COPY NUMBER VARIATION AND MENTAL RETARDATION

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COPY NUMBER VARIATION AND MENTAL RETARDATION

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Contents

Abbreviations

Array CGH	Array-based Comparative Genomic Hybridization
BAC	Bacterial Artificial Chromosome
bp	base pair
CGH	Comparative Genomic Hybridization
CMT1A	Charcot-Marie-Tooth Disease Type 1A
CNV	Copy Number Variation/ Copy Number Variant
DECIPHER	DatabasE of Chromosome Imbalance and Phenotype in Humans using Ensembl Resources
DGS	DiGeorge Syndrome
DNA	DeoxyriboNucleic Acid
ECARUCA	European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations
EMG	Electromyography
FISH	Fluorescence In Situ Hybridization
FoSTeS	Fork Stalling and Template Switching
HMM	Hidden Markov Model
HNPP	Hereditary Neuropathy with liability to Pressure Palsies
IQ	Intelligence Quotient
kb	kilobase (thousand base pairs)
LCR	Low-Copy Repeat
LINEs	Long Interspersed Nuclear Elements
Mb	Megabase (million base pairs)
MDLS	Miller-Dieker Lissencephaly Syndrome
MIM	Mendelian Inheritance in Man
MLPA	Multiplex Ligation dependent Probe Amplification
MRI	Magnetic Resonance Imaging
NAHR	Non-Allelic Homologous Recombination
NHEJ	Non-Homologous End Joining
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase Chain Reaction
PTLS	Potocki-Lupski Syndrome
PWS	Prader-Willi Syndrome
SMS	Smith-Magenis Syndrome
SNP	Single Nucleotide Polymorphism
UCSC	University of California, Santa Cruz
VCFS	VeloCardioFacial Syndrome
WHS	Wolf-Hirschhorn Syndrome
WBS	Williams-Beuren Syndrome
ZonMW	Nederlandse organisatie voor gezondheidsonderzoek en zorginnovatie

Chapter 1

General introduction and outline of the thesis

1.1 Mental Retardation

Definition

Mental retardation is a highly diverse disorder in terms of both cognitive and non-cognitive functions. A non-verbal adult in an institution who is fully dependent on support in his/her activities and a child with Down syndrome in a regular elementary school are just two extremes of the wide clinical manifestation of mental retardation. Mental retardation has many faces and worldwide it is defined in various different ways. The most widely used definition is provided by the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR), published by the American Psychiatric Association.[1] Other well-known definitions have been formulated by the American Association on Intellectual and Developmental Disabilities (AAIDD), formerly the American Association on Mental Retardation (AAMR),[2] and in the World Health Organization's International Classification of Diseases (ICD-10).[3]

The diagnostic criteria for mental retardation according to the DSM-IV-TR include: (i) significant sub-average intellectual functioning, (ii) concurrent deficits or impairments in present adaptive functioning, and (iii) the onset before the age 18 years.[1] Similarly, in the definition of the AAIDD mental retardation or intellectual disability is defined as a disorder characterized by significant limitations both in intellectual functioning and in adaptive behavior as expressed in conceptual, social and practical adaptive skills, originating before the age of 18 years.[2] The ICD-10 defines mental retardation as a 'condition of arrested or incomplete development of the mind, which is especially characterized by impairment of skills manifested during the developmental period', skills which contribute to the overall level of intelligence, i.e., cognitive, language, motor and social abilities.[3]

With regard to the intellectual criterion for the diagnosis of mental retardation, intelligence is generally defined by an Intelligent Quotient (IQ) test score of approximately 70 or below. However, both the DSM-IV-TR and the AAIDD take into account a five-point measurement error inherent to the more commonly used intelligence tests and, therefore, individuals with IQ scores between 70 and 75 can also be diagnosed as having mental retardation if they exhibit significant deficits in adaptive functioning.[1,2] In children younger than 5 years of age standardized IQ testing is not reliable and, therefore, the term global developmental delay is usually reserved for these cases, whereas the term mental retardation or intellectual disability is applied to older children. Global developmental delay is defined as a significant delay in two or more domains, including gross or fine motor development, speech/language, cognition, social/personal development and activities of daily living, and it is thought to predict the future manifestation of mental retardation.[4]

Adaptive skills are the conceptual, social, and practical skills that people have learned to function in their everyday lives, e.g. communication, self-care, home living, social/ interpersonal skills, use of community resources, self-direction, functional academic skills, work, leisure, health and safety.[2] In both the DSM-IV-TR and the AAIDD definition, adaptive behavior should be limited in at least two of these areas.[1,2]

The commonly used definitions discussed above are inclusive and tend to capture limitations in different aspects, emphasizing the vast impact on everyday functioning for people with mental retardation and their families. In addition to the impairments in intellectual and adaptive functioning, a large proportion of mentally retarded children is delayed in reaching motor or speech developmental milestones, such as rolling over, sitting up, standing and walking, and suffer from a wide range of associated disabilities such as epilepsy, motor impairment, sensory (vision and/or hearing) impairment and behavioral and/or psychiatric disorders, such as aggression, self-injury and mood disorders.[5]

Additionally, mental retardation is also frequently associated with congenital malformations and dysmorphisms (syndromic mental retardation). Congenital malformations can be defined as developmental abnormalities resulting from an intrinsically abnormal developmental process,[6] e.g. malformation of the limbs, heart and nervous system. The latter may include neural tube defects, polymicrogyria, holoprosencephaly and agenesis of the corpus callosum, and may be directly related to the mental retardation. Dysmorphisms are defined as deviations of outward body form,[7] such as hypertelorism and clinodactyly. Specific combinations of congenital malformations and/or dysmorphic features may direct a clinician towards a syndrome, i.e., a particular set of developmental anomalies occurring together in a recognizable and consistent pattern that is known (or assumed) to be the result of a single cause.[6]

Classification and prevalence

The severity of mental retardation is often classified based on ranges in IQ score. The DSM-IV-TR and ICD-10 classify four different degrees of mental retardation: mild, moderate, severe and profound (**Table 1.1**), although individuals with severe and profound mental retardation are often grouped together in a single category. According to DSM-IV-TR, the segments of mental retardation are distributed from a high proportion of mild mental retardation (85%) to a low proportion of profound mental retardation (1-2%).[1] Also a binary classification of severity is often used, with mild mental retardation defined by an IQ score of 50-70 and severe mental retardation by an IQ score below 50.

Table 1.1: Levels of mental retardation as classified by DSM-IV-TR and ICD-10 based on IQ scores

	DSM-IV-TR classification		ICD-10 classification	
Level of MR	code	IQ level	code	IQ level
Mild	317	50-55 to 70	F70	50-69
Moderate	318.0	35-40 to 50-55	F71	35-49
Severe	318.1	20-25 to 35-40	F72	20-34
Profound	318.2	<20-25	F73	<20

Note.—The DSM-IV-TR takes into account a measurement error of approximately five-points, inherent in the more commonly used intelligence tests.[1]

In contrast, the AAIDD classification system focuses on the intensity of the support required, thus stressing the overall impact of mental retardation on everyday life. Accordingly, the level

of mental retardation is classified in four groups: intermittent, limited, extensive and pervasive support. To some extent, the DSM-IV-TR and the ICD-10 classification levels also reflect the four AAIDD classification levels. However, the AAIDD system is more comprehensive, requiring interdisciplinary teams to determine the types of support required across multiple dimensions.

The prevalence of mental retardation shows considerable variation in different studies. Leonard and Wen incorporated data on the rate of severe (IQ <50) and mild (IQ 50-70) mental retardation, adapted from a review by Roeleveld *et al*.[8] and data from prevalence studies reported between 1997 and 2002.[9] The prevalence of severe mental retardation was relatively constant and suggested to be 3-4 per 1,000 children, with a range of 1 to 7 per 1,000.[9] The prevalence of mild mental retardation showed even more variation (2-85 per 1,000), with an average prevalence of approximately 33 per 1,000.[9] It should be noted that the wide range of rates of mental retardation reported in the literature may be attributable to differences in definition, classification and methods of identifying persons with mental retardation, rather than true differences in prevalence.[8]

Etiology of mental retardation

Mental retardation may be caused by a wide range of factors that, together, contribute to its pathogenesis. In different studies on the etiology of mental retardation the diagnostic yield appeared to be highly variable (reviewed by Curry *et al*.,[10] Leonard and Wen,[9] and Moog[11]). This variation is likely attributable to differences in methodology, classification and particularly the use of the term diagnosis, thus hampering comparisons of clinical studies on the etiology of mental retardation.[11]

In 2003, a comprehensive study on the etiology of mental retardation was performed by Stevenson *et al*.[12] The study was based on a cohort drawn from a service delivery population, including 10,997 individuals with mental retardation. Although not perfect, this study provides a general idea on the frequencies of the main causative categories (**Figure 1.1**). A specific cause for mental retardation was found in 44% of the individuals in the cohort, which is in line with previous reports indicating that even after extensive investigation about half of the individuals with mental retardation remain without a diagnosis.[9-11,13-15]

Environmental factors were implicated in approximately 16% of the cohort, similar to the percentage of exogenous causes reported in a meta-analysis by Curry *et al*.[10] In some other studies, e.g. Rauch *et al*., mental retardation was assigned to environmental factors in a much lower percentage.[14] This may be due to an underrepresentation of this category among individuals referred to specialized clinical genetic centers. Environmental factors may include prenatal causes, such as excess maternal alcohol consumption, drug abuse and cigarette smoking during pregnancy, but also numerous other prenatal and perinatal conditions that can cause brain damage, such as prematurity and maternal infections, including rubella, toxoplasmosis and cytomegalovirus. In addition, postnatal emotional deprivation, malnutrition and infectious diseases, such as meningitis and encephalitis, may cause mental retardation.

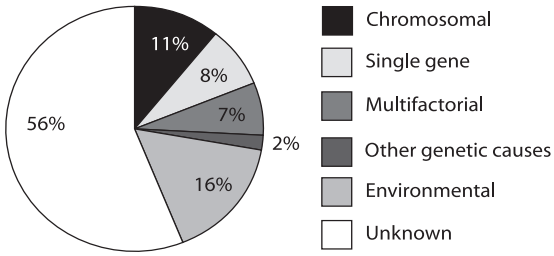

Figure 1.1: Causes of mental retardation**.** Adapted from Stevenson *et al*.[12]

Genetic factors represent another important causative category in mental retardation. Stevenson *et al.* reported that genetic conditions may account for up to 28% of the causes.[12] The Online Mendelian Inheritance in Man (OMIM) database for genetic conditions (http://www.ncbi.nlm.nih.gov/omim/) contains 1,498 entries in which mental retardation is reported (June 2008). Similarly, the Winter-Baraitser Dysmorphology Database contains information on over 1,680 syndromes associated with mental retardation and/or developmental delay, including single gene disorders, sporadic conditions and syndromes caused by environmental agents.

Overall, genetic disorders can be divided into multifactorial disorders, single-gene disorders and chromosomal disorders. Multifactorial disorders result from the action of one or multiple genes in combination with environmental factors. Numerous birth defects that cause deformities of the central nervous system leading to mental retardation, such as neural tube defects, hydrocephalus, agenesis of the corpus callosum and Dandy–Walker malformation, can be assigned to the multifactorial category.

For the total of 1,498 mental retardation entries in OMIM, 300 genes are currently recorded. Chelly *et al*. proposed a 'synapse-based' hypothesis for the pathogenesis of mental retardation.[16] In this hypothesis, mental retardation results from defects in synaptic structure and/or function, and neuronal connectivity, caused by dysfunction of proteins encoded by genes involved in a large spectrum of specific pathways and cellular processes.[16] Examples of distinct functional subclasses of proteins encoded by mental retardation-related genes include transcription (*TCF4*, *SOX3*) and chromatin-remodeling factors (*CBP*, *MECP2, ATRX*), transmembrane proteins (*TM4SF2*, *IL1RAPL*), microtubule- and actin-associated proteins (*DCX*, *SHROOM4*) and regulators and/or effectors of RhoGTPase pathways (*OPHN1*, *FGD1*, *PAK3*, *ARHGEF6*) (reviewed by Nokelainen and Flint,[17] Chiurazzi *et al*.,[18] Raymond and Tarpey,[19] and Chelly *et al*.[16])

The most common single gene disorder associated with mental retardation is the fragile X syndrome (MIM #300624). The fragile X syndrome is caused by trinucleotide repeat expansions within the Fragile X Mental Retardation 1 (*FMR1*) gene and is the most prevalent cause of heritable mental retardation with a frequency of approximately 1 in 6,000 males.[13,20] Single genes that affect cognitive functions have predominantly been found on the X chromosome. At present, more than 80 genes on the X chromosome are known to be involved in mental retardation (http://xlmr.interfree.it/home.htm).[18] This may be due to the gene richness of the

X chromosome, but also to the ease to identify families with X-linked inheritance patterns of mental retardation.[21] Therefore, it is likely that the majority of autosomal mental retardation genes still await identification. The identification of autosomal dominant genes, however, is complicated by the fact that autosomal dominant disorders manifest themselves mostly in isolated cases. Moreover, the genetic heterogeneity of mental retardation and the scarcity of large pedigrees hamper the identification of autosomal recessive mental retardation genes. So far, only five genes, *PRSS12* on 4q26 (MIM #249500),[22] *CRBN* on 3p26 (MIM #607417),[23] *CC2D1A* on 19p13.12 (MIM #608443),[24] *GRIK2* on 6q16.3 (MIM #611092)[25] and *CC2D2A* on 4p15.33-p15.2[26] have been reported to cause autosomal recessive mental retardation without additional abnormalities (non-syndromic mental retardation).
Genetic disorders most commonly found in individuals with mental retardation are chromosome aberrations.[12] With an estimated incidence of 1 per 800 births, trisomy 21, which causes Down syndrome (MIM #190685), is the most common mental retardation-associated chromosome abnormality.[27-29] Besides trisomies and other changes in overall copy number (aneuploidies), chromosome aberrations can be grouped into those encompassing changes in copy number over specific regions (segmental aneuploidies) and those encompassing balanced structural rearrangements.

1.2 Identification of chromosome aberrations in mental retardation

The detection of chromosome aberrations in patients with mental retardation started with the determination of the human diploid chromosome number as 46 in 1956.[30-32] This discovery enabled the identification of numerical chromosome aberrations in individuals with mental retardation and/or congenital malformations such as trisomy 21 in Down syndrome (MIM #190685),[33] 45,X in Turner syndrome,[34] 47,XXY in Klinefelter syndrome,[35] trisomy 13 in Patau syndrome[36] and trisomy 18 in Edwards syndrome.[37] The subsequent implementation of chromosome banding techniques and the improvement of cell culture methods from the 1970s on resulted in an enormous increase in the detection of both segmental aneupoidies and structural rearrangements, causally related to mental retardation and/or malformation syndromes.[38] Since the 1970s, G-banding has been the main tool for the detection of microscopically visible chromosome aberrations (>5-10 Mb) and, based on this technology, numerous mental retardation-related unbalanced chromosome aberrations such as deletions, duplications, translocations and inversions have been reported and categorized.[39]

Through the application of high-resolution chromosome banding techniques the level of resolution of chromosome analysis was increased to <5 Mb.[40] The method includes the fixation of cells at an early stage of mitosis (prometaphase) when the chromosomes are not fully contracted yet.[41] The subsequent recognition of genome alterations in the 10^4 to 10^6 bp range was enabled by the introduction of restriction fragment length polymorphism (RFLP) analysis,[42] pulsed-field gel electrophoresis (PFGE),[43] and fluorescence *in situ* hybridization

(FISH).[44] For FISH analyses, large fragments of DNA such as those cloned into bacterial artificial chromosomes (BACs; ~40,000 to 200,000 bp) are labeled with a fluorescent dye and hybridized to either metaphase spreads or interphase nuclei. Subsequently, fluorescence microscopy can be used to assess if and where the fluorescent probe has bound to the chromosome, thus allowing the detection and localization of specific DNA fragments. FISH can be used to dissect in detail (sub)microscopic chromosomal aberrations. However, large-scale FISH studies are time consuming, since they often require separate hybridizations and extensive microscopic analyses. In the early 1990s comparative genomic hybridization (CGH) was developed.[45] In this application, equal amounts of patient DNA and normal reference DNA are fluorescently labeled with different colors and hybridized to normal human metaphase chromosomes. The DNA copy-number is proportional to the test/reference fluorescence ratio. However, the detection of small, cryptic aberrations is limited, because metaphase chromosomes are used as the hybridization target.[45]
Several quantitative polymerase chain reaction (Q-PCR)-based technologies have also been developed for the identification of submicroscopic chromosome aberrations, including multiplex amplifiable probe hybridization (MAPH),[46] quantitative fluorescent PCR (QF-PCR),[47] and multiplex ligationdependent probe amplification (MLPA).[48] However, similar to the FISH technique mentioned above, these quantitative PCR-based approaches are targeted and, as such, only allow the screening of a limited number of loci in a single experiment. More recently developed FISH-based techniques using multiple fluorescence dyes, such as multiplex-FISH (M-FISH)[49] and spectral karyotyping (SKY),[50] enabled the simultaneous visualization of all chromosomes, thus allowing the identification of (complex) chromosome rearrangements without prior knowledge of the regions involved ('chromosome painting'). Still, however, these techniques are labor intensive, have a limited resolution and are difficult to automate.

The introduction of molecular cytogenetic tools, mainly DNA microarrays,[51,52] enabled the bridging of the technical divide between molecular genetics and cytogenetics. DNA microarrays allow the detection of submicroscopic genomic variation at a resolution far below 5 Mb. Submicroscopic genomic variants that alter chromosomal structure are often referred to as structural variation.[53] Structural variation includes inversions and translocations, but also changes that alter DNA copy number, i.e. deletions, insertions and duplications, which are collectively termed—copy number variations (CNVs)—.[53] By definition, the minimum size of structural variation is 1kb and, although no maximum size is determined, structural variation and CNV are commonly used in the context of submicroscopic aberrations (**Figure 1.2**).[53]

The high-throughput genome-wide detection of submicroscopic interstitial structural variation associated with mental retardation became feasible with the introduction of array-based comparative genomic hybridization (array CGH),[51,52] also referred to as 'molecular karyotyping'. Molecular karyotyping refers to all technologies that allow a genome-wide detection of chromosome aberrations at the submicroscopic level.[55,56] The initial array CGH platforms were

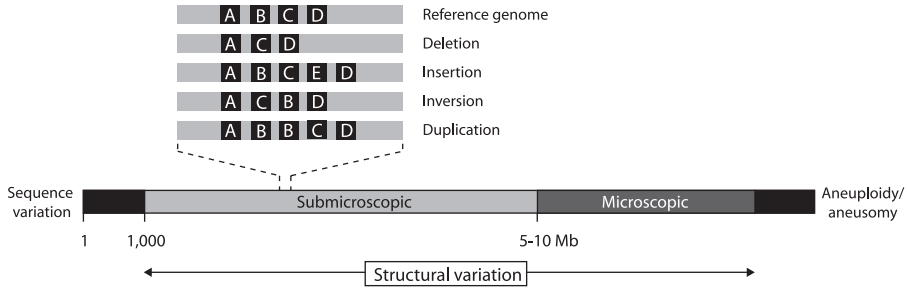

Figure 1.2: From chromosome to DNA level. The genome manifests a size continuum of genomic variants from single base deletions or duplications to whole chromosomal aneuploidies.[54] Structural variation includes variants that alter chromosomal structure, both balanced changes and variants that alter DNA copy number [copy number variation (CNV)]. Different genomic technologies are required to interrogate genomic variants at different levels, from single basepair changes using DNA sequencing to the identification of microscopically visible chromosomal aberrations through conventional karyotyping.

clone-based platforms that used genomic fragments from large insert clones such as cosmids, P1 clones, phage artificial chromosome (PAC) clones[52] and bacterial artificial chromosome (BAC) clones,[51] as probes. The development of these clone-based microarrays was mediated by (i) the generation of genome-wide clone resources integrated into the finished human genome sequence, (ii) the development of high throughput microarray-based platforms, and (iii) the optimization of CGH protocols and data analysis systems.[57] The main principle of array CGH is outlined in **Figure 1.3**.

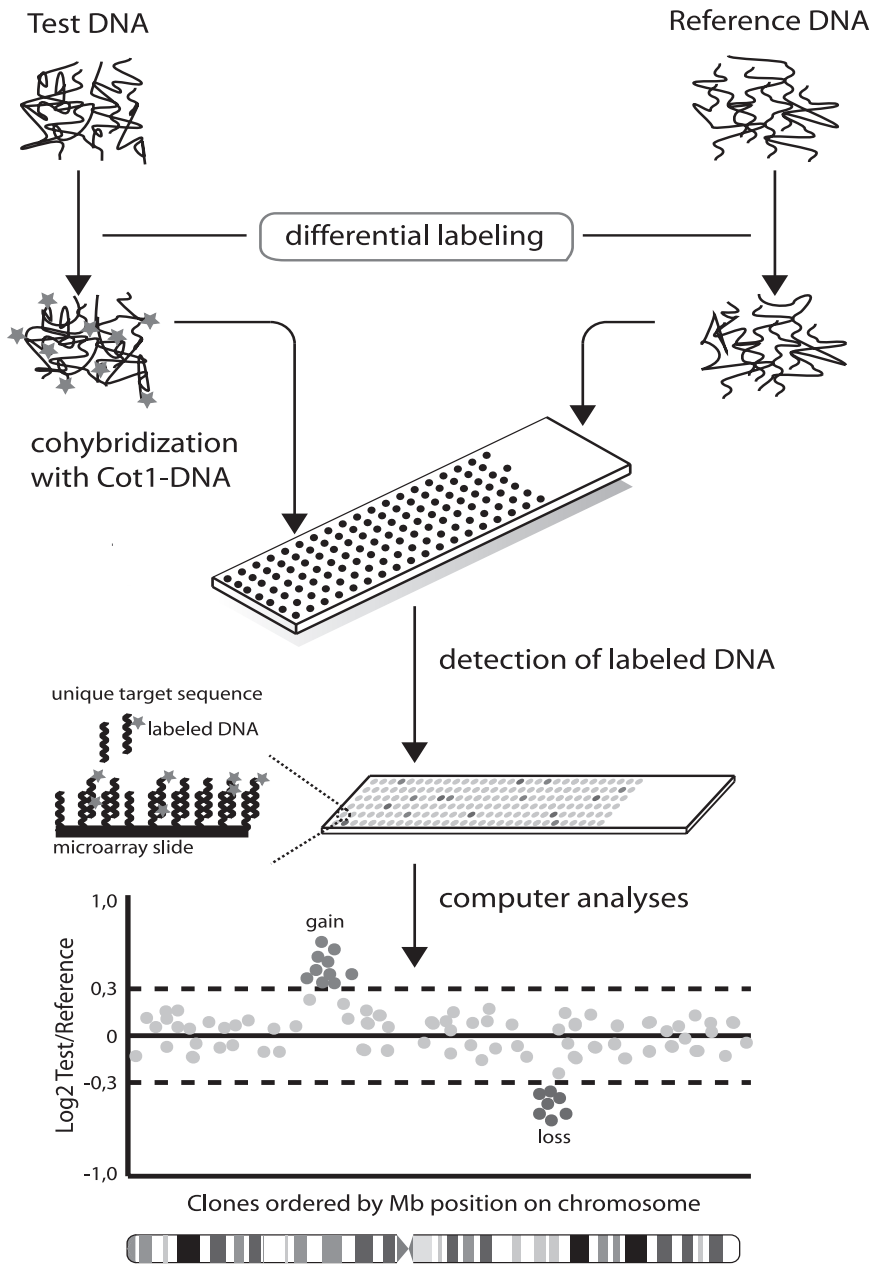

Figure 1.3: The principle of array CGH. Equal amounts of isolated and fragmented genomic test DNA and reference DNA are differentially labeled using fluorescent dyes. Subsequently, test (patient DNA; left panel) and reference samples (reference DNA; right panel) are mixed with Cot-1 DNA, co-precipitated, and resuspended in a hybridization solution. After denaturation the DNA mix is hybridized to the DNA on the array. After several washing steps, images of the fluorescent signals are captured and the ratio of test over reference signals is quantified computationally and plotted for each probe on the array. For color figure see page 190.

1.3 Clinical consequences of submicroscopic copy number variation

Subtelomeric copy number variation

Chromosome rearrangements of the telomeres are one of the most commonly observed structural abnormalities detected by conventional chromosome analyses in individuals with mental retardation. In 1963, Lejeune et al. described deletions of the short arm of chromosome 5 (5p-) in patients with similar phenotypic features, thus defining the Cri-du-Chat syndrome (MIM #123450).[58,59] Later terminal deletions of 4p were identified in patients with Wolf-Hirschhorn syndrome (MIM #194190)[60,61] and terminal deletions of 17p in patients with Miller-Dieker syndrome (MIM #247200).[62-64]

Since the descriptions of the Cri-du-Chat, Wolf–Hirschhorn and Miller–Dieker syndromes, clinical disorders caused by terminal microscopically visible deletions have been reported for numerous human chromosomes.[39] Moreover, in 1995, Flint *et al*. reported the identification of cryptic, submicroscopic structural rearrangements involving subtelomeric regions in individuals with mental retardation.[65]

Subtelomeres are defined as genomic regions adjacent to the telomeres. The telomeres themselves are complex regions harboring repetitive DNA sequences consisting of long $(TTAGGG)_{n.}$ repeats.[66] The subtelomeres are divided by interstitial degenerate $(TTAGGG)_n$ repeats into distal and proximal sequences that are repeated near the ends of multiple chromosomes (**Figure 1.4**).[67] The complex structure of the subtelomeres is a potential source of phenotypic diversity and may have a role in the reconstitution of telomeres in the absence of telomerase. However, it may also mediate rearrangements of the ends of the chromosomes.[68] These latter rearrangements are likely to have clinical consequences as the highest gene densities in the human genome are encountered in the regions just adjacent to telomeres.[69]

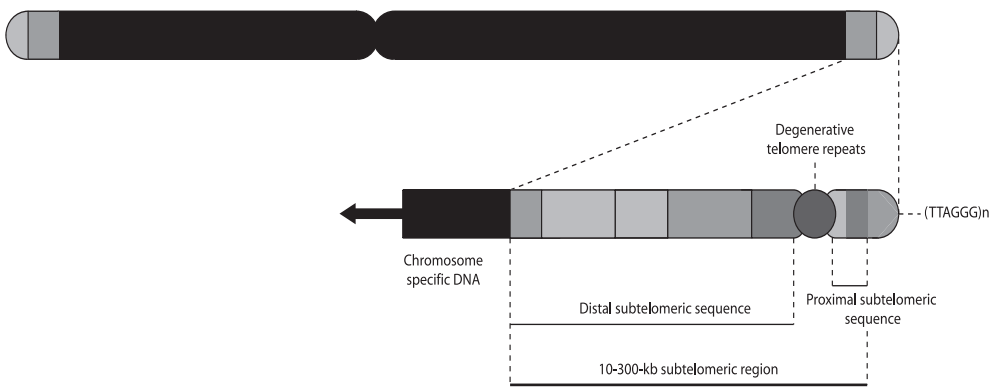

Figure 1.4: Structure of the subtelomeric region. Subtelomeres are the genomic regions just adjacent to the telomeres and are subdivided by interstitial degenerate $(TTAGGG)_n$ repeats into distal and proximal subtelomeric sequences that are repeated near the end of multiple chromosomes.[67]

After the first report of submicroscopic CNVs in the subtelomeres of individuals with unexplained mental retardation,[65] technological improvements in cytogenetics, such as the development of a 12-color FISH assay, termed M-TEL,[70] and the introduction of the Multiprobe™ slide,[71] soon allowed the interrogation of all subtelomeres into a routine procedure. The first survey of a large cohort of 466 children with idiopathic mental retardation and a normal G-banded karyotype revealed subtelomeric CNVs in 5% of them, of which almost 50% was inherited from a balanced carrier.[72] Since then, numerous studies have been performed showing that subtelomeric rearrangements contribute significantly to idiopathic mental retardation (reviewed by Biesecker,[73] de Vries *et al*.,[74] Flint and Knight,[75] and Rooms *et al*.[76]) In the majority of these studies FISH was used to identify subtelomeric rearrangements, but also other techniques were employed such as microsatellite marker analysis,[77,78] MAPH,[46] MLPA (**Chapter 2.1**),[79,80] and array CGH.[81,82] In total, subtelomeric rearrangements were identified in 5-6% of ~3,800 individuals with unexplained mental retardation.[76] As most of these initial reports represent proof-of-principle studies, the patient cohorts may have been preselected. In 2006, FISH analyses among a large cohort of 11,668 individuals with unexplained mental retardation revealed clinically significant telomere aberrations in ~2.5% of the cases, of which 60% was inherited from a normal parent carrying a balanced translocation.[83] A similar diagnostic yield was obtained from nearly 7,000 consecutive clinical cases analyzed by targeted array CGH.[84] Taken together, these latter two studies encompass the results of almost 19,000 subtelomere tests. The most common submicroscopic subtelomeric CNVs identified in these studies, are discussed below.

1p

One of the best-studied subtelomeric regions is 1p36. The 1p36 microdeletion syndrome (MIM #607872) is the most frequently observed subtelomeric deletion and, with an incidence of one in 5,000 births, one of the most commonly occurring mental retardation syndrome in humans.[85-87] Clinical features characteristic for the 1p36 microdeletion syndrome include developmental delay/mental retardation, epilepsy, hypotonia, hearing loss, microcephaly,

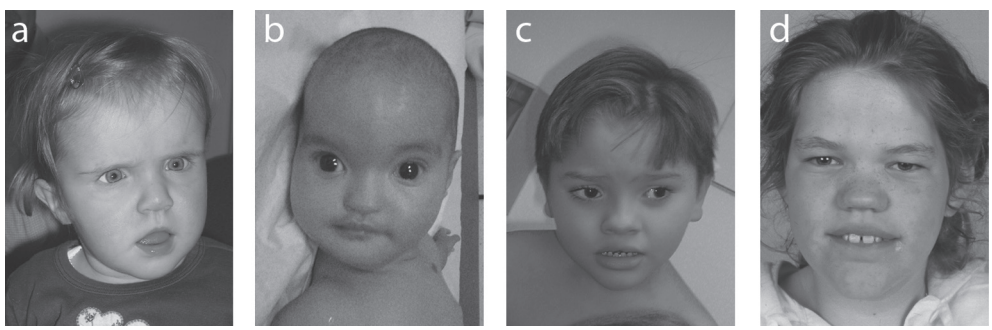

Figure 1.5: Clinical photographs of patients with subtelomere deletions. (**a**) 1pter deletion (**b**) 4pter deletion (**c**) 9qter deletion (**d**) 22qter deletion. Informed consent was obtained for publication of photographs. For color figure see page 191.

brachycephaly and facial dysmorphisms including straight eyebrows, deep-set eyes, a flat nasal bridge, a flat nose, asymmetric ears and a pointed chin (**Figure 1.5a**). In addition, cardiac defects and cleft lip/palate are common features.[86,88,89] Molecular cytogenetic analyses revealed that terminal 1p36 deletion sizes vary widely from ~1 Mb to more than 10 Mb and that the deletions comprise terminal deletions, derivative chromosomes, complex rearrangements and interstitial deletions.[85,89,90]

2q

Isolated, primarily subtelomeric deletions with breakpoint at or within chromosome 2q37 have been reported in almost 100 individuals.[91] Individuals with subtelomeric 2q37 deletions commonly present with mild-moderate mental retardation, epilepsy, autistic features and facial characteristics, including prominent forehead, thin, highly arched eyebrows, a depressed nasal bridge, full cheeks, hypoplastic alae nasi, prominent columella, a thin upper lip and various minor anomalies of the pinnae.[91] Brachymetaphalangism is reported in ~50% of the cases.[92] In addition, central nervous system anomalies and ocular-, cardiac-, gastrointestinal-, renal- and other genitourinary malformations have been noted in nearly one-third of the patients. There is an extensive phenotypic variability, and a clear genotype-phenotype correlation has not been observed. Patients with the most distal deletion present with mental retardation, short stature, obesity and bracymetaphalangia. These features, resembling the physical anomalies found in Albright hereditary osteodystrophy (MIM #103580), have been designated Albright hereditary osteodystrophy (AHO)-like syndrome or brachydactyly-mental retardation syndrome (MIM %600430). The minimal deleted region in patients with AHO-like brachymetaphalangism has been narrowed down to ~3 Mb.[92]

4p

Subtelomeric deletions of 4p result in a clinically well recognizable characteristic phenotype known as the Wolf-Hirschhorn syndrome (WHS, MIM #194190).[60,61] WHS occurs in approximately one in 50,000 births.[93] The major features are low birth weight, postnatal failure to thrive, microcephaly and mental retardation, hypertelorism, prominent glabella (Greek warrior helmet), a broad nasal tip, a bilateral cleft lip and a short philtrum **(Figure 1.5b**).[94] The WHS critical region (WHSCR) is located in 4p16.3 and in approximately 25% of the patients the deletion comprising this region is not detectable using conventional chromosome analysis.[74] The shortest region of overlap of the deletions observed in WHS patients has been confined to a stretch of 165 kb, including the *WHSC1* and *WHSC2* genes.[95] Moreover, a second critical interval of 300 to 600 kb on 4p16.3 (WHSCR2), contiguous distally with the WHSCR, was defined by Zollino *et al*. in patients with a mild phenotype.[96]

7q

Common clinical features in children with cryptic 7q36 deletions include developmental delay, holoprosencephaly, microcephaly, facial dysmorphisms and heart anomalies.

Holoprosencephaly associated with the subtelomeric 7q36 genomic segment is caused by loss of the Sonic Hedgehog gene (*SHH*).[97] *SHH* encodes the human sonic hedgehog homolog, a protein that is instrumental in patterning the early embryo and has been implicated in normal development of a variety of organ systems, including the brain and spinal cord, the eye, craniofacial structures and the limbs (reviewed by Ming *et al*.[98]) Haploinsufficiency of another gene included in this region, the *HLXB9* homeobox gene, causes Currarino syndrome (MIM #176450), which is characterized by the triad sacral dysgenesis, anorectal atresia and a presacral mass.[99,100] Holoprosencephaly and Curranino syndrome exhibit remarkable clinical variability and the expression of features of the holoprosencephaly spectrum and of the Currarino syndrome due to deletions of 7q36 can be minimal.[101]

8p

Distal 8p23.1 deletions are associated with a mild phenotype that can extend into the normal range.[102] Deletions of 8p23.1pter were first identified in a boy with mental slowness, behavioral problems and seizures, in his sister and father who had minimal phenotypic abnormalities with borderline to normal intelligence and, prenatally, in a fetus and its phenotypically normal father.[103,104] Subsequently, a *de novo* terminal deletion of 8p23.1pter was ascertained in a girl with initial motor and language delays, but average cognitive development and intellectual ability after close monitoring over a period of 5 years.[105] De Vries *et al*. reported two mildly retarded, non-dysmorphic cousins with behavioral problems including inappropriate sexual behavior and pyromania and a submicroscopic 8pter deletion caused by a familial t(8;20)(p23;p13).[106] In addition, several patients with a microscopically visible 8p23.1pter deletion and normal IQ scores have been reported.[103-105]

Interstitial deletion of 8p23.1, mediated by clusters of olfactory receptor/ defensin repeats, is a genomic disorder characterized by developmental delay, behavioral problems, including hyperactivity and impulsiveness, congenital heart disease, diaphragmatic hernia and a Fryns syndrome-like condition.[107-110] Some of these deletions are larger than the common rearrangement and may even extend to include the 8p subtelomeric region.[110] The congenital heart disease characteristic for this deletion is attributable to haploinsufficiency of the *GATA4* gene.[111]

9q

Common features seen in the 9q34 deletion syndrome are severe mental retardation, hypotonia, brachycephaly, a flat face with hypertelorism, synophrys, anteverted nares, a cupid bow or a tented upper lip, an everted lower lip, prognathism, macroglossia, conotruncal heart defects and behavioral problems (**Figure 1.5c)**.[112,113] Comprehensive mutation analysis of the *EHMT1* gene in patients with clinical presentations reminiscent of the 9q subtelomeric deletion syndrome revealed two *de novo* mutations, indicating that haploinsufficiency of *EHMT1* is causative for the 9q subtelomeric deletion syndrome.[112]

16p

Deletions of the 16p13.3 region give rise to α-thalassemia-mental retardation linked to the chromosome 16 syndrome (ATR-16; MIM #141750).[114] In addition to the α-thalassaemia, the most consistent physical findings in pure 16pter monosomy are mild mental retardation, hypotonia, a high forehead, hypertelorism, a broad or prominent nasal bridge and talipes equinovarus (clubfoot).[115] Genital abnormalities are also seen, particularly undescended testes in males. Also nonspecific difficulties in the perinatal period are relatively common, possibly due to neuromuscular hypotonia.[115] A similar disorder, the X-linked α-thalassemia-mental retardation syndrome (ATR-X, MIM #301040), is caused by mutations in the ATRX gene.[116] Familial ATR-16 has been described resulting from an inherited cryptic subtelomeric translocation, t(3;16)(q29;p13.3), inherited in a seemingly autosomal dominant fashion with reduced penetrance.[117] Haploinsufficiency of the α-1 (*HBA1*) and α-2 (*HBA2*) globin genes is thought to cause the α-thalassaemia, whereas haploinsufficiency of the transcriptional regulator *SOX8* has been suggested to contribute to the mental retardation in ATR-16.[118]

22q

One of the first syndromes caused by a submicroscopic subtelomeric CNV identified among unselected individuals with idiopathic mental retardation was the subtelomeric 22q13.3 microdeletion syndrome (MIM #606232).[119] Subtelomeric 22q13.3 deletions range in size from 130 kb to 9.0 Mb, but comparison of clinical features to deletion sizes showed few correlations (**Chapter 2.2**).[120,121] The 22q13.3 deletion syndrome is characterized by neonatal hypotonia, global developmental delay, normal to accelerated growth, minor dysmorphic features (**Figure 1.5d**), absent to severely delayed speech development and autistic behavior.[122-124] It has been suggested that haploinsufficiency of *SHANK3/ProSAP2,* encoding the synaptic scaffolding protein SHANK3, is responsible for the clinical disorder.[125] Moreover, it has been shown that mutations in *SHANK3* and CNVs encompassing *SHANK*, are also associated with autism spectrum disorder.[123,125-127]

1.3.2. Interstitial copy number variation

Elucidation of the underlying cause of known syndromes

The introduction of novel techniques such as high resolution prometaphase chromosome banding, RFLP, PFGE and FISH allowed the detection of genomic aberrations that could not be detected by conventional chromosome analyses (**Chapter 1.2**). Using these techniques, cryptic CNVs were found to be the cause of several previously described malformation syndromes, such as deletion of 15q11q13 in Prader-Willi syndrome (PWS, MIM #176270) and Angelman syndrome (AS, MIM #105830), deletion of 8q24.11q24.13 in trichorhinophalangeal syndrome type II (TRPS2, MIM #150230), deletion of 11p13 in the Wilms tumor aniridia genitourinary-anomalies mental retardation syndrome (WAGR, MIM #194072) and deletion of 22q11.2 in DiGeorge/velocardiofacial syndrome (DGS/VCFS, MIM, #188400/#192430) (**Table 1.2**).

Table 1.2: Mental retardation syndromes commonly caused by submicroscopic CNVs: their chromosomal location and genes involved

Syndrom	CNV	Chromosomal location	Common Size (Mb)	Gene(s) involved	MIM
Williams-Beuren	del	7q11.23	1.5	ELN[6]	194050
TRPS2	del	8q23.3q24.1	–	TRPS*1*, EXT1[c]	150230
WAGR	del	11p13	–	PAX6, WT1[d]	194072
Prader-Willi/Angelman	del	15ql1.2ql3[a]	5	UBE3A[c]	176270/105830
Smith-Magenis	del	17pl1.2	3.7	RA11	182290
DiGeorge/velocardiofacial	del	22ql1.2	3	TBX1[f]	188400/192430
Pelizaeus-Merzbacher	dup/del	Xp22	–	PLP1	312080

[a] Paternal in origin in PWS; maternal in origin in AS.[128]
[b] *ELN* mutations cause supravalvular aortic stenosis.[129]
[c] Mutations in *TRPS1* and *EXT1* cause TRPS1 and multiple exostoses type I, respectively.[130,131]
[d] *PAX6* mutations cause aniridia type II; loss of *WT1* results in genitourinary anomalies and increased risk for Wilms' tumor.[132,133]
[e] In approximately 25% AS is caused by mutations in the ubiquitin-protein ligase E3A gene (*UBE3A*).[134]
[f] *TBX1* mutations result in five major components of the 22q11.2 microdeletion syndrome.[135]

The latter recurrent microdeletions share a common etiologic mechanism, i.e., non-allelic homologous recombination (NAHR) between low-copy repeats (LCRs) that flank the rearranged unique genomic segment.[136,137] NAHR is based on mispairing between non-allelic homologous LCRs and subsequent crossing over between DNA strands during meiosis, resulting in duplication or deletion of the intervening sequence that may range in size from a few kb to several Mb.[136,137] Clinical human disorders that are caused by rearrangements predisposed by structural characteristics of the genome are referred to as 'genomic disorders'.[136] The rearrangements associated with these genomic disorders include deletions, duplications and inversions and lead to the complete loss or gain of a gene(s) sensitive to a dosage effect or, alternatively, to disruption of its structural integrity.[84,136-138] Next to NAHR, other molecular mechanisms might underlie these rearrangements, such as non-homologous end joining (NHEJ)[139] and replication Fork Stalling and Template Switching (FoSTeS).[140]

Identification of new interstitial CNVs in mental retardation

From the identification of microdeletions in clinical syndromes and the detection of cryptic subtelomeric aberrations using FISH and PCR-based analyses, it could be expected that submicroscopic chromosome aberrations should also be present at various other sites within the human genome, in particular in patients with mental retardation. The first probe collections for the measurement of chromosome anomalies across the human genome were assembled in 2001.[141] The subsequently developed microarrays contained approximately 3,400 BAC clones covering the entire genome with an average spacing of one clone per Mb.[142]

Several pilot studies showed the diagnostic value of the genome-wide 1 Mb resolution microarrays in mental retardation.[142-144] In these studies *de novo* submicroscopic genome

imbalances were identified in approximately 10-15% of individuals with mental retardation.[142-144] The clinical usefulness of genome profiling was underscored in larger cohorts of patients with unexplained mental retardation using similar microarrays, tiling-resolution BAC microarrays and, subsequently, 100k SNP microarrays.[121,145-151]
In genome-wide studies among unselected individuals with unexplained mental retardation, the diagnostic yield of clinically relevant CNVs was approximately 11% (**Table 1.3**).[121,142-157] Obviously, the diagnostic yield of genome-wide diagnostic approaches largely depends on the previous cytogenetic studies performed, patient selection and the microarray platforms used.[158]

The exact clinical interpretation of the CNVs observed, however, is challenging and many questions remain (**Chapter 3.1**). One of the major difficulties is caused by the fact that genomes from normal individuals show extensive benign genomic copy number variation.[159-161] It is generally assumed now that CNV is the most prevalent type of structural variation in the human genome and that it contributes significantly to genetic heterogeneity.[162] A first-generation map of CNVs in healthy individuals showed that up to approximately 12% of the human genome involves CNVs.[160] In genome-wide approaches for the diagnosis of mental retardation, these CNVs challenge the interpretation of the test results and the translation into clinical phenotypes. Since as yet little is known about the clinical consequences of these submicroscopic CNVs, they severely hamper the genetic counseling in families. Targeted genomic microarrays designed to interrogate regions of known clinical significance can be used in order to facilitate the clinical interpretation of submicroscopic CNVs in a diagnostic setting.[163,164] However, in contrast to

Table 1.3: Diagnostic yield of genome-wide microarray studies in mental retardation using different platforms

Study	Reference	Number	Platform	Diagnostic yield (%)
1	Vissers (2003)	20	1 Mb BAC array	10
2	Shaw-Smith (2004)	50	1 Mb BAC array	14
3	De Vries (2005)	100	32K BAC array	10
4	Schoumans (2005)	41	1 Mb BAC array	9.1
5	Tyson (2005)	22	1 and 3 Mb BAC array	9.8
6	Menten (2006)	140	1 Mb BAC array	16.7
7	Miyake (2006)	30	1,5Mb BAC array	13.6
8	Friedmann (2006)	100	100K oligo array	13.6
9	Krepischi-Santos (2006)	95	1 Mb BAC array	10
10	Rosenberg (2006)	81	1 Mb BAC array	13.7
11	Engels (2007)	60	500 kb BAC array	6.7
12	Hoyer (2007)	104	100K oligo array	12.0
13	Fan (2007)	100	44K oligo array	8.7
14	Aradhya (2007)	20	44K oligo array, 1 Mb BAC array	40
15	Wagenstaller (2007)	67	100K oligo array	14.9
16	Thuresson 92007)	48	1 Mb BAC array	4.2
17	Koolen (in press)[a]	386	32K BAC array	9.1
	Total	**1,364**		**11.2**

[a] The results of de Vries *et al*.[121] are included.

genome-wide microarrays, targeted approaches fail to detect sporadic CNVs related to mental retardation, as the corresponding probes are not present on these microarrays **(Chapter 3.2**).

