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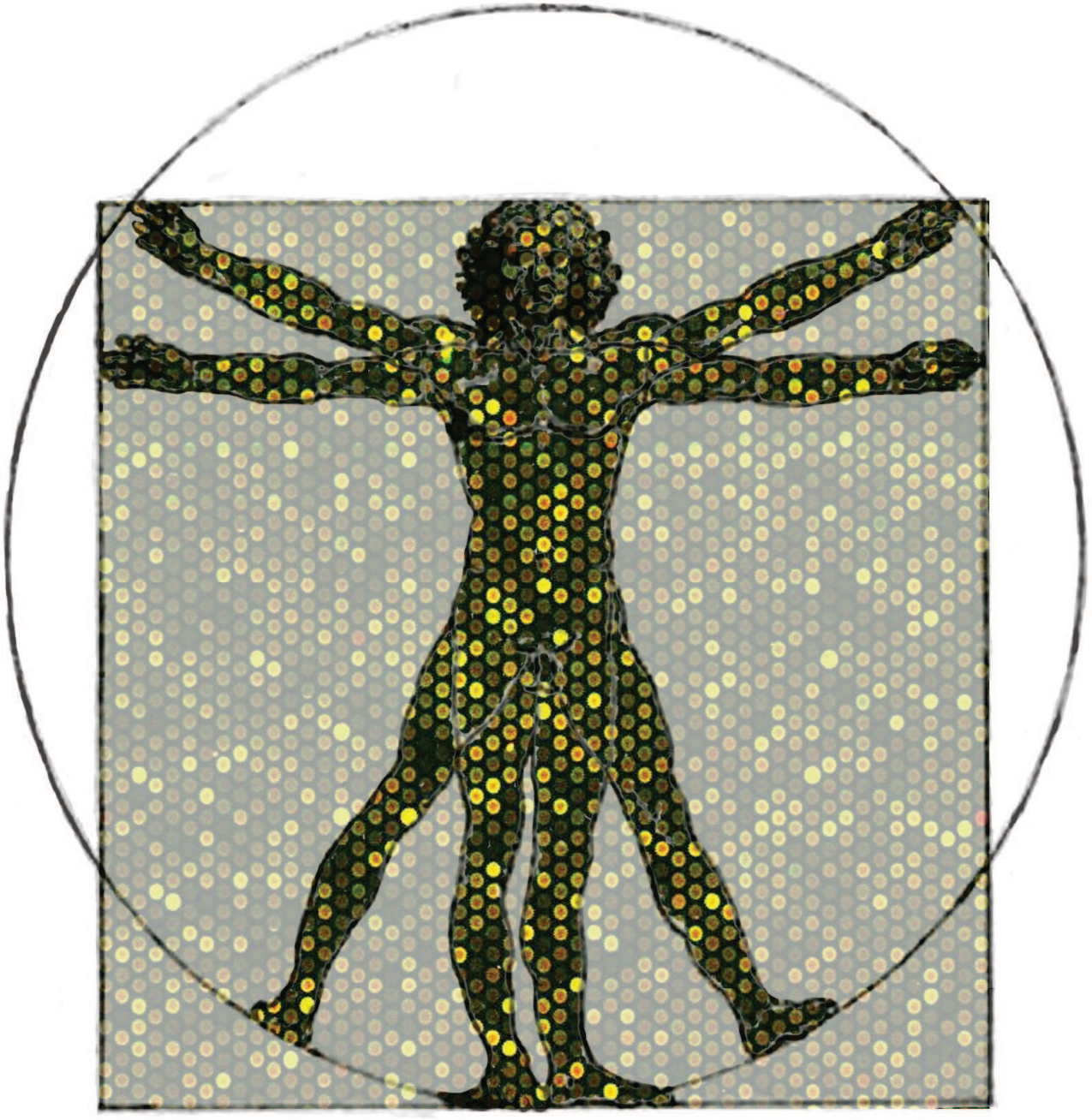
High resolution DNA copy number analysis of constitutional chromosomal aberrations in human genomic disorders



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Björn Menten

2007

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FACULTY OF MEDICINE AND HEALTH SCIENCES

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Understanding is always a journey, never a destination

Richard Fortey, 2004

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From Chromosomes to Base Pairs

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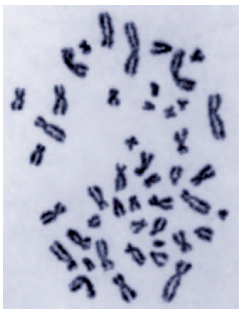
THE FIRST ILLUSTRATIONS of human chromosomes were published in 1882 by Walther Flemming and in 1888 the term 'chromosome' (Greek for 'stained body') was coined by Heinrich von Waldeyer [1]. In the beginning of the 20th century, Theodor Boveri and Walter Sutton independently proposed that chromosomes carry the hereditary factors, or genes, and Walter Sutton, who combined cytology and genetics, was the first to refer to his work as 'cytogenetics'. In 1923, Thomas Painter reported the observation of 24 chromosomes in testicular cells and supposed human somatic cells contained 48 chromosomes [2]. This figure of 48 became generally accepted in the years thereafter and in the end was taken as a given fact by most cytogeneticists for over three decades. The correct number was only established in 1956 by Tjio and Levan (Figure 1.1) (3 years after the publication of the DNA double Helix by Watson and Crick) with this hesitant statement [3]:

> 'Before a renewed, careful control has been made of the chromosome number in spermatogonial mitoses of man we do not wish to generalize our present findings into a statement that the chromosome number of man is 2n = 46, but it is hard to avoid the conclusion that this would be the most natural explanation of our observations.'



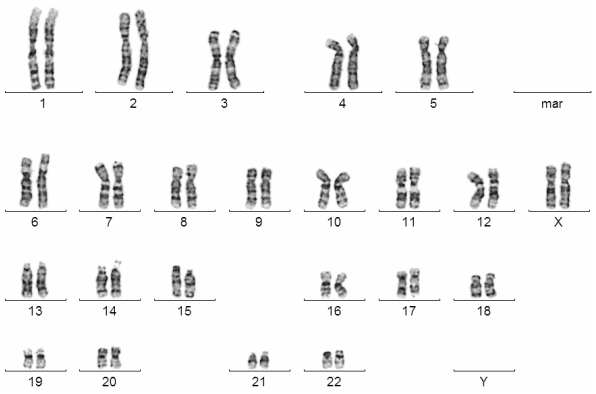


Figure 1.1 – *(left) The metaphase spread from the original publication from Tjio and Levan, establishing the human chromosome number as 46 [3]. (bottom) A normal G-banded female karyotype.*

This discovery was the result of a beautiful example of serendipity: prior to slide preparation, accidental use of a hypotonic rather than an isotonic solution was used to cell suspensions, causing the cells to swell through osmosis [4]. This new technique for chromosome spreading made it possible to unambiguously count the number of chromosomes, and order them in 7 groups, based on their length and centromeric position and heralded the birth of a new discipline: medical cytogenetics.

