviewed by Wyandt, 2004). With the introduction of the whole-genome high-resolution screening technique, a similar problem has appeared; this technique is able to detect smaller abnormalities and thereby also new variants of unknown clinical relevance (**chapters 2** and **3**). It has been estimated that up to 12% of the human genome is involved in CNVs (Redon et al., 2006). Large studies on healthy individuals have revealed many new benign CNVs which are collected in the Database of Genomic Variants (DGV) (Iafrate et al., 2004; Sebat et al., 2004; Sharp et al., 2005; Tuzun et al., 2005; Feuk et al., 2006; Friedman et al., 2006; Hinds et al., 2006; Locke et al., 2006; McCarroll et al., 2006; Redon et al., 2006; Conrad and Hurles, 2007). This database is a valuable tool for geneticists for comparing CNVs that they find in their patients. Although this database is still growing, many rare CNVs identified in MR patients are not reported. As more data becomes available in the next few years, it will become clear whether CNVs that are at present classified as potentially pathogenic are pathogenic or not. Databases like DECIPHER and ECARUCA, which collect CNVs detected in patients can help unravel novel disease-causing CNVs much faster.

In March 2010 the DGV contained 14478 CNV loci and is still regularly updated. The data submitted to this database are not subjected to an editorial screening, the only requirements being that the data is published as a scientific manuscript and that the CNV was identified in a non-disease control sample. The contributors are therefore able to use different methods with a variety of detection rates, error rates, and genomic coverage. In some contributing studies no distinction is made between gains and losses; these CNVs should therefore be excluded for comparison. Caution should be exercised in the interpretation of the submitted CNVs.

Manufacturers producing high-resolution array techniques are developing new arrays with even higher resolution. Recently we have tested the latest available 2.7M (Affymetrix) array. These arrays contain 2.7 million markers, including 400.000 SNP probes and 2.3 million CNV probes. Not only the resolution but also the laboratory procedures are constantly being improved. In diagnostic laboratories, procedures with fewer steps and more automation are highly encouraged, since this means less hands-on time and less possibility of sample mix-up. However, a higher resolution will, per definition, also detect more CNVs of unknown clinical significance.

Next generation sequencing technologies will eventually replace the array technologies in the genetic diagnostic flow of MR. Sequencing will be able to detect mutations as well as deletions, duplications, inversions and translocations. At present, sequencing of whole genomes is feasible and in a few years also affordable; however, analysis of the data is not yet suitable for a large scale. Bioinformaticians will be necessary to solve this problem. Implementation of sequencing will furthermore introduce the same problems on an even larger scale, as the whole-genome high-resolution arrays do at present. Large numbers of new variants will be identified, most of which are probably not disease causing and many others that may be associated with other unexpected diseases. The challenge to distinguish between harmless variants and variants causing or contributing to disease will become even more daunting.

In the series of articles presented in this thesis we have studied patients with MR and/or congenital malformations. The question we have tried to answer by studying the genome of the patient is whether we can find a cause for the signs and symptoms observed in the patient. The SNP array was successfully used for the detection of novel CNVs and has replaced the conventional karyotyping in the routine diagnostic flow in

MR patients in our diagnostic setting. We are therefore able to make a diagnosis in a higher number of MR patients, thus improving medical care and genetic counselling. However, a major complexity is the finding of potentially pathogenic CNVs for which the clinical significance is not immediately clear.

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Summary
Samenvatting

Summary

Conventional karyotyping has been used as the standard cytogenetic technique since the 1970s. With conventional karyotyping one can detect aberrations larger than 5 – 10 Mb. To identify smaller copy number variants (CNVs) new molecular cytogenetic techniques have been developed. One of these techniques is the array Comparative Genome Hybridization (aCGH), which is based on the hybridization of different fluorescent labelled patient DNA and reference DNA to immobilized DNA fragments. The first arrays contained relatively large DNA fragments (100-300 kb) and the resolution depended on the size of the probes. Other arrays were developed containing oligonucleotide probes of 25 to 60 bp, oligonucleotide aCGH and Single Nucleotide Polymorphism (SNP) arrays. The resolution of these arrays does not depend on the size of the probes but on the number of probes on a slide. The SNP array, which was initially developed for linkage and association studies, is now widely used for the detection of submicroscopic CNVs and regions of Loss of Heterozygosity (LOH).

With conventional karyotyping one can detect chromosomal aberrations in approximately 5% of patients with mental retardation. In **chapter 2** we have described the use of SNP arrays for the detection of submicroscopic CNVs in patients with idiopathic mental retardation (MR). In total, 318 patients were screened using different array platforms. Aberrations, including known syndromes, potentially pathogenic CNVs, large regions of LOH and mosaic trisomies, were found in 22.6 % of the patients. This study has demonstrated that high-resolution whole-genome array techniques have a higher diagnostic yield as compared to conventional karyotyping. However, it has also shown that the finding of many potentially pathogenic CNVs makes diagnosis difficult and it will probably be many years before we can determine which CNVs are pathogenic and which are benign. We have implemented the SNP array in our diagnostic setting and we now use it as the first step, instead of conventional karyotyping, for analysing MR patients.

In **chapter 3.1** we have further delineated a previously found recurrent 16p11.2 deletion. Deletions of this region were initially identified in a cohort of patients with autism. We identified 13 patients with the same deletion in a group of 4284 MR patients. Some of the healthy parents were carriers of the same CNV. These findings suggested that the recurrent 16p11.2 deletion is associated with a variable phenotype.

In **chapter 3.2**, 13 MR patients with a previously identified apparently balanced translocation or inversion were analyzed with the SNP array. Disruption of genes at breakpoints of such rearrangements, or the presence of small CNVs around the breakpoints or elsewhere in the genome can be the cause of the MR. We detected additional CNVs in eight patients; in three patients these were near the breakpoints of the rearrangement and in five patients the CNVs were unrelated to the balanced rearrangement. With this data we have demonstrated that it is necessary to use conventional karyotyping in combination with high-resolution array screening to unravel complex karyotypes.

In **chapter 3.3** we have reported five mentally retarded males with a CNV on the X-chromosome. We were able to classify the CNVs as pathogenic in only two of the patients but it was difficult to determine pathogenicity for the other relatively small duplications. To be able to classify all of them we need to study more patients or healthy individuals with (approximately) the same CNVs.

In **chapter 3.4** three cases of mosaicism with a normal cell line and an unbalanced autosomal reciprocal translocation are described. Mosaicisms with such

cell lines are very rare. The exact mechanism responsible for these karyotypes is not clear. We have demonstrated that high-resolution whole-genome arrays can reveal more mosaicisms, unbalanced translocations or a combination of both.

In **chapter 4** we have described the use of SNP arrays in unravelling complex chromosome aberrations. In **chapter 4.1** we determined the size and nature of the extra material that had been detected by conventional karyotyping on chromosome 9. Fine mapping by SNP array analysis identified a 400 kb duplication, 2.5 Mb triplication and 130 kb duplication of 9q34.3. This highlights the value of combining conventional karyotyping with the array technologies. In **chapter 4.2** a familial translocation was detected by SNP array analysis and FISH. Two sibs inherited different derivative chromosomes and both showed different phenotypes. The index patient had MR and some minor dysmorphic features, while her brother was apparently healthy. This chapter underscores the importance of reporting chromosomal imbalances, even if they are found in individuals with an apparently normal phenotype.

That the SNP array is not only useful for the detection of CNVs in MR patients is shown in **chapter 5**. Blepharophimosis- Ptosis- Epicanthus inversus syndrome (BPES) is a rare syndrome that includes an eyelid malformation. Mutations in the *FOXL2* gene are responsible for the majority of BPES cases. However, in a small group of patients the cause remains unknown. In 9 out of 27 patients with a BPES-like phenotype we identified CNVs that had not been previously identified (**chapter 5.1**). Sotos syndrome includes overgrowth, facial dysmorphism and learning impairment. In 60 – 90 % of the Sotos patients haploinsufficiency of the *NSD1* gene is responsible for the phenotype. In 4 out of 26 patients with a Sotos-like syndrome (without a mutation in *NSD1)*, we identified a CNV that had previously not been identified (**chapter 5.2**). The findings of these two studies indicate that the whole-genome screening is a powerful technique to search for new candidate genes in different diseases.

In **chapter 6**, the findings of the work described in this thesis are discussed and future perspectives are presented. The identification of novel disease causing CNVs in MR patients has increased the possibilities for MR diagnosis and genetic counselling. Yet, the finding of a high number of potentially pathogenic CNVs introduces a major challenge. It is likely that the next generation sequence technologies will eventually replace the array technique and will pave the way towards the identification of more new MR disease genes and disorders.