Delineation of new interstitial mental retardation syndromes

The implementation of novel cytogenetic and molecular techniques resulted in the identification of recurrent disease-associated CNVs. Often, these recurrent rearrangements result from local structural characteristics of the genome (see above). The first recurrent rearrangement that was shown to result from such genomic architectural features was a 1.5-Mb DNA duplication of the 17p12p11.2 region associated with Charcot-Marie-Tooth disease type 1A (CMT1A, MIM #118220).[165,166] The duplication results from NAHR mediated by flanking LCRs, leading to a gene dosage effect of the *PMP22* gene.[167-169] Point mutations in the same gene can lead to the same phenotype.[170-172] Reciprocal deletions were identified in patients with a milder phenotype, i.e., hereditary neuropathy with liability to pressure palsies (HNPP, MIM #162500).[173] Similarly, reciprocal duplications of known genomic disorders associated with mental retardation are predicted to occur. The Potocki-Lupski syndrome (PLS, MIM #610883), resulting from homologous recombination reciprocal of the Smith-Magenis syndrome-associated microdeletion 17p11.2, was the first predicted mental retardation microduplication syndrome described.[174,175] Similarly, reciprocal duplications of the WBS critical region at 7q11.23 and the DGS/VCFS region at 22q11.2 have been associated with mental retardation.[176-179]

High resolution cytogenetic analyses and/or additional molecular/cytogenetic analyses also revealed mental retardation syndromes such as the Potocki-Shaffer syndrome (PSS, MIM #601224), caused by an interstitial deletion of 11p11.2p12 and recurrent 10q22q23 deletions, associated with cognitive and behavioral abnormalities.[180,181]

The implementation of microarray technologies resulted in the identification of numerous overlapping submicroscopic CNVs in individuals with mental retardation. In retrospect, common clinical features could be determined and, subsequently, a clinical syndromes associated with mental retardation could be defined. This 'genotype-first' approach,[182] or 'reverse phenotypics', by which individuals with unexplained mental retardation are characterized by a similar genomic aberration before a common clinical presentation is defined, has proven to be successful considering the constantly increasing list of newly detected microdeletion/microduplication syndromes (**Table 1.4**). Various interstitial microdeletion/microduplication syndromes associated with mental retardation that were recently recognized are briefly discussed below.

Table 1.4: Newly recognized interstitial microdeletion/duplication syndromes associated with mental retardation.

Name	Size (Mb)[a]	LCR	MIM	Clinical features
1q41q42 microdeletion	1.2	-	-	MR, seizures, various dysmorphisms, cleft palate, diaphragmatic hernia
2p1516.1 microdeletion	3.9	-	-	MR, MC, receding forehead, ptosis, telecanthus, short palpebral fissures, downslanting palpebral fissures, broad/high nasal bridge, long/straight eyelashes, smooth and long philtrum, smooth upper vermillion border, everted lower lip, high narrow palate, hydronephrosis, optic nerve hypoplasia
3q29 microdeletion	1.6	+	609425	MR, mild FD, including high nasal bridge and short philtrum
3q29 microduplication	1.6	+	611936	Mild/moderate MR, MC, obesity
7q11.23 duplication	1.5	+	609757	MR, speech and language delay, autism spectrum disorders, mild FD
9q22.3 microdeletion	6.5	-	-	MR, hyperactivity, overgrowth, trigonocephaly, macrocephaly, FD
12q14 microdeletion	3.4	-	-	Mild MR, failure to thrive, proportionate short stature and osteopoikilosis
14q12 microdeletion	0.4	-	-	MR, widely spaced eyes, short nose with flat nasal bridge, long philtrum, Cupid's bow of the upper lip, full lower lip, auricular anomalies
15q13.3 microdeletion	1.5	+	612001	MR, epilepsy, FD, digital dysmorphisms
15q24 microdeletion	1.7	+	-	MR, growth retardation, MC, digital abnormalities, genital abnormalities, hypospadias, loose connective tissue, high frontal hairline, broad medial eyebrows, downslanted palpebral fissures, long philtrum
16p11.2p12.2 microdeletion	7.1	+	-	MR, flat facies, downslanting palpebral fissures, low-set and malformed ears, eye anomalies, orofacial clefting, heart defects, frequent ear infections, short stature, minor hand and foot anomalies, feeding difficulties, hypotonia
17p11.2 microduplication[b] cardiovascular anomalies	3.7	+	610883	MR, infantile hypotonia, failure to thrive, autistic features, sleep apnea, and structural
17q21.31 microdeletion	0.5	+	610443	MR, hypotonia, long hypotonic face with ptosis, large and low-set ears, tubular or pear-shaped nose with bulbous nasal tip, long columella with hypoplastic alae nasi, broad chin
22q11.2 microduplication	3.7	+	608363	Highly variable. MR, FD, e.g. widely spaced eyes and downslanting palpebral fissures, velopharyngeal insufficiency, conotruncal heart disease
22q11.2 distal microdeletion	1.4 - 2.1	+	611867	MR, prematurity, prenatal/postnatal growth delay, mild skeletal abnormalities, arched eyebrows, deep-set eyes, smooth philtrum, thin upper lip, hypoplastic alae nasi, small pointed chin
Xq28 microduplication	0.4 - 0.8	-	-	MR, severe hypotonia, progressive lower-limb spasticity, absent or very limited speech

Note. —The 8p23.1 syndrome is described in Chapter 1.3.1, as some deletions extend to include the 8p telomere. MR, mental retardation; MC, microcephaly; FD, facial dysmorphisms

[a] Common region

[b] Potocki-Lupski syndrome

1q41q42 microdeletion

Using a targeted microarray covering regions commonly rearranged in chromosome abnormalities and genes involved in important developmental pathways, Shaffer *et al.* identified seven recurrent *de novo* deletions of 1q41q42 that include the *DISP1* gene, which is involved in the sonic hedgehog pathway (SHH; see above 7q36 deletion syndrome).[182] The deletions range in size from 2.7 – 9.1 Mb with a smallest region of overlap of 1.2 Mb. Although none of these patients showed frank holoprosencephaly, many exhibited other midline defects (cleft palate, diaphragmatic hernia), developmental delay or mental retardation, seizures and dysmorphic features.

2p15p16.1 microdeletion

Through whole genome array CGH screening of subjects with idiopathic intellectual disability, Rajcan-Separovic *et al*. identified two unrelated individuals with an overlapping *de novo* interstitial microdeletion at 2p15-2p16.1 with respective sizes of 4.5 and 5.7 Mb.[183] De Leeuw *et al*. reported a similar patient with a corresponding 3.9 Mb deletion at 2p15-2p16.1.[184] These individuals shared a number of malformations and rather specific dysmorphic features, including microcephaly, facial dysmorphisms (bitemporal narrowing, a receding short forehead, ptosis, telecanthus, short palpebral fissures, downslanting palpebral fissures, a broad/high nasal bridge, long, straight eyelashes, a smooth and long philtrum, a smooth upper vermillion border, an everted lower lip, a high narrow palate) (**Figure 1.6a**), hydronephrosis and optic nerve hypoplasia, as well as moderate to severe mental retardation, indicating that they share a newly recognized microdeletion syndrome. Chabchoub *et al*. identified a 570-kb *de novo* microdeletion at 2p15 in a patient with mild intellectual disability, a heart defect, an ectomorphic habitus and similar facial dysmorphic features, but without the microcephaly, kidney anomalies, autistic disorder, or optic nerve hypoplasia.[185]

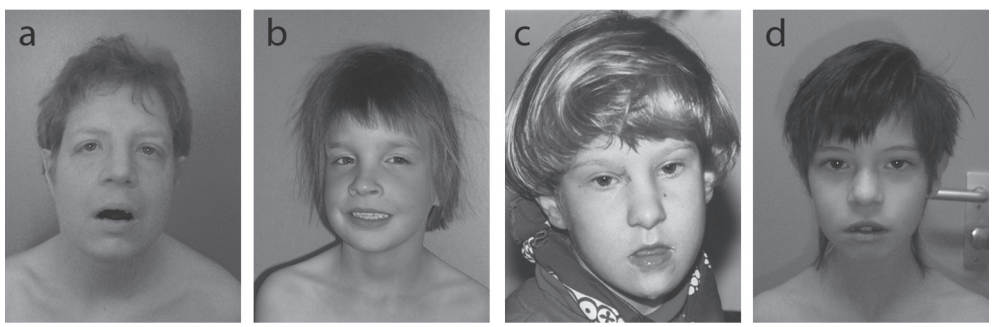

Figure 1.6: Patients with newly recognized interstitial microdeletion syndromes. (**a**) 2p15p16.1 microdeletion, (**b**) 3q29 microdeletion (**c**) 17q21.31 microdeletion (**d**) distal 22q11 microdeletion. Informed consent was obtained for publication of photographs. For color figure see page 191.

3q29 microdeletion

Subtelomere screening using FISH and MLPA revealed the presence of a terminal deletion of 3q29 in six individuals with mild to moderate mental retardation, a long and narrow face, a short philtrum and a high nasal bridge (**Figure 1.6b**).[186] Further delineation of the deletions using FISH revealed that they were interstitial, as the most distally located ~700 kb was retained in all cases. In addition, the deletions were almost identical in size, and the presence of two highly homolgous LCRs on either side of the deletion breakpoint suggested that NAHR is the likely mechanism underlying this syndrome. Ballif *et al*. identified 14 individuals with microdeletions of 3q29, including a family with a mildly affected mother and two affected children, among 14,698 individuals with idiopathic mental retardation that were analyzed by array CGH.[187] Eleven individuals had typical 1.6 Mb deletions and three individuals had deletions that flank, span, or partially overlap the commonly deleted region. The clinical presentations of individuals with microdeletions of 3q29 varied widely, with mild to moderate mental retardation/developmental delay, microcephaly, and mild dysmorphic features (including a high nasal bridge and a short philtrum) the only features common to the majority.[187]

3q29 microduplication

A reciprocal duplication of the recurrent 3q29 microdeletion was identified in a three-generation family in which five members exhibited mild to moderate mental retardation and minor dysmorphic features.[188] Ballif *et al*. identified five reciprocal duplication products of the 3q29 microdeletion.[187] Like that of the 3q29 microdeletion, the phenotypes of the reciprocal microduplication varied considerably, with mental retardation, microcephaly and obesity being the only common features.[187]

9q22.32q22.33 microdeletion

In a genome-wide array CGH-based screening of patients with unexplained overgrowth syndromes Redon *et al*. identified two children with nearly identical 6.5 Mb long *de novo* interstitial deletions at 9q22.32q22.33.[189] Although the deletion boundaries were different in the two patients, they suggested that microdeletions of 9q22.32q22.33 represent a recognizable syndrome, as both affected individuals presented with similar clinical features, including psychomotor delay, hyperactivity, overgrowth, trigonocephaly, macrocephaly and distinctive facial features.[189]

15q13.3 microdeletion

Through whole-genome array CGH-based screening of 757 individuals with mental retardation and/or congenital anomalies Sharp *et al*. identified two identical *de novo* 1.5 Mb deletions in 15q13.3.[190] Subsequently, they screened 1,040 individuals with mental retardation of unknown etiology using quantitative PCR (Q-PCR) assays targeted to the 15q13.3 region. By doing so, they identified nine affected individuals, including six probands: two with *de novo* deletions, two who inherited the deletion from an affected parent and two with an unknown mode

of inheritance. The patients had mental retardation, epilepsy and variable facial and digital dysmorphisms in common. The proximal breakpoint of the largest deletion is contiguous with breakpoint 3 (BP3) of the PWS and AS region, extending 3.95 Mb distally to BP5. A smaller 1.5 Mb deletion had a proximal breakpoint within the larger deletion (BP4) and shared the same distal breakpoint (BP5). This recurrent 1.5 Mb deletion encompasses six genes, including a candidate gene for epilepsy (*CHRNA7*).

15q24 microdeletion

De Vries *et al.* reported a 15q24 deletion identified in a boy with mental retardation[121] and, subsequently, Sharp *et al*. identified another patient with a *de novo* microdeletion of 15q24 among a cohort of 290 children with idiopathic mental retardation.[191] After this, two more patients with submicroscopic deletions of this region were identified and all four individuals shared several clinical features, including mental retardation, growth retardation, microcepahly, digital abnormalities, genital abnormalities, hypospadias and loose connective tissue. In addition, similar facial dysmorphisms were noted, including high frontal hairline, broad medial eyebrows, downslanted palpebral fissures and a long philtrum, indicating that the 15q24 deletions represent a clinical syndrome.[192] A further 15q24 microdeletion case showed similar phenotypic features, although microcephaly and growth deficiency were absent.[193] The deletion in the patients varied from 1.7 to 3.9 Mb in size. The breakpoints were located in nearly identical segmental duplications, which turned NAHR into the most likely underlying mechanism

16p11.2p12.2 microdeletion

Ballif *et al.* screened the pericentromeric regions of the genome and identified recurrent *de novo* interstitial deletions of 16p11.2p12.2 in four patients.[194] The common clinical features included distinct facial features, i.e., flat facies, downslanting palpebral fissures, low-set and malformed ears and eye anomalies. Other features were orofacial clefting, heart defects, frequent ear infections with potential hearing loss, a short stature, minor hand and foot anomalies, feeding difficulties, hypotonia and cognitive and developmental delays.[194] The deletions, ranging from 7.1 to 8.7 Mb in size, shared a common distal breakpoint, but varied in their proximal breakpoints. The complex structure of the 16p11.2p12.2 region, including LCRs that flank some of the deletion breakpoints, suggest that NAHR may be the mechanism underlying these deletions.

17q21.31 microdeletion

Three groups simultaneously described the identification of a microdeletion syndrome encompassing 17q21.31. Recurrent overlapping *de novo* microdeletions in 17q21.31 were identified in patients with mental retardation using array CGH and MLPA (**Chapter 3.3**).[191,195,196] Clinical comparison of these patients revealed marked phenotypic similarities, i.e., mental retardation, hypotonia and characteristic facial features, including a long hypotonic face with

ptosis, large and low-set ears, a tubular or pear-shaped nose with a bulbous nasal tip, long columella with hypoplastic alae nasi and a broad chin (**Figure 1.6c**).[195] The identification of more patients with the same aberration showed that the 17q21.31 microdeletion syndrome (MIM *610443) is a frequent cause of mental retardation and allowed the detailed clinical and molecular delineation this syndrome (**Chapter 3.4**).[197]

22q11.2 distal micodeletion

Using array CGH analysis, Ben-Shachar *et al*. defined a recurrent genomic disorder at 22q11.2, distinct from the DGS/VCFS critical region.[198] They detected six *de novo* deletions within 22q11.2, located distal to the approximately 3 Mb common 22q11.2 deletion region. The rearrangements shared clustered breakpoints and either a ~1.4 Mb or a ~2.1 Mb recurrent deletion flanked by LCRs. The patients presented with prematurity, prenatal and postnatal growth delay, developmental delay and mild skeletal abnormalities, and characteristic facial dysmorphic features, including arched eyebrows, deep-set eyes, a smooth philtrum, a thin upper lip, hypoplastic alae nasi and a small, pointed chin (**Figure 1.6d**). Two patients had a cardiovascular malformation and one patient had a cleft palate. Although there is some clinical overlap with DGS/VCFS, the distal chromosome 22q11.2 deletion syndrome (MIM #611867) represents a novel genomic disorder.

Xq28 duplication

By array CGH, van Esch *et al*. identified a small duplication at Xq28 in a large family with a severe form of mental retardation associated with progressive spasticity.[199] Subsequently, Q-PCR analysis among patients with similar clinical features revealed three additional duplications at Xq28. The duplications varied in size from 0.4 to 0.8 Mb encompassing the *LICAM* and *MECP2* genes. No consistent flanking centromeric or telomeric breakpoints could be identified.[199] Individuals with a Xq28 duplication encompassing *MECP2* frequently present with severe mental retardation, severe hypotonia and in the majority of patients a progressive lower-limb spasticity and absence or very limited speech.[199,200] In addition, seizures and recurrent severe infections are common.[199,200] Loss-of-function mutations of *MECP2* are associated with Rett syndrome (MIM #312750) in females and increased gene dosage of *MECP2* is the most likely explanation for the severe mental retardation in patients with submicroscopic Xp28 duplications.[199]

The above-mentioned syndromes emphasize the great potential of the development of novel technologies and show that the detection of submicroscopic CNVs might result in the identification of numerous novel disorders associated with mental retardation. Apart from these syndromes, several other novel genomic disorders have been identified, such as a microdeletion syndrome on 12q14, associated with osteopikilosis, short stature and mental retardation[201] and recurrent deletions of 14q11.2.[202] Moreover, reciprocal 16p13.1 duplications and deletions predisposing to autism and/or mental retardation have been described, similar

to the association between autism and microdeletion or microduplication at 16p11.2.[203,204] Additionally, recurrent interstitial CNVs in the subtelomeric region of the long arm of chromosome 1 have been described.[205-207] The identification of these interstitial CNVs close to the telomere, but also e.g., the complex nature of many subtelomeric 1p36 deletions[85,89,90] illustrates that the discrimination between subtelomeric- and interstitial submicroscopic CNVs is in fact artificial and mainly reflects the historical context in which the respective CNVs have been identified.

1.4 Outline of the thesis

At the onset of the work described in this thesis, the genetic diagnosis of mental retardation remained unknown in about 50% of the patients. Next to a number of gross chromosomal anomalies detected by conventional cytogenetic analysis, also several submicroscopic chromosomal anomalies (smaller than ~5-10 Mb) had already been identified in several well-defined clinical syndromes using FISH and/or PCR-based techniques. Among these anomalies, subtelomeric rearrangements were found to represent an important cause of mental retardation. In addition, several pilot-studies indicated that also interstitial submicroscopic chromosome aberrations might significantly contribute to the etiology of mental retardation. Subsequently, genomic microarrays found their way into the clinical workup of individuals with mental retardation, and they further emphasized the importance of structural genomic variation —mainly CNVs— in mental retardation. The identification of these causes of mental retardation is of major importance for the patients and their families, as an explanation of the disease may provide insight in its occurrence and the clinical features associated with it. This, in turn, may lead to better genetic counseling within families and a better clinical management of the patients.

The general goal of this thesis was to obtain a better understanding of the genetic basis of mental retardation and congenital malformations by aiming at the following objectives:

I. Determination of the frequency of submicroscopic CNVs in mental retardation.

Using MLPA, we determined the frequency of submicroscopic CNVs at the subtelomeres among a cohort of individuals with unexplained mental retardation (*n=210*) (**Chapter 2.1**). In order to establish the frequency of interstitial CNVs in mental retardation we studied affected individuals (*n=386*) using whole-genome tiling resolution BAC arrays (**Chapter 3.1**). The frequency of CNVs associated with mental retardation was tested by MLPA analysis targeting to candidate regions for mental retardation among a large European patient cohort (**Chapter 3.2**).

II. Identification and characterization of mental retardation syndromes.

The identification of overlapping submicroscopic deletions and duplications in individuals with mental retardation and similar clinical features, allows the definition of new mental retardation syndromes. Implementation of subtelomeric DNA copy number analysis in the diagnostic workup of patients with mental retardation resulted in the recognition of several distinct clinical entities, such as the 22q13 microdeletion syndrome (MIM #606232). We studied the size and nature of 22q13 deletions using high-resolution chromosome specific array CGH and investigated the relationships between clinical features and deletion sizes (**Chapter 2.2**).

We identified three overlapping interstitial submicroscopic deletions on 17q21.31 in patients with unexplained mental retardation (**Chapter 3.3**). Clinical comparison of the patients revealed marked phenotypic similarities, indicating that this microdeletion underlies a clinical syndrome. Subsequently, we studied a cohort of individuals with the 17q21.31 microdeletion syndrome (*n=22*), allowing a delineation of the critical region (**Chapter 3.4**). Based on detailed clinical information of all deletion carriers, the clinical phenotype of the syndrome could be established and an estimate of the prevalence of the 17q21.31 microdeletion syndrome could be obtained.

In **Chapter 4** the implications of this work are discussed and an outline of future avenues towards the detection of additional mental retardation-associated syndromes and its clinical diagnostic application are provided.

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Chapter 2

Subtelomeric copy number variation in mental retardation

2.1 Screening for subtelomeric rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplicifaction (MLPA)
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2.2 Molecular characterization of patients with subtelomeric 22q abnormalities using chromosome specific arrray-based comparative genomic hybridization
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Chapter 2.1

Screening for subtelomeric rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplification (MLPA)

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Abstract

Background: Subtelomeric rearrangements contribute to idiopathic mental retardation and human malformations, sometimes as distinct mental retardation syndromes. However, for most subtelomeric defects a characteristic clinical phenotype remains to be elucidated.

Objective: To screen for submicroscopic subtelomeric aberrations using multiplex ligation dependent probe amplification (MLPA).

Methods: 210 individuals with unexplained mental retardation were studied. A new set of subtelomeric probes, the SALSA P036 human telomere test kit, was used.

Results: A subtelomeric aberration was identified in 14 patients (6.7%) (10 deletions and four duplications). Five deletions were *de novo*; four were inherited from phenotypically normal parents, suggesting that these were polymorphisms. For one deletion, DNA samples of the parents were not available. Two *de novo* submicroscopic duplications were detected (dup 5qter, dup 12pter), while the other duplications (dup 18qter and dup 22qter) were inherited from phenotypically similarly affected parents. All clinically relevant aberrations (*de novo* or inherited from similarly affected parents) occurred in patients with a clinical score of $\geq$3 using an established checklist for subtelomeric rearrangements. Testing of patients with a clinical score of $\geq$3 increased the diagnostic yield twofold to 12.4%. Abnormalities with clinical relevance occurred in 6.3%, 5.1%, and 1.7% of mildly, moderately, and severely retarded patients, respectively, indicating that testing for subtelomeric aberrations among mildly retarded individuals is necessary.

Conclusions: The value of MLPA is confirmed. Subtelomeric screening can be offered to all mentally retarded patients, although clinical preselection increases the percentage of chromosomal aberrations detected. Duplications may be a more common cause of mental retardation than has been appreciated.

Introduction

Chromosomal rearrangements involving subtelomeric regions are a common cause of idiopathic mental retardation (reviewed by Knight and Flint[1] and De Vries *et al.*.[2]) Subtelomeric rearrangements have been reported to occur in approximately 5% of patients with unexplained mental retardation.[2] Some subtelomeric submicroscopic deletions result in well defined mental retardation syndromes, such as monosomy 1p36, Wolf–Hirschhorn syndrome (4p-), and cri-du-chat syndrome (5p-), but for most subtelomeric defects a characteristic phenotype remains to be defined.[2] For this reason, screening of all subtelomeres is a valuable diagnostic tool. Multiprobe FISH (fluorescence *in situ* hybridization), using telomeric probes on metaphase chromosomes is commonly used for detecting subtelomeric abnormalities.[3-5] This reliable method, however, remains labor intensive and therefore expensive for routine diagnostic testing. New techniques, such as multiplex amplifiable probe hybridization (MAPH) and array based comparative genomic hybridization (array CGH), are proven to be suitable for the detection of subtelomeric chromosome aberrations.[6,7] However, MAPH, requires immobilization of sample nucleic acids[8] and array CGH is labor intensive and requires expensive equipment. Recently, multiplex ligation dependent probe amplification (MLPA) was applied to detect subtelomeric defects in 75 patients with mental retardation of unknown cause.[9] In the current study MLPA was used with a new set of subtelomeric probes designed by Schouten *et al.*[8] for the detection of submicroscopic aberrations in a larger sample of clinically well defined patients with idiopathic mental retardation. We show that MLPA is a reliable technique to detect submicroscopic telomeric copy number changes, rendering it suitable for routine diagnostic screening in mentally retarded patients.

Methods

Patients

The diagnostic capacity of MLPA for detecting subtelomeric chromosome aberrations was tested by screening 210 patients with unexplained mental retardation. The patients were all referred to the department of human genetics, University Medical Centre Nijmegen for subtelomeric analysis. A total of 137 patients had been evaluated by one of the clinical geneticists at our centre. The remaining patients were referred by other medical specialists, mainly pediatricians. All patients had a normal G banded karyotype at a 550 band level using standard procedures, and no clinical syndrome had been recognized. The level of mental retardation (mild, IQ 50 to 70; moderate, IQ 30 to 50; severe, IQ <30) and the score on the checklist for submicroscopic subtelomeric rearrangements developed by de Vries *et al.*[10,11] were obtained retrospectively from notes by the referring specialist. The incidence of subtelomeric aberrations was assessed for the level of mental retardation and for the subtelomeric aberration checklist score. In case of a subtelomeric aberration, DNA samples of the parents were requested for further testing. The MLPA kit was first validated using 15 DNA samples from patients with 16 known (sub) microscopic defects involving the subtelomeric region.

Multiplex ligation dependent probe amplification

MLPA probes

A specifically designed set of probes for testing for subtelomeric chromosomal imbalances, SALSA P036 human telomere test kit (MRC-Holland, Amsterdam, Netherlands; http://www.mrc-holland.com) was used for subtelomere screening. Probe preparation has been described previously.[8] The MLPA mix contained probes for each subtelomeric region (**Table 2.1.1**) except for the short arms of the acrocentric chromosomes. For the latter, probe recognition sequences on the q arm, in one of the first genes following the repeated sequences of the centromere, were used. Because these probes were not subtelomeric, they were not included in our analysis.

MLPA analysis

Genomic DNA of each patient was isolated using standard procedures.[14] MLPA analysis was carried out as described by Schouten *et al.*,[8] with slight modifications. Briefly, 200 to 400 ng

Table 2.1.1: Subtelomeric MLPA probes and their distance to the telomere

	Salsa P036 MLPA subtelomere probes of the p arms			Salsa P036 MLPA subtelomere probes of the q arms		
Telomere	Length (nt)	Gene detected	Distance to telomere (Mb)	Length (nt)	Gene detected	Distance to telomere (Mb)
1	130	CAB45	1.07	306	KIAA1720	0.20
2	137	ACP1	0.25	314	CAPN10	1.76
3	144	CHL1	0.34	322	BDH	0.74
4	151	FLJ20265	0.50	330	FAT	3.41a
5	158	PDCD6	0.37	338	MGC16175	0.24
6	165	IRF4	0.34	346	PSMB1	0.21
7	172	CENTA1	0.70	354	VIPR2	0.23
8	179	FBXO25	0.40	362	KIAA0150	1.58
9	186	DMRT1	0.84	370	MRPL41	0.71
10	194	KIAA0934	0.44	378	CYP2E1	0.22
11	202	MUC2	1.09	386	KIAA0056	0.85
12	210	SLC6A12	0.17	394	KIAA1545	0.68
13	218	PSPC1	18.14	402	F7	1.32
14	226	HEI10	18.78	410	KIAA0284	1.99
15	234	CYFIP1	20.52	418	ALDH1A3	1.07
16	242	POLR3K	0.04	426	TUBB4	0.30
17	250	RPH3AL	0.21	434	TBCD	0.32
18	258	USP14	0.19	442	FLJ21172	0.22
19	266	CDC34	0.49	450	LOC125905	0.18
20	274	SOX12	0.30	458	EEF1A2	0.89
21	282	RBM11	14.51	466	S100B	0.10
22	290	BID	16.60	474	RABL2B	0.06
X/Y	298	SHOX	0.54	482	SYBL1	0.13

The SALSA P036 human telomere test kit was used. The unique length of the amplification product of each probe (in nucleotide [nt]) and the gene detected are shown. The distance to the telomere for each probe recognition sequence was determined using the UCSC Human Genome Browser and Blat Search, July 2003 Freeze.
[a] Proximal to the D4Z4 repeat associated with FSHD.[12,13]

of DNA sample was diluted with milliQ to 8 ml and heated at 98˚C for five minutes (GeneAmp PCR System 9700, Applied Biosystems, Foster City, California, USA). After addition of the probe mix (1.5 ml per sample), which was mixed 1:1 with a salt solution (1.5 M KCl; 300 mM Tris-HCl, pH 8.5; 1 mM EDTA), samples were heated for one minute at 95˚C and incubated overnight at 60˚C. Next the Ligation-65 mix (following the supplier's instructions) was added and incubated for 15 minutes at 54˚C. Ligase-65 was inactivated by heating at 98˚C for one minute. The ligation products were amplified by polymerase chain reaction (PCR) using the common primer set with the 6-FAM label distributed by the supplier. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3100 genetic analyzer, using Genescan analysis software (version 3.7) and Genotyper software, all from Applied Biosystems. Subtelomeric screening of 48 samples using MLPA took approximately 1.5 days, including four hours of hands-on time. Each subtelomeric rearrangement was detected by at least one additional MLPA analysis.

Statistical analysis/data processing

The signal strength of the PCR products was determined by Genotyper software (Applied Biosystems). A spreadsheet was developed in Microsoft™ Excel in order to process the sample data efficiently. First, the data were normalized by dividing each probe's signal strength by the average signal strength of the sample. This normalized peak pattern was divided by the average peak pattern of all the samples in the same experiment. The resulting values were approximately 1.0 for every wild type peak, 0.5 for heterozygous deletions, and 1.5 for heterozygous duplications. As a quality check for the probes, we computed the coefficient of variation (cv) of the normalized signal strength over the controls. If a particular probe had a cv of more than 10% over all samples tested, the results of the analysis for that particular probe were discarded. However, this was never the case with the Salsa P036 probe set. The analysis for a particular sample was repeated if the cv over all probes was more than 15% (~18% of all tests). Twenty control samples (40 alleles) were run to exclude the presence of common polymorphisms and to test the feasibility of the statistical analysis.

Confirmation experiments

FISH analysis using first and second generation sets of telomere specific clones[5,15] and Vysis probes (Vysis, Downes Grove, Illinois, USA) was carried out to confirm the aberrations identified by MLPA. Copy number changes were checked for *de novo* occurrence by MLPA and FISH analyses in both parents. In case the aberration could not be detected by FISH, DNA samples from the parents were only tested by MLPA. FISH analyses were done using routine methods. Fixed chromosome suspensions were prepared from cultured peripheral blood lymphocytes obtained from patients and parents. Labeling of the probes, slide preparation, and hybridization were carried out using a standard protocol. A Leica DMRA fluorescence microscope, equipped with appropriate filters, was used for visual examination of the slides. The images were captured by a cooled CCD camera (SenSys) coupled to a Leica computer and

analyzed by a CW4000 software package. Inverted DAPI staining and a chromosome specific centromere probe were used for chromosome identification.

Results

In this study MLPA was used to detect subtelomeric aberrations in a group of 210 patients with unexplained mental retardation. An improved set of subtelomeric probes—the SALSA P036 human telomere test kit—was applied. The sensitivity of the probe set was first determined by testing DNA samples from patients with a known chromosomal defect. These known defects were either cytogenetically visible or detected by FISH. Positive controls were available for 1pter, 1qter, 2qter, 3pter, 4pter, 7pter, 9pter, 10qter, 16qter, 18qter, 19qter, 22qter, and for the probes in the pseudoautosomal regions of Xpter, Ypter, Xqter, and Yqter. In all cases the genomic defect was confirmed by MLPA (data not shown).

The initial subtelomeric screening in patients with idiopathic mental retardation showed a subtelomeric rearrangement in 19 patients. Remarkably, a duplication of the 10qter MLPA probe was detected in five patients. These duplications could not be confirmed by FISH analysis. Parental analysis of two of the cases by the same technique revealed an identical duplication in a phenotypically normal father and mother in different families. In addition, the patients showed no clinical resemblance. For these reasons the 10qter duplication was considered most likely to be a polymorphism and was therefore not included in further analyses. Thus 14 patients (6.7%) with a subtelomeric rearrangement remained: 10 deletions and four duplications. Four MLPA profiles of subtelomeric aberrations are shown in **Figure 2.1.1**. Five deletions were *de novo* (1p (twice), 3q, 4p, 10q) and all could be confirmed by FISH. Four deletions (2p, 11p, 12p, 16q) were also present in phenotypically normal parents and these deletions could not be confirmed by FISH. In case 10 with a 22qter deletion, the parents were not available for testing. Two *de novo* subtelomeric duplications (5q, 12p) were detected. The other duplications identified by

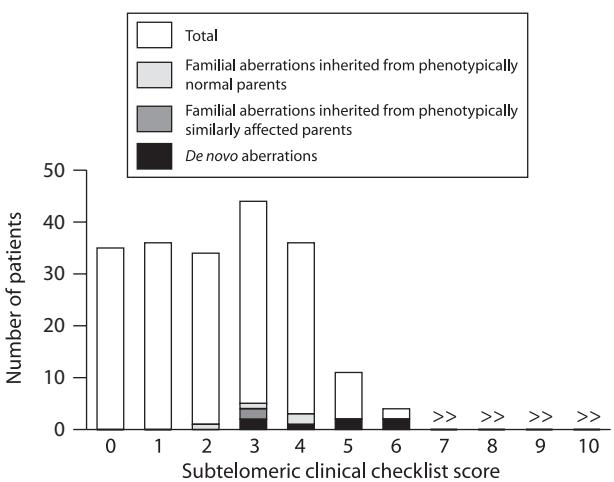

Figure 2.1.1: Detection of subtelomeric aberrations by multiplex ligation dependent probe amplification (MLPA). In each figure the profile of the patient is shown in line 1. The profiles of the father and mother are depicted in lines 2 and 3, respectively. The rectangle indicates the position of the aberrant MLPA probe. (**a**) *De novo* 1pter deletion (case 1). (**b**) Maternal inherited 11pter deletion (case 7). (**c**) *De novo* 12pter duplication (case 12). (**d**) 18qter duplication inherited from the mother (case 13).

MLPA (18q, 22q) were inherited from phenotypically similarly affected parents. For the 22qter duplication, FISH analysis showed that this was the result of a submicroscopic unbalanced translocation (t(21;22)), whereas the other direct duplications identified by MLPA could not be confirmed by FISH. **Table 2.1.2** shows overviews of the respective submicroscopic deletions and duplications identified by MLPA.

Table 2.1.2: Submicroscopic deletions and duplications identified by multiplex ligation dependent probe amplification (MLPA)

Case	MLPA result	FISH confirmation	Parents	Clinical features[a]	Score[b]
Submicroscopic deletions					
1	del 1pter	Yes	de novo	Moderate MR; facial dysmorphisms; ventricular septal defect; hearing loss	6
2	del 1pter	Yes	de novo	Severe MR; facial dysmorphisms ; vesico-ureteric reflux; urachus cyst	3
3	del 3qter	Yes	de novo	Mild MR; scaphocephaly; facial dysmorphisms; pectus carinatum	4
4	del 4pter	Yes	de novo	Moderate MR; short stature; microcephaly; plagiocephaly; facial dysmorphisms; hydronephrosis; hypospadias	4
5	del 10qter	Yes	de novo	Mild MR; facial dysmorphisms; vesico-ureteric reflux; hearing loss	5
6	del 2pter	No	Inherited Paternal	Moderate MR; behavior problems; microcephaly; facial dysmorphisms	4
7	del 11pter	No	Inherited Maternal	Moderate MR; brachycephaly; facial dysmorphisms	2
8	del 12pter	No	Inherited Paternal	Severe MR; hypotonia; microcephaly; holoprosencephaly; corpus callosum dysgenesis	4
9	del 16qter	No	Inherited Paternal	Moderate MR; facial dysmorphisms	3
10	del 22qter	Yes	Not available	Severe MR ; no clinical information available	
Submicroscopic duplications					
11	dup 5qter[c]	No	de novo	Mild MR; facial dysmorphisms; hearing loss; epilepsy; brachycephaly; hydrocephalus	3
12	dup 12pter[c]	No	de novo	Moderate MR; short stature; facial dysmorphisms; hypermobility; pectus excavatum	6
13	dup 18qter[c]	No	Inherited Maternal	Mild MR; short stature	3
14	dup 22qter	Yes, der(21) t(21;22)	Inherited Paternal[d]	Moderate MR; short stature; facial dysmorphisms; seizures; behavior disorders; micro- and trigonocephaly	3

[a] For more details, see results section.
[b] Subtelomeric clinical checklist score.[10]
[c] Submicroscopic aberration not reported before.
[d] Inherited from phenotypically similarly affected parents.
FISH, fluorescence *in situ* hybridization; MLPA, multiplex ligation dependent probe amplification; MR, mental retardation.

The degree of mental retardation in the study group was mild in 31% of the cases (63/201), moderate in 39% (78/ 201), and severe in 30% (60/ 201). In nine cases the level of mental retardation was unspecified. Clinically relevant aberrations (*de novo* or inherited from a phenotypically similarly affected parent) occurred in 6.3%, 5.1%, and 1.7% of the mildly, moderately, and severely retarded patients, respectively. **Figure 2.1.2** shows the clinical score on the checklist for subtelomeric rearrangements reported by de Vries *et al.*[10] and the subtelomeric anomalies per group. All aberrations with clinical relevance were identified in patients with a clinical score of $\geq$3. Clinical information was insufficient for determining a score in 10 cases. A brief clinical description of the cases in which a chromosomal imbalance was identified is given below. If any phenotypic or chromosomal abnormality was present in either parent, this is included in the case description. For case 10 (del 22qter), a 14 year old boy, additional clinical features were not available, nor were the parents available for further testing.

***De novo* submicroscopic subtelomeric deletions identified by MLPA**

Case 1 (del 1pter) was a moderately mentally retarded 18 month old girl (born at 40 weeks' gestation; birth weight 3,060 g, 15th centile). She presented with psychomotor delay and growth failure. She had a perimembranous ventricular septal defect, hearing loss, and several dysmorphic features including a large anterior fontanelle, prominent broad forehead, flat midface, deep set eyes, strabismus, downslant of the palpebral fissures, slight hypertelorism, small low set posteriorly rotated ears, small nose, and flat nasal bridge. In addition she had a flat palate with wide alveolar ridges, downturned corners of the mouth, pointed chin, a short neck, and small broad hands with abnormal implant of the thumbs. At the age of 11.5 months her height was 70 cm (5th centile) and her occipitofrontal head circumference (OFC) was 44.5 cm (16th centile).

Case 2 (del 1pter) was a severely mentally retarded five year old girl (born at 37 weeks' gestation; birth weight 2,765 g, 30th centile) who presented with delayed psychomotor development. Her dysmorphic features included almond shaped eyes, upward slant of the palpebral fissures, flat nasal bridge, bifid uvula, a small U shaped curve in the hard palate, bilateral epicanthic folds, downturned corners of the mouth, mild retrognathia, and a short perineum. She also had a urachus cyst which was corrected surgically and vesicoureteric reflux for which she was treated with prophylactic antibiotics. At four years and three months her height was 109.5 cm (75th centile), her weight was 28.6 kg (+4 SD), and her OFC was 49.9 cm (50th centile).