Soon, the first association between disease and chromosomes was made when Jérôme Lejeune observed that patients with Down syndrome had an extra chromosome 21 (trisomy 21) [5]. The next year, trisomy 13 was recognized as the cause for Patau Syndrome [6] and trisomy 18

for Edwards Syndrome [7]. Shortly after these observations, two frequent disorders of sex differentiation were shown to be caused by abnormalities in the number of sex chromosomes. Turner syndrome is caused by monosomy X [8] and Klinefelter syndrome is caused by an extra X chromosome in males (47,XXY karyotype) [9]. This rudimentary technique of chromosome spreading also led to the identification of the first specific chromosomal aberration associated with human malignancy. Peter Nowell and David Hungerford described the Philadelphia chromosome, a supposed deletion of chromosome 22, in patients with chronic myelogenous leukemia [10]. In the ensuing years, human cytogenetics evolved by several technological advances that combined innovations in molecular biology, chemistry and instrumentation. Peter Nowell identified phytohemagglutinin, a kidney bean extract, as a mitogen for lymphocytes [11]. This important discovery made it possible to use peripheral blood for chromosome analysis, instead of, more invasive, bone marrow aspirates or fibroblasts. In 1963, it was again Jérôme Lejeune who noted that patients with the Cri-du-Chat syndrome, a specific syndrome in which children suffered from severe mental retardation and a specific cat-like cry, all lacked the terminal piece of the short arm of chromosome 5. The first deletion syndrome was identified [12]. A second milestone in the development of human cytogenetics, resulted from the study of plant chromosomes by Torbjörn Caspersson; a fluorescent dye (quinacrine) was used to differentially stain chromosomes. Following this simple staining procedure, a chromosome specific banding pattern (Q-banding) was obtained [13]. This banding greatly facilitated the recognition of chromosomes and most importantly the detection of smaller structural defects. Other banding methods (e.g. G-, R-, C-, and NOR-banding) rapidly appeared and extended the methodological abilities of cytogenetics [14]. A third wave of innovation in medical cytogenetics resulted from the introduction of a culture technique that allowed synchronization of the cell cycle and collection of dividing cells in prometaphase. These elongated prometaphase chromosomes were of superior quality, exhibiting a much larger number of chromosome bands and thus offering a considerable increase in resolution (Figure 1.1) [15, 16]. These new developments again lead to a boost in clinical cytogenetics: the underlying genetic chromosomal aberrations were identified for DiGeorge syndrome [17, 18] and Williams-Beuren syndrome [19] and the concept of the microdeletion or contiguous gene syndrome was born [20]. Apart from elucidating the genetic cause of known syndromes, also new syndromes were described. Smith et al. reported a 17p11 interstitial deletion in nine unrelated patients with specific clinical characteristics; a syndrome now referred to as Smith-Magenis syndrome [21].

Through the analysis of chromosome banding patterns, thousands of recurrent and non-recurrent chromosomal abnormalities have been identified in patients with mental retardation and congenital disorders [22], leading to a better understanding of the molecular causes of these disorders and a better patient management.

Molecular Cytogenetics

Fluorescence *In Situ* Hybridization

Molecular genetics came of age more than a decade after the introduction of cytogenetics in clinical medicine. The tremendous opportunities for diagnosis of monogenic disorders, in particular after the discovery of PCR-based methods and improved sequencing strategies [23–26], quickly overshadowed cytogenetics as

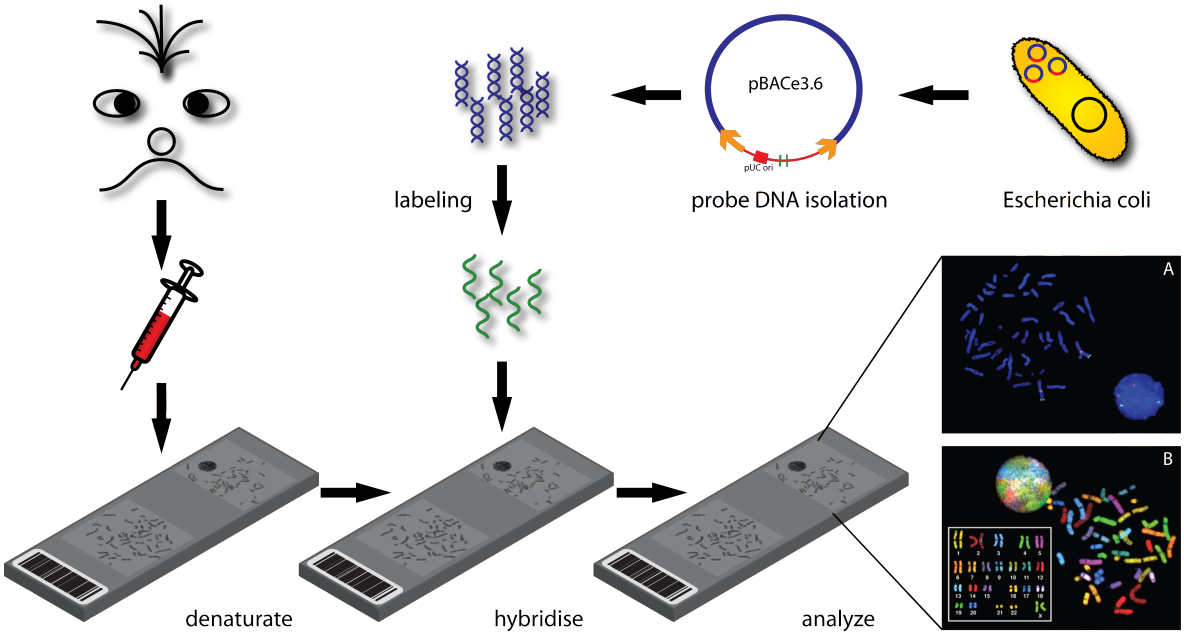


Figure 1.2 – *Principle of FISH: probe DNA is propagated in transfected E. coli, isolated, amplified and labeled either directly or indirectly with respectively a fluorescent reporter or hapten. Labeled probe DNA is subsequently hybridized onto patients' metaphase chromosomes or interphase nuclei. (A) Interphase and metaphase FISH, with probe for 3q telomere (green) and chromosome 3 centromere (red); (B) multi-color FISH, with all chromosomes labeled in a different color*

a discipline. During the late 80s of the previous century, cytogenetics was a technique receiving little attention and enthusiasm from clinical geneticists. This however dramatically changed after the introduction of fluorescent *in situ* hybridization (FISH) which bridged the gap between both disciplines and lead to renewed interest in cytogenetics and in particular in the 'hybrid' specialism: molecular cytogenetics. Although in situ hybridization techniques were already described in 1969 [27, 28], the breakthrough came with the introduction of fluorescent labeled probes in 1982 [29, 30]. Molecular cytogenetics is based on the principle of hybridizing DNA probes to the genome (Figure 1.2). Although initially limited by the availability of specific DNA probes, FISH rapidly evolved as an important technique for the fast and sensitive detection of numerical as well as structural defects both on metaphase chromosomes as interphase nuclei [31–33]. The technique further evolved and several applications emerged such as multiplex-FISH, analyses of subtelomeric regions and comparative genomic hybridization.