Samenvatting

Conventionele karyotypering met behulp van de lichtmicroscoop is de standaardtechniek die sinds de jaren '70 gebruikt wordt voor het detecteren van chromosomale afwijkingen bij patiënten met een verstandelijke beperking (mentale retardatie) en aangeboren afwijkingen. Een voordeel van deze techniek is dat alle chromosomen in een celkern in één microscopisch veld zichtbaar gemaakt kunnen worden en met elkaar vergeleken kunnen worden. Echter, een belangrijk nadeel van deze techniek is de lage resolutie. Een afwijking in een chromosoom moet namelijk wel rond de 5 – 10 miljoen bouwstenen (baseparen) omvatten om zichtbaar te zijn door de microscoop. Conventionele karyotypering kan in ongeveer 5% van de patiënten met mentale retardatie een chromosomale afwijking (verdubbeling (duplicatie) of verlies (deletie) van gehele of delen van chromosomen) detecteren. Een techniek die kleinere afwijkingen kan opsporen is de Fluorescente *In Situ* Hybridisatie (FISH), deze techniek wordt gebruikt voor het opsporen van een specifieke deletie en kan alleen gebruikt worden bij verdenking van een klinisch syndroom. Een test die alle uiteinden van de chromosomen kan analyseren is de Multiple Ligation-dependent Probe Amplification (MLPA). Beide technieken lenen zich echter niet voor analyse van het gehele genoom.

Om kleinere chromosomale afwijkingen, de zogenaamde varianten in kopieaantal, op te sporen in het hele genoom zijn er in de loop der jaren verschillende nieuwe technieken ontwikkeld. Eén van deze technieken is de Single Nucleotide Polymorphism (SNP) array. Deze techniek combineert de drie bovengenoemde onderzoeken in één experiment. Met de SNP array kan naar honderdduizenden plekken verdeeld over alle chromosomen gekeken worden.

De array gebaseerde techniek wordt tegenwoordig wereldwijd gebruikt voor het opsporen van varianten in kopieaantal bij patiënten met mentale retardatie en vervangt al in vele laboratoria de hierboven genoemde technieken (conventionele karyotypering, FISH en subtelomeren MLPA) bij deze groep patiënten.

In **hoofdstuk 2** van dit proefschrift beschrijven we het gebruik van de SNP arrays voor het opsporen van varianten in kopie aantal in 318 patiënten met onverklaarde mentale retardatie. In 22.6 % van deze patiënten werd een verandering gedetecteerd, waaronder bekende syndromen, mogelijk ziekteveroorzakende verschillen in kopieaantal, grote gebieden met verlies van heterozygositeit en mozaïek trisomieën. Deze studie toont aan dat hoge-resolutie genoom-brede array technieken een hogere diagnostische opbrengst hebben dan de conventionele karyotypering. Deze bevinding is in overeenkomst met de literatuur. Echter, deze studie laat ook zien dat het vinden van mogelijk ziekteveroorzakende varianten in kopieaantal het stellen van een diagnose moeilijk kan maken en het in veel gevallen waarschijnlijk jaren gaat duren voordat duidelijk wordt of de variant ziekteveroorzakend is of niet. In ons laboratorium is de SNP array ingevoerd als diagnostische test en wordt gebruikt als eerste test, in plaats van conventionele karyotypering, bij patiënten met mentale retardatie en/of aangeboren afwijkingen.

De array techniek maakt het mogelijk om grote aantallen patiënten op genoom-brede schaal te onderzoeken, waardoor de kans op het ontdekken van nieuwe syndromen verhoogd is. In de literatuur verscheen een nieuwe microdeletie, die autisme zou veroorzaken, in de korte arm van chromosoom 16, band 16p11.2 In **hoofdstuk 3.1** beschrijven wij dat deze deletie ook voorkomt bij patiënten met mentale retardatie. Wij verzamelden 13 patiënten met deze deletie in een groep van 4284 mentale retardatie patiënten. Bij zeven van de 13 patiënten is een gezonde ouder

drager van dezelfde deletie. Deze bevindingen laten zien dat het maken van een genotype-fenotype correlatie niet eenvoudig is en suggereren dat de 16p11.2 deletie geassocieerd is met een variabel fenotype inclusief autisme, mentale retardatie, en een normaal fenotype.

Op SNP arrays werd DNA van dertien patienten getest bij wie tijdens conventionele karyotypering een ogenschijnlijk gebalanceerde reciproke translocatie of inversie was gevonden, **hoofdstuk 3.2**. Verstoring van genen rond de breukpunten van deze afwijkingen, of de aanwezigheid van kleine varianten in kopie aantal rond het breukpunt of ergens anders op het genoom kunnen de oorzaak zijn van de mentale retardatie bij deze patiënten. We ontdekten varianten in acht patiënten; in drie waren deze rond de breukpunten van de translocatie of inversie en in vijf waren er deleties of duplicaties ergens anders in het genoom aanwezig. Hiermee toonden we aan dat de combinatie van conventionele karyotypering en hoge-resolutie arrays nodig blijft om complexe karyotypes te ontrafelen.

In **hoofdstuk 3.3** beschrijven we zeven varianten, deleties en duplicaties, gevonden op het X-chromosoom van mannen met mentale retardatie. Alleen voor twee van deze varianten kon vastgesteld worden dat ze pathogeen waren, bij de andere relatief kleine varianten was dit niet mogelijk. Om de eventuele klinische betekenis van de mogelijk pathogene veranderingen nader te definiëren zullen meer patiënten of gezonde personen bestudeerd moeten worden die (ongeveer) dezelfde variant vertonen.

In **hoofdstuk 3.4** worden drie families besproken waarbij één familielid drager is van een ongebalanceerde autosomale translocatie in mozaïeke vorm. Naast de cellijn met de ongebalanceerde translocatie hebben deze personen een normale cellijn. Bij twee van deze personen is de ongebalanceerde translocatie hoogst waarschijnlijk na de bevruchting ontstaan. Bij de derde persoon is de moeder draagster van de gebalanceerde vorm van de translocatie. Dit is erg zeldzaam en het precieze mechanisme voor het ontstaan van dit mozaïek is (nog) niet bekend.

In **hoofdstuk 4** worden een aantal casussen gepresenteerd. In **hoofdstuk 4.1** hebben we de grootte en oorsprong van een eerder met conventionele karyotypering gevonden chromosoom 9 afwijking kunnen bepalen. De SNP array toonde aan dat deze afwijking bestond uit een 400 kb duplicatie, een 2.5 Mb triplicatie en een 130 kb duplicatie op chromosoomband 9q34.3. In **hoofdstuk 4.2** beschrijven we een familiaire translocatie die gevonden is met behulp van de SNP array en FISH onderzoek. Deze cryptische translocatie is niet zichtbaar met conventionele karyotypering. Broer en zus erfden allebei een andere ongebalanceerde vorm van de translocatie van moeder en hadden allebei een ander klinisch fenotype. De zus is mentaal geretardeerd en heeft enkele aangeboren afwijkingen, terwijl haar broer ogenschijnlijk gezond is. Dit hoofdstuk onderstreept het belang van het rapporteren van chromosomale afwijkingen, ook al worden ze gevonden in gezonde personen.

Dat de SNP array niet alleen bruikbaar is voor het opsporen van varianten in kopie aantal bij patiënten met mentale retardatie laten we zien in **hoofdstuk 5**. Blepharophimosis- Ptosis- Epicanthus inversus Syndroom (BPES) is een zeldzaam syndroom dat wordt gekenmerkt door een ooglidvernauwing. Mutaties in het *FOXL2* gen zijn verantwoordelijk voor de meerderheid van de BPES patiënten. Echter, in een kleine groep van patiënten blijft de oorzaak onbekend. In 9 van de 27 door ons onderzochte BPES-achtige patiënten vonden we een chromosomale verandering die niet eerder ontdekt was en welke mogelijke een verklaring is voor het fenotype (**hoofdstuk 5.1**).

Sotos syndroom wordt gekenmerkt door overgroei, een grote schedel, een bijzondere vorm van het aangezicht en leerachterstand. In 60-90% van de Sotos patiënten is haploinsufficientie (het verlies van één werkend kopie van een gen) van het *NSD1* gen verantwoordelijk voor het klinisch fenotype. In 4 van de 26 door ons onderzochte Sotos-achtige patiënten vonden we een chromosomale verandering die niet eerder ontdekt was (**hoofdstuk 5.2**). De bevindingen van de twee bovengenoemde studies geven aan dat de genoom-brede array een geschikte techniek is voor het vinden van delen van chromosomen die genen kunnen bevatten voor verschillende syndromen of ziekten.