Case 3 (del 3qter) was a mildly mentally retarded six year old girl (born at 40 weeks' gestation; birth weight 2,930 g, 10th centile). She presented with hearing loss and psychomotor delay. She had scaphocephaly. Computed tomography of the cerebrum revealed a normal brain structure. At six years and two months her height was 1.17 cm (30th centile) and her OFC was 51 cm (50th centile). Besides the scaphocephaly, other dysmorphic features included frontal bossing, slightly downslanting palpebral fissures, low set posteriorly rotated ears, broad nostrils, smooth philtrum, everted lower lip, high palate, pectus carinatum, clinodactyly of the fifth fingers, long

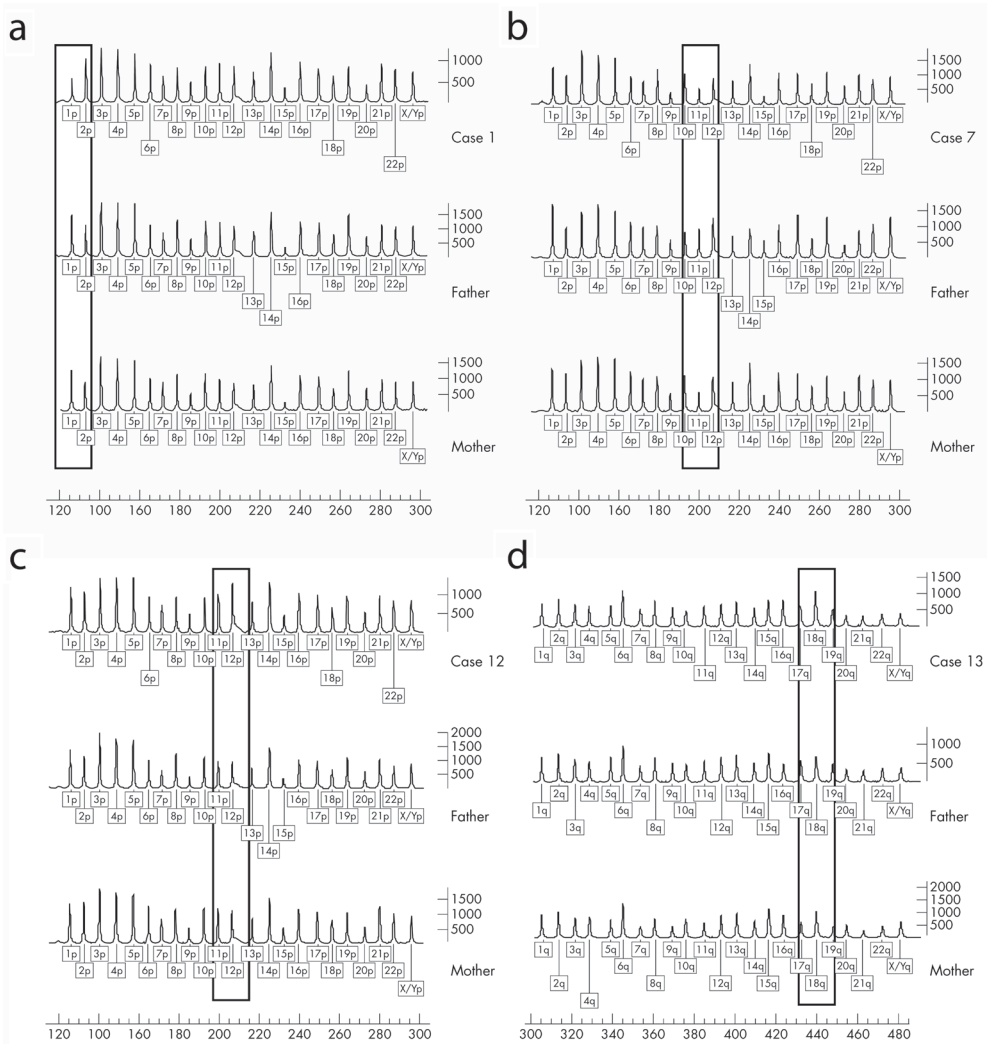

Figure 2.1.2: Distribution of subtelomeric aberrations using the subtelomeric clinical checklist score developed by de Vries *et al*.[10]

tapering fingers, and clinodactyly of toes three to five. *Case 4* (del 4pter) was a moderately mentally retarded five year old boy (born at 37+6 weeks' gestation; birth weight 2,105 g, -2.5 SD) who presented with psychomotor delay, growth failure, and severe feeding difficulties which required percutaneous endoscopic gastrostomy. He had unilateral hydronephrosis, astigmatism, and exotropia of the left eye. At the age of four years and 10 months his height was 97 cm (-3 SD) and his OFC was 46.2 cm (-3 SD). Dysmorphic features included plagiocephaly, frontal bossing, proptosis, epicanthus, hypertelorism, prominent glabella, wide nasal bridge, short philtrum, high palate, short neck, and hypospadias. In retrospect, the Wolf–Hirschhorn

syndrome was the likely clinical diagnosis, although his psychomotor development was better than in previously reported cases and epilepsy was not present.
Case 5 (del 10qter) was a mildly mentally retarded two year old girl (born at 40+ weeks' gestation; birth weight 2,335 g, -2.5 SD). Delay in psychomotor development was noticed. At the age of 15 months her height was 70 cm (-3 SD) and her OFC was 44.5 cm (10th centile). Dysmorphic features included bilateral epicanthic folds, strabismus convergens (which was surgically corrected), posteriorly rotated left ear, and a small right ear with prominent helix. She underwent unilateral ureteric reimplantation because of left sided grade IV vesico-ureteric reflux with decreased function of the kidney. In addition she had a mild conductive hearing loss (40 dB) of the right ear.

Familial submicroscopic subtelomeric deletions identified by MLPA
Case 6 (del 2pter) was a moderately mentally retarded six year old boy (born at 40 weeks' gestation; birth weight 3,220 g, 20th centile). He presented with mild developmental delay, hyperactivity, and aggressive behavior. Dysmorphic features included microcephaly, midface hypoplasia, bilateral epicanthic folds, small nose, smooth philtrum with a thin upper lip, short fifth fingers, and camptodactyly of the third left toe. Because of severe prenatal maternal alcohol abuse, fetal alcohol syndrome was suggested. At the age of five years and 10 months his height was 123 cm (85th centile) and his OFC was 48.5 cm (2nd centile).
Case 7 (del 11pter) was a moderately mentally retarded two year old girl born at full term (birth weight 2,940 g, 30th centile). She presented with psychomotor delay and feeding difficulties. She had a triangular face, brachycephaly, deep set eyes, strabismus alternans, slight upslanting of the palpebral fissures, straight eyebrows, dysplastic helices, clinodactyly of the fifth digits of the hands, and hypoplastic nails of the second toes.
Case 8 (del 12pter) was a severely mentally retarded 10 month old boy (born at 35+6 weeks' gestation; birth weight 2,835 g, 50th centile). He was kept in hospital after delivery because of respiratory insufficiency and maternal fever during labor. He had microcephaly (-2.5 SD) and generalized hypotonia. Magnetic resonance imaging (MRI) of the cerebrum revealed semilobar holoprosencephaly with fusion of the frontal lobes and corpus callosum dysgenesis. Apart from a high palate no dysmorphic features were noted.
Case 9 (del 16qter) was a moderately mentally retarded nine year old boy born a term (birth weight 3,800 g, 75th centile). He presented with psychomotor retardation with prominent speech delay. He had a triangular face, broad forehead, flat midface, slightly upslanting palpebral fissures, telecanthus, broad nasal bridge, large posteriorly rotated ears, and a prominent chin. He was admitted once to hospital because of haematuria of unknown cause. At nine years and two months his height was 132 cm (10th centile) and his OFC was 55 cm (86th centile).

De novo submicroscopic subtelomeric duplications identified by MLPA

Case 11 (dup 5qter) was a mildly mentally retarded three year old girl (41+4 weeks' gestation) with bilateral mixed hearing loss (60 dB), epilepsy, a left sided choroidal defect, and a lateral neck fistula/dimple at the lower jaw. Psychomotor development was delayed, with initial walking at 26 months. MRI of the cerebrum showed enlargement of the intra- and extracerebral spaces. At the age of two years and 11 months her height was 95 cm (50th centile) and her OFC was 51 cm (85th centile). Dysmorphic features included brachycephaly, frontal bossing, midfacial dysplasia, narrow palate, small nose, small mouth, slight retrognathia, and small posteriorly rotated ears. Case 12 (dup 12pter) was a moderately mentally retarded 12 year old girl (born at 37+4 weeks' gestation; birth weight 2,580 g, 30th centile) who presented with growth failure and hypermobility of the joints. She had feeding difficulties and constipation. At 12 years and four months of age her height was 141 cm (-2.5 SD) and her OFC was 51 cm (3rd centile). Dysmorphic features included high broad forehead, upslanting palpebral fissures, telecanthus, broad nasal bridge, short philtrum with a smooth upper lip, small maxilla, high palate, hypermobility of the wrists and the finger joints, and thin slightly hyperelastic skin. She had broad fingertips and bilateral clinodactyly of the fifth finger. In addition she had mild pectus excavatum and a strikingly furrowed tongue.

Familial submicroscopic subtelomeric duplications identified by MLPA

Case 13 (dup 18qter) was a mildly mentally retarded eight year old boy (39+5 weeks' gestation; birth weight 2,500 g, -2.5 SD). He was referred because of growth failure and mild mental retardation. At 7.5 years his height was 112.7 cm (-3.5 SD). Microcephaly was noted, with an OFC of 48 cm (-2.5 SD). His mother had a similar 18qter duplication and she had attended a special school for learning difficulties. Her adult height was 153 cm (-2.5 SD) with striking microcephaly (-4 SD).

Case 14 (dup 22qter) was a moderately mentally retarded five year old boy, born at term (birth weight 2,330 g, 3rd centile) to unrelated parents. The first year of life was complicated by feeding problems, failure to thrive and frequent attacks of syncope. He presented at two years with delayed psychomotor development, seizures, hyperactivity, and dysmorphic features, including micro- and trigonocephaly, deep set eyes, flat midface, depressed nasal bridge, prominent upper lip, and downturned corners of the mouth. At the age of three years and 11 months his height and OFC were 93 cm (-3 SD) and 45.5 cm (-3.5 SD), respectively. MRI of the brain revealed post-haemorrhagic ventricular dilatation. Analysis of the father, who had similar clinical features (microcephaly, sparse hair, hypertelorism, and prominent upper lip), showed the same unbalanced submicroscopic translocation, der(21)t(21;22)(p10;q13.3), as was present in the boy.

Discussion

In this study we present the results of the subtelomeric screening by MLPA in a group ofs 210 patients with unexplained mental retardation. A duplication of 10qter was identified in five of these patients on initial testing. This aberration was also present in two phenotypically normal parents and was regarded as a polymorphism and excluded from further analyses. Apart from the 10qter duplication, aberrations were identified in 6.7% of the patients screened: 10 deletions and four duplications. Clinically relevant submicroscopic aberrations were identified in nine patients (4.3%): seven *de novo* aberrations (five deletions and two duplications) and two duplications inherited from similarly affected parents. In the group of patients with mild, moderate, and severe mental retardation, clinically relevant anomalies occurred in 6.3%, 5.1%, and 1.7% of the cases, respectively.

It is of note that the greatest frequency of abnormalities was detected among mildly mentally retarded patients, in contrast to previous findings by Knight *et al.*, who reported abnormalities in 7.4% among moderately or severely retarded individuals, and only in 0.5% among mildly retarded individuals.[4] This might be explained by the increased detection of smaller aberrations and by the identification of submicroscopic duplications that cause less severe phenotypes in general. Our data support testing for subtelomeric aberrations in individuals with mild mental retardation.

In addition, rearrangements with clinical relevance were all found in patients with a clinical score of $\geqslant 3$ using the checklist for subtelomeric rearrangements composed by de Vries *et al*.[10] This 0–10 checklist was developed to help preselection of cases for subtelomeric testing and consists of five items: family history of mental retardation, prenatal onset of growth retardation, postnatal growth abnormalities, two or more facial dysmorphic features, and one or more non-facial dysmorphic features or congenital abnormalities. Testing of patients with a clinical score of >3 increased the diagnostic yield twofold to 12.4% (12/97).

The results of the current study show that clinical preselection of cases for subtelomeric screening is beneficial. The frequency of abnormalities in this study is comparable to previous studies, in which subtelomeric defects were identified in approximately 5% of the patients,[2,16] although direct telomeric duplications were not included in the previous studies. A novel finding in our study is that subtelomeric direct duplications are a relatively frequent cause of isolated as well as familial mental retardation. Previous studies using FISH strategies were largely insensitive to such duplications, except those associated with an unbalanced translocation. We believe that subtelomeric direct duplications may have been underdiagnosed. We identified five *de novo* submicroscopic subtelomeric deletions in this study. Del 1pter (case 1–2), del 4pter (case 4), and del 10qter (case 5) have often been reported before and resembled the previous reports.[2] The patient with a *de novo* 3qter deletion (case 3) had scaphocephaly, which was also seen in her mother (OFC 60 cm; +2.5 SD), grandfather, two of uncles, and a cousin. A *de novo* submicroscopic 3qter deletion has only been reported once.[17] In microscopically visible terminal deletions of 3q, similar abnormal skull shapes (dolichocephaly and trichonocephaly)

have also been reported.[18] However, the abnormal skull shape seemed to be familial and therefore not to be related to the *de novo* 3qter deletion in our case.

Two *de novo* submicroscopic subtelomeric duplications were identified. The three year old mildly mentally retarded girl with a duplication 5qter (case 11) had to our knowledge the first reported submicroscopic duplication of this region. In addition to facial dysmorphisms, she had hearing loss and epilepsy. Patients with microscopically visible 5qter duplications and unbalanced 5qter duplications with additional deletions of other chromosome ends have been described.[19,20] In addition to mental retardation, growth retardation, seizures, and some overlapping facial characteristics were present, making it likely to be a pathogenic cause in this patient. The 12pter duplication is also the first submicroscopic duplication of this region to be reported. Some of the dysmorphisms found in microscopically visible cases of 12pter duplications[21] could also be observed in our case.

Four subtelomeric deletions were inherited from phenotypically normal parents. The phenotypes described in these cases—del 2pter (case 6), del 11pter (case 7), del 12pter (case 8), and del 16qter (case 9)—were quite different from previously reported cases with deletions in a similar region.[17,22-25] Therefore it is likely that these aberrations are polymorphisms without clinical implications.

Both familial duplications detected in this study were inherited from similarly affected parents. In case 13, a maternal duplication of probe 18qter was identified in a mildly retarded boy presenting with growth failure, and the same features were observed in his mother. She was microcephalic (-4 SD) and had a height of -2.5 SD and learning difficulties. Intrauterine growth retardation and microcephaly have been reported in microscopically visible 18qter duplications,[26] strengthening the impression that the duplication in the 18qter chromosomal segment caused the phenotype in our patient. The duplication in our patient was significantly smaller than those previously reported, which might explain the milder phenotype in our case.

In case 14, MLPA analysis showed a duplication of the 22qter probe. FISH analysis confirmed the duplication and revealed an unbalanced submicroscopic translocation—der(21)t(21;22)(p10;q13.3)—in the proband and his father. The father was mildly mentally retarded and showed similar minor facial anomalies. Duplications including the telomere region of the long arm of chromosome 22 have been described, with some clinical resemblance to the proband, such as intrauterine growth retardation, microcephaly, and hypertelorism.[26]

All *de novo* deletions could be confirmed by FISH analysis. However, the inherited deletions (del 2pter, del 11pter, del 12pter, and del 16qter) could not be confirmed. The MLPA probes were positioned in or close to (~50 kb) the telomere specific FISH clones of the same telomere, except for the 11pter MLPA probe which was mapped more to the centromere (UCSC Genome Browser, July 2003 Freeze).[5] It is most likely that these familial aberrations represent small genomic polymorphisms missed by a FISH probe encompassing the same region. Three duplications (dup 5qter, dup 12pter, and dup 18qter) could not be confirmed by FISH. The duplicated regions were probably too close together on the genome to be detectable by routine FISH. In the current study a single new set of subtelomeric probes was used—the

SALSA P036 human telomere test kit. In general the MLPA probes were either located in the region covered by the telomere specific FISH clone or were closely proximal to this region. For the latter probes, it is possible that terminal aberrations detectable by FISH may be missed by MLPA. In a recent paper, a group of 75 mentally retarded patients was analyzed using two complementary MLPA probe sets, SALSA P019 and P020.[9] When validating these probe sets on our panel with known chromosomal aberrations, we found that several probes in the P019 and P020 kit were too far from the telomere to detect small terminal deletions. The main problem is that the probe on 1p missed small deletions that are relatively common.[27] Furthermore, in addition to the probe on 21q,[9] the probes for 2q, 6p, and 15q in the P019 and P020 kit were polymorphic (data not shown). Thus the P036 kit not only has the advantage that only one kit is needed for all subtelomeric regions, but the individual probes in this kit are also more reliable. A fusion of the three kits with additional subtelomeric probes might eventually offer the best solution for routine diagnostic screening of subtelomeric aberrations, because it will allow more accurate identification and delineation of the subtelomeric copy number changes.

Conclusions

We have confirmed that MLPA is a reliable method for detecting subtelomeric rearrangements. Screening for subtelomeric anomalies by MLPA can be offered to all mentally retarded patients, although clinical preselection increases the percentage of anomalies detected. To exclude polymorphisms, interpretation of the results should always include parental testing and comparison with clinical features of previously reported patients with similar subtelomeric rearrangements. In addition, we found that MLPA detects pure subtelomeric duplications that can easily be missed by routine FISH analysis.

Note added in proof

The duplication 5qter in case 11 could not be confirmed by later MLPA testing when repeated on a new DNA sample and therefore we cannot exclude the possibility that the duplication was the result of a technical artefact. Since the acceptance of this paper, we have found several other duplications in the same subtelomeric region—for example on 15qter and 9pter—confirming that duplications may be more common than previously thought.

Acknowledgements

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Chapter 2.2

Molecular characterization of patients with subtelomeric 22q abnormalities using chromosome specific array-based comparative genomic hybridization

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Abstract

The 22q13 deletion syndrome is associated with global developmental delay, absent or delayed speech, and generalized hypotonia. In this study, the size and nature of 22q13 deletions (*n=9*) were studied in detail by high-resolution chromosome specific array-based comparative genomic hybridization (array CGH). The deletion sizes varied considerably between the different patients, that is, the largest deletion spanning 8.4 Mb with the breakpoint mapping to 22q13.2 and the smallest deletion spanning 3.3 Mb with the breakpoint mapping to 22q13.31. In one case, a unique subtelomeric 3.9 Mb deletion associated with a 2.0 Mb duplication of 22q13 was observed, adding to a growing number of similar cases identified for other chromosome ends. Remarkably, this patient had signs suggestive of retinitis pigmentosa, which has never been reported before in the 22q13 deletion syndrome. The identification of two pairs of recurrent proximal breakpoints on 22q13 suggests that these specific regions may be prone to recombination, due to yet unknown genome architectural features. In addition to the copy number changes on 22q13, a duplication of ~330 kb on 22q11.1 was observed and shown to be a genetic large-scale copy number variation without clinical consequences. The current study failed to reveal relationships between the clinical features and the deletion sizes. Global developmental delay and absent or severely delayed speech were observed in all patients, whereas hypotonia was present in 89% of the cases (8/9). This study underscores the utility of array CGH for characterizing the size and nature of subtelomeric deletions, such as monosomy 22q13, and underlines the considerable variability in deletion size in the 22q13 deletion syndrome regardless of the clinical phenotype.

Introduction

Subtelomeric deletions are a common cause of mental retardation.[1,2] In recent years, screening of subtelomeres for copy number changes in mentally retarded patients has resulted in the recognition of new distinct clinical entities, based on monosomy 1q, 2q, 9q, 14q, and 22q (reviewed in Vries *et al.*[1]) The latter entity is also referred to as the 22q13 deletion syndrome, of which to date at least 100 cases have been reported.[3–6] Common features associated with this syndrome are mild-to-severe global developmental delay, absent or delayed speech, generalized hypotonia, and minor anomalies including dolichocephaly, ptosis, abnormal ears, relatively large hands, and dysplastic toenails.[5]

Here we describe a series of nine patients with monosomy 22q13 in which the size and the nature of the chromosome 22 deletions were studied in detail by high-resolution chromosome-specific array-based comparative genomic hybridization (array CGH).[7]

Materials and methods

Patients and DNA samples

In total, nine patients with subtelomeric deletions of 22q13 were included in this study. Three cases were previously published (case 2,[8] case 7[9] and case 9[10]). DNA samples of the patients were derived from different clinical centers in France, Ireland, the United Kingdom, and the Netherlands (Nijmegen). Clinical information on the patients was obtained from the referring physician. DNA samples of all patients were analyzed using array CGH. In one patient (case 3), the 22qter deletion was initially identified by routine chromosome analysis at a 550-band level and confirmed by fluorescence *in situ* hybridization (FISH) [46, XY, del(22)(q13.2).ish del(22)(TUPLE+, ARSA-)]. Another deletion (case 4) resulted from a *de novo* translocation [46, XY, der(22)t(14;22)(q32.33;q13.31)pat. ish der(22)(N85A3-)], whereas the remaining cases were initially identified by FISH, using the following probes: N85A3 (cases 1–2, 5), ARSA (cases 6–8), D22S163 (case 8), or STS WI-941 and D22S39 (case 9).

Array-based comparative genomic hybridization

High-resolution chromosome 22 array CGH was performed. The microarray consisted of 350 positionally selected chromosome 22 BAC clones (BACPAC Resources), together with 1532 control clones located on other chromosomes. The chromosome 22-specific clones covered the long arm of chromosome 22 (35.1 Mb) with an average spacing of one clone per 100 kb. However, the actual resolution may be less in repeat dense regions. The average clone-insert size was 168 kb, resulting in a 1.7-fold coverage of the long arm of chromosome 22. DOP-PCR products of the BAC clones were spotted in six-fold onto CMT-GAPS-coated glass slides (Ultragaps, Corning) using an OmniGrid 100 arrayer (Genomic Solutions). All steps in the labeling, hybridization, and data-analysis procedure were performed as described previously.[11] In brief, equal amounts of patient and reference genomic DNAs were labeled by random

priming with Cy3-dUTP or Cy5-dUTP (Amersham Biosciences). Labeled test- and reference DNAs were mixed with Cot-1 DNA (Roche), co precipitated and resuspended in a hybridization solution. After denaturation of probe and target DNA, hybridization and post-hybridization washing procedures were performed using a GeneTac Hybridization Station (Genomic Solutions), according to the instructions of the manufacturer. Fluorescence intensity images were acquired using an Affymetrix 428 scanner (Affymetrix), and analyzed by Genepix Pro 5.1 (Axon Instruments).

Statistical data analysis

Data normalization was performed by applying Lowess curve fitting with a smoothing factor of 0.3 as described by Vissers *et al.*[11] Copy number alterations were identified by using a Hidden Markov Model algorithm. Three hidden states represent normal, loss, and gain conditions. The probability to observe a log ratio given its hidden state is modeled by a Gaussian with SD 0.25 and mean 0, -0.4 and 0.4, respectively. The hidden state of adjacent clones is correlated with respect to their distance. The thresholded marginal probability of a clone's hidden state determines if it is normal, a loss or gain. In order to discriminate between causative aberrations and large-scale copy number variations (LCV) we used a data set of 72 normal individuals. The control population was tested by a genome-wide 32,477 clone BAC array which included the same clones as used for the chromosome 22 array.

Results

Seven patients with a submicroscopic 22qter deletion, one patient with an unbalanced translocation and one patient with a microscopically visible 22qter deletion were studied. In **Table 2.2.1**, the main characteristics of the patients are summarized. In addition, minor facial dysmorphisms were noted (**Figure 2.2.1**). High-resolution chromosome 22-specific array CGH confirmed the known copy number changes in all cases and delineated the specific aberrations in detail. **Figure 2.2.2** shows two examples of chromosome 22 array CGH profiles. All deletions analyzed included the most telomeric clones. Interstitial deletions in the subtelomeric region of the long arm of chromosome 22 were not observed. The deletion sizes identified in this study varied considerably between the different patients, that is, the largest deletion spanning 8.4 Mb with breakpoint mapping to 22q13.2qter (68 clones, case 1) and the smallest deletion spanning 3.3 Mb with breakpoints mapping to 22q13.31qter (27 clones, case 9) (**Figure 2.2.3**). Identical proximal breakpoints were localized in cases 2 and 3 (between RP11-786O06 and RP11-236I15) and cases 5 and 6 (between RP11-766K21 and RP11-49A20), resulting in deletion sizes of 7.7 and 5.9 Mb, respectively. In case 3, a del(22)(q13.2qter) was originally identified by routine chromosome analysis. Array CGH analysis confirmed the karyotypic analysis and established the deletion size to 7.7 Mb. Case 4 carried a translocation between chromosomes 14 and 22 [46,XY,der(22)t(14;22)(q32.33;q13.31)]. Array CGH analysis revealed a 6.6 Mb deletion at 22q13.31qter. In case 7, a deletion of 5.0 Mb was identified, whereas in case 8 the deletion

Table 2.2.1: Main characteristics of 22q13 deletion cases presented in this study

Patients	1	2[8]	3	4[a]	5	6	7[9]	8	9[10]
Deletion size (Mb)	8.4	7.7	7.7	6.6	5.9	5.9	5.0	3.9	3.3
Duplication size (Mb)	–	–	–	–	–	–	–	2.0	–
Clinical characteristics									
Sex	F	M	M	M	F	F	M	F	F
Global developmental delay	+	+	+	+	+	+	+	+	+
Normal to accelerated growth	+	+	+	+	+	+	+	–	+
Absent/severely delayed speech	+	+	+	+	+	+	+	+	+
Hypotonia	+	+	+	+	+	+	+	–	+
Chewing behavior	+	–	–	–	–	–	+	–	–
Dolichocepahaly	–	–	–	–	–	–	–	–	–
Ptosis	–	–	–	–	–	–	–	–	–
Prominent/dysplastic ears	+	+	+	+	+	+	+	+	–
Prominent/pointed chin	+	–	–	+	+	+	+	-	–
Relatively large, fleshy hands	+	–	–	–	+	+	+	+	–
Abnormal toenail growth	+	+	+	–	+	–	+	–	–

F, female; M, male; +, feature present; -, feature absent.
[a] 46,XY,der(22)t(14;22).

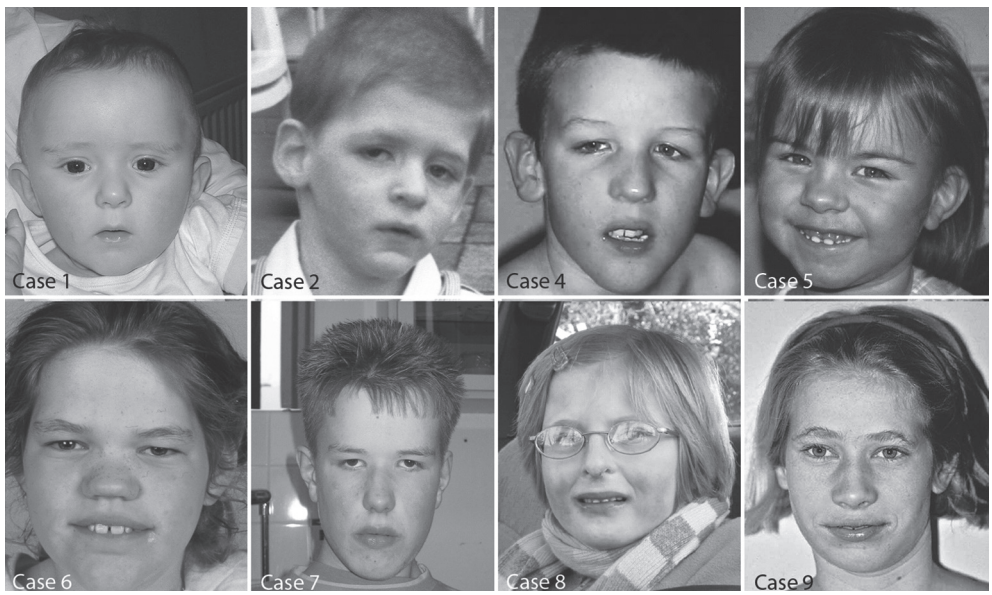

Figure 2.2.1: Patients with 22q13 deletion syndrome. Note the known facial features of the 22q13 deletion syndrome, prominent dysplastic ears (visible in cases 1, 2, 4 and 5), and a prominent/ pointed chin (cases 1 and 4– 7). For color figure see page 192.

spanned 3.9 Mb. The latter 22q13 deletion was associated with a duplication of 2.0 Mb, including 13 clones adjacent to the deleted region (**Figure 2.2.2b**).

In addition to these subtelomeric copy number alterations, a duplication of ~330 kb on 22q11.1 was detected in the same patient. Duplications in the same region next to the centromere were identified in two other cases in this series (cases 4 and 7). In the control panel of 72 normal

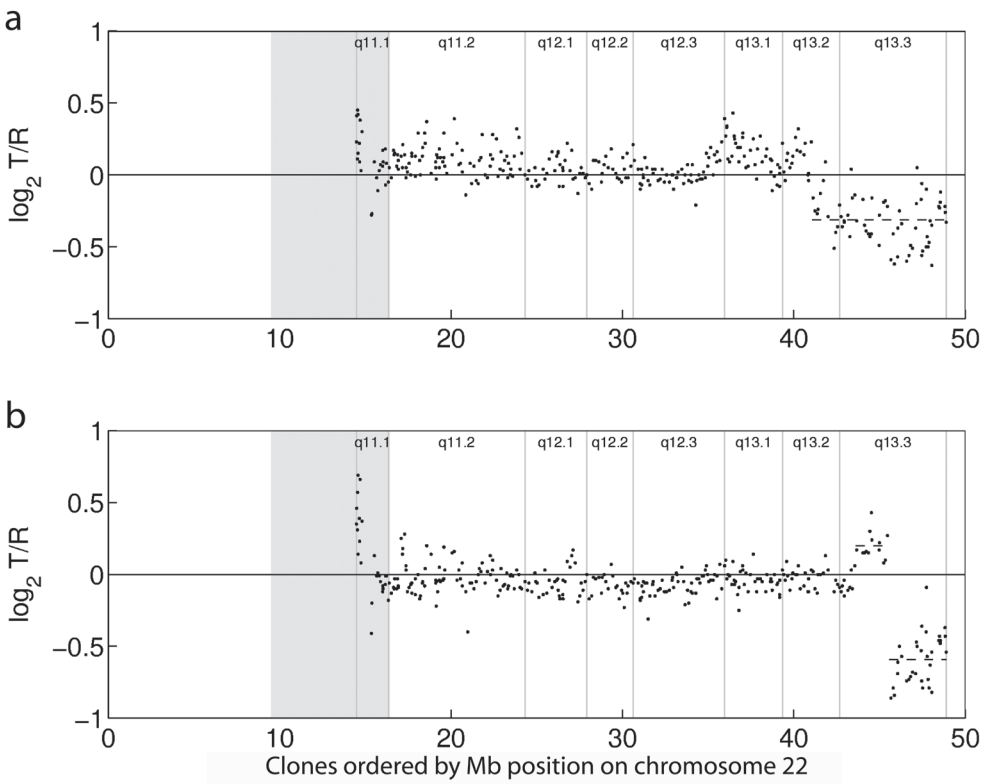

Figure 2.2.2: Examples of chromosome- 22 profiles obtained by array CGH. Arrays contained 350 cloned chromosome- 22 genomic DNA targets (indicated by small circles representing the mean $\log_2$-transformed and Lowess-normalized T/R intensity ratios), ordered from q11.1 to qter on the basis of physical mapping positions obtained from the May 2004 freeze of the UCSC Genome Browser. The centromere is indicated by a hatched area. (**a**), case 1: del(22)(q13.2); (**b**), case 8: del(22)(q13.31). Note the duplication of 2.0 Mb on 22q13.31 and the duplication on 22q11.1.

individuals tested by a tiling resolution genome-wide BAC array, copy number gain of the same region was observed in five individuals, whereas loss of the chromosomal segment was seen in 21 controls (de Vries, submitted).

Discussion

For detailed analyses of patients with known 22q13 deletions, a tiling resolution array was constructed with a 1.7-fold coverage of the long arm of chromosome 22. Buckley *et al.*[12] demonstrated the utility of such a comprehensive chromosome 22 array by profiling acral melanoma, dermatofibrosarcoma, DiGeorge syndrome, and neurofibromatosis 2. The chromosome 22 tiling resolution array had an average clone spacing of 100 kb, resulting in a resolution that is 30 times higher than high-resolution karyotyping. The array did not cover

the most telomeric 635 kb of the chromosome, which includes the gene *SHANK3*/*ProSAP2*. Haploinsufficiency of this gene has been proposed to be responsible for the major neurological features of the 22q13 deletion syndrome.[6,13,14] However, FISH analysis using subtelomeric clones, confirmed the extension of all deletions found till the subtelomeric region.

In the present study, a considerable difference in deletion sizes was noted, which is in accordance with the results of Luciani *et al.*[3] which showed an extremely variable 22qter deletion size, extending from 160 kb to 9 Mb. Interestingly, a complex chromosome 22 rearrangement was observed in case 8, with a unique combination of a deletion and a duplication of 22q13. The detection of the deletion-duplication in this patient adds to a growing number of similar cases identified for other chromosomes, including deletion-duplications in 1p, 2q, 4p, and 8p.[15–18] Giglio *et al.* demonstrated that the formation of the inverted duplications of 8p associated with a terminal deletion is caused by nonallelic homologous recombination (NAHR) between two olfactory receptor-gene clusters.[19] In addition, Ballif *et al.* described two terminal deletions of 1p36 associated with cryptic interrupted inverted duplications.[18] This type of chromosome rearrangement may be more common than previously thought. The detection of cryptic duplication associated with terminal deletions is greatly improved by high-resolution copy number screening using array CGH. Further studies are needed to determine the mechanisms underlying these rearrangements. Ballif *et al.*[18] proposed a premeiotic model in which a terminally deleted chromosome is generated in the germ line and passes through at least one breakage–fusion–bridge cycle in which uncapped sister chromatids are fused by nonhomologous end joining (NHEJ), resulting in gametes with terminal deletions associated with cryptic interrupted inverted duplications. The identification of recurrent proximal breakpoints in cases 2 and 3 (between RP11- 786O06 and RP11-236I15) and cases 5 and 6 (between RP11-564B15 and RP11-673D06), suggests that these regions may be prone to recombination, due to the presence of yet unknown genome architectural features. Segmental duplications were present at or close to the recurrent breakpoints and also to the other, non-recurrent breakpoints in our series. To a large extent, chromosome rearrangement breakpoints are located in intervals containing complex genomic architecture, such as AT-rich palindromes or low copy repeats (LCRs).[20] Through the process of NAHR, LCRs can lead to translocations, inversions, duplications and interstitial deletions.[21] However, the mechanisms for generating and/or stabilizing terminally deleted chromosomes are poorly understood. NEHJ, possibly stimulated by LCRs or other repetitive sequences, may be one of the causative mechanisms for the terminal 22q13 deletions in our series.

In addition to the known 22qter deletions, a submicroscopic duplication on 22q11.1 next to the centromere was identified in three cases (cases 4, 7 and 8). In the control population of 72 normal individuals, copy number gains in the same region were identified in five individuals, whereas losses were observed in 21 controls (de Vries; submitted), indicating that this anomaly represents a LCV. This LCV at 22q11.1 was previously reported by Sebat *et al.*[22] (http://projects.tcag.ca/variation/) and stresses the variation of the human genome and the importance of parental and control analysis in case a submicroscopic alteration is identified.

Location	Cytoband	Clone	1	2	3	4	5	6	7	8	9
	q13.2	RP11-420C16									
	q13.2	RP11-657N06									
	q13.2	RP11-744N13				EXCL					
	q13.2	RP11-415M09									EXCL
41 Mb	q13.2	RP11-651I4									
	q13.2	RP11-241G19		EXCL							EXCL
	q13.2	RP11-60H08	LOSS								
	q13.2	RP11-400M07	LOSS			EXCL					
	q13.2	RP11-804K19	LOSS								
	q13.2	RP11-582G11	LOSS								EXCL
	q13.2	RP11-794G14	LOSS			EXCL					
	q13.2	RP11-786O06	LOSS								
	q13.2	RP11-236I15	LOSS	LOSS	LOSS						
42 Mb	q13.2	RP11-67I21	LOSS	LOSS	LOSS						
	q13.2	CTD-2010M18	EXCL	LOSS	LOSS				EXCL	EXCL	EXCL
	q13.2	RP11-50E02	LOSS	LOSS	LOSS	EXCL					
	q13.2	RP11-731M01	LOSS	LOSS	LOSS	EXCL					
	q13.2	RP11-80L20	LOSS	LOSS	LOSS						
	q13.31	RP11-535F10	LOSS	LOSS	LOSS	EXCL					
	q13.31	RP11-746K24	LOSS	LOSS	LOSS						
	q13.31	RP11-792K05	LOSS	LOSS	LOSS						
	q13.31	RP11-135L16	LOSS	LOSS	LOSS	LOSS					
43 Mb	q13.31	RP11-357F14	LOSS	LOSS	LOSS	EXCL					
	q13.31	RP11-41J01	LOSS	LOSS	EXCL	EXCL					
	q13.31	RP11-590C21	LOSS	LOSS	LOSS	LOSS					
	q13.31	CTD-2008L15	LOSS	LOSS	EXCL	EXCL					
	q13.31	RP11-766K21	LOSS	LOSS	LOSS	LOSS					
	q13.31	RP11-49A20	LOSS	LOSS	EXCL	EXCL	LOSS	LOSS			
44 Mb	q13.31	RP11-673D06	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS		GAIN	
	q13.31	RP11-660F09	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS		GAIN	
	q13.31	RP11-23B21	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS		GAIN	
	q13.31	RP11-585K21	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS		GAIN	
	q13.31	RP11-379E18	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS		GAIN	
	q13.31	RP11-192I08	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS		GAIN	
	q13.31	RP11-699K18	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS		GAIN	
	q13.31	RP11-435J19	LOSS	EXCL	LOSS	LOSS	EXCL	LOSS	LOSS	GAIN	EXCL
	q13.31	RP11-953P12	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	GAIN	
45 Mb	q13.31	RP11-704E16	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	GAIN	
	q13.31	RP11-313M05	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	GAIN	
	q13.31	RP11-620A14	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	GAIN	
	q13.31	RP11-234M14	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	GAIN	
	q13.31	RP11-427B17	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	
	q13.31	RP11-751L10	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	
46 Mb	q13.31	RP11-506A05	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS	EXCL
	q13.31	RP11-607P17	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	EXCL
	q13.31	RP11-5G12	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	
	q13.31	RP11-704G20	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	
	q13.31	RP11-720H05	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.31	RP11-320G02	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.31	RP11-689F11	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.32	RP11-328I12	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
47 Mb	q13.32	RP11-799D02	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.32	RP11-693J18	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.32	RP11-690L04	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.32	CTD-2260N21	LOSS	LOSS	EXCL	EXCL	LOSS	LOSS	EXCL	LOSS	LOSS
	q13.32	RP11-262A13	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	EXCL
	q13.32	RP11-53E22	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.32	CTD-2006M09	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.32	RP11-693A20	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.32	RP11-242B05	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	EXCL
	q13.32	RP11-636D02	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS	EXCL
	q13.32	RP11-255N20	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS
	q13.33	RP11-12O20	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-551M20	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
48 Mb	q13.33	RP11-687O19	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	EXCL
	q13.33	RP11-792P08	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-314N05	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-94B12	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-329B15	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-125K03	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-734K06	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-232E17	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	LOSS	LOSS
	q13.33	RP11-931F19	LOSS	LOSS	LOSS	LOSS	EXCL	LOSS	LOSS	LOSS	EXCL
	q13.33	RP11-428L10	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS	LOSS

Figure 2.2.3: Overview of the array CGH results for the nine patients with monosomy 22qter. Arrays contained 350 chromosome-22 DNA BAC clones, ordered from q11.1 to qter on the basis of physical mapping positions obtained from the May 2004 freeze of the UCSC Genome Browser. The distal 9 Mb of the long arm of chromosome 22 is depicted in the figure. Copy number alterations were identified, using a Hidden Markov Model algorithm. Three hidden states represent normal (empty), loss (LOSS) and gain (GAIN) conditions. Individual clones that did not pass quality control criteria were excluded (EXCL).

In the current study, no relation between clinical features and deletion size could be observed. The clinical features observed were consistent with the common clinical phenotype associated with the 22q13 deletion syndrome,[5] although dolichocephaly and ptosis were not found in the current study. Global developmental delay and absent or severely delayed speech were observed in all patients. Hypotonia was present in 89% of the cases (8/9). A pointed chin was present in cases 1 and 4–7. Wilson *et al.*[6] previously suggested a candidate gene for this feature in the proximal region of 22q13, however this could not be confirmed in our series. In addition, in case 8, ophthalmic assessment showed myopia and salt-and-pepper retinal changes suggestive of retinitis pigmentosa. These eye anomalies, which have not been reported in the 22q13 deletion syndrome before, are possibly attributable to the 2 Mb 22q13.31 duplication in this patient. Relationship between the deletion size and clinical features could not be observed. However, case 9 with the smallest 22q13 deletion (3.3 Mb), did not show any of the dysmorphic features commonly described in the 22q13 deletion syndrome. Facial dysmorphic features in this patient included upslanting palpebral fissures, a moderate hypertrophic nasal root, and thick lips.[10] These findings underline the study of Wilson *et al.*, in which no significant correlation with the size of the deletion could be demonstrated for most clinical features and support the idea that a gene in the 3.3 Mb minimal deleted region (notably *SHANK3*/ *ProSAP2*) may be the major candidate gene in the 22q13 deletion syndrome.[3–6]

In conclusion, this study underscores the utility of array CGH for further characterization of the size and nature of subtelomeric deletions. In addition, these results confirm the considerable differences in deletion size observed in patients with the 22q13 deletion syndrome, regardless of the clinical phenotype.

Acknowledgements

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Interstitial copy number variation in mental retardation

3.1 Genomic microarrays in mental retardation: a practical workflow for diagnostic applications.
Hum Mutat (in press).

3.2 Identification of non-recurrent submicroscopic genome imbalances: the advantage of genome-wide microarrays over targeted approaches.
Eur J Hum Genet 2008;16:395-400.

3.3 A new chromosome 17q21.31 microdeletion syndrome associated with a common inversion polymorphism.
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J Med Genet (in press).

Chapter 3.1

Genomic microarrays in mental retardation: a practical workflow for diagnostic applications

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**equally contributed*

Hum Mutat (in press).

Abstract

Microarray-based copy number analysis has found its way into routine clinical practice, predominantly for the diagnosis of patients with unexplained mental retardation. However, the clinical interpretation of submicroscopic copy number variants (CNVs) is complicated by the fact that many CNVs are also present in the general population. Here we introduce and discuss a workflow that can be used in routine diagnostics to assess the clinical significance of the CNVs identified. We applied this scheme to our cohort of 386 individuals with unexplained mental retardation tested by genome-wide tiling resolution DNA microarray and to 978 additional patients with mental retardation reported in 15 genome-wide microarray studies extracted from the literature. In our cohort of 386 patients we identified 25 clinically significant copy number losses (median size 2.6 Mb), 9 copy number gains (median size 2.0 Mb), and one mosaic numerical chromosome aberration. Accordingly, the overall diagnostic yield of clinically significant CNVs was 9.1%. Taken together, our cohort and the patients described in the literature include a total of 1,364 analyses of DNA copy number in which a total of 11.2% (71.9% losses, 19.6% gains, 8.5% complex) could be identified, reflecting the overall diagnostic yield of clinically significant CNVs in individuals with unexplained mental retardation.

Introduction

Microarray technology was pioneered more than a decade ago and has now become a routine tool in basic and applied research. Five years ago, this technology was used for the first time in the field of clinical genetics, when we described microarray-based copy number analysis of all human telomeres in patients with mental retardation,[1] a common disorder for which the genetic diagnosis is mostly lacking. At that time, we suggested that "the robustness and relative simplicity of this array-based telomere copy number screening makes it highly suited for introduction into the clinic as a rapid and sensitive automated diagnostic procedure." Since then, microarrays have indeed found their way into the clinical setting, although the majority of applications now target not only the telomeres, but also other clinically relevant genomic regions,[2-4] or the entire genome at varying resolution levels,[5-7] for reviews see Menten *et al*.[8] and Veltman.[9] The power of microarray technology is used to its fullest for unbiased whole genome copy number analysis. Initially, the clone-based genomic microarrays developed were only available to researchers with dedicated microarray facilities. These microarrays have now been largely replaced by commercially available microarrays using oligonucleotide probes that can easily be implemented in clinical diagnostic laboratories. The latter platforms provide higher genome coverage than most clone-based genomic microarrays, and can be produced in large quantities according to industrial quality standards. The latest microarrays achieve kilobase level resolution, a major leap forward as compared to the megabase level resolution for conventional chromosome analysis.