Multicolor FISH

By making use of various combinations and concentrations of fluorescent dyes, every chromosome could be visualized in a different color. Three different techniques make use of this principle, spectral karyotyping (SKY) [34], Multiplex-Fluorescence *In Situ* Hybridization (M-FISH) (Figure 1.2.) [35] and COmbined Binary RAtio labeling (COBRA) [36]. These techniques were very helpful in the characterization of complex chromosomal rearrangements often seen in malignancies [37], but also in some patients with complex constitutional rearrangements [38].

Subtelomeric Aberrations

In the mid 1990s, it was noted that the subtelomeric regions of chromosomes are gene-rich and susceptible for chromosomal aberrations [39]. These aberrations, leading to loss of several genes at the tip of the chromosomes, were shown to be an important cause for mental retardation and congenital abnormalities. Using FISH with subtelomeric specific probes, all human telomeres could be investigated. Deletions of these telomeric ends were noted in 3 to 10% of patients with mental retardation and congenital abnormalities [40–42]. Based on the clinical presentation of patients with subtelomeric abnormalities, a checklist for selection of patients was developed. Important parameters seemed to be a familial history of mental retardation and the presence of congenital abnormalities (growth abnormalities, dysmorphic features and/or congenital abnormalities) [43]. These characteristics are often referred to as the 'chromosomal phenotype'. The clinical features of patients with subtelomeric deletions were described, and it was noted that some subtelomeric deletions didn't seem to have any phenotypical effect at all (reviewed in [44], Balikova et al. in press). Although several improvements were made to the FISH-based technique [45–49], the procedure remained very labour intensive and expensive. To obtain faster and cheaper results, molecular strategies like MLPA (multiplex ligation-dependant probe amplification) and MAPH (multiplex amplifiable probe hybridization) were developed for the detection of subtelomeric aberrations [50–53]. Some groups developed subtelomeric arrays [54, 55], enabling a rapid investigation of all telomeres and the direct delineation of the size of telomeric aberrations.

Because of the high incidence of subtelomeric deletions in patients with mental retardation and/or congenital abnormalities (MR/CA), it was anticipated that not only subtelomeric deletions, but also submicroscopic interstitial deletions were an imported cause for MR/CA. Unfortunately, at that time, no whole genome high resolution screening tools were available to investigate this hypothesis.

Comparative Genomic Hybridization

A variant of the FISH technique was developed by the group of Ollie and Anna Kallioniemi, Dan Pinkel and Joe Gray, and the group of Peter Lichter [56,57]. Instead of hybridizing probes to metaphase spreads from the patient, the DNA of the patient and a reference genome are differentially labeled with fluorophores and hybridized onto normal control metaphase spreads. With dedicated software, a fluorescence ratio is calculated along the axis of each of the chromosomes and this ratio is a measurement for the relative copy number status of each region in the DNA of the patient compared to the reference DNA. This technique made it possible to detect chromosomal gains and losses in DNA samples where no karyotype could be obtained (e.g. some tumor samples).

Microarray Comparative Genomic hybridization

In 1997 a variant of CGH was developed [58,59]. Instead of hybridizing the test and reference DNA to normal metaphase spreads, a microarray of DNA probes immobilized on a glass slide is used. The fluorescence ratio of each spot on the array is again a measurement for the relative copy number status of the locus of that particular probe in the test DNA compared to the reference DNA. Despite its relatively early introduction, it took several years before this array CGH methodology became more widely used. This

was mostly due to the restriction in availability of appropriate reporters as well as technical problems related to various aspects of the procedure [60, 61]. Array CGH is now more widely applied due to the availability of large sets of validated clones (eg. BAC sets developed for the Human Genome Project [62]) and the availability of commercial platforms, increasing steadily in density and resolution (BlueGnome, Spectral Genomics). Apart from BAC clone tiling path arrays which attain a resolution up to 75 kb [63, 64], short oligonucleotide sequences are increasingly being used allowing further increase of resolution (Affymetrix, Agilent, Nimblegen). For smaller genomes or specific sequences, arrays have been developed which can even detect single base-pair alterations [65, 66]. This new technique called 'array-based sequencing' makes it possible to resequence whole stretches of DNA for € 1500/Mb.

Closing the Gap

While cytogenetics has evolved to molecular cytogenetics, and array platforms are now available to screen the entire genome for SNP changes [67–69] or deletions down to 6 kb, the gap between cytogenetics and molecular genetics is closing. 'The goal is to do a genome in minutes or seconds for a $ 1000,' says sequencing pioneer Craig Venter (Nature September 2002), and several leading scientists believe that technical innovations will make it possible to achieve this within the next 10 to 15 years [70]. The costs will be insignificant compared to the medical costs that are involved in the diagnosis and treatment of patients with constitutional or somatic genetic aberrations.

Bioinformatics

Starting with the first gene sequenced by the group of Walter Fiers [23], sequence data were exponentially generated worldwide. Consequently, the need for databases to store and explore all these data emerged. Over the years, huge amounts of transcriptome, proteome, microRNAome, methylome, ... data were generated demanding for powerful bioinformatics tools to easily annotate and explore all this information (regulatory sequences, repetitive sequences, homology etc.). Some of these databases as well as software tools are grouped in the 'National Center for Biotechnology Information' (NCBI), the Ensembl and UCSC Genome Bioinformatics group.

With the advent of microarrays and mass-spectrometry, another source of high throughput data came available, with subsequently the need for specific algorithms and tools to store, analyze and interpret these data [71–74].

Bioinformatics encompasses the development and application of computational tools and approaches to acquire, store, organize, archive, analyze, or visualize biological, medical behavioral or health data. Bioinformatics involves the use of applied mathematics, informatics, statistics, computer science, artificial intelligence, chemistry and biochemistry to solve biological problems. Several programming languages were developed or adjusted to address these new needs. BioPerl is a collection of Perl modules that facilitate the development of Perl scripts for bioinformatics applications and has played an essential role in the Human Genome Project. Perl (Practical Extraction and Report Language) was first developed by Larry Wall for data extraction (1987) and is sometimes called: 'The Swiss army knife of programming languages'. A second very popular scripting language in bioinfor-

matics is R. R is a programming language and software environment for statistical computing. Specific tools for bioinformatics are grouped in the open development software project for the analysis and comprehension of genomic data: bioconductor [75]. Whereas Perl was mostly used for genome annotation etc. most R packages in bioconductor are microarray related. A third programming language often used in bioinformatics is PHP (PHP Hypertext Preprocessor), a scripting language originally designed for producing dynamic web pages.