In **hoofdstuk 6** worden de in dit proefschrift beschreven resultaten besproken en worden toekomstperspectieven voor het diagnostische onderzoek bij mentale retardatie en aangeboren afwijkingen beschreven. Het identificeren van nieuwe ziekte veroorzakende chromosomale varianten in patiënten met mentale retardatie is verbeterd. Daartegenover staat dat het vinden van het hoge aantal mogelijk pathogene varianten in kopie aantal een enorme uitdaging heeft geïntroduceerd, namelijk om te komen tot een juiste en goed onderbouwde interpretatie van deze varianten in het genoom.

Het is zeer waarschijnlijk dat uiteindelijk de nieuwe generatie sequencing technieken de arrays zullen gaan vervangen en deze zullen op hun beurt weer helpen nieuwe mentale retardatie genen en syndromen te identificeren. Echter, het aantal varianten in het genoom dat wordt gevonden zal dan nog veel groter zijn, de interpretatie van die variatie is een nog grotere uitdaging.

Curriculum Vitae

Curriculum Vitae

Antoinet Gijsbers werd geboren te Beuningen op 21 januari 1981. Ze behaalde het VWO diploma in 2000 aan het Pax Christi College te Druten. Aansluitend begon zij met de studie Medische Biologie aan de Universiteit van Amsterdam. De eerste stage werd verricht bij de afdeling Klinische Genetica onder leiding van Dr. Heleen Schuring-Blom, in het AMC. De afstudeerstage werd vervuld op de afdeling Biological Lead Optimization van Solvay Pharmaceuticals te Weesp, onder leiding van Dr. Hiskias Keizer. In 2005 behaalde ze haar Master of Science titel.

Vanaf oktober 2005 was zij bijna een jaar werkzaam als analist op de afdeling Klinische Genetica van het Leids Universitair Medisch Centrum. In oktober 2006 is zij begonnen als onderzoeker in opleiding op dezelfde afdeling onder de leiding van Dr. Claudia Ruivenkamp, Prof. Dr. Martijn Breuning en Prof. Dr. Bert Bakker, met als onderwerp het opsporen van nieuwe genetische afwijkingen bij patiënten met mentale retardatie.

Sinds 1 mei 2010 is Antoinet Gijsbers werkzaam als Klinisch Cytogeneticus in opleiding op de afdeling Klinische Genetica van het Leids Universitair Medisch Centrum.

List of publications

List of publications

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Dankwoord

Dankwoord
De afgelopen vier jaar heb ik uitgekeken naar het moment dat ik zou kunnen zeggen: het is me gelukt! Nu is het zover!!!! Promoveren is een grote uitdaging en gaat samen met pieken en dalen.

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Appendix (color figures)

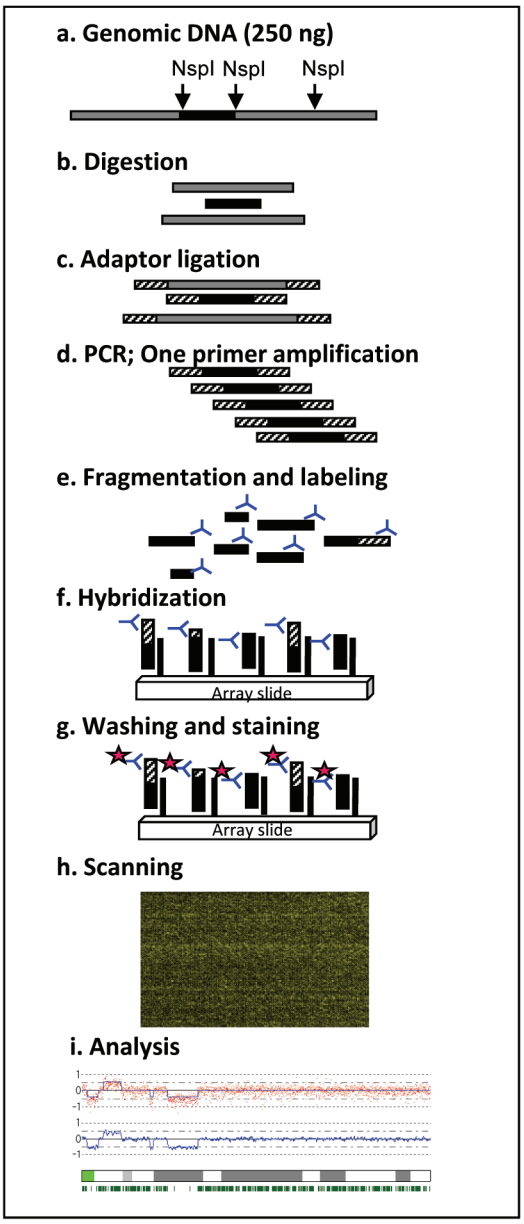


Figure 1.1 Schematic overview of the Affymetrix platform procedure

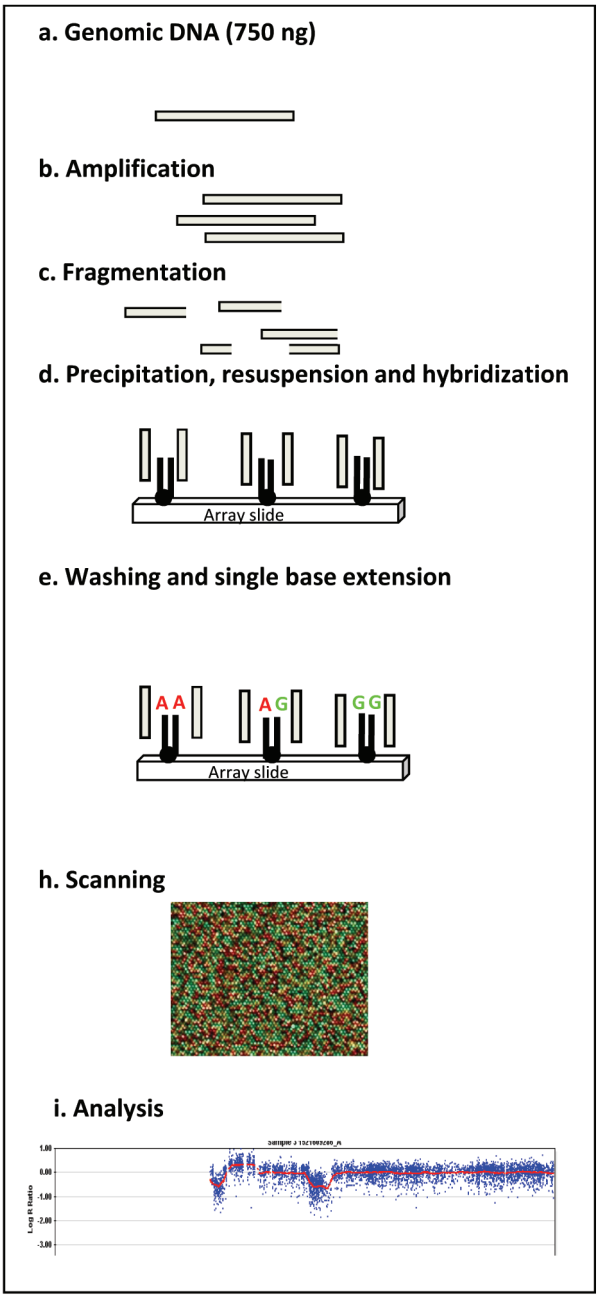


Figure 1.2 Schematic overview of the Illumina platform procedure

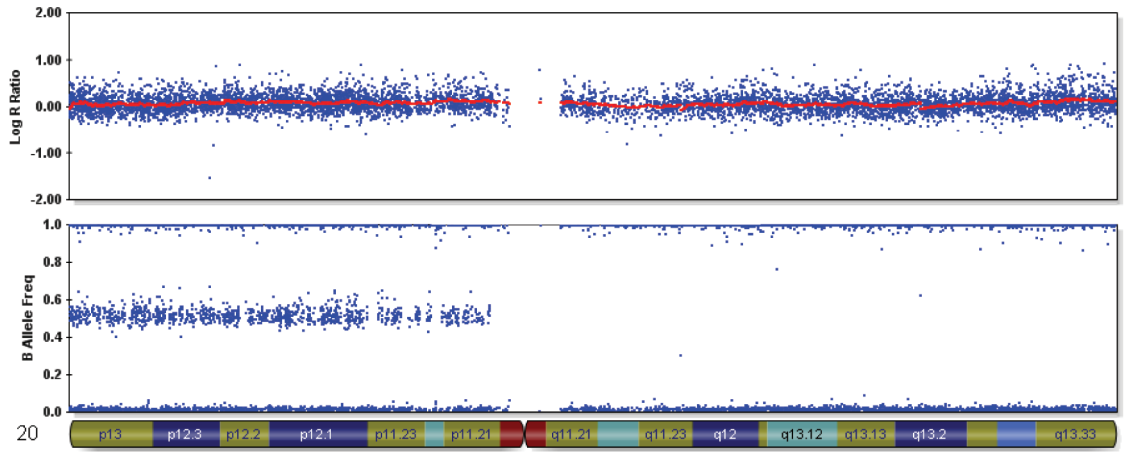


Figure 2.1 37.26 Mb region of LOH on chromosome 20q in case BC311 detected with the Illumina 317K BeadChip. Beadstudio logRratio estimate for each individual SNP in the first plot and genotype call for every SNP in the second plot. The X-axis shows the position on the chromosome.