There appear to be no major technical shortcomings that preclude widespread implementation of this microarray-based copy number analysis in routine clinical settings. Clinical interpretation of the copy number changes identified, however, is still challenging and many questions remain.[10] One of the major difficulties is the fact that genomes from apparently healthy individuals also show wide-scale genomic copy number variation,[11-13] reviewed in Pinto *et al*.[14] Disease-causing copy number variants (CNVs) can be distinguished from variations without direct clinical significance by studying unaffected parents as well as large normal control cohorts. But what can we do if one or both parents are unavailable for testing inheritance? What are the chances of finding a rare inherited variant without clinical significance that is not present in the control cohort? How large does the control cohort have to be in order to exclude low frequency CNVs and can control data from multiple sources be used, as collected for example in the Database of Genomic Variation (http://projects.tcag.ca/variation/)?[13] Most disease-associated CNVs identified so far appear to be unique and it is therefore difficult to predict long-term clinical outcome. Finally, independent validation of CNVs identified by microarrays is preferable, but often time-consuming, and it may be questioned whether it is still necessary for all cases. In this review we will present data from our own diagnostic cohort of 386 patients with unexplained mental retardation tested onto tiling resolution BAC arrays, as well as data on 978 patients extracted from the literature. We will discuss the ways by which different groups come to clinical interpretations and propose a practical workflow that can

be used in routine clinical diagnostics, independent of the microarray platform used and the prior experience. We will not discuss the use of different microarray platforms and their effect on CNV detection, as several genomic microarray platform comparison studies have recently been published.[15-17] In addition, we will only focus on studies that reported on the application of genome-wide microarrays, as the clinical interpretation of CNVs identified in these studies is most challenging.

Microarray-based CNV analysis in mental retardation, description of studies included in this review

Our results are derived from genomic copy number profiling among a cohort of 386 individuals with unexplained mental retardation using tiling resolution 32k BAC array CGH (100 of which were previously reported).[5] Microarray preparation, analysis and basic interpretation were performed as described elsewhere.[5] CNVs were identified using a Hidden Markov Model, requiring a minimum of three adjacently mapped BACs to be present within a CNV for further follow-up. Based on the average insert size of ~170 kb and the clone cover size of 47 kb,[18] the effective resolving power using this criterion is ~300 kb. The minimal number of adjacently located clones might lead to underestimation of smaller, but true CNVs, but minimizes the number of false positives. A total of 109 unaffected individuals, mostly parents of mentally retarded children, were analyzed on the same microarrays for establishing a control cohort. All CNVs that were considered to be clinically relevant were validated by multiplex ligation-dependent probe amplification (MLPA) and/or fluorescence *in situ* hybridization (FISH) technology.[5] MLPA and or FISH technology was also used to test DNA from the parents, if available.

Many other groups have now reported on the use of genomic microarrays in the clinical workup of mental retardation (listed in **Table 3.1.1**). While the majority has used microarrays consisting of collections of large-insert clones (mostly with an approximate coverage of one

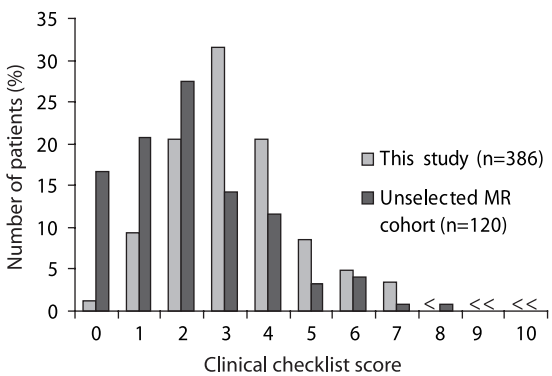

Figure 3.1.1: Clinical preselection of patients. A total of 120 unselected patients (dark gray) and 386 patients tested in the present study (light gray) were scored using a clinical scoring system.[19,20] The mean score of the unselected group was 2.2 $\pm$ 1.7 which was significantly less than the mean score (3.2 $\pm$ 1.5) of the 386 patients tested in the current study ($P < .0001$ by Mann-Whitney *U* test), indicating that we are using a preselected cohort.

Table 3.1.1: Overview of CNV studies using genomic microarrays in mental retardation

Study	Reference	Number	Group	Prior studies	Targets	Number	Resolution	Follow-up studies
1	Vissers, 2003	20	MR and additional dysmorphisms (Checklist score >3)[a]	KT	BAC	3569	1 Mb	FISH
2	Shaw-Smith, 2004	50	Learning disability and dysmorphisms	KT, tel FISH (17), genotyping (24)	BAC	3500	1 Mb	FISH, genotyping
3	de Vries, 2005	100	MR with/or without congenital malformations and/or dysmorphism	KT, tel MLPA	BAC	32447	100 kb	FISH, MLPA
4	Schoumans, 2005	41	MR (mild to severe) and dysmorphic features, malformations, and/or a family history (Checklist score >3)[a]	KT, tel FISH (30) or SKY (11)	BAC	2600	1 Mb	FISH
5	Tyson, 2005	22	MR (mild to severe) and nonsyndromic pattern of dysmorphic features	KT, tel FISH (13)	BAC	1003 or, 2600	1 Mb 3 Mb	FISH, microsatellite DNA analysis, real-time PCR
6	Menten, 2006	140	MR with one or more major congenital malformation or dysmorphism, or both	KT, tel MLPA or FISH (31)	BAC	3431	1 Mb	FISH, RT qPCR
7	Miyake, 2006	30	MR associated with some dysmorpic features features	KT, normal in 29	BAC	2173	1.5 Mb	FISH
8	Friedman, 2006	100	MR (moderate-severe) and > additional clinical features: one major malformation, microcephaly, abnormal growth, or multiple minor anomalies	KT	oligo	100K	30 kb	FISH
9	Krepischi-Santos, 2006	95	Syndromic phenotype; almost all patients presented MR	KT	BAC	3500	1 Mb	FISH, MLPA
10	Rosenberg, 2006	81	MR (mild to severe) and cranial/facial dysmorphisms and at least one additional dysmorphic feature	KT	BAC	3500	1 Mb	FISH, MAPH
11	Engels, 2007	60	MR mostly combined with congenital anomalies	KT, tel FISH	BAC	6000 or 8000	500 kb	FISH
12	Hoyer, 2007	104	MR, with or without multiple congenital anomalies	KT, tel FISH	oligo	100K	30 kb	FISH, qPCR for selected deletions <100kb and dup
13	Fan, 2007	100	MR (Checklist score >3)[a]	KT	oligo	44290	30-35 kb	FISH, BaCGH or OaCGH244K
14	Aradhya, 2007	20	MR and dysmorphic features or congenital anomalies or growth retardation	KT, tel FISH (18)	oligo, BAC	44290, 2600	30-35 kb, 1 Mb	FISH
15	Wagenstaller, 2007	67	MR with/or without additional symptoms	KT, tel FISH (42)	oligo	100K	30 kb	qPCR
16	Thuresson, 2007	48	MR with/or without congenital malformations and/or dysmorphism	KT, tel FISH	BAC	3500	1 Mb	FISH, MLPA
17	This study[b]	386	MR with/or without congenital malformations and/or dysmorphism	KT, tel MLPA	BAC	32447	100 kb	FISH, MLPA
	Total	1,364						

[a] Checklist = 0-10 Clinical checklist score.[19,20]
[b] The results of de Vries *et al.*[5] are included in the current study.

clone per megabase), oligonucleotide based microarrays are becoming more widely used. The published microarray studies differ in various aspects.

Firstly, different methods have been applied to reliably identify CNVs. In some studies every aberrant clone was followed-up, whereas in other studies a minimal number of adjacently located clones or oligonucleotides was used, or a minimal genomic size was considered as a threshold for further follow-up.

Secondly, the majority of these studies can be regarded as proof-of-principle studies, since the number of patients included was often (far) below 100. These low numbers may reflect patient selection, which is likely to differ between these studies, although, unfortunately, most studies do not report on the use of standard clinical checklists. In the study by de Vries *et al.* we indicated that indeed our patient cohort was not representative for the overall referral of mentally retarded patients for cytogenetic analysis.[5] This is changing now that microarrays are increasingly implemented in routine clinical diagnostic settings, although the currently included 386 patients still represent a preselected cohort ($P < 0.001$ by Mann-Whitney *U* test) (**Figure 3.1.1**).

Thirdly, patients included have often undergone different cytogenetic and/or molecular tests prior to microarray analysis. Karyotyping was performed in all studies prior to microarray analysis, and often subtelomeric abnormalities were excluded, as well as known microdeletion or microduplication syndromes in selected cases.

Although, these different approaches hamper the comparison of the published studies in many ways, the basic workflow used to come to a clinical diagnosis is similar in the majority of cases. In all studies, if possible, parental samples were tested for testing *de novo* occurrence. In addition, efforts were made in almost all studies to compare the CNVs obtained, to collections of CNVs identified in unaffected control individuals, apart from the few studies which were published prior to the publication of widespread copy number variation in healthy individuals.[7,21] Also, the fact that various genomic regions were known to be associated with well-known mental retardation syndromes was often used in the interpretation. Finally, various methods were used to validate microarray-based CNVs linked to mental retardation, ranging from FISH to qPCR to (a re-analysis of) karyotyping. In **Figure 3.1.2**, we propose a workflow for the clinical interpretation of CNVs in individuals with mental retardation. On the basis of this workflow we classified all CNVs reported in the various studies listed in **Table 3.1.1** either as (1) common CNVs not directly related to mental retardation, (2) rare CNVs related to mental retardation, or (3) CNVs of unknown clinical significance.

Common CNVs, not related to mental retardation

In order to differentiate between CNVs that are likely implicated in the etiology of mental retardation and CNVs that are not involved in the disease phenotype, it is essential to recognize common variation also identified in normal controls. It has been estimated that CNVs cover ~12% of the human genome.[12] Discriminating between common CNVs and rare CNVs is

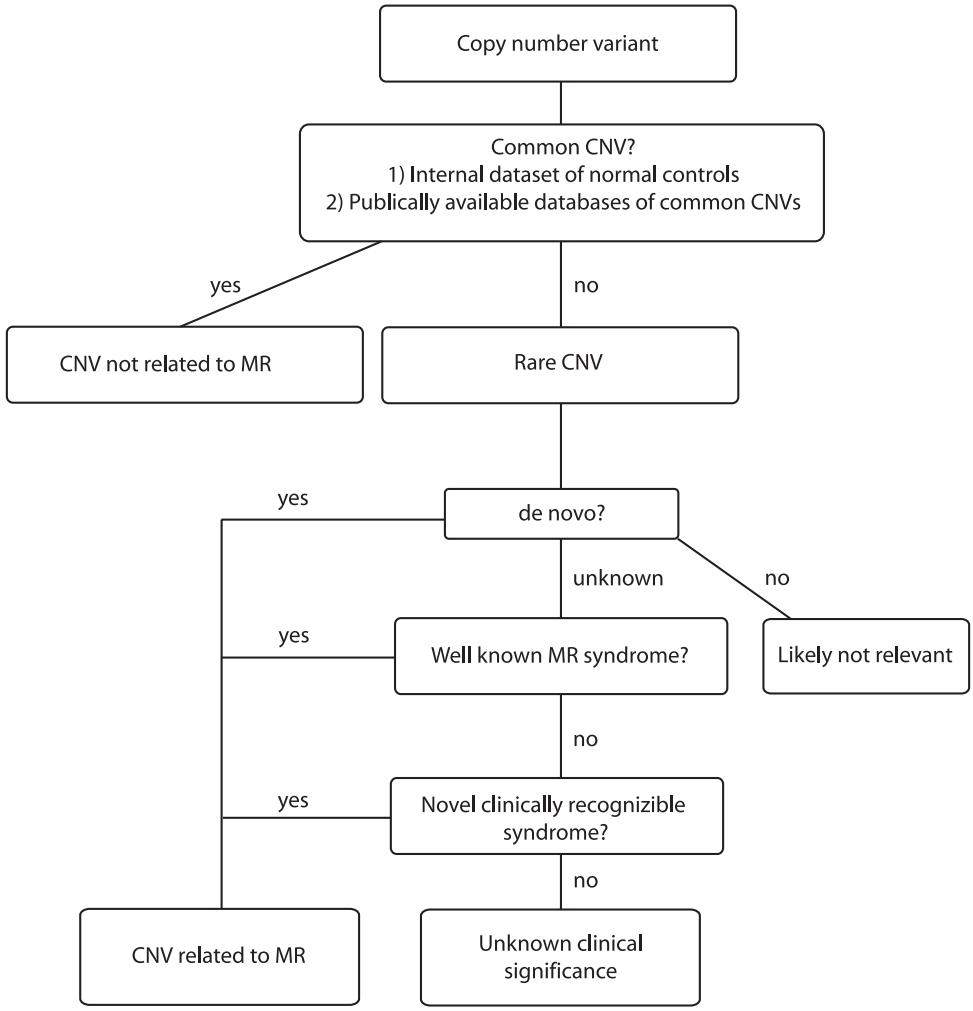

Figure 3.1.2: Workflow for the decision making of CNVs in individuals with mental retardation

a first step in the diagnostic process after having identified CNVs in a patient by microarray analysis. This first step is essential for all data obtained from microarray platforms with more than ~3,000 probes, as these platforms will reveal many common CNVs not related to disease. Although our current knowledge about the clinical implications of common CNVs is far from complete, it is unlikely that CNVs frequently found in normal controls are directly related to mental retardation. As yet, however, it cannot be excluded that they contribute to the severity of the phenotype.

CNVs identified in healthy controls have been documented in publicly available databases, such as the Database of Genomic Variants.[13] It is important to realize that these databases have been created mainly for research purposes. Most CNVs are identified by high-resolution microarray

or sequencing strategies. Only a minority of reported CNVs has been validated, and therefore, the percentage of false-positive CNVs is unknown. Moreover, it is often difficult to determine the size of a genomic region that is affected by CNV from these databases, especially when low-resolution microarray platforms have been used. The benign CNVs previously reported are often actually smaller than was previously thought, partly due to the older/low resolution microarrays used when they were discovered.[22] With the advent of high-resolution microarray and sequencing platforms increasingly smaller CNVs are detected. In a study, using a two-stage high-resolution array CGH approach on 50 healthy males, the majority of CNVs was smaller than 20 kb.[23] These results indicate that it will soon be difficult to find a megabase of genomic sequence that does not show CNVs in healthy controls. Therefore, one should be careful in comparing CNVs identified in patients to those in control databases by considering not only the presence or absence, but also the amount of overlap with CNVs identified in control cohorts.

As the interpretation of the publicly available datasets is challenging, we created an independent control cohort using the same microarray platform for analyzing patients and

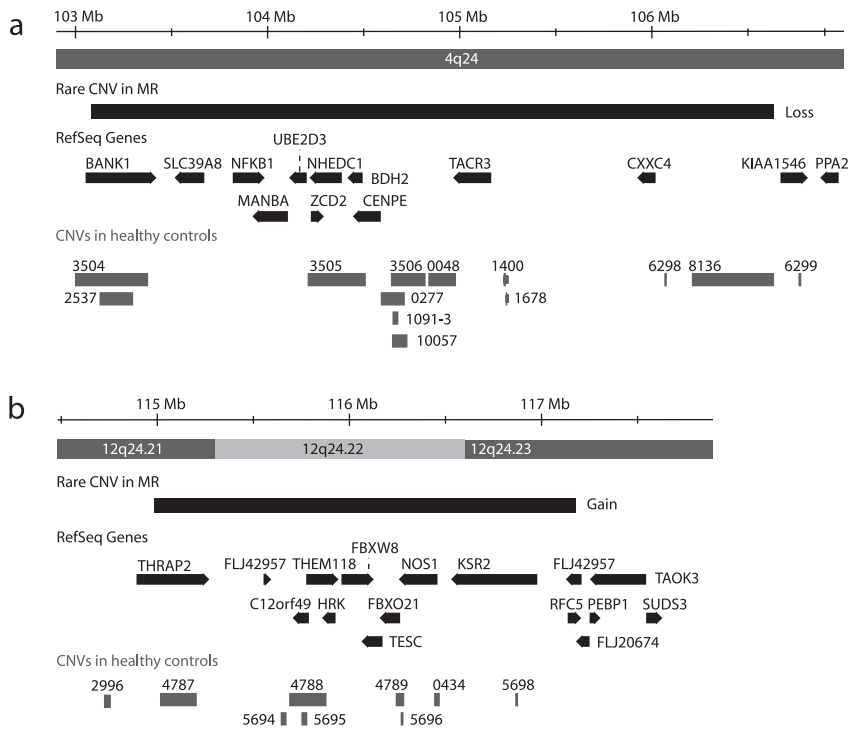

Figure 3.1.3: Rare CNVs versus common CNVs in healthy controls. Two examples of CNVs identified in patients with mental retardation that show overlap with CNVs in healthy controls. (**a**) Case 13, 3.21 Mb loss at 4q24 and (**b**) Case 21, 1.96 Mb gain at 12q24.21q24.23. Both CNVs do not overlap for more than 50% with CNVs in control datasets, and the non-overlapping (unique) genomic segment present in the patient CNVs is larger than 100 kb in size. Therefore, these CNVs are considered to be rare CNVs for which follow-up is needed to determine the clinical significance.

controls. Ideally, such an internal dataset should contain copy number profiles from unrelated normal controls, but for practical and financial reasons we included data obtained from testing unaffected parents of individuals with mental retardation. This dataset consists of 109 samples analyzed by tiling resolution 32k BAC array, of which 98 were published by de Vries *et al*.[5]

We have used both the Database of Genomic Variants as well as our internal control dataset to determine whether a CNV identified in a patient is likely to be a common variant. For inclusion in this analysis we decided that a common CNV should be reported at least three times, preferably in different datasets, and that it should be reported in the same orientation (loss/gain) as that observed in the patient. Furthermore, a CNV observed in the patient is considered to overlap significantly with known CNVs if this overlap exceeds 50% and the non-overlapping (unique) genomic segment is less than 100 kb in size (**Figure 3.1.3**). If we use the approach described above, the large majority of CNVs identified onto tiling resolution 32k BAC arrays fall into the class of common CNVs and, therefore, no validation is needed for these CNVs. This notion, significantly reduces the number of patients for which validation is required.

Most other groups have also started to report on the presence or absence of CNVs in healthy control cohorts, either as published separately or as deposited in the Database of Genomic Variation. Investigators sometimes prefer to look specifically at control data obtained on the same microarray platform because of the ease of comparison. The advantage of this approach is that a one-to-one comparison can be made as the same clones or oligonucleotides have been analyzed in both patients and controls, thus circumventing problems with comparing genomic sizes. Rosenberg *et al*. also compared the CNVs to an in-house dataset of 100 normal controls, encompassing a mix of data from unaffected individuals and data from normal chromosomes of cases with an abnormal karyotype.[24] There are no generally accepted guidelines for dealing with overlap between CNVs in patients and CNVs present in control cohorts. Wagenstaller *et al*. for example state that "All regions that considerably overlapped with known CNVs provided by the Database of Genomic Variants had been excluded",[25] without defining what "considerably overlapped" means.

Rare CNVs of clinical significance

Validation

After having excluded common CNVs, the remaining CNVs can be considered candidates for causing mental retardation. Before determining the clinical significance of such rare CNVs, we have validated the CNVs by an independent technology. For the present study, we have chosen to use MLPA, with specifically designed synthetic probe sets, and/or FISH on fixed metaphase spreads. Other published studies have also validated their results using a second independent technique, mostly FISH, MLPA, real-time PCR, qPCR, microsatellite analysis, or other microarray platforms (**Table 3.1.1**).

From a technical point of view, and depending on the experience present in the diagnostic laboratory, validation of CNVs spanning more than ~50-100 targets may not be necessary, as it is unlikely that >50-100 adjacent targets show an abnormal copy number by chance. For example, for an oligonucleotide array containing 250,000 targets, this would mean that CNVs larger than ~500 Kb in size require no validation by another technique. From a counseling perspective, however, follow-up FISH in the patient and the parents is valuable for the estimation of the recurrence risk, as it might reliably detect balanced rearrangements in healthy carriers.

***De novo* CNVs**

The technique used for validation can also be applied for parental testing, which is often a crucial test to determine causality of an observed CNV, especially when the genomic region involved has not yet been reported to be associated with mental retardation. The use of control CNV data may provide a first clue about the possibility that a CNV is associated with disease, but does not provide a definite answer. Control CNV data are currently too limited to exclude the presence of uncommon CNVs without clinical significance. In addition, control CNV data are not available for all ethnic populations, while we know that there are many population-specific CNVs.[12,26] The clinical significance of a *de novo* event is stressed by the interrogation of CNVs among normal individuals. Of 12,060 biallelic CNV genotypes identified in the HapMap collection by Redon *et al*., only 0.2% exhibited Mendelian discordance, which according to the authors probably reflects the genotyping error rate rather than the rate of *de novo* events at these loci.[12]

Using the above criteria, in our diagnostic cohort of 386 individuals with mental retardation, *de novo* CNVs were identified in 29 patients (**Table 3.1.2**). In total, we found 21 *de novo* copy number losses (median size 1.57 Mb), seven *de novo* copy number gains (median size 2.60 Mb), including a complex duplication rearrangement[27] and one mosaic numerical chromosome aberration.

Also in most other published studies the clinical relevance of CNVs is determined mostly by establishing whether a CNV occurred *de novo* or is inherited from an unaffected parent. On average, *de novo* CNVs were found in 9.2% (126/1,364) of the patients (**Table 3.1.1**), varying from 4.2%[28] up to 30%.[29] This considerable variation can be explained by differences in microarray platform used, differences in patient inclusion criteria, as well as previous molecular/cytogenetic tests performed, but also reflects the availability of parental samples, rather than the true diagnostic yield of the study.

CNVs overlapping critical regions of well-defined genomic disorders

In some cases, it is impossible to obtain DNA samples from both parents of a patient, hampering the clinical interpretation of rare CNVs. Parental samples are, however, not always needed to establish the clinical significance of a CNV. In the case of mental retardation, CNVs can be

Table 3.1.2: Rare CNVs of clinical significance or candidate alterations

Patient	CNV	Chr	Location	Start (Mb)[a]	End (Mb)[a]	Size (Mb)	Clones	Genes	LCR	Confirmed[b]	Origin	Clinical features (Score)[c]	OMIM/ novel syndrome
CNVs related to mental retardation													
1	loss	1	1p36.11p35.3	27.1	28.00	0.90	12	23	-	+	de novo	Severe MR, FD, VM, cataract (1)	
2	loss	1	1p34.3p34.2	39.22	43.07	3.85	40	53	-	+	de novo	Severe MR, FD, GR, MC, delayed brain myelinization (7)[f,g]	
3	loss	1	1q43q44	238.36	242.23	3.87	50	22	-	+	unknown	Moderate MR, FD, GR, MR, epilepsy, absent speech, ACC, persisting cavum septum pellucidum, VM (4)	1q44 microdeletion
4	loss	1	1q44q44	239.93	243.39	3.46	47	20	-	+	de novo	Severe MR, FD, MC, epilepsy, delayed myelinisation, ACC, irregularly implanted toes, hirsutism (4)	1q44 microdeletion
5	loss	1	1q44q44	240.16	241.96	1.80	28	12	-	+	de novo	Severe MR, epilepsy, GR, MC, FD, papil coloboma, talipes equinovarus (4)	1q44 microdeletion
6	loss	2	2p16.3p16.3	48.08	49.59	1.50	17	7	-	+	de novo	Moderate MR, MC, microphtalmia, retina coloboma, FD (4)	
7	loss	2	2p15p16.1	57.87	61.47	3.60	43	12	-	+d	de novo	Moderate MR, MC, FD, opticus hypoplasia (5)[h]	2p15p16.1 microdeletion
8	gain	2	2q23.1q23.1	148.9	149.44	0.54	7	2	-	+	de novo	Mild MR, HT (3)	
9	loss	2	2q23.1q23.2	149.17	150.09	0.92	11	3	-	+	de novo	Severe MR, FD, GR, MC, BP, epilepsy (3)[f]	
10	loss	3	3q27.1q29	184.31	196.73	12.42	131	105	-	+	de novo	Severe MR, FD, GR, MC, hypogenitalism, hypoplastic kidneys, deafness (6)[f]	
11	loss	3	3q29q29	197.21	198.47	1.27	21	29	-	+	unknown	Severe MR, FD, cataract, myopia, macula abnormalities (3)	MIM 609425
12	gain	4	4p16.13q16.13	0.71	2.09	1.38	22	40	-	+	de novo	Modereate MR, HT, FD, iris coloboma, anal atresia (4)	
13	loss	4	4q24q24	103.21	106.42	3.21	27	11	-	+	de novo	Mild MR, FD, polydactyly (3)	
14	gain	5	5q35.1q35.1	170.52	171.52	1.00	14	6	-	+	de novo	Mild MR, semilobular holo-prosencephaly, finger-like thumbs, polydactyly (3)[f,i]	
15	loss	7	7q22.3q22.1	96.83	100.27	3.44	34	97	-	+	de novo	Moderate MR (4)	
16	gain	8	mos trisomy 8	0	146.26	146.28	1439	821	-	+e	de novo	Mild MR, BP, psychotic episisodes (2)	

Table 3.1.2: Continued

Patient	CNV	Chr	Location	Start (Mb)[a]	End (Mb)[a]	Size (Mb)	Clones	Genes	LCR	Confirmed[b]	Origin	Clinical features (Score)[c]	OMIM/ novel syndrome
17	loss	9	9q31.1q31.1	99.74	102.58	2.85	31	17	-	+	de novo	Moderate MR, FD, MC, transposition of the great vessels (4)[h]	
18	loss	9	9q33.1q33.1	115.3	115.78	0.48	6	1	-	+	de novo	Mild MR, FD, macrocephaly, autistic spectrum (3)[f]	
19	loss	10	10q22.3q23.2	81.86	88.86	7.00	69	35	-	+	de novo	Moderate MR (4)	
20	loss	11	11q14.1q14.1	77.8	85.08	7.28	66	19	-	+	de novo	Mild MR FD (5)[f]	
21	gain	12	12q24.21q24.23	114.91	116.87	1.96	38	10	-	+	de novo	Severe MR, FD, GR, MC, BP (7)[f,j]	
22	gain	15	15q11.2q13.1	21.16	26.58	5.42	75	24	+	+	unknown	Severe MR, GR, obesitas, MC, FD, BP (5)	MIM 209850
23	loss	15	15q24.1q24.2	72.2	73.74	1.54	20	38	+	+	unknown	Mild MR, FD, GR, MC (6)[f,k]	15q24 microdeletion
24	loss	16	16p13.2p13.2	8.87	9.88	1.01	10	5	-	+	de novo	Moderate MR, FD, epilepsy, talipes equinovarus, cryptorchidism (4)	
25	gain	17	17p13.2p13.1	4.1	7.16	3.06	30	84	-	+	de novo	Moderate MR, FD (3)[f,l]	
25	gain	17	17p13.1p13.1	7.7	9.27	1.57	29	40	-	+	de novo	Moderate MR, FD (3)[f,l]	
25	gain	17	17p12.1p12	12.64	15.70	3.07	27	16	-	+	de novo	Moderate MR, FD (3)[f,l]	
25	gain	17	17p11.2p11.2	18.55	20.28	1.73	22	33	+	+	de novo	Moderate MR, FD (3)[f,l]	
26	gain	17	17p11.2p11.2	16.56	20.29	3.73	53	83	+	+	de novo	Moderate MR, MC, FD (3)	MIM 610883
27	loss	17	17q21.31q21.31	41.07	41.58	0.51	8	5	+	+	de novo	Moderate MR, FD, hip dysplasia, VM (4)[m]	MIM 610443
28	loss	19	19q13.11q13.12	39.47	41.92	2.45	30	84	-	+	de novo	Severe MR, GR, hypermetropia, HT, MC, FD, VM (2)	
29	loss	19	19q13.42q13.42	60.63	63.01	2.38	27	77	-	+	de novo	Moderate MR, FD (3)	
30	loss	21	21q22.11q22.11	33.55	34.22	0.66	8	12	-	+	unknown	Severe MR, FD, oculair albinisme, epilepsy, inguinal hernia (1)	
31	loss	22	22q11.21q11.21	17.37	19.97	2.60	33	67	+	+	de novo	Mild MR, FD, obesitas, epilepsy, polydactyly of the hands (2)	MIM 188400/192430
32	loss	22	22q11.21q11.21	17.37	20.12	2.75	34	72	+	+	de novo	Mild MR, FD, GR (3)[f]	MIM 188400/192430
33	loss	22	22q11.21q11.21	17.37	19.97	2.60	33	67	+	+	unknown	Severe MR, FD, GR, BP, cataract, kyphosis (6)	MIM 188400/192430
34	gain	22	22q11.21q11.21	18.98	20.19	1.20	19	34	+	+	unknown	Severe MR, epilepsy, buphtalmos, PDD NOS (2)	MIM 608363

Table 3.1.2: Continued

Patient	CNV	Chr	Location	Start (Mb)[a]	End (Mb)[a]	Size (Mb)	Clones	Genes	LCR	Confirmed[b]	Origin	Clinical features (Score)[c]	OMIM/ novel syndrome
35	gain	X	Xq13.2q21.1	72.26	79.27	7.02	54	36	-	+	de novo	Severe MR, FD, GR (3)	
Candidate CNVs													
36	gain	1	1q21.1q21.1	143.53	145.38	1.84	26	30	-	+	unknown	Moderate MR, FD, GR (5)[f]	
37	gain	2	2p13p14	66.6	70.73	4.14	50	38	-	+	unknown	Moderate MR, FD, BP, PE (5)	
38	loss	3	3p14.2p14.1	63.64	65.07	1.43	15	7	-	+	unknown	Severe MR, GR, BP, FD (2)	
39	loss	3	3p14.1p14.1	67.82	68.15	0.33	3	1	-	+	unknown	Moderate MR (2)[f]	
40	loss	3	3p12.2p12.2	81.67	81.82	0.15	4	1	-	+	unknown	Severe MR, MC, epilepsy, spastic quadriplegia, scoliosis, HT, FD, tapering fingers, syndactyly (2)	
41	loss	3	3q26.1q26.1	165.39	165.64	0.25	8	0	-	+	unknown	Severe MR, HT, MC, FD, hernia umbilicalis, arachnodactyly, flexion contractures, rocker bottom feet, camptodactyly (4)	
42	gain	4	4p16.3p16.3	0.95	1.61	0.66	13	16	-	+	unknown	Moderate MR, MC, HT, BP, FD (4)	
43	gain	6	6q15q15	90.93	91.42	0.49	8	2	-	+	unknown	Moderate MR, autistiform behaviour, ADHD, macrocephaly, FD, PE, kidney anomalies (4)	
44	gain	6	6q24.1q24.1	141.14	142.71	1.57	15	4	-	+	unknown	Severe MR (3)	
45	gain	7	7p13p13	43.79	43.96	0.17	5	8	-	+	unknown	Severe MR, FD, MD, hydronephrosis (3)	
46	gain	7	7q21.13	87.99	89.44	1.46	27	4	-	+	unknown	Mild MR, FD (2)	
47	gain	17	17q25.1q25.1	69.32	70.08	0.76	9	10	-	+	unknown	Moderate MR, FD, BP, GR, MC, kyphoscoliose (4)	
48	gain	X	Xq24q24	117.39	117.57	0.19	4	1	-	+	maternal	Moderate MR, macrocepahy, HT, FD, PE (2)	
49	gain	X	Xq25q26.3	128.43	133.13	4.70	47	29	-	+	maternal	Moderate MR, GR, MC, FD, cleft palate, hypospadias, cryptorchidism (7)	

Note.—Mos. = mosaicism; MR = mental retardation; FD = facial dysmorphism; VM = ventriculomegaly; GR = growth retardation; HT = hypotonia; MC = microcephaly; ACC = agenesis corpus callosum; BP = behavioral problems; PE = pectus excavatum. Extensive clinical descriptions can be found at www.ecaruca.net. [a] Mb position of the clone midpoint; [b] FISH and or MLPA confirmation; [c] 0-10 Clinical checklist score[19] in parenthesis; [d] FISH: deletion confirmed in 66.7% of cells (*n=400*); [e] FISH: trisomy chromosome 8 in 7% of interphase nuclei (*n=300*); [f] de Vries *et al.*[5]; [g] Vermeer *et al.*[30]; [h] de Leeuw *et al.*[31]; [i] Koolen *et al.*[32]; [j] Ruiter *et al.*[33]; [k] Sharp *et al.*[34]; [l] Vissers *et al.*[27]; [m] Koolen *et al.*[35]

Table 3.1.3: The diagnostic yield of genome-wide microarray studies in mental retardation

		De novo CNVs				CNVs related to MR[a]			
Reference	Patients	Loss	Gain	Complex[b]	Yield (% OMIM related)	Loss	Gain	Complex[b]	Yield (% OMIM related)
Vissers, 2003	20	2	0	0	10 (50)	2	0	0	10 (50)
Shaw-Smith, 2004	50	6	1	0	14 (28.6)	6	1	0	14 (28.6)
Schoumans, 2005	22	1	1	0	9.1 (0)	1	1	0	9.1 (0)
Tyson, 2005	41	4	0	0	9.8 (25.0)	4	0	0	9.8 (25.0)
Menten, 2006	30	1	1	0	6.7 (50)	3	1	1	16.7 (60)
Miyake, 2006	81	5	2	0	8.6 (42.9)	6	3	2	13.6 (45.5)
Friedman, 2006	140	9	3	5	12.1 (35.3)	10	4	5	13.6 (42.1)
Krepischi-Santos, 2006	100	8	2	0	10 (0)	8	2	0	10 (0)
Rosenberg, 2006	95	10	0	1	11.6 (72.7)	10	2	1	13.7(76.9)
Engels, 2007	60	3	0	0	5 (0)	3	1	0	6.7 (25)
Hoyer, 2007	100	7	0	0	7 (14.3)	9	2	1	12.0 (50)
Fan, 2007	104	7	1	1	8.7 (33.3)	7	1	1	8.7 (33.3)
Aradhya, 2007	20	5	1	0	30 (83.3)	7	1	0	40 (87.5)
Wagenstaller, 2007	67	7	1	0	11.9 (12.5)	8	2	0	14.9 (20)
Thuresson, 2007	48	1	1	0	4.2 (50)	1	1	0	4.2 (50)
This study[c]	386	21	6	2	7.5 (13.8)	25	8	2	9.1 (22.9)
Total	1,364	97	20	9	9.2 (30)	110	30	13	11.2 (37.9)

[a] De novo, OMIM syndrome or associated with a novel clinically recognizable syndrome

[b] Numerical anomalies, translocations, multiple CNVs

[c] The results of de Vries *et al*.[5] are included in the current study

considered pathogenic when they overlap with genomic regions known to cause well-defined mental retardation syndromes, as defined by the database of the Online Mendelian Inheritance in Man (OMIM) (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim). For diagnostic purposes, these CNVs should at least include the critical region of the syndrome and, if known, the causative gene(s). In addition, the clinical features of the patient should be consistent with the phenotype of the syndrome involved, although a wide variation in phenotypic expression should be considered. Even in case a CNV associated with a well known syndrome is found to be inherited from one of the parents, it does not rule out the pathogenicity of the CNV. Mosaicism in the parents, variable expression and incomplete penetrance have previously been described, for example in the DiGeorge/velocardiofacial syndrome (DGS/VCFS [MIM 188400/192430]).[36]

In our study we observed eight cases in which a rare CNV overlapped with an OMIM mental retardation syndrome (**Table 3.1.2**). In all these cases, the critical disease region was included in the CNV and the phenotype matched, at least in part, with the syndrome associated with the genomic abnormality. Also, the other published studies considered a CNV pathogenic if it overlapped with that of a known clinical syndrome. In the combined studies, CNVs deposited in the OMIM database comprise 37.9% of the CNVs related to mental retardation (**Table 3.1.3**). Deletions of the 1p36 genomic segment (MIM 607872) and deletions of the 22q11.21 genomic segment, involved in DGS/VCFS (MIM 188400/192430) are the most frequent pathogenic

Table 3.1.4: OMIM associated CNVs identified in combined microarray studies

Chr	Syndrome	OMIM	Number
1	Monosomy 1p36 syndrome	607872	8
2	Brachydactyly mental retardation syndrome	600430	1
3	3q29 microdeletion syndrome	609425	2
4	Wolf-Hirschhorn Syndrome	194190	2
5	Sotos syndrome	117550	1
7	Williams-Beuren syndrome	194050	2
8	Nablus mask-like facial syndrome	608156	1
9	9q34 deletion syndrome	610253	2
15	Susceptibility to autism; AUTS4	608636	1
15	Prader-Willi/Angelman syndrome	176270/105830	2
17	Miller-Dieker syndrome	247200	1
17	17q21.31 microdeletion syndrome	610443	6
17	Potocki-Lupski syndrome	610883	5
17	Smith-Magenis syndrome	182290	1
18	18qter deletion syndrome	601808	1
18	Pitt-Hopkins syndrome	610954	1
22	22q13 microdeletion syndrome	606232	3
22	22q11.2 microduplication syndrome	608363	7
22	DiGeorge/velocardiofacial syndrome	188400/192430	7
X	Leri-Weill dyschondrostosis	127300	1
X	XLMR, MRGH	300123	1
X	FG syndrome 5	300581	1
X	XLMR. MRX3	309541	1
X	Pelizaeus-Merzbacher disease	312080	1
X	MECP2 duplication	312750	1

CNVs. The reciprocal 22q11.21 duplication (MIM 608363) was also identified in multiple cases, as well as the recently identified 17q21.31 microdeletion syndrome.[35,37,38] An overview of the CNVs associated with an OMIM mental retardation syndrome in all studies is provided in **Table 3.1.4**.

Novel clinically recognizable recurrent CNVs

The application of genomic microarrays has resulted in the identification of several novel recurrent disease causing CNVs, not all of which have an OMIM entry yet. International databases, such as ECARUCA (www.ECARUCA.net) and DECIPHER (www.sanger.ac.uk/PostGenomics/decipher/), capture cytogenetic and clinical information of patients with rare CNVs and are helpful in identifying overlapping copy number changes. However, these novel recurrent CNVs should be interpreted cautiously in a diagnostic setting, because the numbers are often small and in many cases no recurrent breakpoints have been identified, since the overlapping CNVs may differ in size. Moreover, the clinical features of many novel recurrent CNVs have not been studied in detail, which complicates the interpretation of the clinical significance of copy number changes in these regions. As a result, if no parental samples are available and the CNV does not overlap with that of a known genomic disorder, we consider a CNV to be clinically significant if it overlaps with the minimal critical region of at least two recurrent *de novo* CNVs identified in patients with mental retardation. Importantly, similar clinical features should support the pathogenicity of the aberrations.

In our study, we identified five CNVs overlapping with novel recurrent CNVs for which a phenotypic overlap has been described (1q44,[39-41] 2p15-16.1,[31,42,43] 3q29,[44] 10q22q23,[45] and 15q24[5,34,46]). Also, recurrent CNVs that define novel clinically recognizable syndromes were identified in the combined studies, such as copy number losses at 6p25,[47] 8p23.1,[48] 12p14,[49] 14q11.2,[6,50] and 16p11.2[51] and it is to be expected that on the basis of genomic profiling, many other new syndromes will be defined in the near future.

CNVs of unknown clinical significance

The clinical significance of rare CNVs remains to be established in case DNA of one or both parents is unavailable and the CNV is not associated with a known mental retardation syndrome and/or does not overlap with a recurrent mental retardation region. In the present study, for 14 rare CNVs (14/49= 28.6%), we were not able to define the clinical significance, which is comparable to the amount of genetic variants of uncertain significance or unclassified variants in *BRCA1* and *BRCA2* mutation analyses.[52] In a recent study, variants of uncertain significance, even accounted for 67% of the mutations identified in DNA mismatch repair genes in a series of early onset colorectal cancer cases.[53] **Table 3.1.2** shows all CNVs with unknown clinical significance identified in our study and summarizes the clinical phenotype of the patients. One could speculate on the clinical significance of these CNVs by looking at the genomic size and the gene content of the genomic region involved, i.e., the larger a CNV, the more likely it

is that it would have been identified in normal control cohorts if it does not result in a clinical phenotype. Thus, with the rapid increase in control CNV data, it becomes more and more likely that a rare CNV spanning over 1 Mb is related to the disease phenotype. Similarly, rare CNVs encompassing numerous (protein-coding) genes are more likely to be causative than CNVs that do not span such genes, especially when these genes are known to be dosage-sensitive. In all cases, however, a straightforward clinical diagnosis cannot be reached by this approach before we know more about the biological pathways underlying mental retardation.

CNVs on the X chromosome

CNVs on the X chromosome deserve special attention as the dosage effect obviously differs in males and females. Common CNVs on the X chromosome can be excluded and *de novo* occurrence, the involvement of regions associated with well-known syndromes or novel recurrent CNVs, are considered indicative for the pathogenicity of the CNV. The exception is that causal CNVs in a male patient can be inherited from a healthy mother. In our cohort, we identified rare CNVs on the X chromosome in three cases. A *de novo* 7.0 Mb gain at Xq13.2q21.1 was considered to be causally related to the phenotype in the male patient. The other two gains, a 190 kb gain at Xq24q24 and a 4.7 Mb gain at Xq25q26.3, were found in male patients and also in their non-affected mothers. Although, these CNVs might contribute to the phenotype of the patients, we are unable to draw firm conclusions on their clinical significance. Overall, genome-wide microarray studies reported on 17 CNVs on the X chromosome, of which 10 CNVs in male patients, were inherited from a normal mother. Rosenberg *et al*. stressed the clinical significance of an inherited CNV by showing segregation of the CNV with the disease in a family,[24] whereas Wagenstaller *et al*. showed a non-random X-inactivation pattern in a healthy mother with the chromosome carrying the CNV being inactivated.[25] However, segregation studies and X-chromosome inactivation analyses are not always feasible within a diagnostic setting. As a result, these rare CNVs on the X-chromosome should often be added to the group of CNVs with unknown clinical significance.