Although PHP is not specifically designed for bioinformatics, it is often used due to its flexible nature and ease of use. Several packages are available for database management, graphical visualizations, mathematical functions, and bioinformatics tools, making PHP a widely used general-purpose scripting language especially suited for web tools (http://www.php.net).
One of the main reasons the Human Genome Project finished earlier than foreseen, was the development of these bioinformatics tools, as well as an enormous improvement in computational power in the last decades.

High Resolution DNA Copy Number Analysis

Introduction

CHROMOSOMAL ABERRATIONS leading to loss or gain of genetic material are an important cause of constitutional genetic disorders. In addition, acquired chromosome changes are a common finding in human malignancies. Standard tools to detect these chromosomal imbalances are classical methods such as karyotyping and fluorescence *in situ* hybridization as indicated above.

Despite the significant improvements made in cytogenetic technologies, a number of limitations are inherent to these methods. First of all, karyotyping requires skilled and experienced laboratory technicians for the delicate procedure of slide preparation and chromosome analysis. A second important limitation is the resolution which is limited to 5 to 10 Mb, at best [16]. Consequently, all chromosome changes detected upon karyotyping implicate large genomic regions encompassing many genes, whereas smaller changes are inevitably overlooked. Moreover, the procedure requires dividing cells, a major drawback of the technique. However, a major advantage of karyotyping remains the fact that in one single experiment the entire genome can be surveyed for gains and losses of genetic material and the possibility to detect balanced chromosomal rearrangements. Fluorescence *in situ* hybridization (FISH) analysis is frequently used in the targeted detection of chromosomal gains and losses. The resolution of FISH is much higher compared to karyotyping and aberrations down to 10 kb can be easily detected [76]. This technique however implies an *a priori* knowledge of the genomic region of interest and only a limited amount of targets can be investigated at the same time. For many recurrent microdeletion syndromes with specific phenotypical characteristics such as the 22q11.2 deletion syndrome, Smith-Magenis syndrome and Williams-Beuren syndrome, FISH has been very helpful in the genetic confirmation of the clinical diagnosis.

Molecular Karyotyping

Comparative genomic hybridization (CGH) allows the genome wide detection of chromosomal gains and losses without the need of mitotic active cells. Like karyotyping however, resolution is limited to 5-10 Mb [77]. Recent technical advances enabled an important improvement of this technique by hybridizing fluorescently labeled test and control genomes to DNA probes immobilized on a glass substrate rather than to metaphase spreads (Figure 1.3). Different libraries of large insert clones (mainly bacterial artificial chromosomes, BACs) were constructed for The Human Genome Project [62]. In initial experiments these large insert clones or alternatively, cDNA clones were used as probes to spot on glass slides and construct the first microarrays for the detection of genomic imbalances [58, 59, 78]. These microarrays were initially used to detect chromosomal amplifications in cancer [79], but after refinement of the technology it became feasible to detect single copy number changes, and microarrays were believed

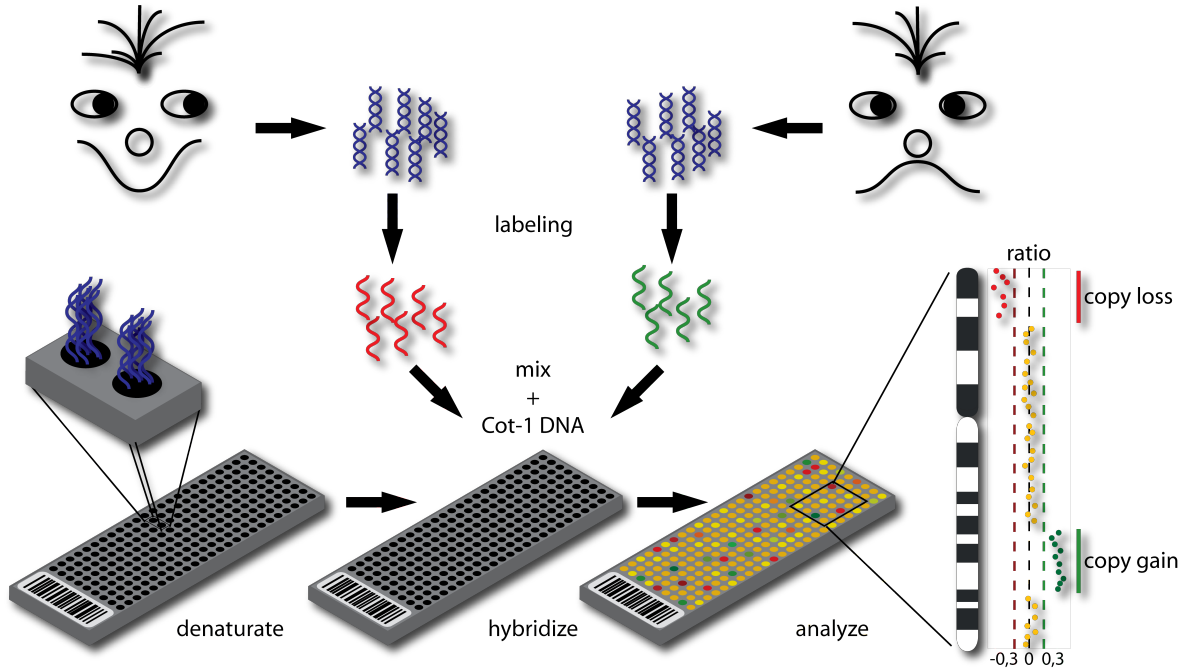


Figure 1.3 – *Principle of array CGH; test and control DNA is differentially labeled with fluorochromes. The DNA is denaturated and mixed with Cot-1 DNA to block repetitive sequences. The mixture is subsequently put on a glass slide with immobilized DNA reporters and hybridized. Fluorescence intensities are measured with a laser scanner and dedicated software.*

to be the ultimate tool in the search for small chromosomal aberrations in human malignancy and constitutional disorders [80–84]. In analogy to conventional karyotyping, the genome wide detection of copy number changes using microarrays was coined 'molecular karyotyping' [61].

Construction of Microarrays

For the construction of CGH microarrays, BAC and PAC clones are propagated in bacteria, purified and then spotted onto a glass slide. As this approach is labor intensive - BACs and PACs are single-copy vectors, yielding only low amounts of DNA - several strategies have been developed to amplify small amounts of cultured and purified DNA. A first technique is degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) [85, 86]. Using partially degenerated primers, all probe DNA is amplified with one primer-mix. This technique was further refined by Fiegler et al. [84] in order to preferentially amplify human DNA and not contaminating *E. coli* DNA, using human specific sequences. A second technique used to this purpose was ligation-mediated PCR [82, 87, 88]. In this method probe DNA is first cut by one or two specific restriction endonucleases followed by attachment of an 'adaptor' to the sticky ends. Finally, all clones are amplified with one single universal primer, complementary to the adaptor. A last technique uses the bacteriophage ϕ29 polymerase for rolling circle amplification. This polymerase can perform strand displacement, proof-reading and isothermal amplification of minute amounts of circular and large fragments of linear DNA with random hexamer primers [89–91].