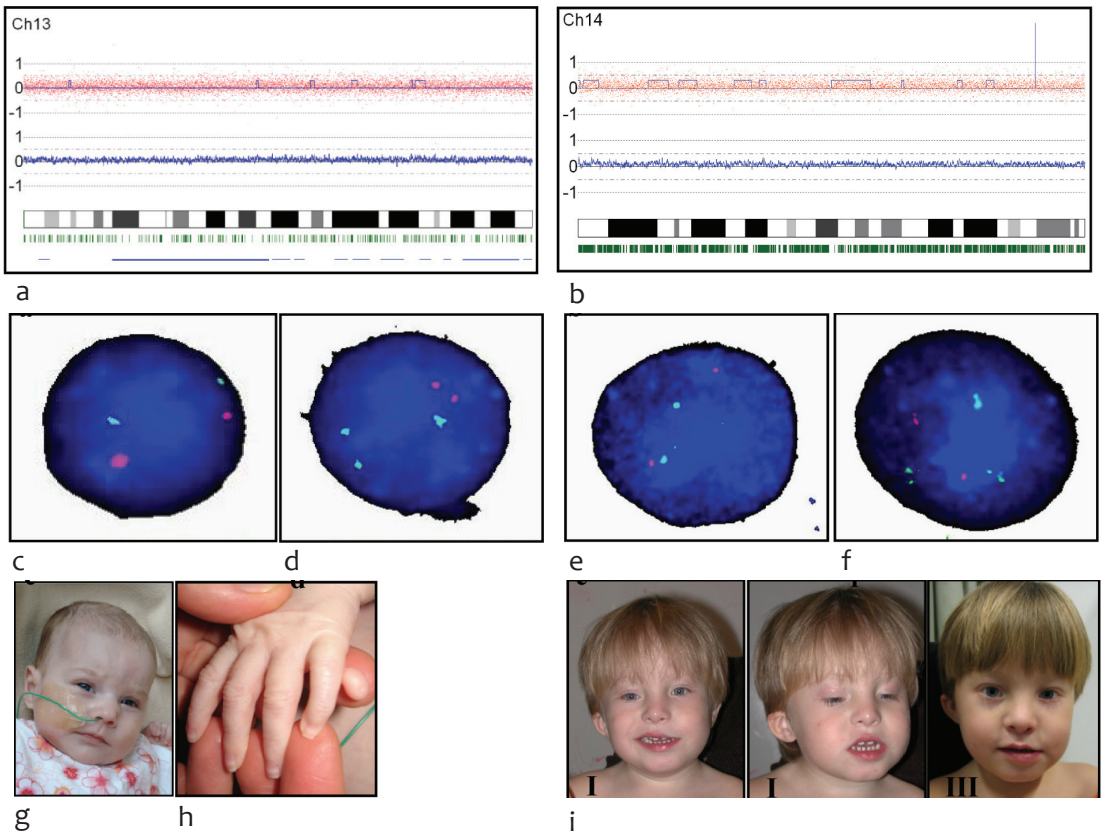


Figure 2.2 (a) CNAG copy number analysis for patient CR355 using the Affymetrix 262K GeneChip. LogR-ratio estimate for each individual SNP in the first plot and for an average of 10 SNPs in the second plot. Both plots show a slight increase in logRratio for whole chromosome 13. Blue line in first plot: copy-number estimate calculated with the Hidden Markov Model. The X-axis shows the position on the chromosome. Green stripes: heterozygous SNP calls. (b) CNAG copy number analysis output for patient CR377 using the Affymetrix 262K GeneChip. Both plots show a slight increase in logRratio for chromosome 14. (c) FISH experiment (probes LSI13 (green) and LSI21 (red), Vysis) showing a normal cell. (d) FISH experiment showing the presence of a mosaic trisomy 13 in 18% of the 200 cells analyzed. (e) FISH experiment (probes LSI CCNDI, 11q13 (red) and LSI IGH, 14q32 (green), Vysis) showing a normal cell. (f) FISH experiment showing the presence of a mosaic trisomy 14 in 9% of the 200 cells analyzed. (g) Facial picture of patient CR355. Facial dysmorphisms included upslant of palpebral fissures, a broad nasal bridge and uplifted earlobes. (h) Picture of postaxial polydactyly of the left hand of CR355. (i) Facial pictures of case CR377, 3 years and 7 months (I, II), and 4 years and 8 months (III). Note marked asymmetry when smiling, asymmetric upslanted palpebral fissures, left sided epicanthus, hypertelorism, low-set and small right ear.

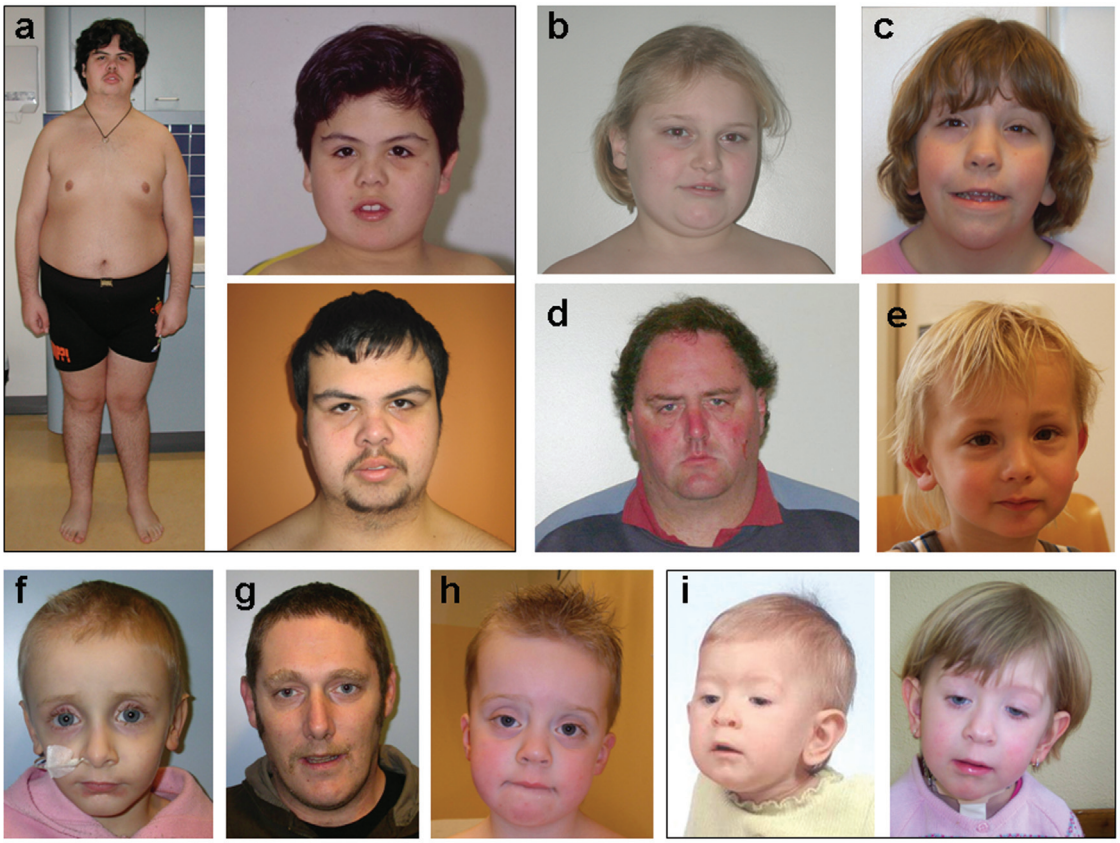


Figure 3.1.1 Phenotypical characteristics of cases with a 16p11.2 deletion. (a) case 2 (overview aged 16 years (left), face aged 7 years (top) and 17 years, (b) case 3, (c) case 6, (d) case 10, (e) case 11, (f) case 12, (g) father of case 12, (h) case 13, (i) case 14 (at the age of 9 months (left) and 4 years). Some of the cases share facial characteristics (long nose in cases 6, 12 and, father of case 12) (c, f, g); narrow palpebral fissures in cases 6 and 14(c, i); periorbital fulness in cases 2, 3, and 11(a, b, e); ptosis in cases 13 and 14 (h, i). Overall however, patients show no common facial features.