Conclusions

Developments in genomic microarray technology have revolutionized the study of human genomic copy number variation. Technological restrictions have been largely overcome and further enhancements in genomic microarray analysis will soon allow the reliable analysis of all CNVs throughout the genome at a kilobase or even single exon level. In recent years, microarray-based copy number analysis has found its way into routine clinical practice and will soon replace conventional karyotyping as the first test in the genetic diagnosis of patients with mental retardation. However, the interpretation of CNVs in routine clinical diagnostics is still a complex process, due to the presence of a high variability in relative large segments of the human genome, hampering the clinical interpretation of the CNVs identified. Here

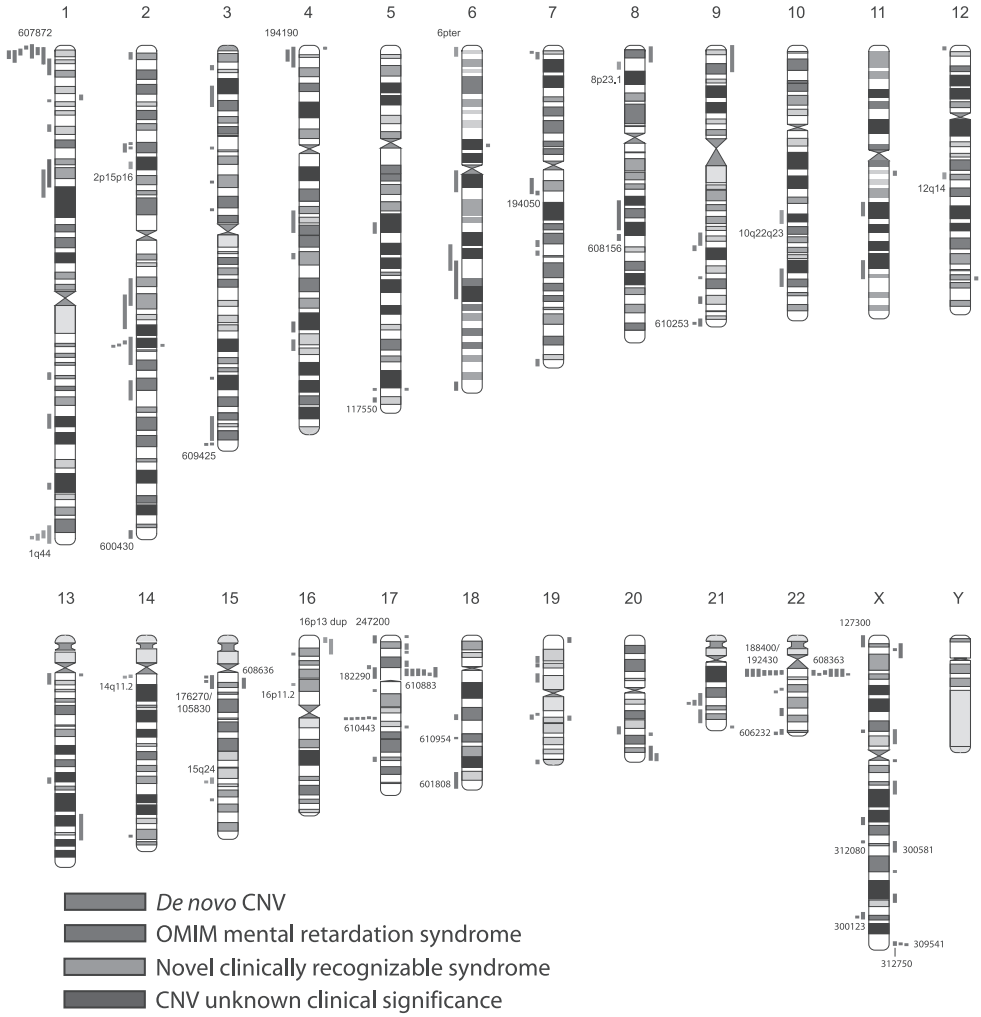

Figure 3.1.4: Overview of all CNVs reported in genome-wide microarrays studies in mental retardation. CNVs are represented by colored bars. Copy number losses and copy number gains are depicted, respectively, on the left hand side and the right hand side of the chromosomes. Red bars, CNVs associated with a well-known OMIM syndromes. Orange bars, novel recurrent CNVs. Green bars, *de novo* CNVs not known to the previous categories and blue bars, CNVs of unknown clinical significance. For color figure see page 193.

we present a practical workflow that can be used in routine clinical diagnostics to assess the clinical significance of CNVs identified. This workflow discriminates between common CNVs and rare CNVs based upon publicly available databases collecting CNV in control cohorts and internal control datasets. Next, the rare CNVs are checked for *de novo* occurrence and the genomic regions involved are screened against a list of known microdeletion/duplication syndromes as well as novel recurrent CNVs associated with mental retardation. Based on this workflow, we reclassified all CNVs described in genome-wide microarray studies on mental

retardation. **Figure 3.1.4** shows an overview of all rare CNVs related to mental retardation (either *de novo*, an OMIM mental retardation syndrome, or associated with a novel clinically recognizable syndrome) and CNVs of unknown clinical significance. The overall yield of clinically significant CNVs is 9.1% in our cohort of 386 patients (25 losses, median size 2.6 Mb; 9 gains, median size 2.0 Mb, 1 mosaic trisomy 8), and 11.2% in the combined published genome-wide microarray studies. Although comparison of the diagnostic yield of these studies is limited, the results emphasize the usefulness of genomic microarray technology in patients with mental retardation. The current frequency is likely to be an underestimate given the fact that most of the microarrays used only detect genomic rearrangements >100-300 kb in size. Novel oligonucleotide-based microarrays will allow the high-resolution detection of even intra-genic (exon) deletions and duplications and it is to be expected that the implementation of these platforms in a diagnostic setting will increase the frequency of clinically significant CNVs in patients with mental retardation. It is essential to further study these CNVs and their functional consequences as this will be instrumental for clinical diagnosis, the identification of the causative genes underlying the pathogenesis of mental retardation, and the identification of new mental retardation syndromes.

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Chapter 3.2

Identification of non-recurrent submicroscopic genome imbalances: the advantage of genome-wide microarrays over targeted approaches

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Abstract

Genome-wide analysis of DNA copy-number changes using microarray-based technologies has enabled the detection of *de novo* cryptic chromosome imbalances in approximately 10% of individuals with mental retardation. So far, the majority of these submicroscopic microdeletions/duplications appear to be unique, hampering clinical interpretation and genetic counseling. We hypothesized that the genomic regions involved in these *de novo* submicroscopic aberrations would be candidates for recurrent copy-number changes in individuals with mental retardation. To test this hypothesis, we used multiplex ligation dependent probe amplification (MLPA) to screen for copy number changes at eight genomic candidate regions in a European cohort of 710 individuals with idiopathic mental retardation. By doing so, we failed to detect additional submicroscopic rearrangements, indicating that the anomalies tested are non-recurrent in this cohort of patients. The break points flanking the candidate regions did not contain low copy repeats and/or sequence similarities, thus providing an explanation for its non-recurrent nature. On the basis of these data, we propose that the use of genome-wide microarrays is indicated when testing for copy-number changes in individuals with idiopathic mental retardation.

Introduction

Genome-scanning array technologies, such as microarray-based comparative genomic hybridization (array CGH), enable the detection of interstitial submicroscopic DNA copy-number alterations in individuals with mental retardation (MR) of unknown etiology. *De novo* submicroscopic alterations have been identified in approximately 10% of individuals with MR using both bacterial artificial chromosome (BAC) microarrays[1–8] and single-nucleotide polymorphism-based microarrays.[9]

Using genome-wide microarray strategies, novel recurrent interstitial submicroscopic aberrations have only been reported sparsely in individuals with MR.[10–17] So far, the vast majority of the cryptic microdeletions/duplications identified appear to be unique, which hampers its clinical interpretation and counseling of the families. However, because these genomic imbalances are likely to harbor dosage sensitive genes related to the pathogenesis of MR, we hypothesized that the genomic regions involved in *de novo* submicroscopic aberrations are candidates for recurrent copy-number changes in individuals with idiopathic MR. To test this hypothesis, we subjected eight pre-selected regions to targeted copy-number analysis using multiplex ligation dependent probe amplification (MLPA) in a cohort of 710 individuals with idiopathic MR and compared the efficacy of our targeted MLPA-based approach to genomewide scanning strategies.

Materials and methods

Subjects

In total, 710 mentally retarded individuals with or without facial dysmorphisms or congenital malformations were included in this study. All individuals exhibited normal G-banded karyotypes at 550-band resolution. Genomic DNA was prepared from blood lymphocytes by standard procedures. The DNA samples were derived from Nijmegen, The Netherlands (*n=200*), Oxford, UK (*n=200*), Schwerzenbach, Switzerland (*n=100*), Stockholm, Sweden (*n=80*), Antwerp, Belgium (*n=80*), and Troina, Italy (*n=50*).

Selection of novel submicroscopic aberrations and in silico LCR analysis

We selected eight de novo submicroscopic copy aberrations for testing among individuals with MR. The aberrations varied in size from 480 kb to 12.4 Mb and were dispersed throughout the genome (**Table 3.2.1**). The aberrations were previously identified in a cohort of 100 mentally retarded individuals using genome-wide tiling path resolution array CGH.[1] The 2q23.1q23.2 microdeletion partly overlapped with a microdeletion previously reported by our group.[8,18]

The flanking 400 kb break point regions of the eight candidate regions were screened for the presence of homologous low copy repeats (LCRs) using the Segmental Duplication Database (http://humanparalogy.gs.washington.edu) and BLAST2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/ wblast2.cgi) analyses. The break points were defined by the average start and end

Table 3.2.1: Candidate regions screened for DNA copy-number changes

	Location	Start (Mb)[a]	End (Mb)[a]	Size (Mb)
1	1p34.3p34.2	39.2	43.1	3.85
2	2q23.1q23.2	149.2	150.1	0.92
3	3q27.1q29	184.3	196.7	12.42
4	5q35.1	170.5	171.5	0.97
5	9q31.1	99.7	102.6	2.85
6	9q33.1	115.3	115.8	0.48
7	11q14.1q14.2	77.8	85.1	7.28
8	12q24.21q24.23	114.9	116.9	1.98

[a] On the basis of 32k BAC microarray data (NCBI, Build 35, May 2004).[6]

positions, respectively, of the first and last flanking BAC clone that identified the genome imbalance (based on NCBI, Build 35, May 2004).

Multiplex ligation-dependent probe amplification

For the MLPA screening two to seven probes were designed within exonic sequences in the genomic regions of interest (**Table 3.2.2**) according to a protocol provided by MRC-Holland (http://www.mlpa.com/pages/support_desing_synthetic_ probespag.html). The same probes had also been used for the confirmation of the *de novo* aberrations, previously identified by genome-wide tiling resolution array CGH (**Figure 3.2.1**).[1] The MLPA probes were combined in one MLPA assay in conjunction with four standard control probes in three different genes, *VIPR2*, *MRPL41* and *KIAA0056*. MLPA reactions using 200 ng genomic DNA were performed as described previously.[19,20] All MLPA reagents were obtained from MRC-Holland, Amsterdam, The Netherlands. Amplification products were identified and quantified by electrophoresis on a capillary sequencer (ABI 310, ABI 3100, ABI 3130 or ABI 3730), using GeneMapper software (Applied Biosystems, Foster City, USA). For copy-number quantification data were normalized by dividing each probe's peak area by the average peak area of the control probes of the sample. The normalized peak patterns were divided by the average peak area of all the samples in the same experiment. For all DNA samples, we computed the coefficient of variation (c.v.) of the normalized signal strength over the controls. If a particular sample had a c.v. of more than 15, the result of the analysis for that particular sample was discarded. If a particular probe had a c.v. of more than 15 over all samples tested, the analysis was repeated. Copy-number change detection was based on thresholds for gains and losses of 1.30 and 0.70, respectively. The MLPA analyses were repeated for all samples in which an aberration was identified. For these confirmation experiments, DNA samples of healthy controls were used for the normalization. If available, DNA of positive controls were included in the MLPA assays

Results and discussion

We used MLPA to look for copy-number changes at eight pre-selected genomic regions in a European cohort comprising 710 individuals with idiopathic MR with or without associated

Table 3.2.2: MLPA probe information

Gene	Band	Size	5' hybridization sequence[a]	3' hybridization sequence[b]
KCNQ4	1p34.2	100	CAGGGCAACATCTTCGCCACGTCCGCGCT	GCGCAGCATGCGCTTCCTGCAGATCCTGC
EDN2	1p34.2	124	GCGGGAGCCTCGGTCCACACATTCCAGGTGGAGGAAGAGAT	AGTGTCGTGAGCTGGAGGAACATTGGGAAGGAAGCCCGCGG
ZMYND12	1p34.2	128	CCATGTTTGACCCTTACCGGCCACTGTACGGGCCTTTCTGGAC	TTGGACTCCTTGGGAGTCGTTTCTCGGCCATTTGACCCGTGGG
MACF1	1p34.3	92	GTGGAATGTTTCACTGCTCCCAAGG	AGCGGGTAATGAGAGTGGCACTTAG
MBD5	2q23.1	84	CAAAGAGTGTGACGGAGGGGA	CAAGGAAGGAGGTCTTCCAGC
EPC2	2q23.1	92	GAGGTGGAAGGTGAAGTATTTGTTT	TCACCTGGTTTTTGTTTGCTATCTG
MBD5	2q23.1	100	CACTAACAGAAGGTTTGGAAGCCTACAGC	CGTGTCCGGAAAAGGAACAGAAAGTAAGC
EPC2	2q23.1	108	CAGTTAGTTCAGATGCAAAGGCAGCAACTTGCC	CAGCTTCAGCAGAAACAGCAATCTCAGCATTCC
KIF5C	2q23.1	112	CCGTGTTTGTATTTTCGCCCACTAGGGGAAGCTGC	ATGACCCCCAGCTCATGGGGATCATCCCACGAATT
MBD5	2q23.1	120	CAGAGTCGGGGATTTGGAGAGCTGCTAAGCACTGCAAAG	CAAGACCTGGTCCTAGAGGAGCAGTCTCCAAGTTCCTCA
MBD5	2q23.1	124	CTAAATACCCCAAGCAGTGCAGCTTTTCCTACTGCATCTGC	CGGAAGTAGTTCTGTAAAGAGTCAGCCTGGTTTGCTGGGAA
MAP3K13	3q27.2	84	GCACGATGGCCAACTTTCAGG	AGCACCTGAGCTGCTCCTCTT
LPP	3q28	88	GACGCTGAGATTGACTCCTTGAC	CAGCATCTTGGCTGACCTTGAGT
TP723L	3q28	92	GGAGAATGGGGTGATATTGGAGAAG	CTGCATGATAAGACCTGTGACCTTC
HRASLS	3q29	100	CCTTTACAAGCGCCAAGTCTGTATTCAGC	AGTAAGGCCCTGGTGAAAATGCAGCTCTT
FGF18	5q35.1	108	CCTCAGGTCCCACTGACCGCTTCTCCATCTGTT	TCCCGCAGGTGTTTACACTTCCTGCTGCTGTGC
STK10	5q35.1	112	CTCGCCCTGTGCCACCCCAACTGTGCCTGATAGAC	CTGCCCCAGCGTTCCTGACTTCTTGCTGGCCTGTG
DC-UbP	5q35.1	120	CTTGGCACCGCCAATCAACATGATAGAGGAAAAGAGCGA	CATAGAGACTCTGGATATTCCTGAGCCACCACCCAATTC
STX17	9q31.1	N92	CAGGTGCACTCATCGGGGGAATGGT	AGGGGGTCCTATTGGCCTCCTTGCA
PRG-3	9q31.1	104	CGAATGCACTGACACTTTTCAGGTGCATATC	CAAGGATTCTTCTGTCAGGACGGAGACTTAA
GRIN3A	9q31.1	108	GCCACAGCCACCATGATCCAACCAGAACTTGCT	CTCATTCCCAGCACGATGAACTGCATGGAGGTG
C9orf27	9q33.1	92	CAAAGGCTCTGTGTTAGTGGATTCA	CAAGCAACCTGTACTCCTCAAAGAA
C9orf27	9q33.1	100	GTATGCAGACTCATCCATCCATGAATCTA	GATTGCAGGGATGTGACCTATGTAATGAA
GAB2	11q14.1	84	CTCCAGCCAGCCAACTCTGTT	CACGTTTGAACCCCCTGTGTC
DLG2	11q14.1	112	CTGACGGGCTTTCTGCCCAAGAGATGAGATGAGAG	CCTCCTCACCCCAGCAGATGTCCAGAGCTGATTTA
PICAM	11q14.2	120	CACAGTGTCACCGGCTCTGCCGTATCCAAGACAGTATGC	AAGGCCACGACCCACGAGATCATGGGGCCCAAGAAAAAG
NOS1	12q24.22	112	CACATGTTCGGTGTTCAGCAAATCCAGCCCAATGT	CATTTCTGTTCGTCTCTTCAAGCGCAAAGTTGGGG
KSR2	12q24.23	120	CTGGCTTTCTCATGGCTTACCCATTGTCTCTGCTCTTCT	GTTCCAACTCAGGAGGCAACCTTTCCAAACAAGACTGGA

[a] The 5′ half-probe is preceded by the 5′ universal primer tag.

[b] The 3′ half-probe is preceded by a 5′ phosphate group and followed by the 3′ universal primer tag.

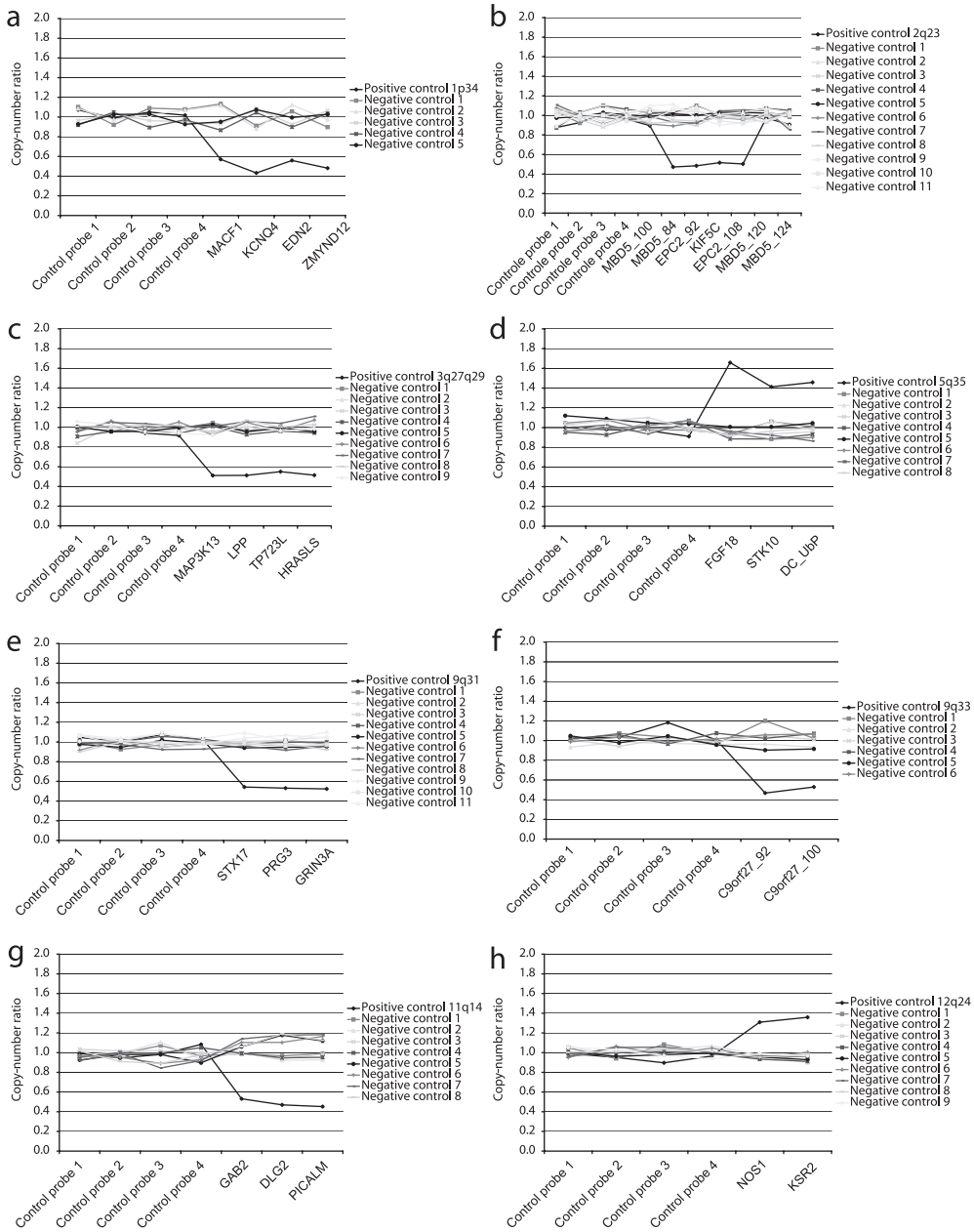

Figure 3.2.1: MLPA validation of submicroscopic genome imbalances. (**a**) Loss 1p34.3p34.2, (**b**) loss 2q23.1q23.2, (**c**) loss 3q27q29, (**d**) gain 5q35, (**e**) loss 9q31.1, (**f**) loss 9q33.1, (**g**) loss 11q14.1q14.2, (**h**) gain 12q24.21q24.23.

dysmorphisms or congenital anomalies. Causative copy-number changes in these eight regions were previously described in single individuals with MR.[1] The eight regions were based upon the unique and *de novo* aberrations that had been found in our initial study among a cohort of 100 MR patients using genome-wide tiling path resolution array CGH.[1] Through the MLPA assay, we failed to detect additional submicroscopic rearrangements at all candidate regions in this patient cohort. **Figure 3.2.2** shows an example of the data obtained by the MLPA copy-number screening of the candidate regions. The loss of the 11q14.1 segment is clearly demonstrated in the positive control sample, whereas in the remaining test samples in the assay, no copy-number changes are found. Subsequently, we screened the flanking break point regions of the preselected candidate regions for the presence of LCRs and/or sequence similarities that might predispose for the occurrence of non-allelic homologous recombination events leading to loss or gain of the intervening DNA sequence.[21]

However, no significant LCRs and/or sequence similarities could be identified. The present study is the first report of a comprehensive screen for interstitial submicroscopic aberrations in a large cohort of individuals with MR using MLPA. Of course, the results might have been different if other regions, such as subtelomeric regions, had been analyzed in this cohort. Although copy-number changes in these latter regions are usually not mediated by LCRs, which is similar to our eight selected regions, they are in regions that have already been associated with recurrent aberrations.

Others have employed a variety of microarray-based targeted approaches to detect recurrent submicroscopic aberrations. Sharp *et al*,[15] for example, generated a segmental duplication BAC microarray targeted to 130 potential rearrangement hot spots in the human genome. By

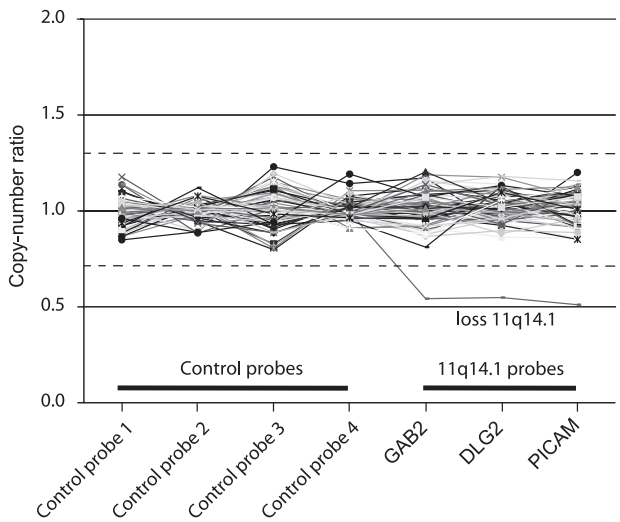

Figure 3.2.2: Copy-number screening at 11q14.1 using multiplex ligation-dependent probe amplification. x-axis, standard control probes and three probes hybridizing to 11q14.1 (for a complete list of probe sequences, see Table 3.2.2). y-axis, normalized copy-number ratios. Copy-number change detection is based on thresholds for gains and losses of 1.30 and 0.70, respectively. The loss of the 11q14.1 segment is clearly demonstrated in the positive control sample, whereas in the remaining test samples in the same assay, no copy-number changes could be identified.

using this targeted approach, they tested 290 individuals with MR and identified 16 pathogenic rearrangements, including four microdeletions in 17q21.31.[22] The phenotypic similarities between the individuals with an overlapping 17q21.31 deletion subsequently pointed to a new microdeletion syndrome.[10,17,22] More recently, several other novel recurrent microdeletions that are mediated by flanking LCRs have been identified. These recurrent aberrations may give rise to new genomic disorders, such as the 15q24 microdeletion syndrome[16] and the 10q22q23 microdeletion syndrome.[23] Targeted microarrays have been developed with target sequences corresponding to genomic regions of known clinical significance, such as the chromosome subtelomeres and regions implicated in well-known human genomic disorders.[24–27] Using these targeted microarrays, Shaffer *et al*[28] found clinically relevant genomic alterations in 5.6% of 1500 consecutive cases referred to the clinic for a variety of developmental problems. Indeed, these targeted microarrays have some advantages over genome-wide microarray scanning technologies, especially in a diagnostic setting, as parental samples are not requisite for the clinical interpretation of the array CGH findings.[29] In addition, in most cases there is ample information available about the clinical consequences of these submicroscopic copy-number alterations, thus facilitating the genetic counseling of families. However, most known microdeletion syndromes, will be clinically recognized by experienced clinical geneticists and can be confirmed by specifically designed FISH tests. Therefore, patients with a recognizable microdeletion syndrome will only rarely be sent in for microarray analysis. Moreover, in contrast to genome-wide microarray approaches, targeted approaches will miss sporadic DNA copy-number changes in MR, as these regions will not be represented on such microarrays.[30] The latter might be overcome if the targeted array is up-dated, regularly, by including all newly and uniquely reported microaberrations. By doing so, one might eventually end up with a whole genome-wide array.

Our study indicates that *de novo* submicroscopic aberrations that are not flanked by genomic architectural features conferring susceptibility to rearrangements appear to be non-recurrent in a large cohort of patients. In the future it is conceivable that advanced technologies and results from large numbers of patient studies will help unravel the majority of genes involved in MR, thereby making targeted testing approaches more viable. However, in the meantime, we recommend the use of genome-wide microarrays when testing idiopathic MR patients for genome imbalance.

Acknowledgements

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Chapter 3.3

A new chromosome 17q21.31 microdeletion syndrome associated with a common inversion polymorphism

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Abstract

Submicroscopic genomic copy number changes have been identified only recently as an important cause of mental retardation. We describe the detection of three interstitial, overlapping 17q21.31 microdeletions in a cohort of 1,200 mentally retarded individuals associated with a clearly recognizable clinical phenotype of mental retardation, hypotonia and a characteristic face. The deletions encompass the *MAPT* and *CRHR1* genes and are associated with a common inversion polymorphism.

Introduction

Mental retardation is the most common developmental disorder, affecting intellectual and adaptive functions with a frequency of approximately 2–3% in the general population. Whole-genome scanning technologies such as array-based comparative genomic hybridization (array CGH)[1] have enabled the detection of interstitial submicroscopic copy number alterations in ~10% of individuals with mental retardation of unknown etiology.[2]

Material and methods

Individuals

We tested 360 mentally retarded individuals for copy number changes using our genome-wide tiling resolution microarray in a diagnostic setting.[2] Previous routine chromosome analysis was normal and subtelomeric MLPA failed to reveal any anomalies (SALSA MLPA kit P036, MRC Holland, Amsterdam, the Netherlands) in all individuals. In addition, we tested a cohort of 840 mentally retarded individuals for copy number changes in the 17q21.31 region by MLPA. DNA samples of these individuals were collected from centers in Oxford, UK (*n=130*), Antwerp, Belgium (*n=130*), Zurich, Switzerland (*n=100*), Troina, Italy (*n=130*), Stockholm, Sweden (*n=70*) and Nijmegen, The Netherlands (*n=280*). We obtained informed consent from the parents, including consent to publish photographs of all subjects described in this study.

Clinical description of individuals with the 17q21.31 deletion

Individual 1

The chromosomes of this 3 year-old-girl were analyzed prenatally and found to be normal. She was born at 35 2/7 weeks gestation with a birth weight of 2,078 g (10th centile) and head circumference of 30.4 cm (5th centile). She was severely hypotonic and her development was considerable delayed: sitting at 3 years and no words at 3 years and 1 month. Magnetic Resonance Imaging (MRI) of the brain at 1 year of age showed widened ventricles and periventricular white matter changes. Electromyography (EMG) was normal, but Brainstem Evoked Response Audiometry (BERA) was abnormal. At 3 months she was treated for a congenital hip dysplasia. Diagnostic testing including DNA analysis for myotonic dystrophy, Spinal Muscular Atrophy (SMA), Prader-Willi syndrome, metabolic screening in blood and urine revealed no abnormalities.

On physical examination at the age of 3 years and 1 month, her height was 95 cm (40th centile), weight 12.5 kg (10th centile for height) and head circumference 49.5 cm (50th centile). She had a long hypotonic face with frontal bossing and bitemporal narrowing, ptosis, blepharophimosis, upward slanting palpebral fissures, epicanthal folds, large low set ears with hypoplastic crus superior, low nasal bridge, bulbous nasal tip, long columella, triangular nostrils, high palate with

broad gums and a broad chin (**Figure 3.3.1a**). Her broad thorax had wide-space nipples and a mild pectus excavatum. She had long fingers, narrow long feet with long toes and hyperlaxity of the joints (Beighton score of 6/10). She was good natured with normal eye contact.

Individual 2

This 17-year-old, moderately mentally retarded woman was born at 38 weeks gestation in breech position with a caesarian section and she had a birth weight of 1,980 g (<3rd centile). In the first 3 weeks she received nasal catheter feeding because of low glucose levels and phototherapy because of hyperbilirubinaemia. She was hypotonic and started walking and speaking after the age of 2 years. She had an IQ of 48 points and attended special schooling. From the age of 1.5 to 3.5 years she had epileptic insults for which antiepileptic drugs were used. An MRI of the cerebrum showed wide ventricles, especially of the temporal horn of the lateral ventricles. In addition, routine chromosome analysis, metabolic screening in blood and urine and EMG, revealed no abnormalities. She developed a scoliosis at 13 years of age. She had normal hearing but mildly impaired vision (+4/+4).

On physical examination at the age of 17 years, she had a low-normal height of 160.8 cm (10th centile). Her weight was 51.2 kg (50th centile for height) and head circumference 53 cm (10th centile). She had a long hypotonic face with ptosis, blepharophimosis, upward slanting palpebral fissures, large ears with hypoplastic crus superior, tubular pear-shaped nose with high nasal bridge and long columella, short philtrum, 2 missing upper teeth, everted lower lip and broad chin (**Figure 3.3.1b**). She had a thoracal scoliosis with a lumbar hyperlordosis. Her hands and fingers were long and slender with a simian crease in the left palm. Her feet had high arches with hallux valgus bilaterally and mild hammer toes. The lower part of the limbs were slender and there was a mild general decrease of strength. In addition, she had mild hyperlaxity of the finger joints (Beighton score of 2/10), and numerous moles on the skin. She had nasal speech and an amiable nature.

Individual 3

This 26-year-old, moderately mentally retarded male was born after an uneventful pregnancy at term with a normal birth weight of 3,120 g (50th centile) but a large head circumference of 37.5 cm (>97th centile). He was notably hypotonic and computed tomography imaging of the brain at 4 months of age showed a communicating hydrocephaly without increased intracranial pressure. Both Somatosensory Sensory Evoked Potentials (SSEP) and BERA studies were abnormal at 6 months but normalized later in life. His development was retarded, walking at 3 years of age and he attended special school. He had an IQ of 40 points at the age of 18 years. He was operated on inguinal hernia and cryptorchidism. A scoliosis developed at 13 years of age. Diagnostic test including routine chromosome, *FMR1* analysis and metabolic screening in blood and urine revealed no abnormalities.

On physical examination at the age of 26 years, he had a low-normal height of 173 cm (10th centile), and a large head circumference of 63 cm (>97th centile). His weight was 68 kg (70th centile for height). He had a long narrow face with a high, broad forehead, blepharophimosis, strabismus divergence, large ears with hypoplastic crus superior and large lobules, tubular pear-shaped nose with bulbous tip and long columella, high palate with broad gums and diastemia frontal upper teeth, and a large broad chin (**Figure 3.3.1c**). The thorax was flat and broad with wide-spaced nipples and a scoliosis.
His hands and fingers were long and hyperlax (Beighton score of 4/10). His feet had high arches with hallux valgus bilaterally and his skin revealed numerous moles. He had nasal speech and a friendly nature.

Array-based comparative hybridization

Microarray preparation, hybridization, and data analysis were performed as described previously.[2] In brief, the array contains 32,447 BAC clones resulting in a complete coverage of the human genome.[3] The array CGH profiles were established through co-hybridization of 500 ng Cy3-dUTP labeled patient DNA and 500 ng sex-mismatched Cy5-dUTP labeled (Amersham Biosciences) reference DNA. After scanning, test-over-reference ratios were determined for each clone, $\log_2$-transformed and normalized by subtracting its local mean $\log_2$ test-over-reference ratio obtained by a weighted median filter. The normalized ratios were analyzed for loss and gain regions by a standard hidden Markov model (HMM).

Copy number screening of the 17q21.31 region by MLPA

We used MLPA[4] to screen for copy number changes at 17q21.31 in the cohort of 840 mentally retarded individuals. A set of uniquely-sized MLPA probes, hybridizing to exon 3 and 13 of *CRHR1*, and to exon 2 of *MAPT* was used. The probes were designed according to a protocol

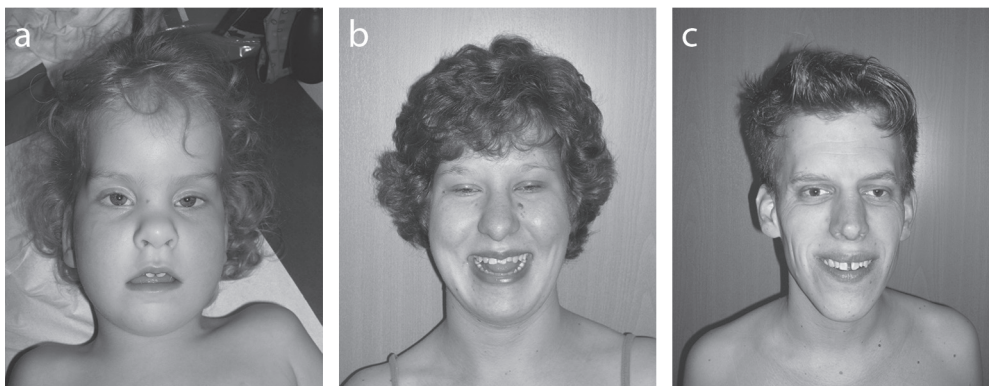

Figure 3.3.1: Clinical characteristics of the 17q21.31 deletion syndrome. (**a**) Patient 1, at 3 years of age. (**b**) patient 2, at 17 years of age, and (**c**) patient 3, at 26 years of age. Note the characteristic facial features, a hypotonic face with ptosis, blepharophimosis, large low set ears, bulbous nasal tip, long collumella with hypoplasic alae nasi, and a broad chin. For color figure see page 194.

provided by MRC-Holland (http://www.mlpa.com/index.htm). The three MLPA probes were combined in one MLPA assay in conjunction with three standard control probes (*VIPR2*, *MRPL41*, and *KIAA0056*). Probe sequences are provided in **Table 3.3.1**. Hybridization, ligation and amplification of the MLPA probes were performed as described before.[4] Amplification products were identified and quantified by capillary electrophoresis on a genetic analyzer (ABI 3730 or 3100), using GeneMapper software (Applied Biosystems).

Data were normalized by dividing each probe's signal strength by the average control probe signal strength of the sample. This normalized peak pattern was divided by the average peak height of all the samples in the same experiment. Copy number change detection was based on thresholds for gains and losses of 1.30 and 0.70 respectively (±3 SD). The MLPA analysis was repeated for all samples in which an aberration was identified. For these confirmation experiments, DNA samples of healthy controls were used for the normalization.

FISH validation studies and *de novo* occurrence.

The 17q21.31 deletions identified by array CGH and MLPA were validated and tested for *de novo* occurrence by fluorescence *in situ* hybridization (FISH) analysis on fixed metaphase spreads from the respective patients and their parents as described before.[2] BAC clones in the aberrant 17q21.31 region were selected and used as probes in the FISH validation assay. In addition, parental DNA samples were tested using the same synthetic MLPA probe set described above.

Genotyping for H1 and H2, and parent-of-origin analysis.

The presence of an intronic 238-bp deletion in intron 9 of the *MAPT* gene, characteristic for the H2 background,[5] was used to genotype individuals with the 17q21.31 deletion and the accompanying parental DNA 1 samples. The 238-bp deletion, if present, was determined by visualizing PCR product on an agarose gel. PCR reactions were performed using primer sequences GGAAGACGTTCTCACTGATCTG (sense) and AGGAGTCTGGCTTCAGTCTCTC (antisense) as described previously[5] in a 25 µl reaction mixture containing 50 ng of template DNA, 10 pmol of each primer, 0.4 mM dNTPs, 1.6 mM $MgCl_2$, and 1.25 U Amplitaq Gold Polymerase (Invitrogen). PCR conditions were as follows: 3 min 94°C followed by 5 cycles 94°C for 30s, 60°C for 30s, 72°C for 45 sec. Subsequently, a 10 cycle touchdown from 60°C to 50°C was performed, followed by 20 cycles 94°C for 30s, 50°C for 30s, 72°C for 45s, with a final extension 72°C for 10 min. The dinucleotide marker, *DG17S142* in intron 9 of the *MAPT* gene, was used for independent validation of the H1/H2 genotyping. Two additional markers, *D17S810* and *D17S920*, were used to study the parental origin of the deletions according to standard procedures

Computational analysis.

The chromosome 17 H1 lineage sequence map, was reconstructed for the region of interest (40.5 Mb – 42.0 Mb) based on the finished human chromosome 17 sequence.[5] Interspersed

Table 3.3.1: MLPA primer sequences

Probe name	5'half-probe sequence	3'half-probe sequence
CRHR1 ex_13	CCAACCCGTGTCAGCTTTCACAGCA	TCAAGCAGTCCACAGCAGTCTGAGC
VIPR2 ex_12*	CGCGCCCAGTCCTTCCTGCAAACGGAG	ACCTCGGTCATCTAGCCCCACCCCTGC
MAPT ex_2	GGACAGGAAAGATCAGGGGGGCTACACCA	TGCACCAAGACCAAGAGGGTGACACGGAC
CRHR1 ex_3	CCCTGCCCTGCCTTTTTCTATGGTGTCCGCTACAATACC	ACAAGTAAGGAAGAAGTGGAGGGTGGACCATCTGCTGGG
MRPL41 ex_1*	GACCCTGACAACCTGGAAAAGTACGGCTTCGAGCC CACACAGGAG	GGAAAGCTCTTCCAGCTCTACCCCAGGAACTTCCTGCGCTAGCTG
KIAA0056 ex_8*	CAGCAATTATGCCAGCCTGACCTACCTTCAGATGGCTTGAAATGGTT	TACTACAGTCTGCATCACTATGTCTGAGACCCTTGTGTTCTCCATCC

* Control probes

repeat sequences within the reconstructed DNA sequence were eliminated by RepeatMasker (http://genome.ucsc.edu) and the repeat masked genomic sequence was analyzed using NCBI BLAST2 for the identification of LCRs (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). All LCRs identified on H1 were in concordance with the previous study of Stefansson *et al*.[6] LCR structures for the H2 lineage were adapted from Stefansson *et al*.[6] The H2 lineage differs from the H1 lineage by a common 900-kb inversion polymorphism, the presence of a 32-kb DNA sequence (LCR17qE) that is present in two copies on the H2 lineage whereas only once on the H1 lineage,[6] and the absence of LCR17qC.

Results and discussion

After obtaining informed consent, we tested 360 mentally retarded individuals within a diagnostic setting for copy number changes using our genome-wide tiling resolution microarray containing 32,477 BAC clones. In one individual, we identified a copy number loss with an approximate size of 600 kb at 17q21.31, encompassing eight BAC clones (**Figure 3.3.2a**). Subsequently, we screened a cohort of 840 mentally retarded individuals for deletions in the 17q21.31 region using multiplex ligation-dependent probe amplification[4] with probes hybridizing to unique sequences in two genes located within this region, *MAPT* and *CRHR1*. By doing so, we identified two additional individuals with microdeletions in 17q21.31 (**Figure 3.3.2b**), with identical genomic sizes and deletion breakpoints at the BAC clone level. One of these breakpoints was identical to that of the index individual, whereas the other breakpoint was located ~100 kb distal to the first (**Figure 3.3.4**). For all individuals, we confirmed the presence of the 17q21.31 deletion by FISH and showed that it arose *de novo* (**Figure 3.3.2c-k**). In addition, the 17q21.31 deletion region has not been reported to show copy number variation in normal individuals (Database of Genomic Variants; http://projects. tcag.ca/variation/).

We identified the deletions at 17q21.31 in a 3-year-old, moderately mentally retarded girl (individual 1), a 17-year-old, moderately mentally retarded woman (individual 2) and a 26-year-old, moderately mentally retarded male (individual 3). In all three individuals, severe hypotonia was present from birth onwards, leading to severely delayed motor development. None of the individuals could stand and/ or walk before the age of three years. Upon physical examination, we noted characteristic facial features. All showed a long hypotonic face with ptosis, blepharophimosis, large, low-set ears, tubular pear-shaped nose with bulbous nasal tip, long columella with hypoplastic alae nasi and a broad chin (**Figure 3.3.1**). In addition, they all had long fingers, nasal speech and displayed an amiable and friendly disposition. The individuals had wide ventricles, as assessed by magnetic resonance imaging before the age of 1 year, whereas individual 1 had additional periventricular white matter abnormalities. Both individual 2 and individual 3 developed a hallux valgus, a pes cavus and, at 13 years of age, a thoracal scoliosis, probably due to marked hypotonia (**Table 3.3.2**).