Resolution

The first mature arrays consisted of a few thousand BAC clones obtaining a resolution of approximately 1 Mb which is an important step forward compared to standard karyotyping [83,84]. As the resolution of array CGH is only dependent on the amount of probes (reporters) spotted on the array and the size of these reporters, further attempts were made to improve the resolution. Tiling path arrays for certain regions and single chromosomes [92] were constructed and subsequently whole genome tiling path BAC arrays were developed [63,64,93]. BAC and PACs have the disadvantage of being relatively large (100-200 kb) limiting the resolution to ˜75 kb [92]. Moreover these large insert clones contain various repeats (ALU repeats, LINEs, SINEs, segmental duplications, ...) which may hamper the analysis if present in high number. Expression arrays using cDNA clones were already described in 1995 [94]. Like BAC/PAC libraries, cDNA libraries are readily available. Moreover, cDNA clones do not contain repeats and allow matching results from DNA copy number analysis with expression studies on the same arrays, which has important advantages compared to BACs [95]. However, as several copy number sensitive regulatory sequences have been identified outside of coding regions [96–98], cDNA arrays will not detect changes implicating these particular regions. Moreover, the signal-to-noise ratio tends to be lower for cDNA arrays compared to BAC arrays, an important drawback which has precluded widespread use of these arrays. The Sanger Institute, trailblazers in array CGH, used the smaller cosmids and fosmids as alternative reporters to BACs enabling a resolution down to 35 kb. Although this technique is well suited for smaller, custom arrays, whole genome analysis would require the culture and subsequent amplification of 150 000 genomic clones, which is logistically extremely demanding. Other groups have started using PCR-based arrays, achieving very high resolutions with repeat-free and non-redundant sequences [99, 100]. Constructing whole genome PCR-arrays requires the design and validation of hundreds of thousands of PCR primers, implicating the need for bioinformatics tools and PCR automation. Moreover, the high cost of primers makes this technique not feasible for a standard genetic laboratory. Alternatively, synthesised oligonucleotides have been used [101–105]. The 'in house' production of such arrays is far from evident and fortunately several companies such as Affymetrix, Nimblegen and Agilent now offer oligonucleotide arrays on a commercial basis. New innovative spotting techniques or even *in situ* syntheses of the oligonucleotides make it possible to obtain up to 385 000 reporters on one microscope slide and hence a theoretical whole genome resolution down to 6 kb (Nimblegen). The practical resolution using these arrays remains however between 40-60 kb due to the need of moving reading windows averaging the probe ratios. Due to repeats in the genome, some regions are less well represented by probes while gene-rich regions are more densely covered with reporters thus providing a higher resolution. Such oligo-arrays can also be used for custom projects in order to interrogate a smaller genomic region rather than the entire genome. Using tiling oligonucleotide arrays, it is even possible to (re)sequence stretches of DNA and hence achieve a resolution up to 1 bp [65, 66]. Some oligonucleotide arrays were first developed for whole genome genotyping experiments with the use of single nucleotide polymorphism (SNP) specific oligonucleotides, but later turned out to be also usable for whole genome copy number analysis (Affymetrix). The advantage of these

arrays is that not only copy number alterations can be investigated, but also uniparental disomy can be detected. These high resolution whole genome DNA copy number analyses have not only provided us a lot of clinical information, but also have revealed a new kind of genomic variation.

Human Genomic Variation

Two unrelated individuals share about 99.9% of their DNA sequences, resulting in a 3 million basepair difference. As the human genome sequence was constructed using mostly DNA from one single individual, the finalization of the human genome sequence in itself did not provide full insight into the extent of this variation. In order to achieve this particular goal, the International HapMap Project, was initiated. This project aims to describe the common patterns of genetic variation, providing a key resource for researchers to find genes affecting health, disease and responses to drugs and environmental factors. Most studies of human genetic variation have focused on single nucleotide polymorphisms (SNPs). Genetic variation can range however from these single nucleotide changes to chromosomal segments extending up to 10 Mb. Recent studies have used high resolution DNA copy number analysis techniques to investigate these larger genetic variations in the population [106]. A total of 1447 copy number variable regions (CNVRs), covering 360 Mb (12% of the entire human genome) were identified. These CNVRs encompass more nucleotides than SNPs identified thus far, underscoring the importance of CNV in genetic diversity and evolution.

Non Allelic Homologous Recombination (NAHR)

Genetic variation is generated by only a limited number of mutational processes. An overview

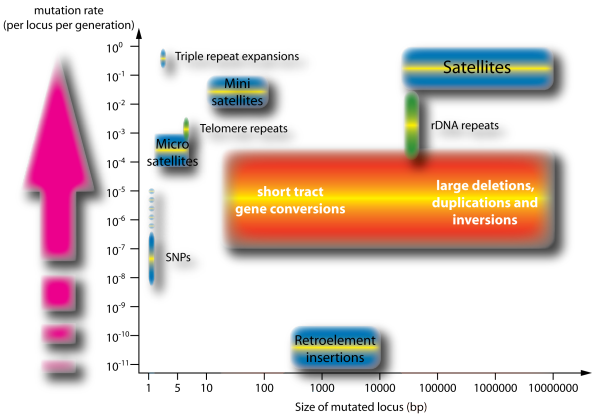


Figure 1.4 – *Mutation processes in the human genome, NAHR (non allelic homologous recombination), SNP (single nucleotide polymorphism)(adapted from www.sanger.ac.uk)*

of these processes is given in figure 1.4.

Diploid species have a significant evolutionary advantage through a process that is known as 'homologous recombination' (HR). Homologous recombination can result in crossover between homologous chromosomes or in gene conversion. Crossing over was first described by Thomas Morgan, and involves the process by which two homologous chromosomes, paired up during meiosis, exchange parts of their DNA. Crossover occurs when homologous sequences, break and then reconnect but to the different end piece. During the process of HR, a Holliday junction is formed, and subsequently genomic material is exchanged. If they break at the same place or locus in the sequence of base pairs, the result is an exchange of chromosomal material between both parental chromosomes. The outcome is the normal crossing over product generating genetic variation. This process is however error prone. Due to sequence homology between repeats, the chromatids may not line up exactly with its corresponding region, leading to unequal crossing over. Such errors are believed to be responsible for the formation of VNTRs (variable number of tandem repeats) occurring through-