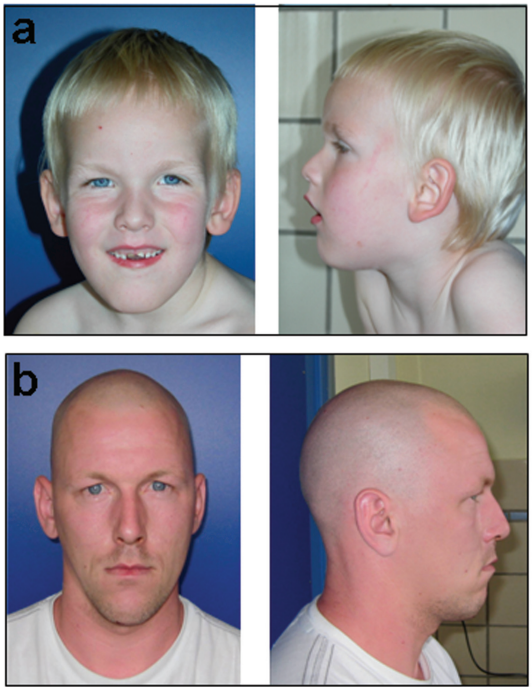


Figure 3.1.2 Facial characteristics of cases with an atypical 16p11.2 deletion. (a) case 15, (b) father of case 15. Note long narrow face, prominent forehead, downslanted and narrow palpebral fissures, and down turned corners of the mouth in both.

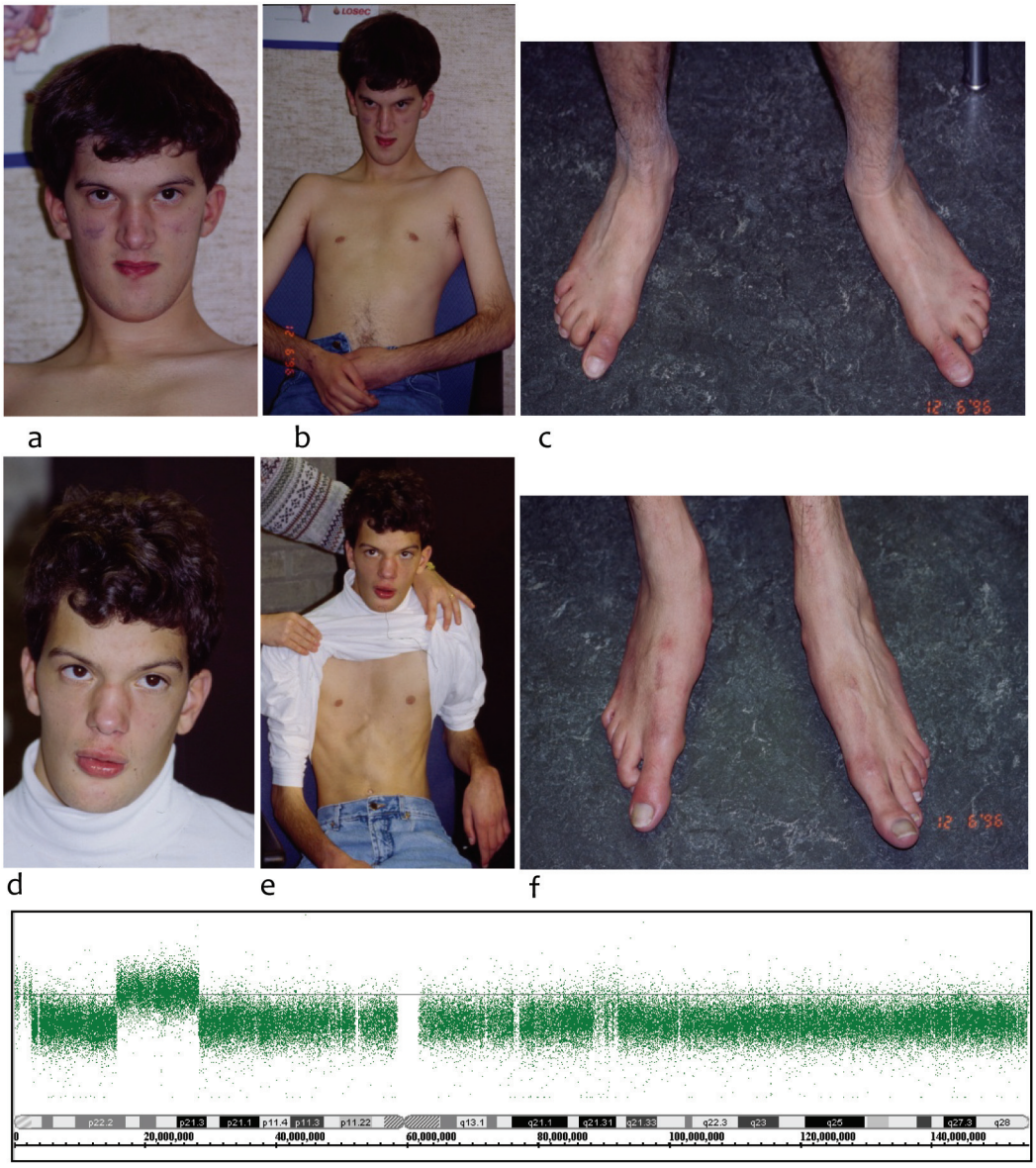


Figure 3.3.1 Patient 1 and his brother. (a) Patient 1 with elongated face and flat midface. (b) Scoliosis of patient 1. (c) Flat, thin and long feet of patient 1. (d) Brother of patient 1 with similar facial appearance. (e) Scoliosis and pectus excavatum of the brother. (f) Flat, thin and long feet with long first digits of the brother. (g) SNP array (Affymetrix Genome-Wide Human SNP Array 6.0) results of patient 1 showing a 12.5 Mb duplication. Log2ratios for each probe are plotted against the position on the X-chromosome. Intensity values are compared with normal males and females resulting in Log2ratios below zero for one copy of the X-chromosome for males. Duplicated regions have a Log2ratio of 0, including the pseudoautosomal regions 1 and 2 (PAR 1 and 2) at Xp22.3 and Xq28.

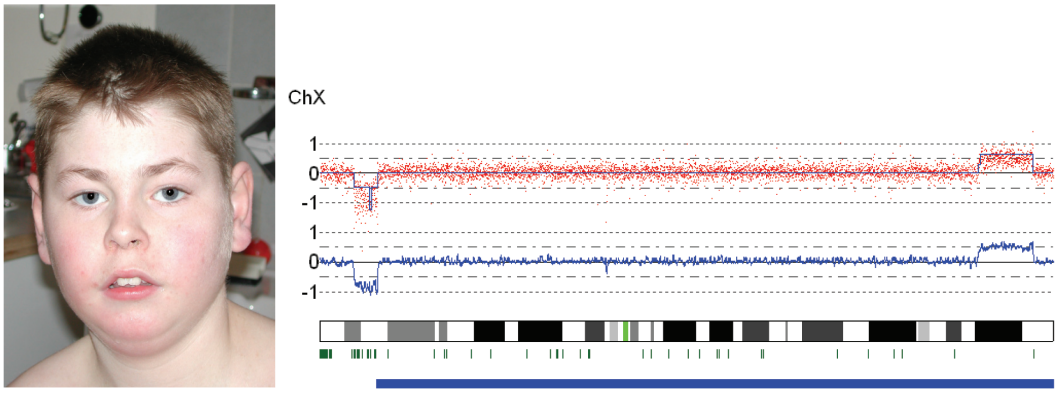


Figure 3.3.2 Patient 3. (a) Facial appearance showing oval eyes, short nose and a relative small chin. (b) SNP array (Affymetrix GeneChip Human Mapping 262K NspI and 238K StyI) results showing the 2.71 Mb deletion and 6.22 Mb duplication. Intensity values are compared with normal males resulting in Log2ratios of zero for one copy of the X-chromosome. Log2ratios for each probe are plotted against the position on the X-chromosome.