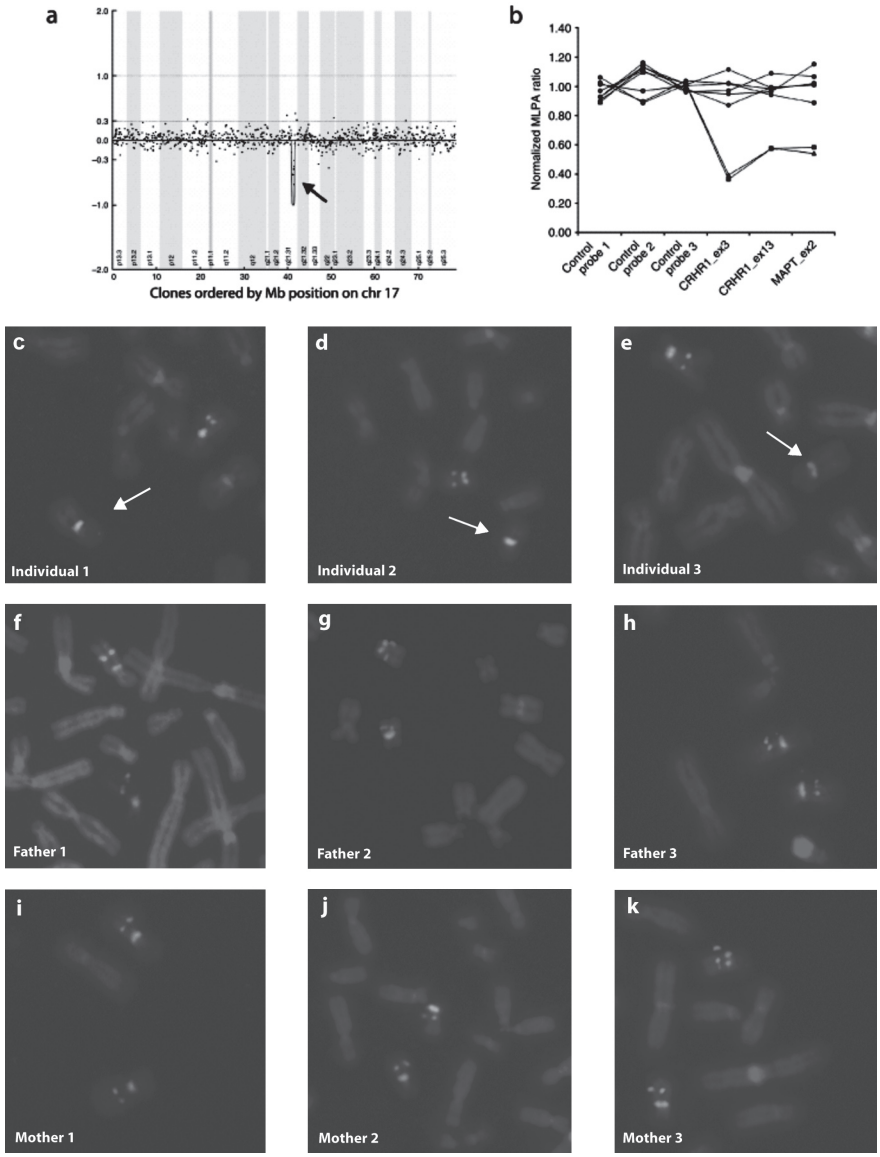

Figure 3.3.2: Analysis of individuals with the 17q21.31 microdeletion syndrome. (**a**) Chromosome 17 array CGH profile of individual 1 with a copy number loss of eight adjacent BAC clones on 17q21.31 (arrow). Clones are ordered on the x axis according to physical mapping positions; log2-transformed test-over-reference (T/R) intensity ratios for each clone are given on the y axis. (**b**) Multiplex ligation-dependent probe amplification (MLPA) analysis, showing a deletion of two probes in CRHR1 and one probe in *MAPT* for both individual 2 (triangles) and individual 3 (squares). The circles represent the MLPA ratios of healthy controls. FISH validation of the 17q21.31 deletion in individuals 1, 2 and 3 (**c-e**) and testing for de novo occurrence in the accompanying parents (**f-k**) using BAC clone RP11-656O14 (red) which is located within the deleted region. The centromere 17 probe was included for reference (green). All individuals show only one signal for RP11-6565O14 (arrow indicating aberrant chromosome 17). The *de novo* occurrence for the deletion was proven in all individuals. For color figure see page 195.

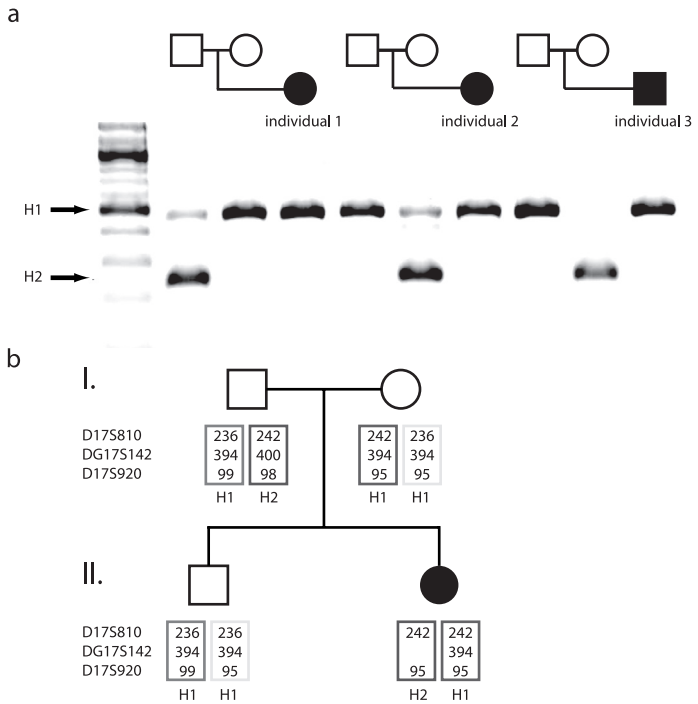

Figure 3.3.3: Genotyping for H1 and H2 lineage and parent-of-origin analysis. (**a**) Genotyping for the H1 (483 bp) and H2 (245 bp) lineages. Far left and far right: 100-bp marker lanes. Individual 3 carries the deletion on the H2 lineage and is of maternal origin. (**b**) Individual 1 and the parents were genotyped using *D17S810*, *DG17S142* and *D17S920* to determine the parental origin of the deletion. The deletion was of paternal origin, and was present on the H2 haplotype. For color figure see page 196.

Notably, the deletions in 17q21.31 are all located within a genomic region that was recently reported to harbor a common 900-kb inversion polymorphism.[6] For this region, two main and highly divergent haplotypes, designated H1 and H2, have been found. The H2 lineage, representing the 900-kb inversion polymorphism, is found at a frequency of 20% in Europeans and can be distinguished from the H1 lineage by genotyping of a dinucleotide marker (*DG17S142*) in intron nine of *MAPT* and by a characteristic 238-bp deletion in the same intron.[6] For all three individuals, one of the parents carried the H2 haplotype: the father of individual 1 and the mother of individual 2 were heterozygous for this haplotype, and the mother of individual 3 was homozygous (**Figure 3.3.3**). Parent-of-origin analysis showed that the deletion occurred on the H2 haplotype in individual 1 and 3, whereas the results for individual 2 were inconclusive (**Figure 3.3.3**). Notably, the H2 haplotype differs from the H1 by a directly oriented low-copy repeat (LCR), LCR17qE (**Figure 3.3.4**) that immediately flanks the breakpoints in all three individuals. This suggests that these deletions have resulted from nonallelic homologous recombination5, mediated by this H2-specific LCR. Consequently, carriers of the H2 lineage are likely to be predisposed to nonallelic homologous recombination, similarly to predisposing

Table 3.3.2: Clinical data from individuals with a 17q21.31 deletion

	Individual 1	Individual 2	Individual 3
Level of MR	Moderate	Moderate	Moderate
Hypotonia	+++	++	+++
Characteristic face			
Long	+	+	+
Blepharophimosis	+	+	+
Ptosis	+	+	+
Tubular pear-shaped nose	+	+	+
Long columella/ hypoplasic alae nasi	+	+	+
Broad nasal tip	+	+	+
Large ears	+	+	+
Broad chin	+	+	+
Friendly/amiable behavior	+	+	+
Brain anomalies	WV, PWM	WV	WV

MR: mental retardation; +: present; -: absent. Hypotonia: +: mild; ++: moderate; +++: severe. Brain anomalies: WV: wide ventricles; PWM: periventricular white matter.

inversion polymorphisms, which have been observed in other microdeletion syndromes such as Williams-Beuren syndrome, Angelman syndrome and Sotos syndrome.[7]

We detected the three individuals with a similar 17q21.31 microdeletion in an unselected European cohort of 1,200 individuals with mental retardation, resulting in an overall detection frequency of 0.3%. As mental retardation occurs in 2–3% of the general population, it can be estimated that the prevalence of this new syndrome is between 1 in 13,000 and 1 in 20,000. This makes it less common than the estimated population prevalence of the 22q11 deletion (DiGeorge-velocardiofacial syndrome) of 1 in 4,000,[8] but similar in frequency to Williams-Beuren syndrome, which has a frequency of 1 in 10,000 to 1 in 20,000.[9] Notably, two other single cases have been reported.[10,11]

The 17q21.31 deletion encompasses two known genes, *CRHR1* (NM_004382) and *MAPT* (NM_005910), and at least two putative genes, *IMP5* (NM_175882) and *STH* (NM_001007532). Loss-of-function mutations have not been reported for any of these genes in humans. However, gain-of-function mutations in *MAPT*, encoding the microtubule-associated protein TAU, cause autosomal dominant forms of frontotemporal dementia and parkinsonism.[12,13] Abnormal filamentous TAU deposits have been reported as a pathological characteristic in several other neurodegenerative diseases.[13] Haploinsufficiency for the microtubule-associated protein TAU may affect axonal elongation and neuronal migration, thereby explaining the major clinical features observed in the 17q21.31 microdeletion-positive individuals (that is, severe hypotonia and moderate mental retardation). In support of this, tau-deficient mice showed muscle weakness and memory disturbance.[14,15]

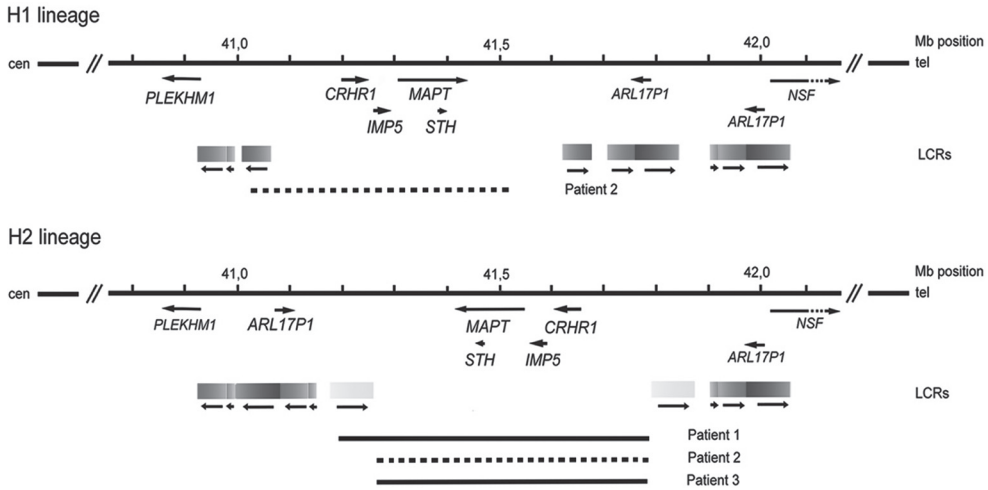

Figure 3.3.4: Transcript map and genomic architecture for the H1 and H2 lineage of the 17q21.31 region. (Dotted) black lines represent the deleted 17q21.31 region identified in the three individuals. Genes located within this region are depicted by black arrows. The H1 and H2 lineage LCR17q structures are depicted as rectangles with colors signifying shared homology and horizontal arrows showing relative orientation (LCR17qA, red; LCR17qB, green; LCR17qC, purple; LCR17qD, blue; LCR17qE, yellow). Note the different genomic orientation of H2, the absence of LCR17qC and presence of LCR17qE. Cen, centromeric; Tel, telomeric. For color figure see page 196.

In conclusion, we report a previously unknown, clinically recognizable syndrome defined by a 17q21.31 microdeletion that includes *MAPT*. The deletion is flanked by LCRs and is associated with a common inversion polymorphism. This observation underscores the relevance of genomic architectural features as the main determinant for the de novo occurrence of recurrent segmental aneuploidies.

Acknowledgements

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Chapter 3.4

Clinical and molecular delineation of the 17q21.31 microdeletion syndrome

Koolen DA[1], Sharp AJ[2,3], Hurst JA[4], Firth HV[5], Knight SJ[6], Goldenberg A[7], Saugier-Veber P[7], Pfundt R[1], Vissers LE[1], Destrée A[8], Grisart B[8], Rooms L[9], Van der Aa N[10], Field M[11], Hackett A[11], Bell K[12], Nowaczyk MJ[13], Mancini GM[14], Poddighe PJ[14], Schwartz CE[15], Rossi E[16], De Gregori M[16], Antonacci-Fulton LL[18], McLellan II MD[18], Garrett JM[18], Wiechert MA[18], Miner TL[18], Crosby S[18], Ciccone R[16], Willatt L[5], Rauch A[19], Zenker M[19], Aradhya S[20], Manning MA[21], Strom TM[22], Wagenstaller J[22], Krepischi-Santos AC[23], Vianna-Morgante AM[23], Rosenberg C[23], Price SM[4], Stewart H[4], Shaw-Smith C[5], Brunner HG[1], Wilkie AO[24], Veltman JA[1], Zuffardi O[16,17], Eichler EE[2,25], de Vries BB[1]

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Abstract

The chromosome 17q21.31 microdeletion syndrome is a novel genomic disorder that has originally been identified using high-resolution genome analyses in patients with unexplained mental retardation. Here we report the molecular and/or clinical characterization of 22 individuals with the 17q21.31 microdeletion syndrome. We estimate the prevalence of the syndrome to be 1 in 16,000 and show that it is highly underdiagnosed. Extensive clinical examination reveals that developmental delay, hypotonia, facial dysmorphisms including a long face, a tubular or pear-shaped nose and a bulbous nasal tip, and a friendly/amiable behaviour are the most characteristic features. Other clinically important features include epilepsy, heart defects (ASD, VSD) and kidney/ urologic anomalies. Using high-resolution oligonucleotide arrays we narrow the 17q21.31 critical region to a 424-kb genomic segment (chr17: 41046729-41470954, hg17) encompassing at least six genes, among which the gene encoding microtubule-associated protein tau (MAPT). Mutation screening of MAPT in 122 individuals with a phenotype suggestive of 17q21.31 deletion carriers, but who do not carry the recurrent deletion, failed to identify any disease-associated variants. In five deletion carriers we identify a <500-bp rearrangement hotspot at the proximal breakpoint contained within an L2 LINE motif and show that in every case examined the parent originating the deletion carries a common 900-kb 17q21.31 inversion polymorphism, indicating that this inversion is a necessary factor for deletion to occur ($p<10^{-5}$). Our data establish the 17q21.31 microdeletion syndrome as a clinically and molecularly well recognizable genomic disorder.

Introduction

Microdeletion syndromes, such as Prader-Willi syndrome, Williams-Beuren syndrome and velocardiofacial syndrome, were initially clinically described before the underlying causative genomic copy number rearrangement was identified. The introduction of microarray-based technology enabled genome profiling in large cohorts of individuals with mental retardation, resulting in the detection of recurrent microdeletions prior to their clinical description. The chromosome 17q21.31 microdeletion syndrome [Mendelian Inheritance in Man (MIM) #610443] is such a new genomic disorder, characterized by a recurrent 500-650 kb deletion involving chromosome 17q21.31.[1-3] The recurrent microdeletion was first identified after screening of large heterogeneous cohorts of individuals with mental retardation using high-resolution microarray screening technologies.[1-3]

So far, fourteen 17q21.31 deletions were reported in the medical literature,[1-8] but for the majority of these cases only limited clinical and molecular data was presented. Further cases and extensive clinical descriptions and molecular studies are needed in order to define the phenotype and genotype of the syndrome.

In all 17q21.31 deletions studied to date the breakpoints map to large clusters of flanking low-copy repeats (LCRs) suggesting that the deletions are stimulated by non-allelic homologous recombination (NAHR),[9] which is further supported by the identification of the reciprocal duplication in a girl with severe psychomotor developmental delay and dysmorphic craniofacial features.[10]

We previously estimated the minimal critical region that is recurrently deleted in individuals with the 17q21.31 microdeletion syndrome to a 478-kb region,[2] encompassing six genes, including the corticotropin-releasing hormone receptor 1 gene (CRHR1) (MIM #122561) and the microtubule-associated protein tau gene (MAPT) (MIM #157140). The deletion interval is also the site of a common ~900 kb inversion polymorphism, associated with two highly divergent haplotypes designated H1 and H2.[11] The H2 lineage, representing the 900 kb inversion polymorphism, is found at a frequency of 20% in the European population.[11] So far, the parents of eight affected individuals have been tested and in all cases at least one of the parents carried the H2 haplotype.[1-3] Therefore, it has been suggested that the offspring of carriers of the H2 lineage are likely to be predisposed to deletion,[12] a phenomenon which has also been described in other microdeletion syndromes, such as Williams-Beuren syndrome, Angelman syndrome, Sotos syndrome,[13] and the recently defined 15q13.3 microdeletion syndrome.[14]

Here we report a collection of 22 individuals with the 17q21.31 microdeletion syndrome. We determine the size of each deletion, allowing detailed delineation of the critical region. We also present the H1/H2 genotypes of the parents, further consolidating the involvement of the H2 haplotype in the deletion. Importantly, based on detailed clinical information of all deletion carriers, the clinical phenotype of the syndrome is characterized and an estimate of the prevalence of the 17q21.31 microdeletion syndrome is obtained.

Materials and methods

Study Subjects

Twenty-two individuals (13 males and 9 females) with a 17q21.31 deletion were included in this study. In three cases the 17q21.31 deletion was suspected, prior to molecular studies, based on the clinical features of the patient. All 19 other cases had been included in broader cohorts of patients with mental retardation screened for genomic copy number changes. The identification of the 17q21.31 deletion in 11 individuals has previously been reported.[1-8] The other cases were identified later using different molecular techniques: multiplex ligation-dependent probe amplification (MLPA),[15,16] 1 Mb resolution BAC array CGH,[6,7] quantitative multiplex PCR of short fluorescent fragments (QMPSF),[17] fluorescence in situ hybridization (FISH), segmental duplication BAC microarray (SD array),[18] chromosomal microarray analysis (CMA) version V6.0 (Baylor),[19] whole-genome oligonucleotide array CGH (Agilent Human Genome CGH Microarray Kit 44A and 244A, Agilent Technologies, Santa Clara, CA),[20] and single nucleotide polymorphism (SNP) micoarray (GeneChip Human Mapping 100K, Array Set, Affymetrix, Inc., Santa Clara, CA.)[21] Clinical information and facial photographs were obtained from the referring clinicians. All legal representatives of the patients gave informed consent for the molecular studies and publication of clinical data.

In total 122 individuals (66 males and 56 females) with learning and/or speech and language delay who had a phenotype suggestive of the 17q21.3 microdeletion syndrome, based on facial characteristics and hypotonia, but who did not carry the recurrent deletion, were included in the study for sequencing of the MAPT gene. Microdeletion of 22q11.2 had been excluded previously in 48 patients.

This study was approved by the Medical Ethics Committee of the Radboud University Nijmegen Medical Center.

Deletion mapping by high-resolution microarray

The 17q21.31 deletions that had not been ascertained by a high-resolution microarray platform and for which genomic DNA was available were further characterized. For this study, 8 cases were tested using a *Nsp1* 250K SNP array, which contained 262,264 25-mer oligonucleotides. All SNP array experiments were performed according to manufacturer's protocols (Affymetrix, Inc., Santa Clara, CA). Copy number estimates were determined using the CNAG software package (v2.0).[22]

In order to fine-map deletion breakpoints we used an ultra-high density custom oligonucleotide array (NimbleGen Systems, Madison, WI). This array consisted of 385,000 isothermal 45-75mer probes covering six genomic regions, including 121,041 probes specifically targeted to two 300-kb intervals (chr17:40800000-41100000 and chr17:41550000-41850000, hg17), corresponding to the putative breakpoints of the 17q21.31 microdeletion (mean density, one probe per 5.2-bp). Hybridizations were performed as described previously.[18,23]

Genotyping for H1 and H2, and parent-of-origin analysis

The presence of a 238-bp deletion in intron 9 of the MAPT gene, characteristic for the H2 background,[11,24,25] and the dinucleotide marker DG17S142 in intron 9 of the MAPT gene[11] were used to genotype individuals with the 17q21.31 deletion and the accompanying parental DNA samples. The 238-bp deletion was determined through visualizing by gel electrophoresis. PCR reactions were performed using primer sequences GGAAGACGTTCTCACTGATCTG (sense) and AGGAGTCTGGCTTCAGTCTCTC (antisense) as described previously.[24] The dinucleotide marker DG17S142, four additional variable number tandem repeats (VNTR) inside the deletion interval, and two flanking short tandem repeats D17S810 and D17S920, were used to study the parental origin of the deletions. All marker analyses were performed according to standard procedures and the size of the peaks were calculated with GeneMapper (v3.7) software (Applied Biosystems).

Mutation screening of *MAPT*

122 patients who presented with features potentially indicative of a deletion of 17q21.31 were identified from clinical records. Each patient was first tested by MLPA (*n=15*) or array CGH using a custom segmental duplication BAC microarray (*n=107*),[2] and found not to carry the recurrent 17q21.31 deletion. A further seven patients with known deletions of 17q21.31[1,2] were also selected. Primers targeting all exons and splice sites of the *MAPT* gene were designed and whole genome amplification of genomic DNA (REPLI-g Kit, Qiagen Inc., Valencia, CA) performed to yield sufficient quantities of DNA for sequencing. High-throughput bidirectional dideoxynucleotide sequencing of PCR-amplified gene products was performed (http://genome.wustl.edu/activity/med_seq/protocols.cgi) at the Genome Sequencing Center (Washington University, St. Louis, USA) and Department of Human Genetics Nijmegen (RUNMC, Nijmegen, the Netherlands) using standard protocols. PolyPhred,[26] and PolyScan[27] software were used to generate an automated report of sequence variations by comparison against reference sequences listed in the NCBI (RefSeq) database. Chromatograms were visually inspected for confirmation of non-synonymous sequence variations.

Results

Identification of novel 17q21.31 deletions

The individuals in whom a 17q21.31 deletion was identified were ordered by age at diagnosis, ranging from 10 months in case 1 to 26 years in case 22 (mean age at diagnosis 9 years). The identification of the 17q21.31 deletion in case 3, case 5-6, 8, 10, 12, 16-17, and case 20-22 had previously been reported.[1-8] In addition, eleven previously unreported 17q21.31 deletions were ascertained: four novel deletions were found using targeted techniques (MLPA, *n=2*; QMPSF, *n=1*; FISH, *n=1*), two deletions by semi-targeted techniques (SD array, *n=1*; CMA V6.0, *n=1*), and five deletions were identified using whole-genome screening technologies (1 Mb resolution BAC array, *n=1*; 100K SNP array, *n=2*; Agilent whole-genome oligonucleotide array, *n=2*). Results of the FISH, MLPA, and QMPSF analyses, and an example of a chromosome 17 profile obtained

using whole-genome microarray technology are shown in **Figure 3.4.1**. Parental samples were available for 21 patients, and showed that in each case the deletion had arisen *de novo*.

Deletion mapping by high-resolution microarray analysis

Refinement of the 17q21.31 deletion intervals was conducted using 250K SNP array on DNA samples from eight patients (case 4, 6, 7, 10, 13-15, 19) and using ultra-high density custom oligonucleotide array targeted to the 17q21.31 region in five cases (cases 5, 8, 17, 21, 22). All cases had an overlapping deletion of the 17q21.31 interval.

The SNP array analyses revealed a 458-kb region (chr17:41012856-41470954, hg17) that is recurrently deleted in the eight affected individuals but for which aneuploidy has not been reported in a control cohort of 240 individuals using similar arrays (unpublished results). The proximal breakpoint resided within a 100 kb region in all cases, whereas the distal breakpoint showed more variation. Note worthily, the distal breakpoint is also a site of frequent copy number variation in controls, most likely confounding the mapping and interpretation of this deletion breakpoint.

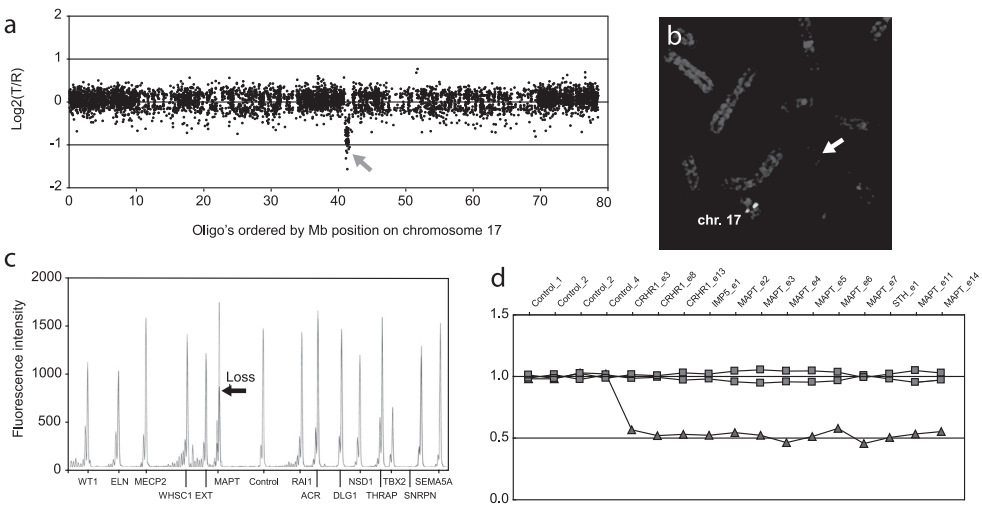

Figure 3.4.1: Identification of the 17q21.31 deletions. (**a**) Chromosome 17 plot of Case 1 obtained by whole-genome oligonucleotide array (Agilent Human Genome CGH Microarray Kit 244A, Agilent Technologies). Chromosome 17 is represented by 5,881 coding and noncoding human sequences (indicated by circles representing the $\log_2$-transformed and normalized test : reference intensity ratios [(Log_2(T/R)], ordered from pter to qter for chromosome 17, in hg17 (NCBI build 35, May 2004). The arrow indicates the presence of a copy number loss at 17q21.31. (**b**) FISH analysis on metaphase spreads of Case 7, using the BAC clones CTD-2324N3 (red) and RP11-413P22 (green) which are both located within the deleted 17q21.31 region. The patient shows only one signal for CTD-2324N3 and RP11-413P22, indicating an aberrant chromosome 17 (arrow). (**c**) Detection of a heterozygous *MAPT* deletion in Case 4 using the microdeletion/ microduplication QMPSF assay. The electropherogram of the patient (in red) was superimposed on that of a normal female control (in blue) by adjusting to the same level the peaks obtained for the control amplicon. The Y-axis displays fluorescence intensity, and the X-axis indicates the genes tested. The heterozygous deletion is detected by a 50% reduction of the *MAPT* peak compared to the normal control (arrow). (**d**) MLPA results for Case 19 (triangles) and two healthy controls (rectangles), showing a deletion of all 13 probes in the *CRHR1, IMP5, MAPT,* and *STH* genes within the 17q21.31 genomic interval. For color figure see page 197.

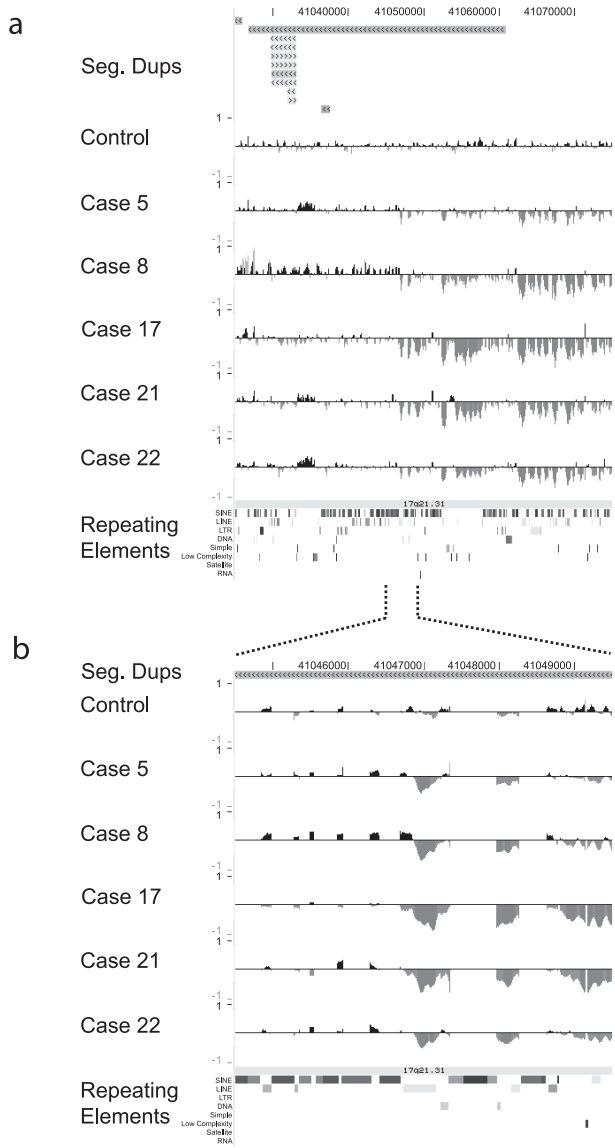

Figure 3.4.2: Ultra-high resolution oligonucleotide array analysis. Results of ultra-high resolution oligonucleotide array analysis of the 17q21.31 microdeletion proximal breakpoint (mean probe density 1 probe/5.2 bp). (**a**) Data from a 50-kb region (chr17:41025000-41075000, hg17) in five unrelated 17q21.31 deletion patients and one control. In each deletion, the proximal breakpoint occured in a segmental duplication of length, 34.2 kb, identity 98.7% (chr17:41026709-41060948). (**b**) Zoomed view showing a 5-kb region (chr17:41044500-41049500). All five patients have breakpoints which are indistinguishable, mapping to within an interval of <500 bp contained within an L2 LINE motif. The highly variable dynamic response of certain probes in this region to report the deletion is likely a result of their different sequence properties.[38] For each individual, deviations of probe $\log_2$-ratios from zero are depicted by gray/black bars, with those exceeding a threshold of 1.5 standard deviations from the mean probe ratio colored green and red to represent relative gains and losses, respectively. Tracks above each plot indicate segmental duplications (gray/yellow bars represent duplicons with 90–98%/98–99% sequence identity, respectively). For color figure see page 198.

To further define the breakpoints we characterized the deletion in five different patients using an ultra-high density custom oligonucleotide array targeted to the 17q21.31 region, with a mean density of one probe per 5.2 bp. These data show that all five patients have proximal deletion breakpoints that map within an interval of <500-bp (chr17:41046729-41047168, hg17), contained within a single L2 LINE motif (Figure 3.4.2). Testing of nine control subjects (including carriers of both the H1 and H2 haplotypes) showed that this hybridization signature was specific to deletion patients and does not represent a copy number variation. In contrast, we were unable to identify a deletion signature at the distal breakpoint which was specific to deletion carriers. On the basis of the location of the L2 LINE element at the proximal side and the copy number variation identified in 240 normal controls at the distal side we refined the critical region to an 424-kb genomic interval (chr17:41046729-41470954, hg17), relative to the H1 lineage.

Genotyping

We performed genotyping of the H1 and H2 haplotypes using a dinucleotide marker (DG17S142) in intron nine of MAPT and a characteristic 238-bp deletion in the same intron.[11,24,25] In all parents tested (n= 42) at least one of the parents carried the H2 inversion and seven parents were homozygous for the H2 inversion. Genotyping of D17S810, DG17S142 and D17S920 and four polymorphic loci, designed in the deleted region, showed that the parent-of-origin carried the H2 inversion polymorphism (**Table 3.4.1** and **Figure 3.4.3**). In total, eight deletions were of maternal origin and 12 were of paternal origin, whereas in two cases marker analysis could not distinguish between the parental chromosomes.

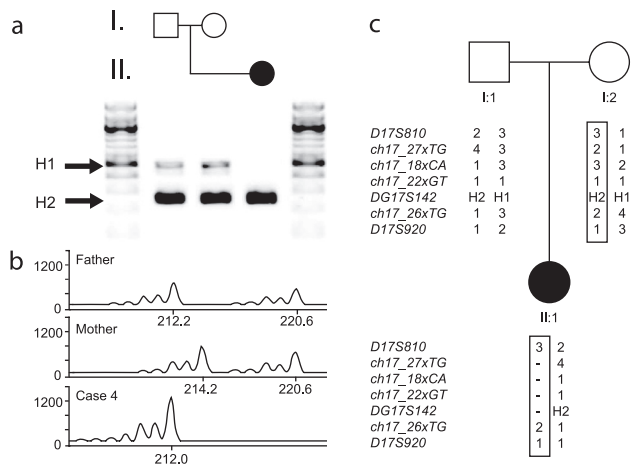

Figure 3.4.3: Genotyping H1 and H2 on 17q21.31 and parent-of-origin analysis. Genotyping results in the family of Case 4. (**a**) PCR results of a 238-bp deletion in intron 9 of *MAPT* indicative for the H2 lineage. Both parents are H1/H2 heterozygotes. Far left and far right: 100-bp marker lanes. (**b**) PCR results of a VNTR (chr17: 41,224,986-41,225,022) within the deleted 17q21.31 region excluding a paternal origin. (**c**) Reconstruction of genomic markers located within and outside the deleted 17q21.31 segment shows the maternal origin of the deletion. Dinucleotide marker *DG17S142* is indicative for the H2 lineage.[11]

Table 3.4.1: Genotyping results

Case	Father	Mother	Parent-of-origin
1	H1/H2	H1/H2	maternal
2	H1/H1	H1/H2	maternal
3	H1/H2	H1/H1	paternal
4	H1/H2	H1/H2	maternal
5	H1/H2	H1/H1	paternal
6	H2/H2	H1/H1	paternal
7	H1/H2	H1/H2	paternal
8	H2/H2	H1/H1	paternal
9	H1/H2	H1/H2	paternal
10	H1/H2	H1/H2	maternal
11	H2/H2	H1/H1	paternal
12	H2/H2	H1/H2	–
13	H1/H1	H1/H2	maternal
14	H1/H2	H1/H1	paternal
15	H1/H2	NA	paternal
16	H1/H2	H1/H1	paternal
17	H1/H2	H1/H1	paternal
18	NA	H1/H2	–
19	H1/H1	H2/H2	maternal
20	H1/H2	H2/H2	paternal
21	H1/H1	H1/H2	maternal
22	H1/H1	H2/H2	maternal

Note.—The parent-of-origin was determined by genotyping for H1/H2 status as well as by other polymorphic markers in the region. For Cases 6, 8, 11, and 16 the parent-of-origin was inferred by SNP array data and extrapolation from child and parental alleles. NA: DNA sample not available. -: marker analyses could not distinguish between the paternal or maternal origin of the deletion.

Mutation screening of *MAPT*

Two novel non-synonymous mutations in *MAPT* were identified among the 122 non-deletion patients tested: (i) an identical heterozygous G>A transition at hg17 position chr17:41443588, resulting in the replacement of valine with isoleucine in an alternatively-spliced exon was detected in two unrelated patients; (ii) a heterozygous G>A transition at hg17 position chr17:41457221, resulting in the replacement of glycine with arginine. Screening of seven 17q21.31 deletion patients did not identify any novel variants.

Clinical Details of the Study Subjects

The clinical features of the patients with a 17q21.31 deletion are listed in **Table 3.4.2**. A summary of the 22 patients is given below.

In the majority of cases (82%) the pregnancy was uneventful. In Case 2 however, the pregnancy was characterized by a placental abruption at seven weeks of gestation, and in Case 9 and 21, the pregnancy was complicated by intrauterine growth retardation. Low birth weight (<3rd centile) was noted in six (27%) individuals. Other birth measurements were within the normal range, although in Case 9 microcephaly was noted, and Case 3 and 14 were small for gestational age. Short stature (<3rd centile) was present in four patients (18%). Case 19 was investigated

for short stature at 11 years of age, with a borderline low growth hormone measurement on provocation testing.
In all patients global psychomotor developmental delay was noted from an early age. The level of developmental delay varied significantly and was estimated from the mild to severe range. For example, in Case 17 the early motor milestones were delayed, but she started to walk at 19 months, whereas Case 10 did not start walking before the age of four years. Eleven patients did not have any words before three years of age. The speech and language development of Case 17 were particularly affected. She did not achieve two-three word sentences until age six. Case 16 communicated primarily with gestures at six years of age, while Case 10 showed no understandable speech during the investigation at the age of six years and two months.
Hypotonia with poor sucking and slow feeding was evident in the neonatal period and during childhood in all but one. In at least six patients feeding difficulties required hospitalization and/or nasogastric tube feeding in the neonatal period.

A history of epilepsy was noted in 50% of the cases. Generalized seizures were present in eight cases (36%). In Case 4 unilateral clonic seizures and hypotonia were observed 48 hours after birth. A parieto-occipital haemorrhagic infarction on the left side with bilateral ventricular haemorrhage was diagnosed and a large thrombus was identified in the terminal portion of the aorta.
Dysmorphic craniofacial features present in more than half of the individuals included a high/broad forehead, long face, upward slanting palpebral fissures, epicanthic folds, an abnormally formed nose (either "tubular" or "pear"-shaped), bulbous nasal tip, large prominent ears, everted lower lip and in addition, abnormal hair pigmentation and texture was observed in 12 individuals (55%). The nose can have a high nasal bridge, a broad nasal root, long columella, hypoplastic and/or thick alae nasi. Ophthalmological evaluation showed strabismus in 45% of patients and hypermetropia was present in 8 cases (36%). Facial photographs of 20 individuals are provided in figure 4. Facial photographs at different ages, spanning a significant period of time, could be obtained for five individuals (**Figure 3.4.5**). The facial characteristics of the patients change with age. In infancy the facial gestalt is mostly characterized by the hypotonia of the face with an open mouth appearance. However, with increasing age there is elongation of the face and broadening of the chin and also the "tubular" or "pear"-shape form of the nose becomes more pronounced.
Slender long fingers were reported in 61% of the patients. In addition, hypoplasia of the hand muscles was described in multiple patients (29%) as well as slender lower limbs (41%), dislocation of the hip(s) (27%), and positional deformities of the feet (27%). Case 13 had in-turned feet with significant pronation and had been fitted with an ankle-foot orthosis. He had progressive contractures in his wrists and ankles and bilateral ulnar deviation of his wrists and deviation of his toes.
Septal heart defects, both atrial septal defects (ASD) and ventricular septal defects (VSD), were found in 6 cases (27%). Kidney and urologic anomalies were found in 7 cases (32%). The latter included vesicoureteric reflux, hydronephrosis, right sided pyelectasis, and a duplex renal system. In Case 6 pyelonephritis and bilateral vesicoureteric reflux grade II was detected. In Case 14 a renal ultrasound at the age of five months showed left hydronephrosis due to a primary pelvi-

Figure 3.4.4: Facial photographs of individuals with a 17q21.31 deletion. Facial photographs of Case 1, at age 10 months; Case 2, at age 1 year; Case 3, at age 2 years; Case 4, at age 3 years; Case 5, at age 3 years; Case 6, at age 3 years 5 months; Case 7, at age 3 years 8 months; Case 8, at age 3 years; Case 9, at age 5 years 8 months; Case 10 at age 5 years; Case 11, at age 3 years 9 months; Case 12, at age 8 years 6 months; Case 14, at age 13 years; Case 15, at age 14 years; Case 16, at 14 years; Case 18, at 16 years; Case 19, at 13 years; Case 20, at age 18 years; Case 21, at 17 years and Case 22, at age 26 years. Informed consent was obtained for publication of photographs. For color figure see page 199.

Figure 3.4.5: Facial photographs at different ages. Facial photographs at different ages (<1, 1-4, 5-6, 7-12, and >12 years of age). Note that the facial characteristics of the patients change with age. With increasing age there is elongation of the face and broadening of the chin and also the "tubular" or "pear"-shape form of the nose becomes more pronounced. For color figure see page 200.

ureteric junction stenosis. Cryptorchidism was reported in seven out of nine male patients (78%) and deformity of the spine requiring treatment was present in eight patients (36%). The spine anomalies mostly included scoliosis, but lordosis and kyphosis were also reported. In Case 13 the scoliosis was progressing rapidly. A brace was required and an X-ray suggested that he might be suffering from a mild degree of restrictive lung disease. Magnetic resonance imaging of the spine showed a compression of the upper cervical spinal cord, due to developmental anomaly in the craniocervical junction and upper cervical canal. Imaging of the spine in Case 19 showed fusion of the C4/5 vertebrae and two small syringes in the thoracic cord.

Table 3.4.2: Clinical features of the 17q21.31 microdeletion syndrome

Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	T(%)
Current age (years)	10/12	1	3	3	3	3^{+5}	3^{+6}	4	5^{+8}	6	6	13	13	13^{+9}	14	14	15	17	18	20	17	26	
Gender	M	M	F	F	F	F	M	F	F	F	F	F	M	M	M	F	F	F	M	M	F	M	
Growth parameters																							
Low birth weight	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	+	+	–	+	+	–	27
Microcephaly	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5
Short stature	–	–	–	–	–	–	–	–	–	+	–	–	–	–	+	+	–	–	–	+	–	–	18
Developmental delay	+	+	+	+	+	+	++	+	++	+	+	+	++	+	+	++	+	+	+	++	+	+	100
Hypotonia (childhood)	+	++	+	++	++	+	+	–	+	++	+	+	+	++	++	+	+	+	+	+	+	++	96
Epilepsy	–	–	+	–	–	+	–	+	–	+	–	+	–	–	–	+	+	+	+	+	+	–	50
Facial features																							
Abnormal hair colour/texture	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	–	–	–	–	55
High/broad forehead	+	–	+	+	+	+	+	–	+	+	–	+	+	+	–	+	–	–	+	+	–	+	68
Long face	–	+		–	+		+		+	+	–	+	+	+	+	+	–	+	–	+	+	+	74
Hypermetropia	–	–	+	–	–	+	–	+	–	–	–	–	–	+	–	+	+	–	–	+	+	–	36
Nasal speech	na	na	na	na	na	na	na		+	na		+	+	+	–	–	–	–	–	–	+	+	50
Pale irides	–	–	–	–	+	–	+	+	–	+	–	+	–	+	–	+	+	+	–	–	+	–	45
Strabismus	+	–	+	–	+	+	+	+	+	–	–	–	–	–	–	+	+	–	–	–	–	+	45
Upward slant	+	+	+	–	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	+	–	68
Blepharophimosis	–	–	+	–	+	–	–	+	–	–	–	–	–	+	–	–	+	+	–	–	+	+	36
Ptosis	–	–	+	–	+	+	+	+	+	+	–	–	–	+	–	+	–	–	–	–	+	+	50
Epicanthal folds	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	68
Tubular or pear–shaped nose	+	+	–	+	+	–	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	82
Bulbous nasal tip	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	95
Large/prominent ears	+	–	–	+	+	+	–	+	+	+	–	–	+	+	+	–	+	–	–	–	+	+	59
Narrow/high palate	+	+	–	–	+	–	–	–	+	–	–	–	+	+	+	–	–	+	+	+	–	+	50
Cleft palate	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	9
Broad chin	–	–	–	–	+	–	+	–	–	+	+	–	+	–	–	+		+	–	–	+	+	42
Extremities																							
Narrow hands	+	+	–	–	+		–		+	–		–	–		–	–	–	–	–	–	+	–	28
Slender/long fingers	+	+	–	–	+		–		+	+		+	–		–	–	–	+	+	+	+	+	61
Hypoplasia hand muscles	–	+	–	–	–		–		+	–		–	+	–	+			–	–	–	+	–	29
Dislocation of the hip(s)	–	–	+	–	+	–	–	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	27
Slender lower limbs	+	–	–	–	+		–		+	+		–	+		–		–	–	+	–	+	–	41

Table 3.4.2: Continued

Positional deformity feet	–	–	–	–	–	–	–	–	+	–	+	–	+	+	–	–	–	–	–	–	+	+	27
Other congenital malfomations																							
Heart defects (VSD/ASD)	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	27
Kidney & urologic anomalies	–	–	–	+	–	+	–	–	–	+	–	+	+	+	–	–	–	+	–	–	–	–	32
Cryptorchidism	+	+					+					–	+	+	–					+		+	78
Scoliosis/ kyphosis	–	–	+	–	–	–	–	–	–	–	–	–	+	+	+	–	–	+	–	+	+	+	36
Pectus excavatum	+	+	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–	23
Ventriculomegaly	+		–	–	+		–		–	–	–		+		–		–	–	+	–	+	+	38
Friendly/amiable behavior	+		+	+	+		–		+	+	+	+	+	+	–		+	+	+	+	+	+	89
Other clinical features			a	b	c		d	e	f		g	h	i;j;k	l	d;m			n;o	m;p	j		q	

+, feature present; ++, severe; –, feature absent; na, not assessable; [a] persistent ductus arteriosus; [b] neonatal haemorrhagic parenchymal infarction, aortic thrombus; [c], periventricular white matter lesions; [d] behavioral problems; [e] metopic ridge; [f] hypothyroidism; [g] sagittal synostosis; [h], bilateral cataract; [i] ulnar deviation of wrists; [j] contractures; [k] compression of the upper cervical spinal cord; [l] severe ichthyosis; [m] agenesis of corpus; callosum; [n] pyloric stenosis; [o] tracheomalacia; [p] c4/5 fused vertebrae; [q] communicating hydrocephalus

In the vast majority of the patients (89%) the behavior was described as friendly, amiable and co-operative with or without frequent laughing. In contrast to this general pattern, the attention span of Case 7 was very short, his interaction with people limited, and he had temper tantrums. Additionally, Case 15 showed behavior problems including hyperactivity alternating with introspection, bad humor, and difficult interaction with other children.