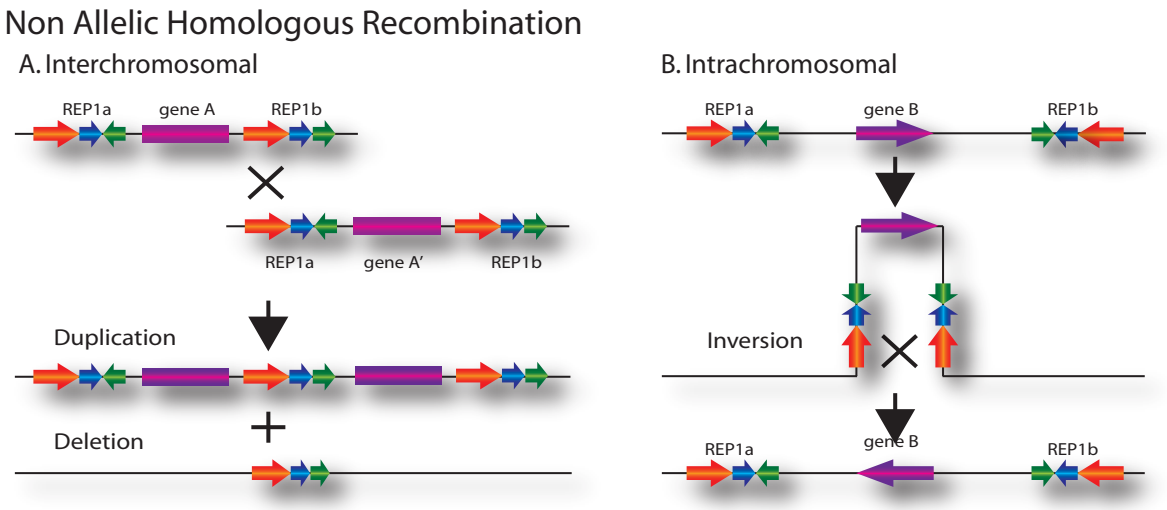


Figure 1.5 – *Principle of NAHR (non allelic homologous recombination)*

out the genome [107]. If the repeat regions are larger in size, they are called Low Copy Repeat elements, or Segmental Duplications [108–110]. Crossing over occurring between such LCRs may result in the duplication of genes on one chromosome and the deletion of these on the other, leading to so called 'genomic disorders'. This process is known as non allelic homologous recombination (NAHR) (Figure 1.5) [111–115]

Nonhomologous End-Joining (NHEJ)

Another mechanism involved in genomic rearrangements is Nonhomologous End-Joining (NHEJ). This mechanism is much less precise, and is held responsible for the various translocations that are associated with cancers, or with non-recurrent microdeletions and translocations [115, 116]. NHEJ is the main pathway for repairing double-stranded DNA breaks in G0, G1 or early S phases of the cell cycle. Some of the enzymes involved in direct joining or NHEJ, are also used to accomplish V(D)J joining for the antibody variable regions [116, 117].

Mental Retardation and Congenital Abnormalities

Introduction

MENTAL RETARDATION occurs in 2-3% of the general population (The Arc 2004, [118–120]) and is defined by the American Association on Intellectual and Developmental Disabilities (AAIDD) as follows:

> 'Mental retardation is a disability characterized by significant limitations both in intellectual functioning and in adaptive behavior as expressed in conceptual, social, and practical adaptive skills. This disability originates before age 18.' (AAMR, 2002)

Intellectual functioning refers to a general mental capability, and intelligence is mostly defined by standardized individually administered tests that are adjusted for age, socio-economical background and result in an intelligence quotient (IQ). Adaptive functioning refers to how effectively individuals cope with everyday life demands. Consideration should be given to the suitability of the instruments to the subject's ethnic and cultural background, education, motivation, cooperation and associated handicaps [121]. Often the term 'developmental delay' or 'developmental disability' is used in stead of 'mental retardation', because the latter one has a negative connotation. According to the Developmental Disabilities Act (Pub. L. 106-402), the term developmental disability means a severe, chronic disability that:

1. is attributed to a mental or physical impairment or a combination of those impairments;
2. occurs before the individual reaches the age of 22;
3. is likely to continue indefinitely
4. results in substantial functional limitations in three or more of the following areas of major life activities; (i) self care, (ii) receptive and expressive language, (iii) learning, (iv) mobility, (v) self-direction, (vi) capacity for independent living, and (vii) economic self-sufficiency; and
5. reflects the individual's need for a combination and sequence of special, interdisciplinary, or generic services, individualized supports, or other forms of assistance that are a long life or extended duration and are individually planned and coordinated.

Mental retardation can occur as an isolated developmental disorder or in combination with other congenital malformations. These associated abnormalities of normal human morphogenesis may express themselves as subtle dysmorphic signs not causing any physical handicap or present as severe disabling and life-threatening malformations such as congenital heart defects.

Diagnosis

The diagnosis of mental retardation requires the evaluation of the person's mental capabilities (IQ) and adaptive skills. The ability to learn, think, reason, live independently and interact or function in society will be ascertained. Intellectual functioning, or IQ, is usually measured by

standardized IQ tests and is usually divided in a non-verbal and verbal IQ. To measure adaptive behavior, professionals look at what a child can do in comparison to other children of his or her age. The most important skills are: daily living skills, such as getting dressed, going to the bathroom, and feeding one's self; communication skills, such as understanding what is said and being able to answer; and social skills with peers, family members, adults, and others. Because standardized IQ tests can not be performed with young children, children are evaluated by assessing the developmental milestones such as the time of laughing, grasping, sitting unsupported, and independent walking [121–124]. The diagnosis of mental retardation usually requires a multidisciplinary approach with involvement of general pediatricians, pediatric neurologists, clinical geneticists, psychologists and developmental therapists. Taking a personal and family history (pedigree) and performing a careful clinical evaluation are essential in the diagnostic process. Neuro-imaging studies as well as electroencephalogram (EEG) recordings and metabolic studies may be important additional investigations. Cytogenetic analysis should be performed in each child with an unexplained form of mental retardation [121–124].

Classification

Mental retardation varies in severity, and the World Health Organization proposed to make a subdivision based on the intelligence quotient (IQ). The definition and classification of mental retardation has changed substantially over the past years, and the latest, published in the International Classification of Diseases 10th revision (ICD-10) [125], is based on the Wechsler Adult Intelligence Scale (WAIS) (Table 1.1). A very similar classification is the one from the American Psychiatric Association, published in the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) [126]. The AAIDD classification system focuses more on the capabilities of the retarded individual than on the limitations. The categories describe the level of support required (intermittent support, limited support, extensive support, and pervasive support). This classification however mirrors the ICD-10 and DSM-IV classification. Sometimes a fifth category is added: borderline mental retardation, with IQ scores ranging between 70 and 80. The majority of patients with mental retardation (up to 85%) have mild MR. About 10% of the mentally retarded population is considered moderately retarded, and only ~5% is classified as severely or profoundly retarded [119,123]. Congenital malformations and dysmorphic signs are usually found in the latter two groups.