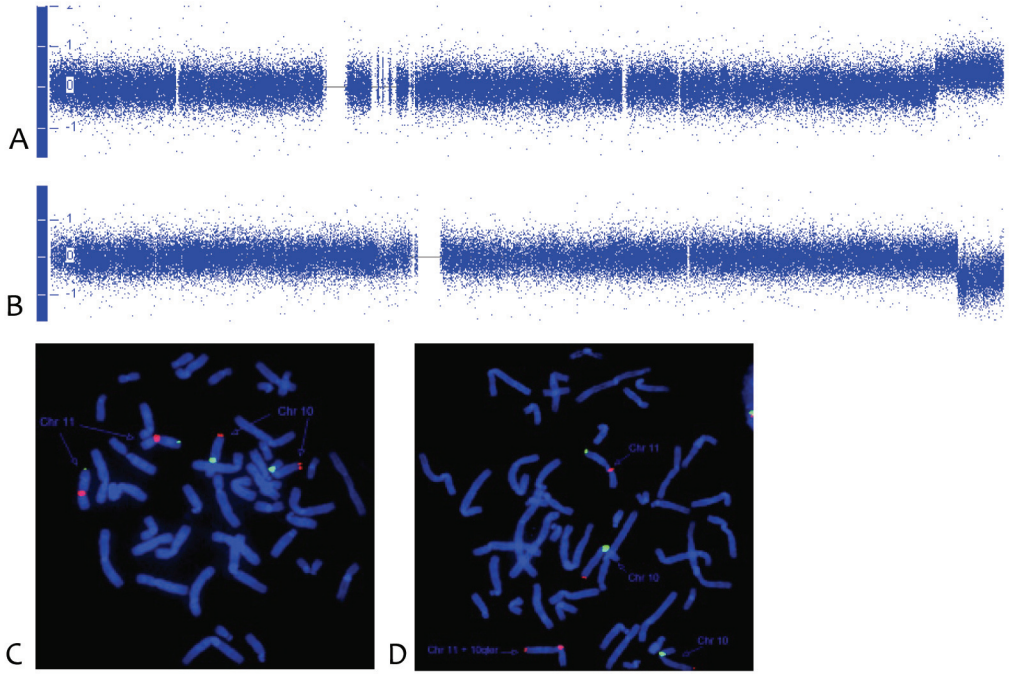
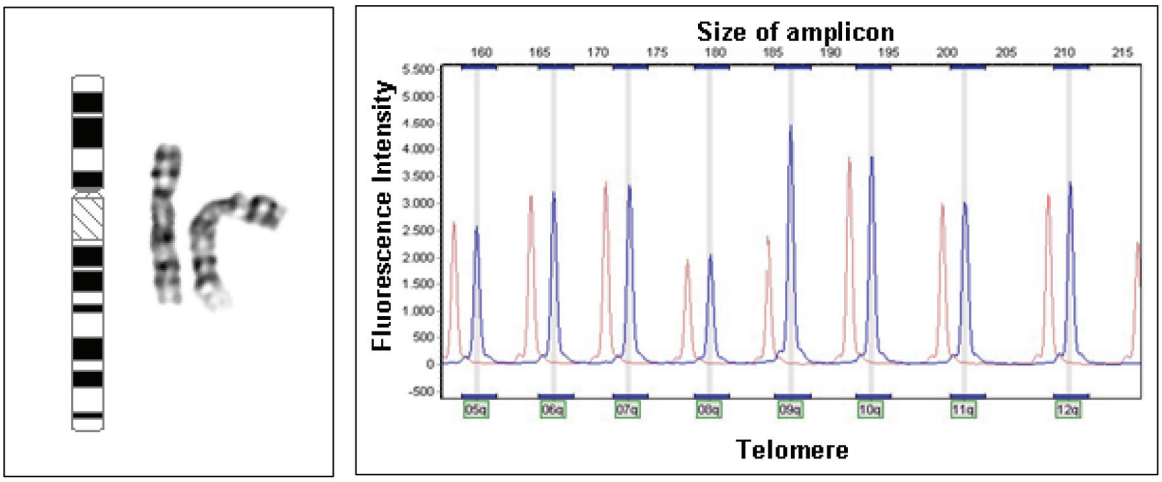
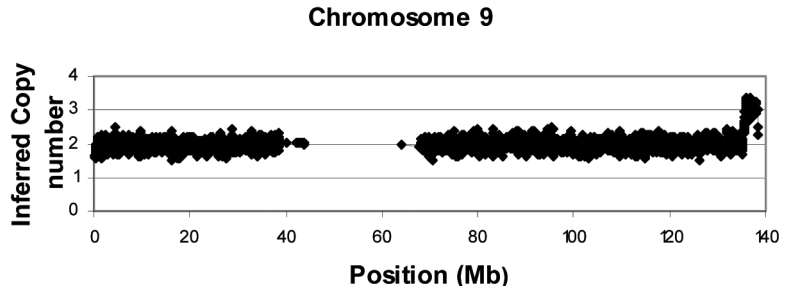


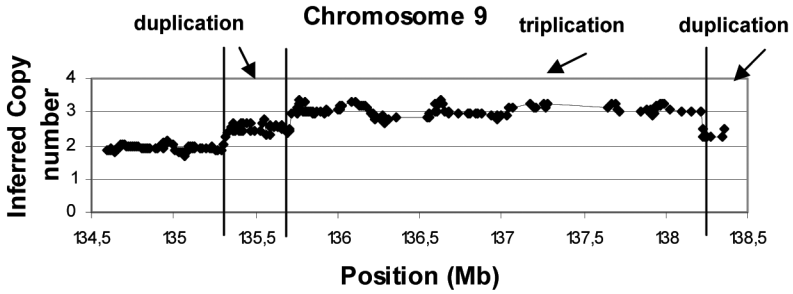
Figure 3.4.2 Case 1. (a) SNP array analysis revealed a duplication of 10.11 Mb on chromosome 10 and (b) a 7.51 Mb deletion on chromosome 11. (c) FISH analysis results showing a normal cell line and (d) a cell line with the der(11)t(10;11)(q26.13;q24.2).



a b



c



d

Figure 4.1.1 Cytogenetic and molecular results. (a) Partial karyotype showing both chromosomes 9. Right: abnormal chromosome 9. (b) MLPA analysis revealing an aberration of the 9q probe. Red peaks: control panel; blue peaks: patient. (c) SNP array analysis of the patient revealed an aberration on the long arm of chromosome 9 of approximately 2.93 Mb. The threshold for deletions and duplications is, respectively, 1.6 and 2.4. (d) SNP array copy number plot focused on the duplicated region of chromosome 9, showing a ~400 kb duplication, a ~2.4 Mb triplication and a ~130 kb duplication.

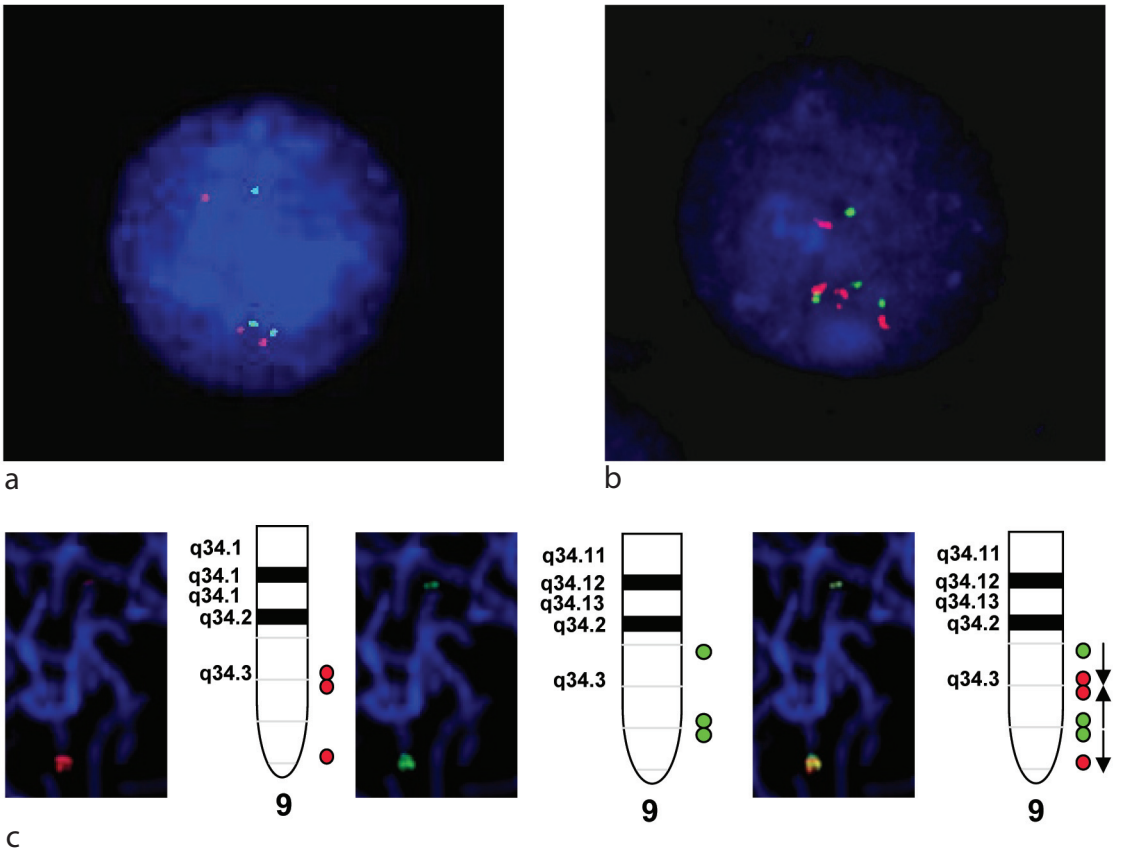


Figure 4.1.2 (a) FISH analysis with RP11-270D17 (green) and GS-135I17 (red) observed a duplication for both the probes confirming the 400 kb and 130 kb duplication, respectively. (b) Hybridization with RP11-413M3 (green, centromeric side of chromosome 9q) and RP11-48E05 (red, telomeric side of chromosome 9q) revealed the presence of four red and four green signals. The signals for the chromosome with the triplicated region showed that the middle repeat is inverted (green-red-red-green-green-red). (c) For probe RP11-48E05 the centromeric signal was of double intensity as compared to the telomeric signal. For probe RP11-413M3 (green) the telomeric signal was stronger than the centromeric signal. This indicates that the repeats of the triplication are normal in the proximal and distal region and inverted in the central region.