Discussion

The 17q21.31 microdeletion syndrome is a new mental retardation syndrome.[1-3] We performed an extensive clinical and molecular characterization of 22 patients. In the majority of cases the microdeletion was identified after microarray-based genomic copy number profiling of large heterogeneous cohorts of individuals with mental retardation. In only three cases the 17q21.31 deletion was suspected based on the clinical features of the patient (cases 7, 8, 18). The remaining 19 cases had been included in broader cohorts including a total of 2,978 patients with mental retardation. Therefore, the prevalence can be estimated to be in the order of 0.64% (95% CI, 0.35-0.93%) of the individuals with unexplained mental retardation. Mental retardation occurs in approximately 2-3% of the general population,[28,29] of which in approximately 50% a diagnosis cannot be made.[30] Therefore, we estimate the prevalence of the 17q21.31 deletion syndrome as approximately 1 in 16,000 individuals. For example, for the Netherlands, this implies that ~12 affected individuals are born each year. So far, only a total of four cases from the Netherlands have been reported, indicating that the 17q21.31 deletion syndrome in individuals with mental retardation is currently highly underdiagnosed.

The 17q21.31 region contains multiple CNVs that are also found in the general population (http://projects.tcag.ca/variation/), complicating the definition of the precise deletion breakpoints. Based on 250K SNP array analyses we could delineate a minimal 424-kb critical region (41046729-41470954 Mb, hg17) that is recurrently deleted in patients, but not in controls. Further refinement of five deletions using an ultra-high density oligonucleotide arrays (mean density, one probe per 5.2 bp) revealed that the proximal breakpoint in all five tested patients is contained within an interval of <500-bp within an L2 LINE motif, representing a possible hotspot for NAHR. Even with the ultra-high density oligonucleotide arrays it is not possible to ascertain the distal breakpoint accurately. The deletion seems to extend more distally in some patients tested, although this might reflect the extreme copy number polymorphism at the distal side, rather than variation in the breakpoints. Future deep sequencing unraveling the H2-sequence and a putative common distal breakpoint, which is currently unknown, will be of major importance to prove NAHR as the underlying mechanism.

The 424 kb critical region encompasses at least six genes, *C17orf69*, *CRHR1* (MIM #122561), *IMP5* (MIM #608284), *MAPT* (MIM #157140), *STH* (MIM #607067), and *KIAA1267*. Haploinsufficiency

of one or more of these genes might underlie the phenotype seen in the 17q21.31 deletion syndrome. The *MAPT* is of particular interest as the gene is highly expressed in brain and is involved in several neurodegenerative diseases, such as frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17),[31,32] progressive supranuclear palsy,[33] and corticobasal degeneration and Alzheimer's disease.[25] Therefore we performed mutation analysis of the entire coding sequence of the *MAPT* (MIM #157140) gene in 122 patients targeted for testing on the basis of clinical features but lacking the characteristic deletion.
Two non-synonymous mutations of uncertain significance were found in this cohort. The first of these, resulting in the replacement of valine with isoleucine, was identified in two unrelated patients. Although not previously reported, the presence of this same variant in two unrelated individuals suggests that this it likely represents a rare polymorphism. Furthermore, the amino acids valine and isoleucine are structurally and biochemically quite similar, suggesting that this substitution is unlikely to significantly affect protein structure. The second variant identified results in the replacement of glycine with arginine at amino acid 389. This same amino acid variant has been previously reported in two families with Pick's disease (frontotemporal dementia, MIM #172700).[34,35] However, this variant only has mild effects on *MAPT* function and in neither of these reports did the mutation segregate with the neurological phenotype, suggesting that this may also represent a rare polymorphism. Although our extensive efforts to identify pathogenic mutations in *MAPT* did not uncover any variants that seem likely to be responsible for the phenotype, no firm conclusions can be made, because parental DNA samples were unavailable for further testing. These data suggest that the 17q21.31 microdeletion syndrome is not caused solely by haploinsufficiency of *MAPT*, although single exon deletions could have been missed in our analyses. We speculate that the phenotype of the 17q21.31 deletion syndrome instead results from haploinsufficiency for one or more other elements within the critical region, perhaps representing a contiguous gene syndrome. This is in contrast to some other genomic disorders in which the associated phenotypes largely or completely result from haploinsufficiency for single genes, e.g. the *RAI1* gene in Smith–Magenis syndrome,[36] and the *UBE3A* gene in Angelman syndrome.[37]

The 17q21.31 genomic interval contains a common 900 kb inversion polymorphism, resulting in a haplotype block with two highly divergent haplotypes designated H1 and H2.11 Genotyping the parents with respect to the H1/H2 lineage showed that in each trio tested the parent originating the deleted chromosome 17 carries at least one H2 chromosome, which is significantly different from the ~20% frequency of the inversion in the European population reported by Stefansson *et al.*[11] ($p<10^{-5}$, Pearson's Chi square test). In total, eight deletions were of maternal origin and 12 were of paternal origin, indicating that there is no significant bias for parental origin of the deletion. The H2 haplotype results in a genomic structure with directly oriented LCR subunits that can undergo a deletion rearrangement via NAHR,38 which suggests that the inversion found in all parents of origin may be a necessary factor for the deletion to occur. Although the H2 allele is a risk factor, the frequency of de novo 17q21.31

microdeletions in carriers of the H2 inversion is low, therefore other as yet poorly understood factors are likely to be important in the generation of the deletion. Moreover, all deletions detected to date are sporadic. Therefore, assuming the H2 allele is requisite for the deletion, based on current knowledge, the occurrence risk for the 17q21.31 deletion in a carrier of the H2 inversion polymorphism might be considered to be in the order of 1/3,200 in European populations. However, from a genetic counseling perspective, the recurrence risk in a family of an affected individual could be higher, because of other factors such as germline mosaicism.

Our analysis of 22 patients with the 17q21.31 deletion syndrome shows a clinically recognizable phenotype. Common features present in more than 50% of the patients that should prompt consideration of this diagnosis include developmental delay, childhood hypotonia, abnormal hair color/texture, high/broad forehead, long face, upward slanting palpebral fissures, epicanthal folds, tubular or pear-shaped nose, bulbous nasal tip, large/prominent ears, slender/long fingers, cryptorchidism, and a friendly/amiable behavior. Other common features that need special medical attention are epilepsy, hypermetropia, pectus excavatum, congenital heart defects (VSD/ASD), kidney and urologic anomalies, dislocation of the hip(s), positional deformities of the feet, and spinal deformities. Hypotonia of the face is most obvious in infancy with an open-mouth appearance, everted lower lip, and a protruding tongue. The typical nose and other facial characteristics can be observed from birth. However, with increasing age, there is a change in phenotype, which is also seen in other mental retardation syndromes, such as Mowat-Wilson syndrome,[39] Noonan syndrome,[40] and Williams-Beuren syndrome.[41]
In conclusion, the molecular and/or clinical characterization of 22 individuals with the 17q21.31 microdeletion syndrome defines the phenotypic features associated with this novel syndrome and provides further insight into the critical region and rearrangement hotspot. Our data further support the hypothesis that the common 17q21.31 inversion polymorphism in the parent-of-origin is a necessary factor for the deletion to occur.

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Chapter 4

General discussion and future prospects

4.1 Frequency of copy number variation in mental retardation

The identification of submicroscopic structural variation in the human genome has revolutionized the diagnostic process of patients with mental retardation. Through the development and implementation of novel methodologies the technical divide between molecular genetic and cytogenetic testing has been bridged and, by doing so, it has been emphasized that submicroscopic structural variants, notably copy number variations (CNVs), are an important cause of mental retardation. At the onset of the work described in this thesis the investigation of subtelomeric regions, mainly by FISH, had already revealed that cryptic subtelomeric CNVs are an important cause of otherwise unexplained cases of mental retardation.[1,2] Subsequent high-throughput FISH-based methods enabled the detection of subtelomeric aberrations in a routine fashion,[3,4] but these methods remained relatively labor intensive and expensive. Therefore, quantitative PCR-based technologies such as MAPH,[5] Q-PCR[6] and MLPA[7] were developed for a more rapid and comprehensive identification of these CNVs. We performed subtelomeric DNA copy number analyses using MLPA in individuals with unexplained mental retardation and identified clinically relevant CNVs in 4.3% of them, demonstrating that MLPA serves as a robust high-throughput method for the detection of subtelomeric rearrangements (**Chapter 2.1**). Moreover, clinical scoring[8] of the patients revealed that subtelomeric screening may be offered to all mentally retarded patients, irrespective of the level of mental retardation. In a total of 33 different studies, encompassing ~3,800 subtelomeric copy number analyses, rearrangements were identified in 5-6% of cases with unexplained mental retardation.[9] However, it should be noted that most of these reports represent proof-of-concept studies employing pre-selected patient cohorts. This notion is underlined by the lower diagnostic yield of 2.5% obtained in two subtelomere studies of almost 19,000 unselected individuals with mental retardation.[10,11] The majority of these patients (~12,000) were tested by FISH, implicating that in tandem microduplications could have been missed. Subtelomeric microduplications, however, appear to be a rare cause of mental retardation. Ballif *et al*. failed to encounter pathogenic microduplications in almost 7,000 patients analyzed by array CGH,[10] and we found pathogenic in tandem microduplications at the subtelomeres in only 0.5% of patients with mental retardation.[12]

In order to establish the frequency of interstitial CNVs in mental retardation, we studied 386 patients using array CGH in conjunction with whole-genome tiling resolution BAC arrays (**Chapter 3.1**). The overall diagnostic yield of clinically significant CNVs in this patient cohort was 9.1%, thus emphasizing the diagnostic importance of genome-wide copy number profiling in patients with unexplained mental retardation. The overall yield of pathogenic CNVs in 1,364 patients reported in the literature was 11.2%. It should be taken into account, however, that similar to the initial subtelomeric studies, these studies are not representative for all patients with unexplained mental retardation. Clinical pre-selection for high resolution screening will become less rigorous in the near future as microarrays are increasingly being

implemented in routine clinical diagnostic settings. Using a clinical scoring system, we showed that the detection rate of 9.1% in our sample set partly reflected clinical pre-selection bias based on phenotypes with additional dysmorphic features and/or growth abnormalities. Clinical characterization of unselected patients with mental retardation using the same clinical scoring system allowed us to extrapolate the diagnostic yield, resulting in an expected yield of interstitial submicroscopic alterations over the genome within an unselected population of mentally retarded patients of ~8%.

Taken together, it has amply been shown now, by us and others, that submicroscopic copy number variation is an important cause of mental retardation. Cryptic subtelomeric and interstitial CNVs larger than 100-300 kb can be detected in ~3.0% and ~8.0% of the patients with unexplained mental retardation, respectively. Accordingly, submicroscopic CNVs account for ~11% of previously unexplained cases of mental retardation, i.e., ~5% of the overall population of patients with mental retardation. This current frequency is likely to be an underestimate given the fact that most of the microarrays used only detect genomic rearrangements larger then 100-300 kb in size. The development of novel SNP-based microarrays (see below) allows high-resolution detection of even intra-genic (exon) deletions and duplications and, thus, will further increase the frequency of CNV detection in patients with mental retardation. Modeling of structural variants, including CNVs, strongly suggested that smaller structural variants are much more common in the genome than larger structural variants.[13] Moreover, validation and refinement of the location of 1,695 structural variants across nine diploid human genomes showed that indeed the majority of the CNVs is 5-10 kb in size or less, and that for several sites (15%) the current reference human genome sequence represents the minor allele.[14] The clinical significance of these small CNVs has still to be elucidated, but it is expected that only a minority will have a direct role in the etiology of mental retardation. However, since they far outnumber CNVs larger than 100-300 kb in size, the absolute contribution of these variants may still be significant (**Figure 4.1**). Consequently, we speculate that CNVs of 1 to 100 kb in size may account for at least another 5% of the causes of mental retardation. Obviously, further testing using high-resolution microarray and/or next-generation sequencing (see below) platforms is required to firmly establish the true frequency of this category of structural variants.

4.2 Clinical interpretation of copy number variation in mental retardation

The major challenge in studying CNVs and other forms of structural genomic variation is to identify the variants that have clinical consequences. Differentiation of CNVs identified by microarray platforms with a dosage-dependent phenotypic effect from those without such an effect is still complicated, mainly since the genomes of normal control individuals also show large-scale copy number variation.[15,22,23] Redon *et al*. identified a total of 1,447 copy number variable regions covering ~12% of the human genome.[15] Given the limited set of reference

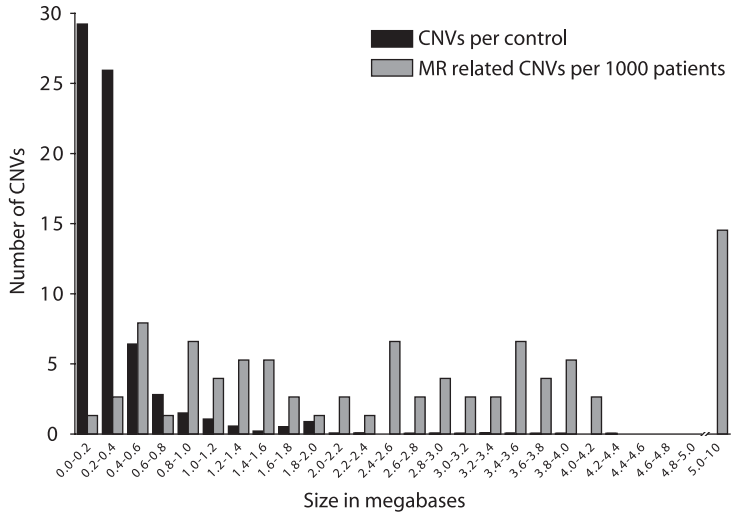

Figure 4.1: Frequency plot of CNV sizes in patient and control cohorts. Black bars, CNVs obtained through a study of 270 individuals from four populations with ancestry in Europe, Africa or Asia (the HapMap collection) using clone-based array CGH.[15] Grey bars, CNVs causally related to mental retardation identified using microarray platforms with similar resolution (*n= 757*).[16-21]

samples assayed, however, the CNVs reported by Redon *et al*. are probably just a tip of the iceberg. New tiling oligonucleotide-based microarrays are expected to reveal ~20 times more CNVs, i.e., 825-1500 CNVs per individual (Matt Hurles, the Wellcome Trust Sanger Institute, personal communication). The Database of Genomic Variants (hg17v2)[22] suggests copy-number variation in ~ 25% of the human genome. As yet, the clinical implications of these CNVs are far from understood. Moreover, many CNVs are population-specific and control CNV data are not available yet for all ethnic populations.[15,24,25]

In **Chapter 3.1**, we propose a practical workflow for the diagnostic interpretation of CNVs. In order to understand the variations discovered by high-resolution microarray and/or next-generation sequencing platforms (see below), thorough clinical characterization of patients and controls is a prerequisite. Currently, publicly available databases documenting CNVs identified in controls are used to discern common CNVs from rare CNVs. These databases, however, have mainly been created for research purposes and only a minority of the reported CNVs has actually been validated. Moreover, it has been shown that the CNVs listed in these databases are often smaller than previously thought, which may partly be attributed to the fact that the data were obtained using relatively low-resolution microarray platforms.[14,26,27] In order to determine whether a CNV identified in a patient is a common variant, we used both publicly available and internal control datasets using the same microarray platform for analyzing both patients and controls (**Chapter 3.1**). It is anticipated that in the end every megabase of the human genome will show CNV in normal controls and, therefore, not only the presence or

absence, but also the amount of overlap with CNVs identified in control cohorts should be considered. By using high quality control data sets, the number of patients for which validation is required may be reduced significantly. From a genetic counseling perspective, however, follow-up studies of patients and parents remain important as they also allow the detection of balanced rearrangements in the parents, which is crucial for estimation of the recurrence risk.

Parental testing is also imperative when the genomic region involved has not yet been reported before as being associated with mental retardation. The clinical significance of *de novo* events is stressed by the low estimated *de novo* locus-specific mutation rates in normal controls (~1.7 x 10^{-6}).[28] Parental samples are, however, not always required to establish the clinical significance of a CNV. For example, CNVs that overlap with genomic regions known to cause mental retardation syndromes, as e.g. defined by the database of Online Mendelian Inheritance in Man (OMIM), can be considered pathogenic. Again, similar to the overlap of CNVs identified in controls, it is important to quantify and qualify the extent of overlap. The CNVs should at least include the critical region of the syndrome and, if known, the causative gene(s). Moreover, the clinical features of the patient have to be consistent with the phenotypic characteristics of the syndrome involved.

In reverse, the identification of a rare CNV in a patient and one of his/her parents does not automatically rule out its pathogenicity. The latter is illustrated by the recurrent microdeletions of 1q21.1 that are associated with a variable spectrum of phenotypic abnormalities and are also identified in apparently unaffected parents.[29] This phenotypic diversity and incomplete penetrance may be explained by multiple CNVs that act in concert or by mutant alleles that exert their effects only within specific genetic backgrounds and/or under specific environmental conditions. Moreover, (i) the parents can be mosaic for the CNV, (ii) an inherited deletion may uncover a recessive mutation in the other allele, (iii) the effect of a CNV may be allele-specific, and (iv) the phenotypic effect of a given CNV may vary depending on the exact copy number, which is difficult to establish accurately with currently available methods.

The current clinical interpretation of CNVs is still limited and for some rare CNVs the clinical significance will remain unclear due to the fact that (i) one or both parents may not be available for further testing and (ii) the CNV has not been associated with a known clinical syndrome. The size of the genomic region involved and its gene content, however, may provide clues for its clinical significance, but obviously more knowledge about the biological pathways underlying mental retardation has to be gained before a straightforward clinical diagnosis can be reached by this approach alone. Therefore, alternative strategies need to be developed to predict the phenotypic consequences of CNVs and to identify putative dosage sensitive genes within CNV regions causing mental retardation and/or additional clinical features. In order to obtain a probabilistic model that predicts the phenotypic effects of CNVs, in-depth bioinformatic analyses are required (see below), which may result in a 'CNV classifier' that can be used for a

clinical interpretation in routine diagnostics and for the identification novel dosage sensitive genes as candidates for mental retardation.

4.3 Identification of syndromes: the 17q21.31 microdeletion syndrome

The application of genomic microarrays has resulted in the identification of several recurrent disease causing CNVs. The number of overlapping CNVs, however, is still limited and in many cases no recurrent breakpoints have been identified. Moreover, the corresponding clinical description of the patients in the literature is often incomplete, thus complicating the delineation of a common phenotype. Now that microarrays become widely implemented in the diagnostic process of mental retardation it is to be expected that, based on genetic data, novel syndromes will increasingly be defined. The identification of overlapping CNVs and the delineation of clinical syndromes will be facilitated by international databases that capture cytogenetic and clinical information from patients with rare CNVs, such as ECARUCA (www.ecaruca.net) and DECIPHER (https://decipher.sanger.ac.uk/). These databases are of major importance and, as the number of cases increases, they will significantly accelerate the clinical interpretation of microarray data.

The implementation of microarray technologies has already resulted in the identification of several microdeletion and microduplication syndromes (**Chapter 1.3**), such as the 3q29 microdeletion syndrome,[30] the 15q13.3 microdeletion syndrome,[31] the 15q24 microdeletion syndrome,[32] and the Xq28 microduplication syndrome.[33] In addition, using array CGH and targeted DNA copy number analyses in a large cohort of patients with mental retardation, we and others identified overlapping submicroscopic deletions at 17q21.31 in patients displaying consistently similar phenotypic features and, thus, defined a novel microdeletion syndrome (**Chapter 3.3**).[34-36]

Using subsequent high-resolution oligonucleotide-based microarray analyses, we narrowed down the critical region of this novel 17q21.31 microdeletion syndrome to a 424 kb genomic segment encompassing at least six genes. It is most likely that one or more of these genes is responsible for the disease phenotype. Mutation screening of one of these genes, *MAPT*, in a cohort of 122 individuals with phenotypes reminiscent to that of 17q21.31 deletion carriers, but without the deletion, failed to identify any disease-associated variants (**Chapter 3.4**). This may be due to the relative small size of the cohort tested or, alternatively, to an inadequate clinical selection of the patients. Now that the phenotype of the 17q21.31 microdeletion syndrome is better defined (**Chapter 3.4**) the pre-selection will be facilitated and, therefore, more clinically well characterized patients can studied. However, the 17q21.31 microdeletion syndrome may also very well represent a contiguous gene syndrome. As such, a mouse model carrying a hemizygous chromosomal deletion that spans a region syntenic to the human

17q21.31 microdeletion region could provide valuable information on the molecular and cellular consequences of dosage alterations in this region. It has previously been shown that through chromosome engineering defined rearrangements can be introduced into the mouse genome.[37] The critical region of the 17q21.31 microdeletion syndrome in human is syntenic to a 400 kb region of mouse chromosome 11qE1, with the order, number, and orientation of the genes being highly conserved, thus making it feasible to establish such a mouse model. Previously such models have been generated for other human genomic disorders such as DGS/VCFS, SMS and PWS (reviewed by van der Weyden and Bradley[37]). Through a model carrying a microdeletion syntenic to the 1.5 Mb human 22q11.2 DGS/VCFS-associated microdeletion, alterations in the brain transcriptome were detected, thus providing evidence that microRNA biogenesis may contribute to the phenotypic deficits in this model.[38] Similarly, in a 17q21.31 microdeletion mouse model, genome-wide expression profiling may allow the identification of differentially expressed genes which, in turn, may provide clues for the underlying biological mechanisms. Alternatively, altered regulation of one or more flanking genes due to a position effect may contribute to the 17q21.31 microdeletion phenotype. This has for example been observed in WBS, where gene expression analyses in patients suggested that not only the aneuploid genes, but also flanking genes that map several megabases away from the genomic rearrangement should be considered as possible contributors to the WBS phenotype.[39] Likewise, altered expression of the *PGRN* gene, located 1.2 Mb centromeric of the recurrent 17q21.31 microdeletion, may contribute to the characteristic phenotype of the patients. *PGRN* encodes the multifunctional growth factor progranulin and loss-of-function mutations in the gene have been identified in patients with the neurodegenerative disorder FTLD-U.[40-42]. Interestingly, haploinsufficiency of progranulin due to a 1.1 Mb microdeletion in the 17q21.31 region was encountered in a girl with mental retardation, poor language, growth hormone deficiency and dysmorphic features (Corrado Romano, personal communication). Preliminary genome-wide expression analyses using EBV-transformed cells derived from 17q21.31 deletion carriers and their parents, however, failed to reveal differential expression for the *PGRN* gene (unpublished data). Ideally, however, this global gene expression profiling should be conducted in brain tissue, as was previously done in both histopathologically affected and unaffected areas of human FTLD-U brains. This revealed 414 upregulated and 210 down-regulated genes in the frontal cortex.[43] Since such brain tissue is not available from 17q21.31 microdeletion carriers, a 17q21.31 deletion mouse model may provide the tool to interrogate the effect of the 17q21.31 deletion on the expression of its associated (flanking) genes. Such a model has already successfully been used for the Potocki-Lupski syndrome (PTLS), which is associated with a microduplication of the 17p11.2 region. In a PTLS mouse model, Dp(11)17/+, direct assessment of the relative transcription levels of genes within and oudside the 17p11.2 genomic interval revealed that not only duplicated genes, but also normal copy number genes that flank the engineered interval may serve as candidates.[44]

Detailed genomic analyses of recurrent 17q21.31 microdeletions has shown that in all these deletions the breakpoints cluster to LCRs or segmental duplications, suggesting that they are

mediated by non-allelic homologous recombination (NAHR) events.[45] This notion is supported by the identification of a reciprocal duplication within this region in a girl with severe psychomotor developmental delay and craniofacial dysmorphisms.[46] In order to provide evidence that NAHR indeed mediates the rearrangements, patient-specific junction fragments should be characterized in detail in order to determine whether there is a recombination hotspot and, if so, whether a homologous recombination event has taken place, possibly accompanied by gene conversions and/or other events that would be consistent with a double strand break repair homologous recombination event.

So far, at least one of the parents of the 17q21.31 microdeletion patients carried the H2 inversion polymorphism haplotype (**Chapters 3.3** and **3.4**). The inversion results in a genomic structure with directly oriented LCR subunits which can undergo a deletion rearrangement via NAHR.[28] This observation indicates that the inversion may be a prerequisite for the deletion to occur. There are increasing indications for a link between common inversion polymorphisms and genomic disorders.[14] For several loci that undergo NAHR it has been shown that deletions in the patients are linked to an inversion in one of the parents, for example in WBS,[47] Angelman syndrome[48] and Sotos syndrome[49]. In addition, inversions were found to be involved in a recurrent t(4;8)(p16;p23) translocation giving rise to WHS with a relatively mild spectrum of dysmorphic features.[50,51] Detailed analyses of 17q21.31 rearrangement rates using sperm-based assays may underscore the hypothesis that the H2-lineage is a prerequisite for the deletion to occur. Turner *et al*. developed such a sperm-based assay to dissect the relative rates of NAHR between different pairs of duplicated sequences and showed that deletions via these sequences are generated at a higher rate than their reciprocal duplications in the male germline.[52] To test the hypothesis that the H2-lineage is a prerequisite for the generation of the 17q21.31 microdeletion, germline rates of *de novo* meiotic deletions and duplications at 17q21.31 in sperm samples of donors homozygous for either the H1 or H2 lineage, and of donors heterozygous for the inversion polymorphisms, should be compared.

4.4 Identification of novel mental retardation genes

To date ~300 different genes are known to give rise to mental retardation when mutated, but their total number may run into the thousands (reviewed by Ropers 2006).[53] Genes linked to mental retardation and cognitive function have predominantly been found on the X chromosome, mainly due to the ease to identify families with an X-linked inheritance pattern.[54,55] To date, mutations in more than 80 genes on the X chromosome have been found to underlie mental retardation.[55] The majority of autosomal mental retardation genes, however, still awaits identification.

Genome-wide genomic profiling of patients with unexplained mental retardation represents a powerful strategy for the identification of novel mental retardation genes. Screening for CNVs

using microarray-based technologies already disclosed the causative genes for several known syndromic and previously elusive mental retardation disorders. The first syndrome for which the causative gene was identified using microarrays was CHARGE syndrome (MIM #214800).[56] Array CGH revealed microdeletions in 8q12 in two patients with CHARGE syndrome. Subsequently, several mutations in the *CHD7* gene, located within the deleted region, were detected in patients with CHARGE syndrome without a 8q12 deletion.[56] Since then, *CHD7* mutations have been identified in the majority of cases with CHARGE syndrome.[57] Similarly, mutations in the *TCF4* gene (18q21.2) were identified in Pitt-Hopkins syndrome (MIM #610954)[58,59] and in the *ZNF674* gene (Xp11.3) in individuals with X-linked mental retardation.[60] Another example of a causative gene that has been identified using a targeted approach based on phenotypic characteristics is *EHMT1*, a gene that underlies the 9q34 microdeletion syndrome (MIM #610253).[61] Similarly, biallelic truncating mutations in the *B3GALTL* gene were identified in patients with Peters-Plus syndrome (MIM #261540) after the detection of a microdeletion in the region 13q12.3q13.1.[62]

The identification of causative genes in known mental retardation syndromes emphasizes the potential of microarray-based mapping strategies to pinpoint candidate autosomal mental retardation genes. The subsequent identification of causative gene mutations is performed in patients selected on the basis of phenotype. The success of this strategy relies on the clinical recognition of a syndrome and the availability of patients with similar overlapping clinical features.

For the identification of recessive mental retardation genes, strategies have been aimed at homozygosity mapping of polymorphic markers in consanguineous families. Here too, technological improvements have been instrumental. Notably, SNP analysis has replaced microsatellite marker analysis, allowing more efficient and more complete homozygosity scans.[63-65] This strategy has e.g. successfully been used for the identification of the autosomal recessive mental retardation gene *CC2D2A*.[66] A major problem with homozygosity mapping in consanguineous families, however, is that it usually reveals large regions encompassing up to 200 genes. Therefore, the identification of genes causing recessive mental retardation by homozygosity mapping in consanguineous families has had limited success. One could speculate that even in outbred populations many patients will be homozygous for a single mutation. The obvious explanation for this assumption lies in the paucity of disease alleles at the population level. Therefore, it is anticipated that high-density SNP arrays can successfully be used for the mapping of autosomal recessive mental retardation genes.

Prioritization of candidate genes for high-throughput sequencing

In general, the CNV regions detected in routine analyses of individuals with unexplained mental retardation serve as candidate regions harboring mental retardation-related genes. Subsequently, sequencing of large patient cohorts with a similar phenotype ('phenocopies')

may result in gene identification through mutation detection. Novel sequencing technologies ('next-generation sequencing') will facilitate the efficient detection of causative mutations and additionally, it will allow the screening of entire genes, including the introns, UTRs and promoter regions. However, throughput in medical (re-)sequencing of hundreds of candidate regions, harboring thousands of candidate genes will still be rate-limiting in the coming years. Therefore, candidate genes and/or other functional elements within these regions should be prioritized for high-throughput sequencing. In-depth bio-informatic analyses can be used to select dosage sensitive genes. Several additional strategies can be applied to further prioritize candidate genes within these regions, such as their specific and restricted expression patterns in brain and their known roles in human neurodegeneration-related pathways. In addition, genes whose mouse orthologues, when disrupted, result in recognizable phenotypes may be enriched in mental retardation-associated CNVs (**Figure 4.2a**).

Also, physical interactions between proteins may be relevant for the prediction of disease-causing genes.[67] When protein A encoded by a gene located in a disease-associated region has been shown to interact with protein B known to be involved in that disease, the likelihood that protein A is also involved is increased by a factor 10.[67] Similarly, if a disease is genetically heterogeneous it becomes more likely that the proteins involved may interact. For several genetically heterogenous disorders, such as Fanconi anemia and limb-girdle muscular dystrophy, it has already been shown that proteins with previously unknown function may actually participate in the same complexes.[68,69] Similarly, genes causing mental retardation have been connected to specific pathways, e.g. the RhoGTPase pathway, or to the formation of specific neuronal complexes such as the postsynaptic density (PSD) complex, which comprises over 1,100 proteins.[70,71] Interestingly, learning and/or memory were affected in 75% of mice with mutations inactivating individual PSD proteins.[72]

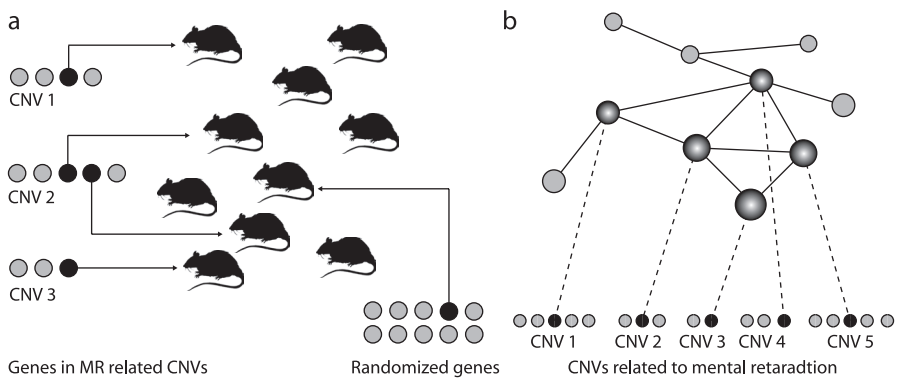

Figure 4.2: Prioritization schemes of candidate genes. (**a**) Genes whose mouse orthologues, when disrupted, result in abnormal phenotypes may be enriched in mental retardation-associated CNVs. (**b)** Interaction network based on protein-protein interactions or gene-gene interactions culled from different databases may reveal novel mental retardation-associated genes.

The protein products of genes within CNVs related to mental retardation can also be linked *in silico* to known protein-protein interaction networks. The genes that, in this way, link disease causing CNVs may serve as interesting candidates for mental retardation (**Figure 4.2b**).

Moreover, evolutionary conserved gene co-expression patterns may be used to prioritize candidate genes.[73] Genes within CNVs causing mental retardation are thought to be dosage sensitive and, therefore, to show a tight regulation of expression in the developing and adult brains of normal individuals. Also, co-expression of genes in different species may indicate that they are part of the same pathway.[74] Together, these features may facilitate the identification of new genes involved in mental retardation.

Several disease-gene prediction programs have already been developed, such as Prioritizer[75] and Endeavour.[76] These programs are based on different data sources, such the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Biomolecular Interaction Network Database (BIND), Reactome, the Human Protein Reference Database (HPRD), the Gene Ontology (GO) database, predicted protein-protein interactions, human yeast two-hybrid interactions and microarray-based co-expression patterns. The efficacy of these and other bio-informatic tools are likely to improve in the near future, as data from different sources can be integrated and more information will be available on the structures and functions of genes and the biological pathways in which they operate.

4.5 Future diagnostics in mental retardation

In recent years, genomic microarray-based profiling technologies have increasingly been implemented in the routine diagnostic workup of individuals with mental retardation. The initial clone-based genomic microarray platforms have already largely been replaced by commercially available oligonucleotide-based platforms. Current high-density oligonucleotide microarrays encompass large numbers of short DNA targets (oligonucleotides) targeting random genomic sequences[77-79] or single nucleotide polymorphisms (SNPs).[18,80-83] The advantages of these platforms are numerous: (i) they provide a higher genome coverage than most clone-based genomic microarrays, (ii) they can be produced in large quantities according to industrial quality standards, (iii) they are available to all investigators, including those without dedicated microarray production facilities, and (iv) they are widely used and thus generate large data sets that facilitate comparison and integration. Most technological restrictions of the initial microarray platforms have been overcome and the resolution of the technology has increased towards the kilobase level (currently: 3-10 kb), which is a major step forward as compared to the ~5-10 Mb level.

It is anticipated that microarray-based genomic profiling technologies will continue to replace conventional karyotyping. Through these technologies also subtelomeric CNVs can be detected

and, therefore, there will no longer be a need for the exclusion of subtelomeric abnormalities e.g. by MLPA prior to whole genome profiling. Obviously, genomic microarrays do not allow for the detection of balanced aberrations, although these aberrations may include cryptic imbalances and/or complex rearrangements.[84,85] With the advent of microarray and next-generation sequencing technologies, more and more cryptic changes at the breakpoints of rearrangements will be identified. It should also be noted that conventional techniques such as karyotyping and FISH may still be valuable for the elucidation of e.g. complex structural chromosome rearrangements, especially when there is a family history positive for mental retardation.

Targeted microarray approaches may have advantages over genome-wide approaches, especially in a diagnostic setting, as parental samples are not required for the clinical interpretation of targeted microarray data. However, through these targeted approaches sporadic DNA copy number changes will be missed, as the corresponding regions will not be represented on such microarrays (**Chapter 3.2**). Moreover, although the interpretation of genome-wide microarray data is not as straightforward as that of targeted microarrays, the results obtained will be helpful for the identification of new syndromes and mechanisms.[86] Targeted (re-)sequencing may efficiently be applied to genes associated with genetically heterogeneous disorders such as Bardet-Biedl (MIM #209900), and clinically related developmental disorders that e.g. have been linked to mutations in the RAS/MEK/ERK signaling pathway such as Cardio-facio-cutaneous syndrome (MIM #115150), Noonan syndrome (MIM #163950) and Costello syndrome (MIM #218040).[87]

Eventually, targeted or genome-wide (re-)sequencing may replace microarray-based profiling technologies as the main molecular tool in the genetic diagnostic process of mental retardation. Major advances in DNA sequencing technologies have recently resulted in the publication of the first two individual human whole-genome sequences.[88,89] The genome of James Watson, co-discoverer of the structure of DNA, was the first genome to be sequenced by a so called next-generation technology.[88] The next-generation sequencing strategies currently in use require a DNA amplification step, which may introduce a bias as some strands of DNA amplify more easily than others. The latest revolution in the field of genome sequencing is single-molecule sequencing and recently Harris *et al*. reported the sequence of a whole viral genome using this approach.[90] Reading the sequence of a single fragment of DNA further simplifies the sequencing process, which ultimately may bring personalized genome sequencing within reach. This, in turn, will allow the comparison of total genomes from patients and parents and the concomitant detection of *de novo* mutational events associated with disease. By using these novel sequencing methodologies, it is anticipated that the majority of single gene disorders associated with mental retardation will be elucidated at the molecular level at an increasingly rapid pace. Sequencing of multiple whole genomes will allow the delineation of the frequency of *de novo* CNVs in humans, and genome-wide SNP and CNV association studies

in large cohorts of fully sequenced individuals with mental retardation is expected to provide new insight into the pathogenesis of mental retardation and other common diseases.
Obviously, there are ample related ethical, legal and social issues that should be addressed within this context.[91] Major considerations are the circumstances under which research results are disclosed to research participants, the obligations that are owed to participant's close genetic relatives and how the future use of patient samples and data obtained by whole-genome sequencing will be dealt with.[91] Ultimately, however, it is anticipated that whole genome sequencing will become part of routine clinical care and an integral part of medical records, facilitating the identification of sequences that are associated with disease such as mental retardation, but also predicting the response to specific medication.

4.6 Concluding remarks

The increasing resolution of genomic analyses tools such as microarray and/or (re-)sequencing platforms will in the near future increase CNV detection in patients with mental retardation. Comprehensive phenotyping and bioinformatic analyses will be imperative for the clinical interpretation of CNVs, including the role of causative mutations, genetic risk factors and modifiers influencing the disease severity. Improvements in our understanding of the role of CNVs in health and disease will lead to a decrease in the number of so called unclassified CNVs. Eventually, up to 25% of all cases of mental retardation may be explained by copy number-dependent gene dosage variations. Moreover, high-throughput (re-)sequencing of candidate genes and other functional elements in large patient cohorts may reveal causative mutations in another 10 to 20% of cases. The identification of overlapping CNVs in patients with similar clinical features will be essential for confirmation of the causal role of these CNVs. Therefore, the continued collection of disease-causing CNVs and their associated phenotypes in databases such as ECARUCA and DECIPHER will be of major importance, not only for the confirmation of pathogenicity, but also for the proper counseling of patients and their families.

Moreover, increased insight into genomic abnormalities and mechanistic pathways underlying mental retardation may result in the development of specifically targeted therapies. In fact, already several strategies are currently being developed. The reduction in metabotropic glutamate receptor (mGluR5) signaling, for example, has been shown to reverse the clinical features of Fragile X syndrome (MIM #300624), thus providing a compelling rationale for the use of mGluR5 antagonists in the treatment of Fragile X syndrome and related disorders.[92] Similarly, discoveries regarding the genetics and pathogenesis of spinal muscular atrophy have revealed potential targets for pharmacotherapy,[93] and the testing of potential therapeutic agents in a mouse model for tuberous sclerosis (TSC, MIM #191100) has suggested that these agents may be used to treat TSC-related brain disease, including infantile spasms.[94] These encouraging results underline the notion that further insight into the pathways affected in mental retardation may pave the way for the development of novel treatment strategies.

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Summary

Mental retardation, a condition that includes limitations in intellectual functioning and lack of skills necessary for daily living, is estimated to affect 2–3% of the population. An etiologic diagnosis, however, can be established in only 50% of all patients. The elucidation of the underlying cause is of importance as it improves the clinical management of patients and allows the disclosure of relevant information to family members. Genetic abnormalities are the most common identifiable cause of mental retardation. Three main categories of genetic disorders associated with mental retardation can be recognized: multifactorial disorders, single-gene disorders and chromosome disorders. Chromosome disorders larger than 5-10 Mb in size can be detected by conventional karyotyping. Other techniques, notably high resolution chromosome banding and fluorescence *in situ* hybridization (FISH), but also amplification methods such as multiplex ligation dependent probe amplification (MLPA), allow the detection of chromosome aberrations below the resolution of conventional karyotyping, so-called submicroscopic aberrations. The introduction of array-based comparative genomic hybridization (array CGH) enabled genome-wide screening for submicroscopic chromosome imbalances, bridging the technical divide between single-gene defects and microscopically visible chromosome aberrations. Array CGH revealed that submicroscopic DNA copy number variation (CNV) is a significant cause of mental retardation. CNV is a type of variation that refers to segments of DNA that are present in variable copies in comparison to a reference genome and may include deletions, duplications and insertions. This thesis aims to provide understanding of the contribution of submicroscopic CNVs in mental retardation by determining the frequency of CNVs in patients with mental retardation and by the identification of mental retardation syndromes.

In **Chapter 2.1** subtelomeric DNA copy number analyses using MLPA in 210 individuals with unexplained mental retardation is described. Clinically relevant CNVs were identified in 4.3% of the patients, confirming the diagnostic value of subtelomeric screening in individuals with mental retardation and demonstrating that MLPA serves as a robust high-throughput technique for the detection of subtelomeric rearrangements.