Table 1.1 – *MR-classification according ICD-10 (World Health Organization, 1994)*

Classification	IQ-score
Mild MR	50-70
Moderate MR	35-50
Severe MR	20-35
Profound MR	0-20

Etiology

The etiology and pathogenesis of mental retardation is still incompletely understood. Although there is no cure for mental retardation, a correct diagnosis with identification of the underlying cause is important for several reasons. The diagnosis is the cornerstone for accurate genetic counseling. It also allows better management and estimation of the prognosis. Early

recognition of the disorder may make preventive measures possible and additional expensive testing and investigations unnecessary. It usually brings sort of relief to the parents and helps them to understand and accept the disabilities of their affected child [121]. Mental retardation may be caused by genetic alterations (mutations), environmental factors or a combination of both. Unfortunately, in at least one-third of all patients, no cause can be identified, even after thorough diagnostic evaluation [119]. Significant advances in laboratory testing over the last two decades have led to an important improvement in the diagnostic yield. Genetic abnormalities are by far the most commonly recognized cause for MR, and can be detected in about 1/3 of patients [127]. Trisomy 21 is the most frequent genetic disorder in this group of patients [119, 127]. Other (non-genetic) causes include prenatal infections, perinatal asphyxia and exposure to teratogenic agents during pregnancy [127].

Non-genetic or acquired causes of mental retardation

In this category three different causes can be distinguished:

Prenatal causes: Maternal infections such as rubella, toxoplasmosis, and cytomegalovirus may be transmitted to the developing fetus, causing mental retardation and congenital abnormalities. High blood pressure (hypertension) or blood poisoning (toxaemia) in the mother can lead to a reduced oxygen flow to the fetus, causing brain damage and subsequently mental retardation. Also drug abuse (e.g. alcohol) and medications (e.g. valproate) taken by the mother can result in mental retardation [127, 128].

Perinatal causes: Perinatal complications leading to oxygen deprivation or brain hemorrhage can have a deleterious effect on the developing brain with mental retardation and cerebral palsy as a result. Prematurity is a well recognized risk factor; premature born babies are at higher risk for exhibiting developmental delay.

Postnatal causes: Infections or injuries of the central nervous system may result in mental retardation. Mental retardation can be a late-occurring sequel of fulminant meningitis and encephalitis. Generalized sepsis also puts the affected infant at risk for developing brain damage and consequently mental retardation. Children living in poverty are at higher risk due to malnutrition, unhealthy living conditions, and improper and inadequate health care. Toxic agents such as lead poisoning and pesticide exposure (organophosphates, carbamates, and pyrethroids) are well known factors causing mental retardation. Unfortunately, child abuse still occurs and may result in mental retardation if brain damage takes place in the developing child.

Genetic Causes of Mental Retardation

Genetic abnormalities that give rise to mental retardation are often divided into three broad categories: chromosomal aberrations, single-gene disorders and multifactorial disorders. This subdivision is somewhat arbitrary since chromosomal aberrations usually involve one or more genes in the deleted/duplicated chromosomal region and therefore could be considered as either monogenic or polygenic in nature. The phenotypic abnormalities may be due to a gene dosage imbalance of several genes or to the pleiotropic effect of one single gene. A good example is the CHARGE syndrome (Coloboma

of the eye, central nervous system anomalies, Heart defects, Atresia of the choanae, Retardation of growth and development, Genital and/or urinary defects, Ear anomalies and/or deafness), a condition initially considered to represent a chromosomal aberration. Researchers worldwide were searching for the causal microdeletion. However after the identification of a microdeletion through high resolution DNA copy number analysis in two CHARGE patients, it turned out that most CHARGE patients had a mutation in a single gene (*CHD7*) located within the deleted interval [129]. CHARGE syndrome represents a typical example of pleiotropy of a single gene defect.

Numerical chromosomal aberrations Most numerical chromosomal aberrations are embryonically lethal resulting in early fetal loss. Fetuses with autosomal trisomies for chromosome 13, 18 and 21 can however survive until birth [130]. Neonates with trisomy 13 and 18 typically die shortly after birth due to severe congenital malformations of the internal organs. Life expectancy of children with trisomy 21 has increased enormously due to a better patient management. Down syndrome or trisomy 21, has an incidence of about 1/800 in live born children, representing the most frequent genetic cause of mental retardation [131]. Down syndrome is characterized by several major and minor anomalies. Most individuals with Down syndrome have mild to moderate mental retardation. The most distinct and recognizable craniofacial features are the flat face with upslanting palpebral fissures with epicanthical folds, the flat nasal bridge, protruding tongue, small ears and redundant skin folds in the neck. Affected infants are usually hypotonic after birth. They have short hands with often a single transversal palmer fold (simian crease). Congenital heart defects are frequently present and hence a thorough heart examination should be performed in every infant diagnosed with Down syndrome. Children with Down syndrome have a higher-than-average risk for acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML) [132,133] and adults with Down syndrome usually develop neuropathological changes typical of Alzheimer's disease by the age of 40 years [134] (reviewed in [135]). Although trisomy 21 was the first recognized chromosomal disorder, thus far only a few genes contributing to specific phenotypic features have been identified [136]. By comparing many individuals with partial trisomy 21, critical regions were defined [137,138]. However, the definition of these regions has been controversial as there are patients with partial triplications outside this region who, nevertheless, manifest some features of Down syndrome [139,140].
Based on transgenic mouse studies, APP, a gene coding for the amyloid precursor protein, has been suspected to play a major role in the cognitive deficits. Overexpression of another gene, the Avian Erythroblastosis Virus E26 Oncogene Homolog 2 (*ETS2*) has been demonstrated to play a role in apoptosis [141]. Transgenic mice overexpressing ets2 developed a smaller thymus and lymphocyte abnormalities, characteristics often observed in patients with Down syndrome [142].

Contiguous Gene Disorders Segmental chromosomal changes can lead to gene dosage imbalances causing mental retardation or congenital abnormalities [115,143]. If indeed several genes are involved in the chromosomal aberration the term contiguous gene syndrome as proposed by Schmickel is often used [20]. For some recurrent chromosomal aberrations, (some of) the genes contributing to major phenotypical de-

fects have been elucidated (Tabel 1.2). As already indicated, a functional annotation of the genome is of utmost importance as it provides insights into the function of genes in normal development. For a number of syndromes, the link between the associated phenotypic features or at least one of several major manifestations of the syndrome is evident. This is the case for example for the *LIS1* gene. Loss of function mutations in this gene cause lissencephaly (agyria-pachygyria), which is an important component of the Miller Dieker syndrome [144]. Another well known example is the retinoblastoma gene, which upon deletion infers a great risk for development for the embryonal eye tumor in such patients [145–147]. For most of the syndromes listed in Table 1.2 however, certain aspects of the phenotype still remain to be explained, i.e. the genes contributing to the malformation, congenital defect or mental retardation remain to be identified. Mouse models can be instrumental in this respect although they have their specific limitation e.g. in regard to facial dysmorphism or simply due to important differences in certain aspects of developmental control between humans and mouse. Moreover, it can be anticipated that haploinsufficiency of certain genes may influence expression of genes located on other chromosomal segments not altered by the chromosomal defect [136]. Inversely, the phenotypic expression (penetrance) of a given microdeletion syndrome may be altered through the effect of so-called modifiers, as illustrated for the VCFS syndrome for which expression and severity seems to be modulated by the *VEGF* gene [148].