Figure 4.1.3 Picture of the female patient at the age of 16 years. Note mild facial asymmetry and narrow palpebral fissures.



Figure 4.2.1 Pictures of the index patient and her apparently healthy brother. (a) (b) Facial picture of the index patient at the age of 3 (a) and 4 (b) years. Note frontal bossing, hypotelorism and thin and long face. (c) Facial picture of her brother at the age of 2 years.

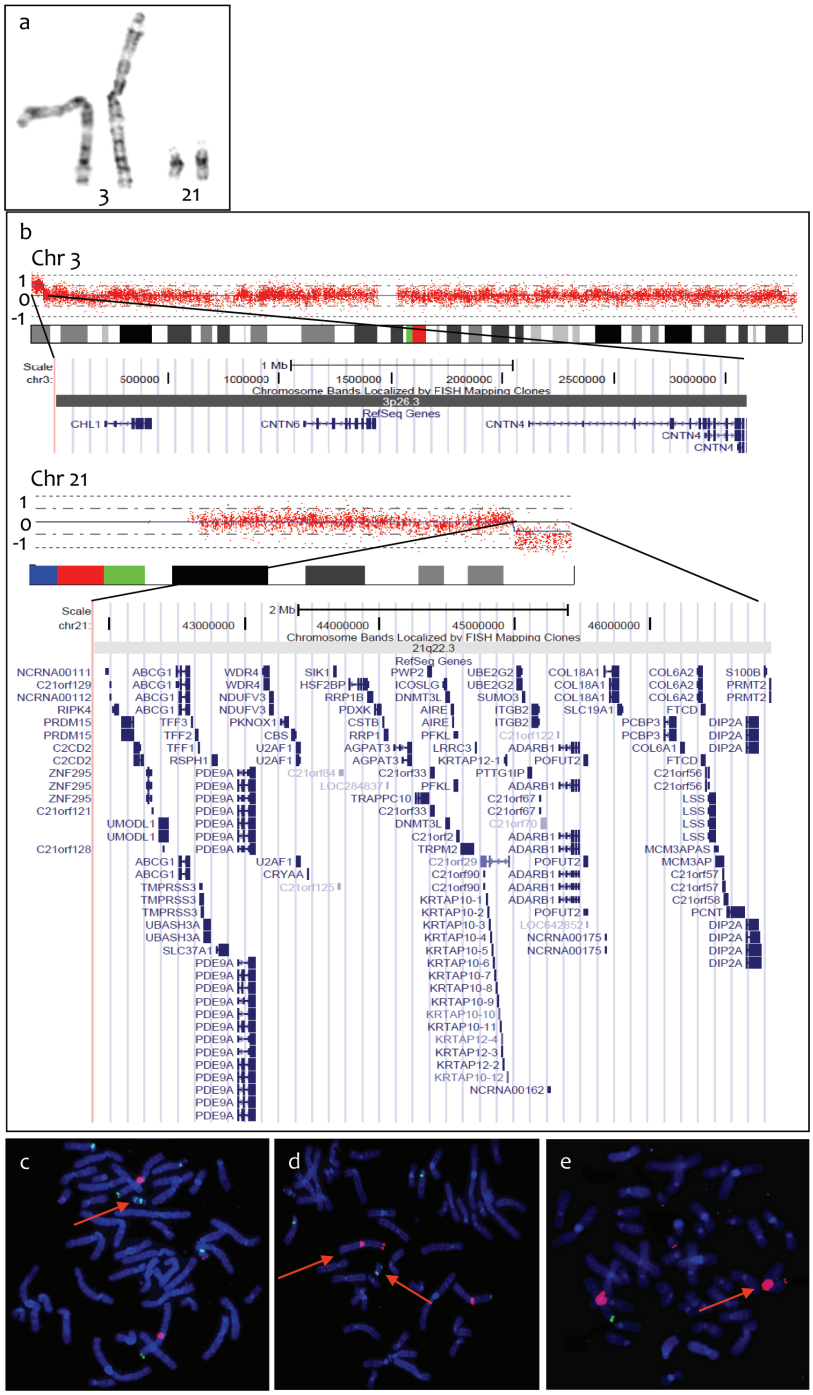


Figure 4.2.2 Cytogenetic and molecular analysis of the familial t(3;21)(p26.3;q22.3). (a) Conventional karyotyping revealed no abnormalities for chromosomes 3 and 21 in the index patient. The arrow indicates the aberrant chromosome 21. (b) SNP array analysis (Affymetrix GeneChip Human Mapping 262K NspI) results for the index patient demonstrating a 3 Mb duplication on the short arm of chromosome 3 and a 5 Mb deletion on the long arm of chromosome 21. Genes involved on both regions, UCSC Human Genome Browser (http://genome.ucsc.edu), Mar 2006 build (hg18). (c) FISH analysis confirmed the presence of the derivative chromosome 21 (black arrow), originating from a translocation between 3p (green; GS-1186B18; Flint) and 21q (red; GS-63H24; Flint). Control probes used for centromere chromosome 3 (red; CEP3; Vysis) and satellites of chromosome 13 and 21 (green; a-sat 13/21; Cytocell). (d) FISH analysis for the mother of the index patient demonstrated that she is carrier of the balanced t(3;21)(p26.3;q22.3) (black arrows) (probes: GS-1186B18, GS-63H24, CEP3 and a-sat 13/21). (e) FISH analysis for the brother of the index patient showing the derivative chromosome 3 (black arrow) (probes: GS-1186B18, GS-63H24, CEP3).

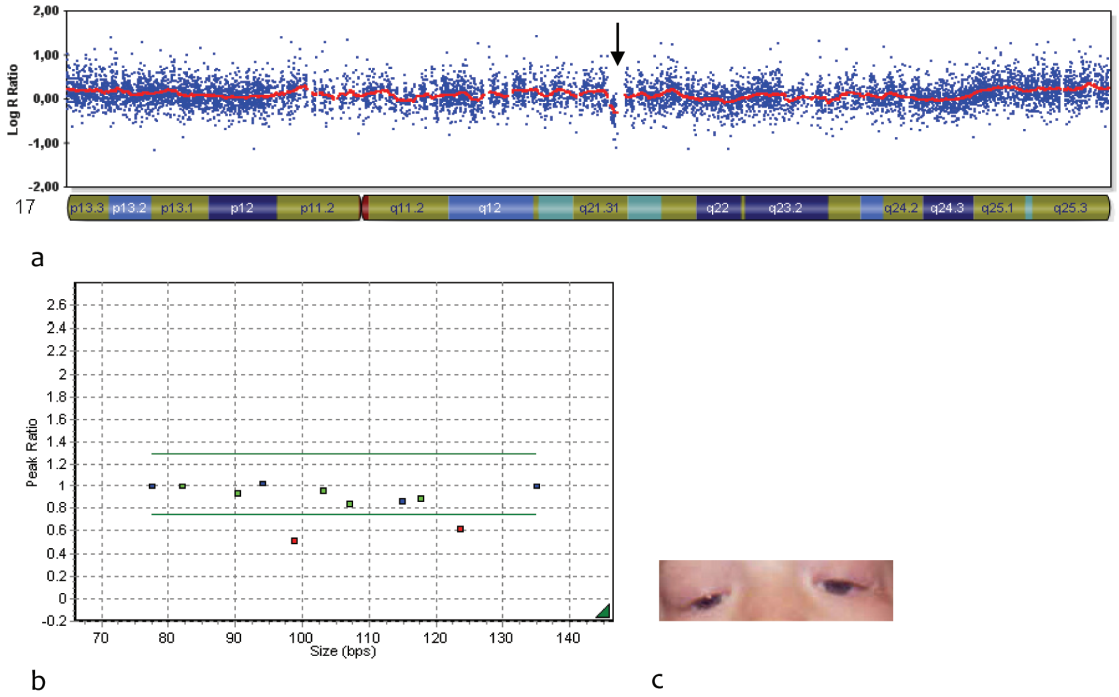


Figure 5.1.1 Case 2 (a) Deletion of chromosome 17q21.31 (485 kb) found with the Illumina's Sentrix HumanHap300 Genotyping Beadchip. (b) MLPA analysis confirmed the deletion. Deleted probes: MAPT and KIAA1267. (c) Picture of the eyes of case 2, characterized by blepharophimosis, bilateral upper lid ptosis, epicanthus inversus and telecanthus.