The implementation of subtelomeric DNA copy number analysis in the diagnostic workup of patients with mental retardation resulted in the recognition of several previously unrecognized clinical syndromes. One of the first distinct syndromes caused by a submicroscopic subtelomeric CNV identified among unselected individuals with idiopathic mental retardation was the subtelomeric 22q13.3 microdeletion syndrome. In **Chapter 2.2** the size and nature of several 22q13.3 microdeletions is studied using high-resolution chromosome-specific array CGH. Moreover, the relationship between clinical features and deletion sizes is investigated. Global developmental delay and absent speech or severely delayed speech development were constant features in all patients. Considerable variability in the size of the 22q13.3 microdeletions was shown without correlations between the genotype and the phenotype.

In **Chapter 3.1** the results from a genome-wide array CGH analysis of a cohort of 386 patients is integrated with those from 15 similar genome-wide studies on 978 patients, reported by other groups. The overall diagnostic yield of clinically significant CNVs in our patient cohort was 9.1% and in combination with data from the literature a collective yield of pathogenic CNVs of 11.2% was assessed. These data strongly underscore the importance of array CGH as a genomic profiling tool for the detection of submicroscopic CNVs in patients with unexplained mental retardation.
Using a clinical scoring system, it was found that the detection rates of subtelomeric CNVs and interstitial CNVs partly reflect clinical pre-selection biases, based on phenotypes with additional dysmorphic features and/or growth abnormalities. After correction for this clinical preselection, a yield of clinically significant CNVs among patients with mental retardation of ~8% was determined. In addition, the challenge of characterizing and classifying genomic CNVs in order to assess their putative implications in mental retardation was addressed and a practical workflow that can be used in a diagnostic setting for the interpretation of CNVs related to mental retardation was proposed.

In **Chapter 3.2** the hypothesis that genomic regions involved in *de novo* submicroscopic aberrations may serve as candidates for recurrent CNVs in individuals with mental retardation is tested. MLPA was used to screen for copy number changes in eight selected candidate regions in a European cohort of 710 individuals with idiopathic mental retardation. No additional submicroscopic rearrangements were determined, indicating that the candidate regions tested were non-recurrently affected in this cohort of patients. These results support the notion that genome-wide analyses are indicated when testing for copy number changes in individuals with idiopathic mental retardation.

In **Chapter 3.3** the identification of a previously unknown microdeletion syndrome involving chromosome region 17q21.31 is reported. Three overlapping 17q21.31 microdeletions were uncovered in a cohort of 1,200 mentally retarded individuals using array CGH and targeted DNA copy number analyses. The presence of low copy repeat sequences flanking the deletion breakpoints suggested that, most likely, nonallelic homologous recombination acts as the underlying mechanism. Subsequent clinical comparison of the patients revealed marked phenotypic similarities, indicating that this novel recurrent genomic anomaly causes a previously unrecognized microdeletion syndrome.

In **Chapter 3.4** the 17q21.31 microdeletion syndrome is further delineated as a clinically and molecularly well-recognizable disorder. Twenty-two individuals with the 17q21.31 microdeletion syndrome were studied. Clinical examination revealed that developmental delay, hypotonia, facial dysmorphisms including a long face, a tubular or pear-shaped nose, a bulbous nasal tip, and a friendly/amiable behavior were the most characteristic features. Other clinically relevant features included epilepsy, heart defects and kidney/ urologic anomalies. The prevalence of

the syndrome was estimated to be 1 in 16,000 and using high-resolution oligonucleotide microarrays the 17q21.31 critical region was narrowed down to a 424 kb genomic segment encompassing the *MAPT* gene. Mutation screening of *MAPT* in 122 individuals with a '17q21.31 deletion-like' phenotype failed to identify any disease-associated variants. In addition, further evidence was provided that a common 900 kb 17q21.31 inversion polymorphism in one of the parents is a necessary factor for the deletion to occur.

In **Chapter 4** the most important results of this thesis are discussed in relation to both molecular genetic and clinical genetic relevance. The increasing resolution of genomic analysis tools will inevitably increase CNV detection in patients with mental retardation in the near future. Comprehensive phenotyping and bioinformatic analyses will be required for the clinical interpretation of these CNVs. The concomitant elucidation of novel genetic causes of mental retardation will provide further insight in its occurrence and its associated clinical features, facilitating a better clinical management of the patient and a better genetic counseling of the family.

Samenvatting

Mentale retardatie gaat gepaard met een verstandelijke handicap en beperkingen in het dagelijks leven en komt bij ongeveer 2-3% van de populatie voor. De precieze oorzaak is echter in ongeveer 50% van de patiënten onbekend. De opheldering van de onderliggende oorzaak is van belang, omdat het mogelijkheden geeft tot verbetering van de begeleiding van de patiënt en gerichte counseling van de betrokken familieleden mogelijk maakt. Genetische aandoeningen zijn de meest voorkomende oorzaak van mentale retardatie. Er bestaan drie belangrijke groepen genetische aandoeningen die samengaan met mentale retardatie: aandoeningen ten gevolge van een combinatie van genetische factoren en omgevingsfactoren (multifactorieel), afwijkingen (=mutaties) in afzonderlijke genen en aandoeningen ten gevolge van chromosoom afwijkingen. Chromosoom afwijkingen groter dan 5-10 miljoen basenparen (Mb) kunnen worden vastgesteld met conventionele karyotypering. Andere technieken, met name chromosoom bandering met hoge resolutie en fluorescence *in situ* hybridization (FISH), maar ook amplificatie methoden zoals multiplex ligation dependent probe amplification (MLPA), maken het mogelijk chromosoom afwijkingen te vinden die kleiner zijn dan de resolutie van conventionele karyotypering, zogenaamde submicroscopische afwijkingen. Met de introductie van array-based comparative genomic hybridization (array CGH) werd het mogelijk om het gehele genoom te screenen op submicroscopische afwijkingen. Hiermee werd een brug geslagen tussen de kloof tussen gen-defecten en microscopisch zichtbare chromosoom afwijkingen. Door de toepassing van array CGH bleken submicroscopische DNA kopie nummer veranderingen een belangrijke oorzaak voor mentale retardatie te vormen. Deze veranderingen hebben betrekking tot delen van het DNA die in vergelijking met een referentie genoom een afwijkend kopie aantal hebben. Dit kunnen zowel deleties, duplicaties als inserties zijn. Dit proefschrift richt zich op het vergroten van het inzicht in de rol van kopie nummer veranderingen in mentale retardatie door het bepalen van de frequentie van submicroscopische kopie nummer veranderingen in patiënten met mentale retardatie en door nieuwe mentale retardatie syndromen te identificeren.

Hoofdstuk 2 beschrijft het onderzoek van de uiteinden van de chromosomen (de subtelomeren) middels MLPA bij 210 personen met onverklaarde mentale retardatie. Klinisch relevante veranderingen werden gevonden in 4,3% van de patiënten, waarmee het diagnostische belang van screening van de subtelomeren bij personen met mentale retardatie bevestigd werd en tevens werd aangetoond dat MLPA een robuuste techniek is voor de detectie van subtelomeer veranderingen.

De invoering van subtelomeer DNA kopie nummer analyse in het diagnostische proces van patiënten met mentale retardatie heeft geleid tot het vinden van verschillende nieuwe klinische syndromen. Een van de eerste goed omschreven syndromen veroorzaakt door een submicroscopische subtelomeer afwijking die gevonden werd in een ongeselecteerde groep patiënten met onbegrepen mentale retardatie is het 22q13.3 microdeletie syndroom. **Hoofdstuk 2.2** beschrijft het onderzoek van de grootte en de aard van verschillende 22q13.3

submicroscopische deleties (microdeleties) door middel van chromosoom specifieke array CGH. Daarnaast werd de relatie onderzocht tussen de klinische kenmerken en de grootte van de deleties. Algemene ontwikkelingsachterstand en afwezige- of sterk vertraagde spraak ontwikkeling waren aanwezig bij alle geteste patiënten. Er bleek echter een aanzienlijk verschil is in de grootte van de 22q13.3 microdeleties, zonder een duidelijke relatie met de klinische kenmerken.

Hoofdstuk 3.1 beschrijft de resultaten van genoomwijde array CGH analyse van een groep van 386 patiënten met mentale retardatie in combinatie met de resultaten van 15 in de literatuur beschreven vergelijkbare genoomwijde studies van in totaal 978 patiënten. In onze groep patiënten werden klinisch significante kopie nummer veranderingen gevonden bij 9,1% van de patiënten en in combinatie met de andere studies leidde dit tot ziekteveroorzakende DNA kopie nummer veranderingen in 11,2% van de geanalyseerde personen. Deze data onderschrijven de waarde van array CGH in de detectie van submicroscopische kopie nummer veranderingen bij patiënten met onverklaarde mentale retardatie.
Door middel van een klinisch scoringssysteem werd aangetoond dat het aantal DNA kopie nummer veranderingen van de subtelomeren en elders in het genoom (interstitieel) voor een deel afhangt van klinische voorselectie van patiënten met bijkomende dysmorfe kenmerken en/of groeistoornissen. Na correctie voor deze klinische voorselectie kon een frequentie van klinisch belangrijke veranderingen bij mensen met een verstandelijke handicap worden vastgesteld van ~8%. Een voorstel voor een klinisch toepasbare diagnostische *workflow* voor de interpretatie van kopie nummer veranderingen gerelateerd aan mentale retardatie wordt voorgesteld.

In **Hoofdstuk 3.2** wordt de hypothese getoetst of bepaalde regio's betrokken bij *de novo* submicroscopische afwijkingen kandidaat regio's kunnen zijn voor kopie nummer veranderingen die ook bij andere personen met mentale retardatie gevonden kunnen worden. Om hier uitspraken over te kunnen doen werd MLPA gebruikt voor het screenen op kopie nummer veranderingen van 8 geselecteerde kandidaat regio's in een Europees cohort van 710 personen met onverklaarde mentale retardatie. Er werden geen nieuwe submicroscopische veranderingen in ons cohort patiënten gevonden, hetgeen betekent dat genoomwijde analyse aangewezen is voor het testen op kopie nummer veranderingen bij personen met onbegrepen mentale retardatie.

In **Hoofdstuk 3.3** wordt de identificatie van een nieuw microdeletie syndroom in de chromosoom 17q21.31 regio beschreven. In een groep van 1200 personen met mentale retardatie werden drie overlappende 17q21.31 microdeleties vastgesteld middels array CGH en gerichte DNA kopie nummer analyse. De aanwezigheid van low copy number repeat sequenties aan weerszijde van de breukpunten van de deletie maken het waarschijnlijk dat nonallelic homologous recombination het onderliggende mechanisme is. Klinische

vergelijking van de patiënten liet vervolgens zien dat zij naast de mentale retardatie duidelijke kenmerken overeenkomstig hebben wat betekent dat deze nieuwe afwijking de basis vormt voor een nieuw microdeletie syndroom.

In **Hoofdstuk 3.4** wordt het 17q21.31 microdeletie syndroom verder uitgewerkt tot een klinisch en moleculair goed gedefinieerde en herkenbare aandoening. Hiertoe werden 22 personen met het 17q21.31 microdeletie syndroom beschreven. Klinisch onderzoek toonde aan dat de meest uitgesproken kenmerken van het syndroom een ontwikkelingsachterstand, hypotonie, faciale dysmorfieën, waaronder een lang gezicht, een 'buis- of peervormige' neus, een bolvormige neuspunt en een vriendelijk voorkomen betreffen. Andere klinisch relevante kenmerken waren epilepsie, hartafwijkingen en nier/ urinewegafwijkingen. De prevalentie van het syndroom wordt geschat op 1 op 16000. Met oligonucleotide microarrays met hoge resolutie werd de kritische 17q21.31 regio terug gebracht tot een segment van 424 duizend basenparen (kb). Deze regio omvat het *MAPT*-gen, maar screening op mutaties in *MAPT* bij 122 personen met '17q21.31 deletie-achtige' kenmerken leidde niet tot veranderingen die met ziekte in verband konden worden gebracht. Wel werd aangetoond dat een veel voorkomend polymorfisme—een 900 kb grote inversie op 17q21.31—bij één van de ouders, een noodzakelijke factor is voor het krijgen van een kind met de 17q21.31 deletie.

In **Hoofdstuk 4** worden de meest belangrijke resultaten van dit proefschrift besproken in relatie tot hun moleculair- en klinisch genetische relevantie. Hogere resolutie van genomische analyse zal ontegenzeggelijk leiden tot een toename in het aantal kopie nummer veranderingen in patiënten met mentale retardatie. Een goede klinische beschrijving en bioinformatica analyses zullen nodig zijn voor de uiteindelijke klinische interpretatie van deze deleties en duplicaties. De opheldering van nieuwe genetische oorzaken van mentale retardatie die hieruit voortkomt zal verder inzicht verschaffen in de klinische kenmerken en een betere klinische begeleiding van de patiënt en counseling van de familieleden mogelijk maken.

Dankwoord

Wat een heerlijk moment; het dankwoord… dat kenmerkt dat de eindstreep van dit proefschrift nu echt nabij is! Een eindsprint van een lange bergetappe. Een die, net als in de wielersport, pieken en dalen kende en soms alles overheerste. Dit deed me op sommige momenten zelfs uit het oog verliezen wat ik hier nou zo leuk aan vond. Gelukkig gaf het op andere momenten ook veel tevredenheid en voldoening. Deze wetenschappelijke tocht heb ik natuurlijk niet alleen volbracht. Ik wil hier dan ook graag een aantal mensen bedanken voor hun bijdrage als 'coach', 'toeschouwer' en 'medesporter'.

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Curriculum vitae

David Aljosja Koolen werd geboren op 22 juni 1976 in 's-Gravenhage en groeide op in Breda. Hij ging naar de Vrije School in Breda en rondde in 1995 zijn VWO af in Zeist. Van 1995 tot 1996 studeerde hij Civiele Techniek aan de Technische Universiteit Delft. Uitgekeken op beton en staal ging hij in 1996 Geneeskunde studeren aan de Radboud Universiteit Nijmegen (voorheen Katholieke Universiteit Nijmegen). Tijdens zijn studie deed hij onderzoek op de afdeling Medische Oncologie onder begeleiding van Prof. dr. C.J.A. Punt. Daarnaast liep hij stage in het Mount Sinai Hospital, Toronto, Canada, in het laboratorium van Prof. dr. I.G. Fantus waar hij onderzoek deed naar de expressie van *PTEN* in insuline resistente vetcellen. Hiervoor ontving hij een subsidie van de Stichting Diabetes Fonds Nederland. Hij legde in november 2002 het artsexamen met goed gevolg af. Hierna werkte hij tot juli 2003 als arts-assistent op de afdeling Interne Geneeskunde van het Canisius Wilhelmina Ziekenhuis in Nijmegen om vervolgens de overstap naar de klinische genetica te maken. Als junioronderzoeker op de afdeling Antropogenetica van het Universitair Medisch Centrum St. Radboud, Nijmegen werkte hij mee aan het opzetten van een Europese database voor cytogenetische en klinische informatie over chromosomale afwijkingen (ECARUCA). In die tijd werd ook een start gemaakt met het promotieonderzoek dat heeft geleid tot dit proefschrift (Promotor: Prof. dr. A. Geurts van Kessel, Copromotor: Dr. B.B.A. de Vries). Sinds december 2004 combineert hij als assistent-geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) zijn promotieonderzoek met de opleiding tot klinisch geneticus (Opleiders: Prof. dr. B.C.J. Hamel en Prof. dr. N.V. van Slobbe-Knoers). Hij is getrouwd met Nadja Joosen.

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Appendix

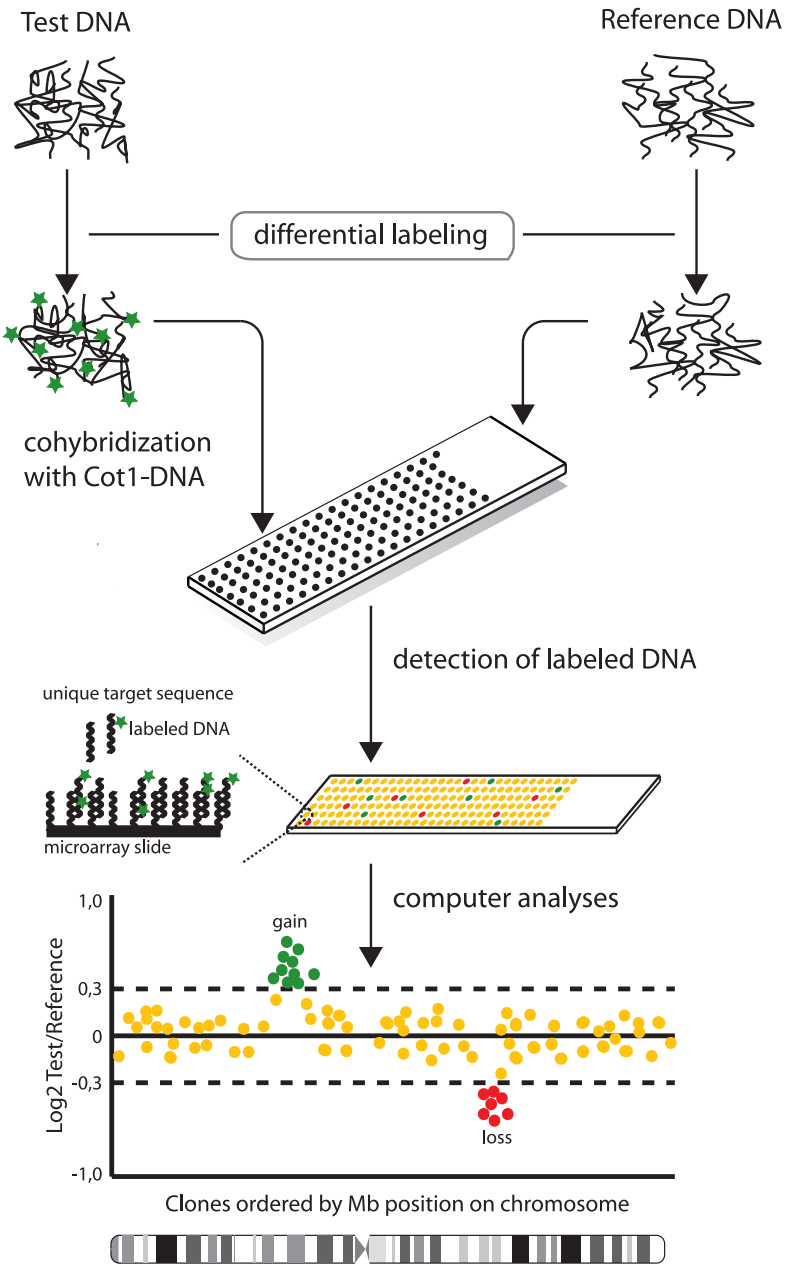

Figure 1.3: The principle of array CGH. Equal amounts of isolated and fragmented genomic test DNA and reference DNA are differentially labeled using fluorescent dyes. Subsequently, test (patient DNA; left panel) and reference samples (reference DNA; right panel) are mixed with Cot-1 DNA, co-precipitated, and resuspended in a hybridization solution. After denaturation the DNA mix is hybridized to the DNA on the array. After several washing steps, images of the fluorescent signals are captured and the ratio of test over reference signals is quantified computationally and plotted for each probe on the array.

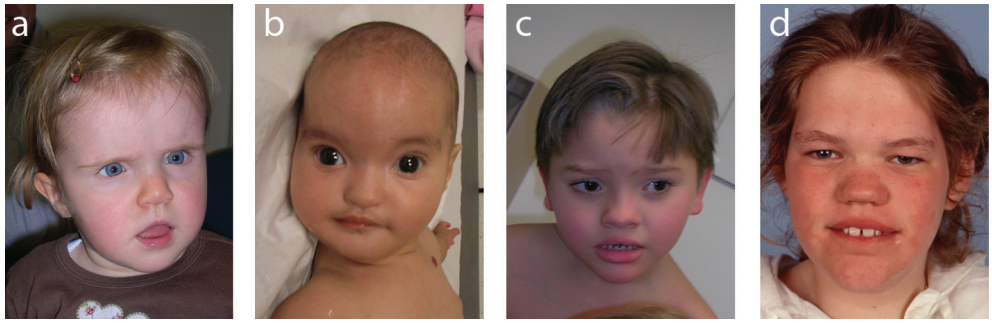

Figure 1.5: Clinical photographs of patients with subtelomere deletions. (**a**) 1pter deletion (**b**) 4pter deletion (**c**) 9qter deletion (**d**) 22qter deletion. Informed consent was obtained for publication of photographs.

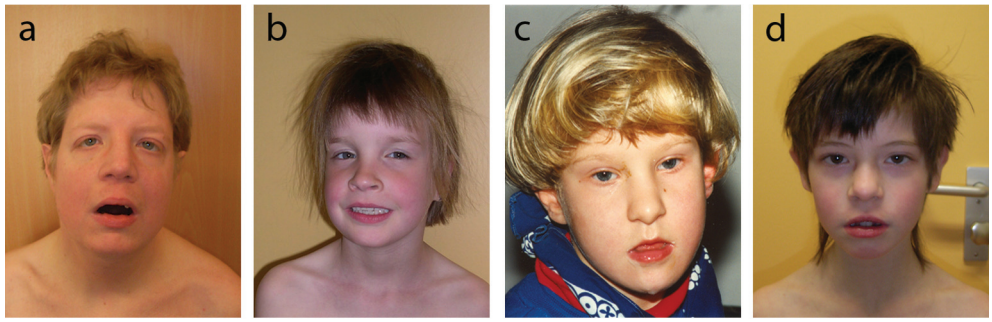

Figure 1.6: Patients with newly recognized interstitial microdeletion syndromes. (**a**) 2p15p16.1 microdeletion, (**b**) 3q29 microdeletion (**c**) 17q21.31 microdeletion (**d**) distal 22q11 microdeletion. Informed consent was obtained for publication of photographs.

Figure 2.2.1: Patients with 22q13 deletion syndrome. Note the known facial features of the 22q13 deletion syndrome, prominent dysplastic ears (visible in cases 1, 2, 4 and 5), and a prominent/ pointed chin (cases 1 and 4– 7).

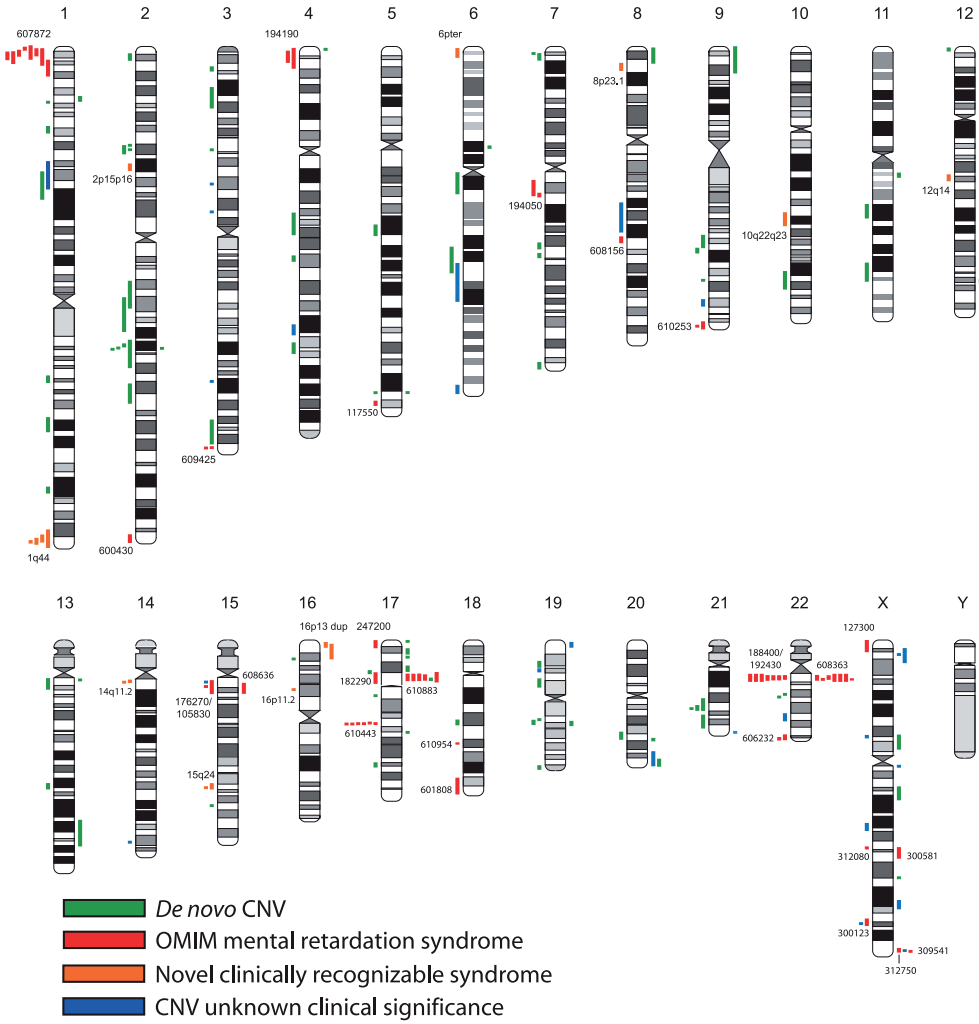

Figure 3.1.4: Overview of all CNVs reported in genome-wide microarrays studies in mental retardation. CNVs are represented by colored bars. Copy number losses and copy number gains are depicted, respectively, on the left hand side and the right hand side of the chromosomes. Red bars, CNVs associated with a well-known OMIM syndromes. Orange bars, novel recurrent CNVs. Green bars, *de novo* CNVs not known to the previous categories and blue bars, CNVs of unknown clinical significance.

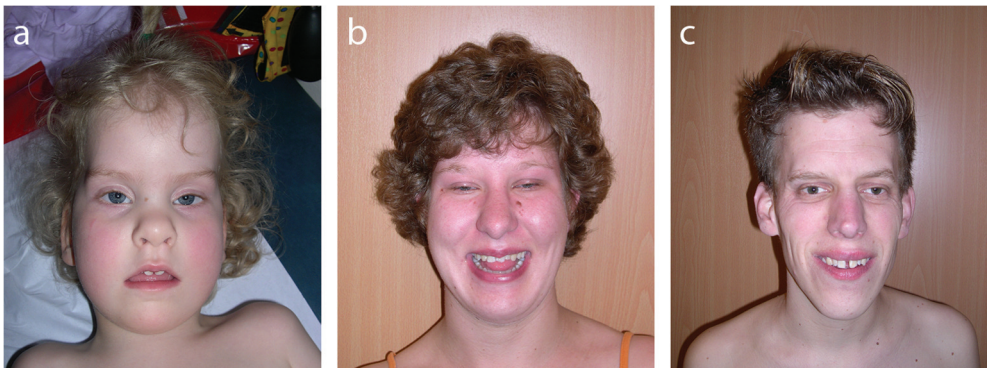

Figure 3.3.1: Clinical characteristics of the 17q21.31 deletion syndrome. (**a**) Patient 1, at 3 years of age. (**b**) patient 2, at 17 years of age, and (**c**) patient 3, at 26 years of age. Note the characteristic facial features, a hypotonic face with ptosis, blepharophimosis, large low set ears, bulbous nasal tip, long collumella with hypoplasic alae nasi, and a broad chin.

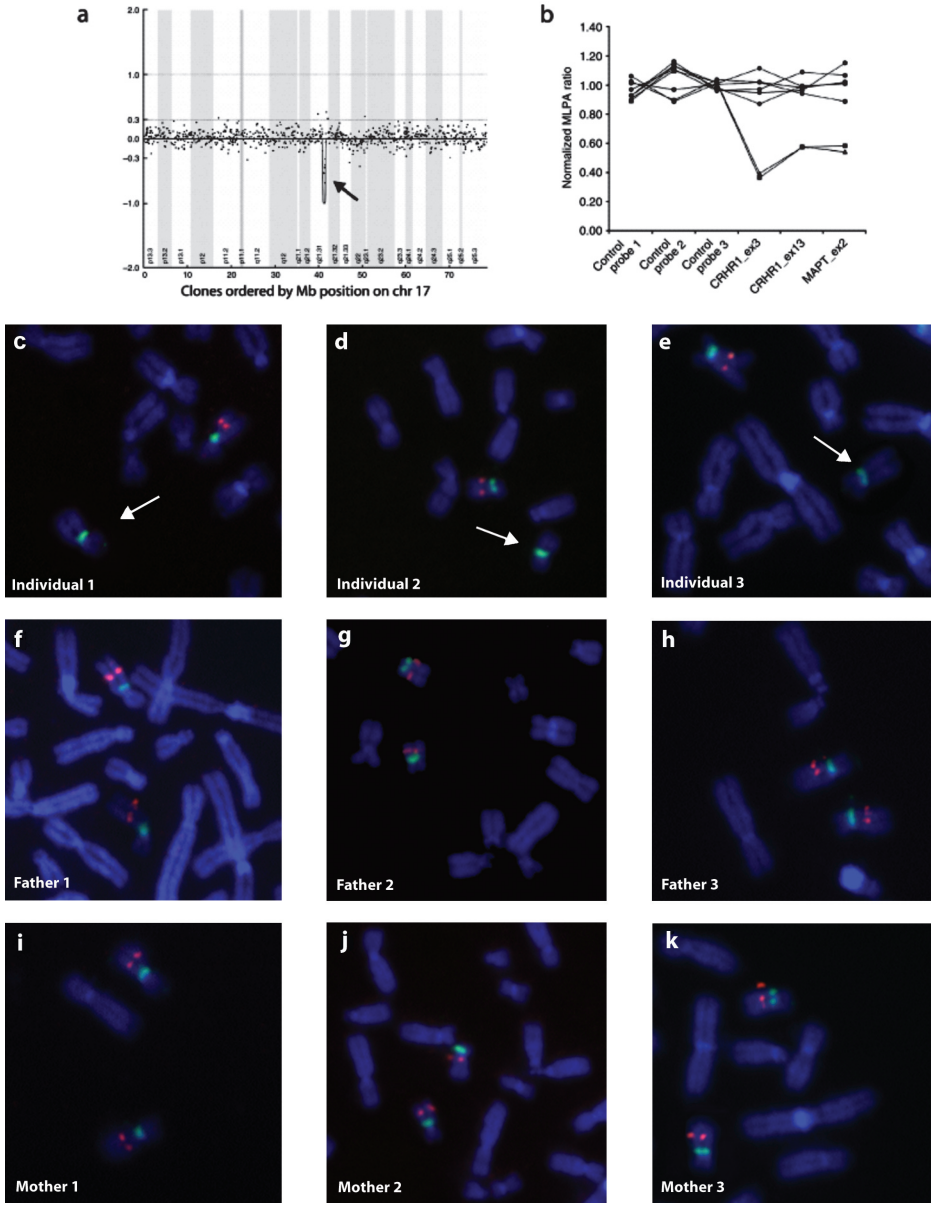

Figure 3.3.2: Analysis of individuals with the 17q21.31 microdeletion syndrome. (**a**) Chromosome 17 array CGH profile of individual 1 with a copy number loss of eight adjacent BAC clones on 17q21.31 (arrow). Clones are ordered on the x axis according to physical mapping positions; log2-transformed test-over-reference (T/R) intensity ratios for each clone are given on the y axis. (**b**) Multiplex ligation-dependent probe amplification (MLPA) analysis, showing a deletion of two probes in CRHR1 and one probe in *MAPT* for both individual 2 (triangles) and individual 3 (squares). The circles represent the MLPA ratios of healthy controls. FISH validation of the 17q21.31 deletion in individuals 1, 2 and 3 (**c-e**) and testing for de novo occurrence in the accompanying parents (**f-k**) using BAC clone RP11-656O14 (red) which is located within the deleted region. The centromere 17 probe was included for reference (green). All individuals show only one signal for RP11-6565O14 (arrow indicating aberrant chromosome 17). The *de novo* occurrence for the deletion was proven in all individuals.

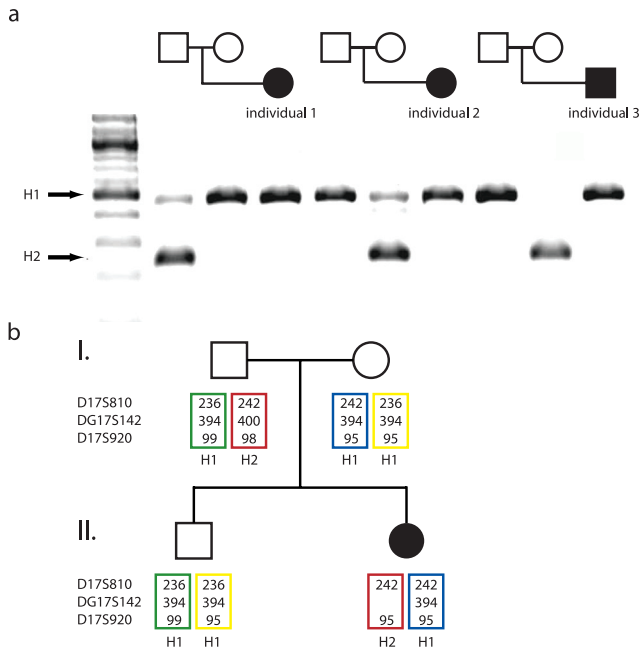

Figure 3.3.3: Genotyping for H1 and H2 lineage and parent-of-origin analysis. (**a**) Genotyping for the H1 (483 bp) and H2 (245 bp) lineages. Far left and far right: 100-bp marker lanes. Individual 3 carries the deletion on the H2 lineage and is of maternal origin. (**b**) Individual 1 and the parents were genotyped using *D17S810*, *DG17S142* and *D17S920* to determine the parental origin of the deletion. The deletion was of paternal origin, and was present on the H2 haplotype.

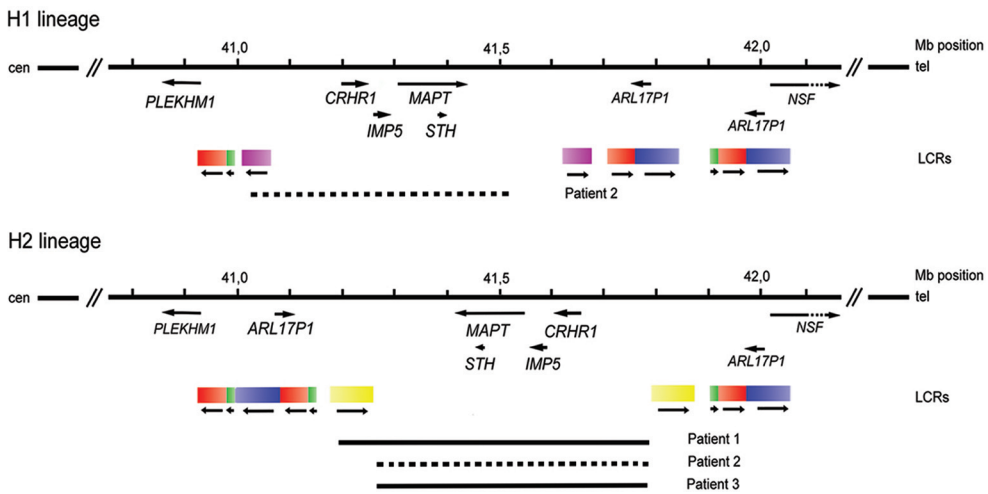

Figure 3.3.4: Transcript map and genomic architecture for the H1 and H2 lineage of the 17q21.31 region. (Dotted) black lines represent the deleted 17q21.31 region identified in the three individuals. Genes located within this region are depicted by black arrows. The H1 and H2 lineage LCR17q structures are depicted as rectangles with colors signifying shared homology and horizontal arrows showing relative orientation (LCR17qA, red; LCR17qB, green; LCR17qC, purple; LCR17qD, blue; LCR17qE, yellow). Note the different genomic orientation of H2, the absence of LCR17qC and presence of LCR17qE. Cen, centromeric; Tel, telomeric.

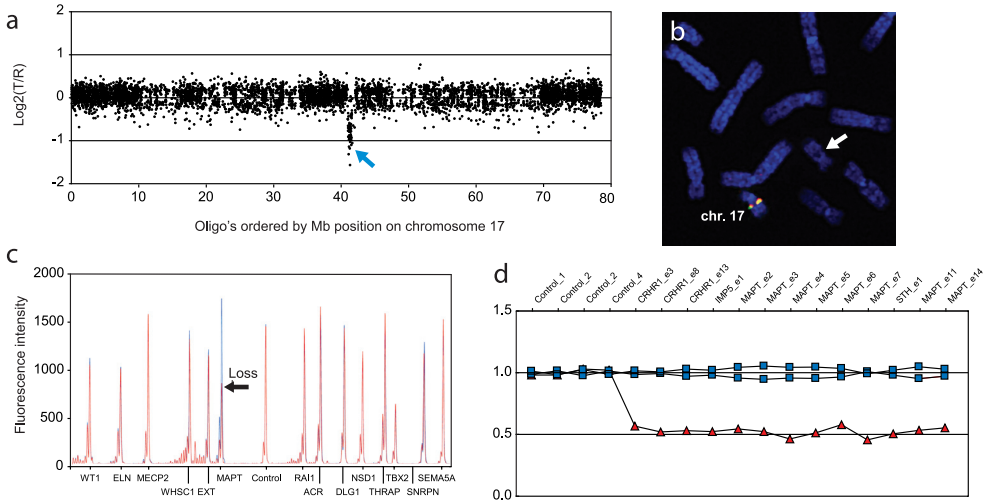

Figure 3.4.1: Identification of the 17q21.31 deletions. (**a**) Chromosome 17 plot of Case 1 obtained by whole-genome oligonucleotide array (Agilent Human Genome CGH Microarray Kit 244A, Agilent Technologies). Chromosome 17 is represented by 5,881 coding and noncoding human sequences (indicated by circles representing the log_2-transformed and normalized test : reference intensity ratios [(Log_2(T/R)], ordered from pter to qter for chromosome 17, in hg17 (NCBI build 35, May 2004). The arrow indicates the presence of a copy number loss at 17q21.31. (**b**) FISH analysis on metaphase spreads of Case 7, using the BAC clones CTD-2324N3 (red) and RP11-413P22 (green) which are both located within the deleted 17q21.31 region. The patient shows only one signal for CTD-2324N3 and RP11-413P22, indicating an aberrant chromosome 17 (arrow). (**c**) Detection of a heterozygous *MAPT* deletion in Case 4 using the microdeletion/ microduplication QMPSF assay. The electropherogram of the patient (in red) was superimposed on that of a normal female control (in blue) by adjusting to the same level the peaks obtained for the control amplicon. The Y-axis displays fluorescence intensity, and the X-axis indicates the genes tested. The heterozygous deletion is detected by a 50% reduction of the *MAPT* peak compared to the normal control (arrow). (**d**) MLPA results for Case 19 (triangles) and two healthy controls (rectangles), showing a deletion of all 13 probes in the *CRHR1*, *IMP5*, *MAPT,* and *STH* genes within the 17q21.31 genomic interval.

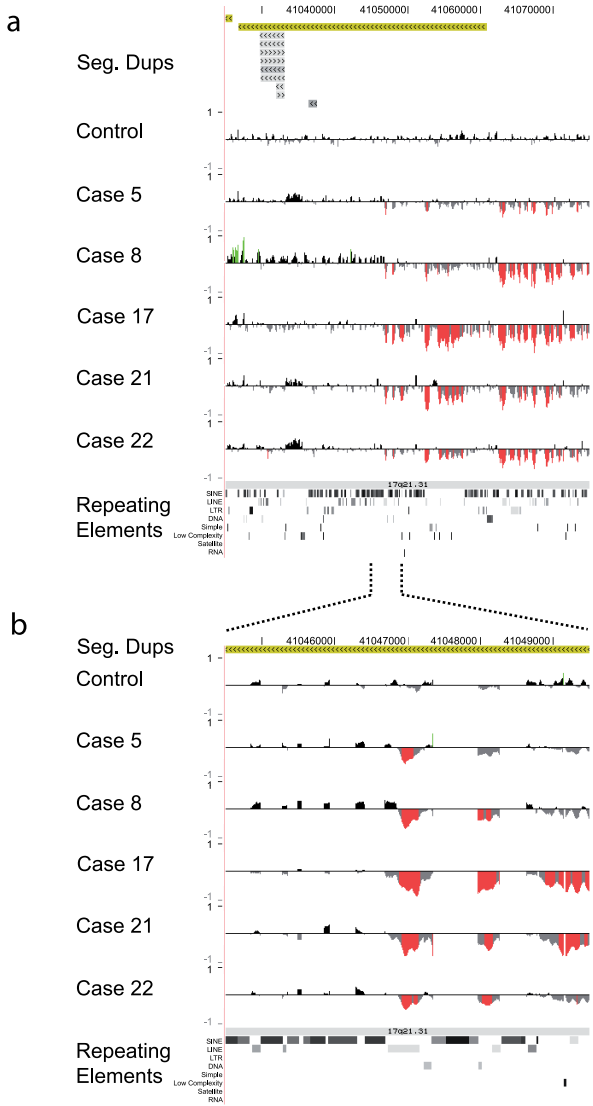

Figure 3.4.2: Ultra-high resolution oligonucleotide array analysis. Results of ultra-high resolution oligonucleotide array analysis of the 17q21.31 microdeletion proximal breakpoint (mean probe density 1 probe/5.2 bp). (**a**) Data from a 50-kb region (chr17:41025000-41075000, hg17) in five unrelated 17q21.31 deletion patients and one control. In each deletion, the proximal breakpoint occured in a segmental duplication of length, 34.2 kb, identity 98.7% (chr17:41026709-41060948). (**b**) Zoomed view showing a 5-kb region (chr17:41044500-41049500). All five patients have breakpoints which are indistinguishable, mapping to within an interval of <500 bp contained within an L2 LINE motif. The highly variable dynamic response of certain probes in this region to report the deletion is likely a result of their different sequence properties.[38] For each individual, deviations of probe $\log_2$-ratios from zero are depicted by gray/black bars, with those exceeding a threshold of 1.5 standard deviations from the mean probe ratio colored green and red to represent relative gains and losses, respectively. Tracks above each plot indicate segmental duplications (gray/yellow bars represent duplicons with 90–98%/98–99% sequence identity, respectively).

Figure 3.4.4: Facial photographs of individuals with a 17q21.31 deletion. Facial photographs of Case 1, at age 10 months; Case 2, at age 1 year; Case 3, at age 2 years; Case 4, at age 3 years; Case 5, at age 3 years; Case 6, at age 3 years 5 months; Case 7, at age 3 years 8 months; Case 8, at age 3 years; Case 9, at age 5 years 8 months; Case 10 at age 5 years; Case 11, at age 3 years 9 months; Case 12, at age 8 years 6 months; Case 14, at age 13 years; Case 15, at age 14 years; Case 16, at 14 years; Case 18, at 16 years; Case 19, at 13 years; Case 20, at age 18 years; Case 21, at 17 years and Case 22, at age 26 years. Informed consent was obtained for publication of photographs.

Figure 3.4.5: Facial photographs at different ages. Facial photographs at different ages (<1, 1-4, 5-6, 7-12, and >12 years of age). Note that the facial characteristics of the patients change with age. With increasing age there is elongation of the face and broadening of the chin and also the "tubular" or "pear"-shape form of the nose becomes more pronounced.