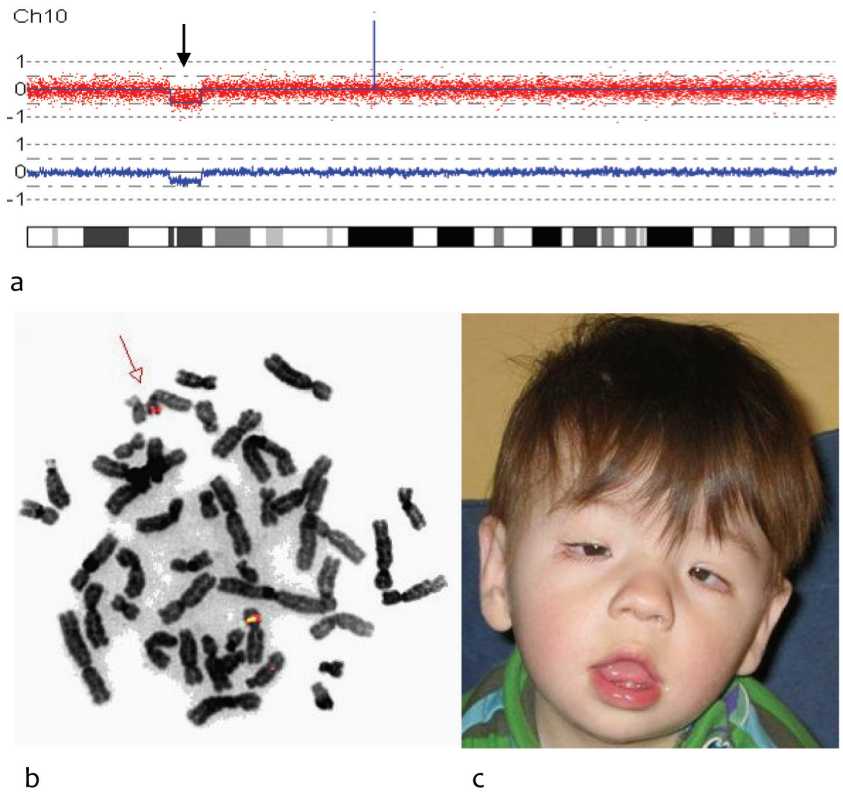


Figure 5.1.2 Case 4 (a) Deletion of chromosome 10p12.33-p12.31 (4.5 Mb) detected with Affymetrix GeneChip Human Mapping 262K NspI .(b) FISH analysis confirmed the deletion. Control probe: RP11-390B4 (red), deleted probe 10p12.31: RP11-177H22 (green). (c) Facial picture of case 4, showing blepharophimosis, ptosis, epicanthal folds, ectropion of the lower eyelid, S-shaped upper eyelid, synophrys.

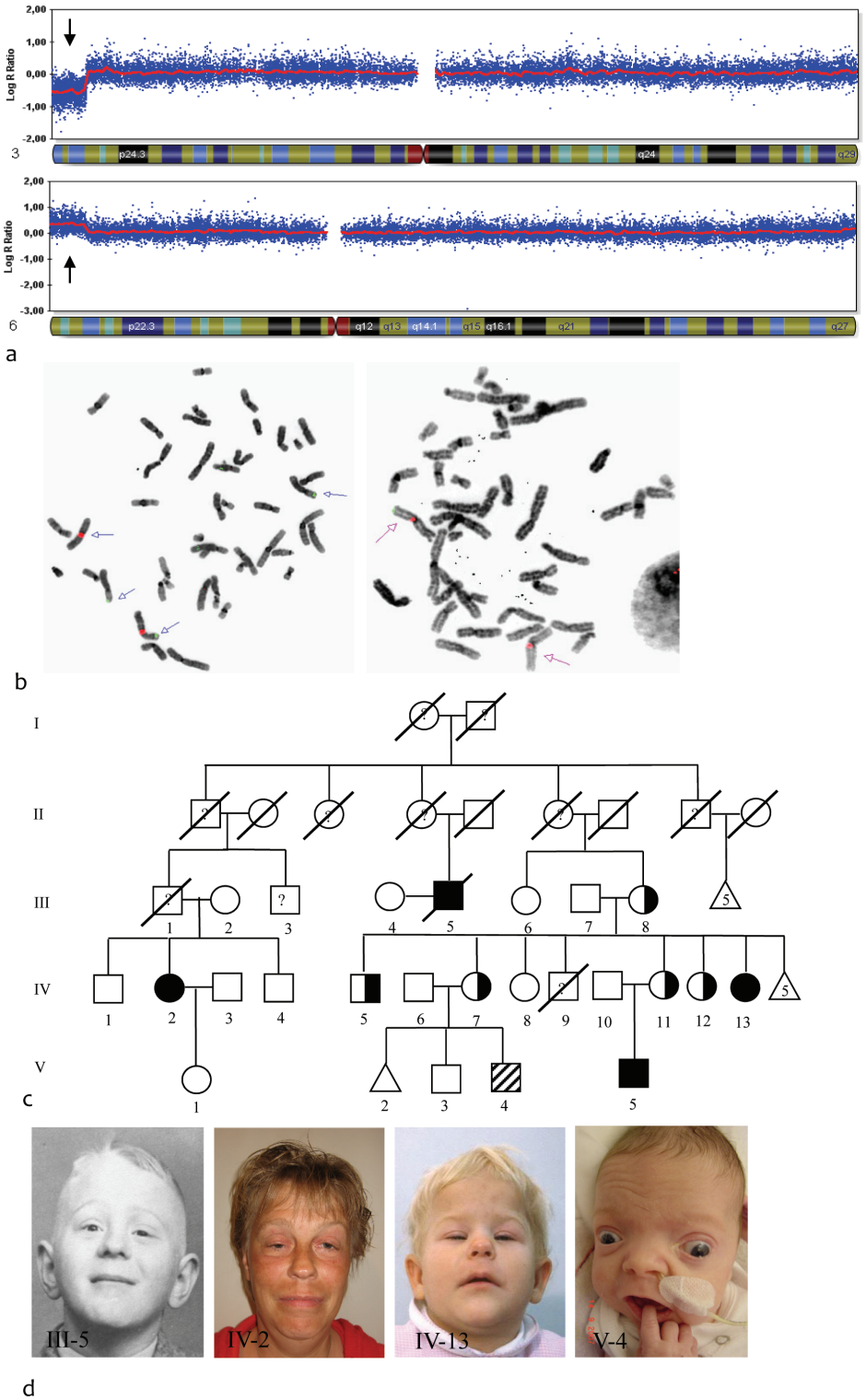


Figure 5.1.3 Case 9 (a) Terminal deletion of chromosome 3p25.3 (8.6 Mb) and terminal duplication of 6p24.3 (7.7 Mb) detected with the Illumina's Sentrix HumanHap300 Genotyping Beadchip. The derivative chromosome 3 was not observed by conventional G-banding. (b) FISH analysis revealed the presence of an unbalanced 3;6 translocation. Left panel, control probe: CEP3 (red) (Vysis), 6pter probe: GS-196I5 (green); right panel, control probe: CEP3 (red) (Vysis), 3pter probe; GS-186B18 (green). (c) Pedigree of the family with the segregating t(3;6) analyzed with FISH experiments. ◧ : balanced t(3;6); ■ : 3p25.3 deletion and 6p24.3 duplication; ▨: 3p25.3 duplication and 6p24.3 deletion. (d) Facial picture of case 9 (IV-2), characterized by blepharophimosis, ptosis, flattened and broad nose. Facial pictures of two relatives (III-5, IV-13) with the same unbalanced translocation as case 9 are presented. These members show similar clinical features. In addition, a facial picture of a relative (V-2) with the opposite unbalanced translocation is shown. No BPES-like features are seen in this child.