New Microdeletion/Microduplication Syndromes
With the implementation of microarrays in clinical cytogenetics, recently several new microdeletion/duplication syndromes have been identified. The Sanger Institute, the Nijmegen group and the group of Evan Eichler in Seattle reported a new recurrent microdeletion syndrome on the long arm of chromosome 17 [149–151]. Notably, these 17q21.31 deletions are located within a genomic region that is known to harbor a common 900-kb inversion polymorphism that suppresses recombination between two ancestral H1 and H2 haplotypes. The H2 lineage, representing the 900-kb inversion polymorphism, is found at a frequency of 20% in Caucasians and can be distinguished from the H1 lineage by a characteristic 238-bp deletion in intron nine of the MAPT gene. These H1 an H2 haplotypes are flanked by Low Copy Repeat (LCR) elements and the orientation of these LCRs is likely to facilitate the generation of this microdeletion by means of non-allelic homologous recombination [149, 150]. A very similar mechanism was already described for the 22q11.2 deletion syndrome (Figure 1.6) [152], Smith-Magenis syndrome [153,154] and Williams-Beuren syndrome [155]. These LCRs can mediate both deletion and duplication events as the expected result from uneven meiotic crossing over and previously illustrated by the deletion and duplication

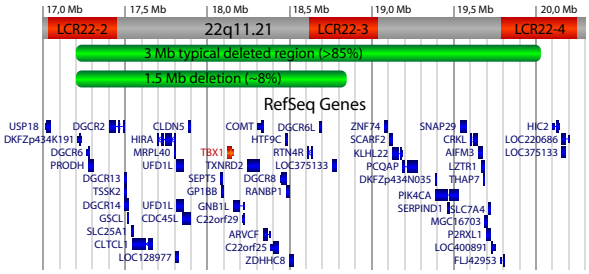


Figure 1.6 – *Del 22q11.2 region on chromosome 22, refseq genes are shown in blue, low copy repeats in red. The 3 Mb typical deleted region which is present in more than 85% of patients with del 22q11.2 syndrome and the 1,5 Mb deleted region are shown in green. The TBX1 gene is shown in red (adapted from [152])*

Table 1.2 – *Contiguous gene syndromes, locus and genes(s) involved*

Syndrome	Locus	Gene(s)
Wolf-Hirschhorn syndrome	4pter	*WHSC1, MSX1*
Williams-Beuren syndrome	7q11.23	*ELN, LIMK1*
8p23.1 deletion syndrome	8p23.1	*GATA4*
Langer-Giedion	8q24.11–q24.13	*EXT1, TRPS1*
WAGR	11p13	*WT1, PAX6*
Prader-Willi/Angelman	15q11-q13	*SNRPN, NDN, UBE3A*
Rubinstein-Taybi	16p13.3	*CREBBP*
Smith-Magenis	17p11.2	*RAI1*
Miller-Dieker	17p13.3	*LIS1*
NF1-microdeletion syndrome	17p	*NF1*
Alagille	20p12	*JAG1*
22q11.2 deletion	22q11.2	*TBX1*

of 17p12 leading to Charcot-Marie-Tooth and HNPP, respectively [156–158]. Recently, the reciprocal of the 7q11.23 deletion (ie the duplication) in Williams-Beuren syndrome was noted in some patients with pronounced speech delay, mental retardation and short stature [159]. Another similar emerging syndrome is the 22q11.2 duplication syndrome [160].

Although it is clear that genomic architectural factors like LCRs play a very important role in the formation of deletions and duplications, some new recurrent microdeletion syndromes have been described without LCRs at or near the breakpoints. A good example is the newly emerging 1p36 deletion syndrome, with an estimated frequency of 1/5000 - 1/10 000 [161, 162]. 1p36 deletion breakpoints are scattered across the 1p36 region, and although most deletions extend up to the telomere, some interstitial deletions have been described as well [163, 164]. Another example is the recently delineated 12q14 microdeletion syndrome discussed in this thesis (paper 4 [165]). Other genomic architectural factors than LCRs are believed to play a role in these 'genomic disorders', but the exact nature of these sequences and mechanisms remain to be elucidated.

Single-Gene Disorders Single-gene or monogenic disorders are caused by mutations in one single gene. Single-gene defects can give rise to a wide variety of diseases or syndromes. Since many genes control normal brain development and normal neuron function, single-gene disorders are also an important cause of mental retardation. Distinction is made between syndromic and non-syndromic (or non-specific) forms of mental retardation. Mental retardation can be categorized as syndromic if it is associated with other physical anomalies that can range from subtle dysmorphic signs to gross malformations of internal organs. The difference between syndromic and non-syndromic mental retardation is not always that obvious. Several disorders have been first described as non-syndromic conditions and have only later been recognized as syndromic disorders because of subtle but distinct clinical features, associated biochemical abnormalities, or specific MRI findings (e.g. oligophrenin 1 mutations (paper 3 [166]). Moreover, different mutations in the same gene may have other phenotyp-

ical consequences, with one mutation associated with nonspecific mental retardation, and another with syndromic MR [167]. The most common single-gene disorder causing mental retardation is the Fragile X syndrome [168].

X-linked Mental Retardation Mental retardation has a male/female ratio of ˜1,4/1 [169–171]. The most likely explanation is the mandatory hemizygosity for the X- chromosome in males. This high ratio has however also lead certain authors suggest that the X-chromosome harbors more genes involved in brain functioning in comparison to the autosomes [172, 173]. Linkage analyses in families with X-linked mental retardation have been proven successful in the identification of the causal genes. By combining these data from many different families in a world wide consortium, regions could be diminished and identification of the causal genes through mutation analysis was made possible [174]. Also structural chromosomal abnormalities have been very helpful in the identification of genes involved in mental retardation [175–178]. Molecular genetic studies can by carried out to identify the disrupted or deleted genes (paper 3 [166]). Since the publication and annotation of the Human Genome Sequence, candidate MR-genes could be identified based on structure, homology and functional domains. At least 140 different forms of syndromic X-linked mental retardation have been described so far and in 66 of these, the causative genetic defect has been identified [174]. Likewise, 22 genes that play a role in non-specific X-linked mental retardation have been identified [179] (Figure 1.7). It is interesting to note that also non-protein-coding genes may play a role in X-linked mental retardation. Examination of the Sanger database of microRNAs reveals that a cluster of microRNAs maps to the Xp11.2-11.3 region, a region that

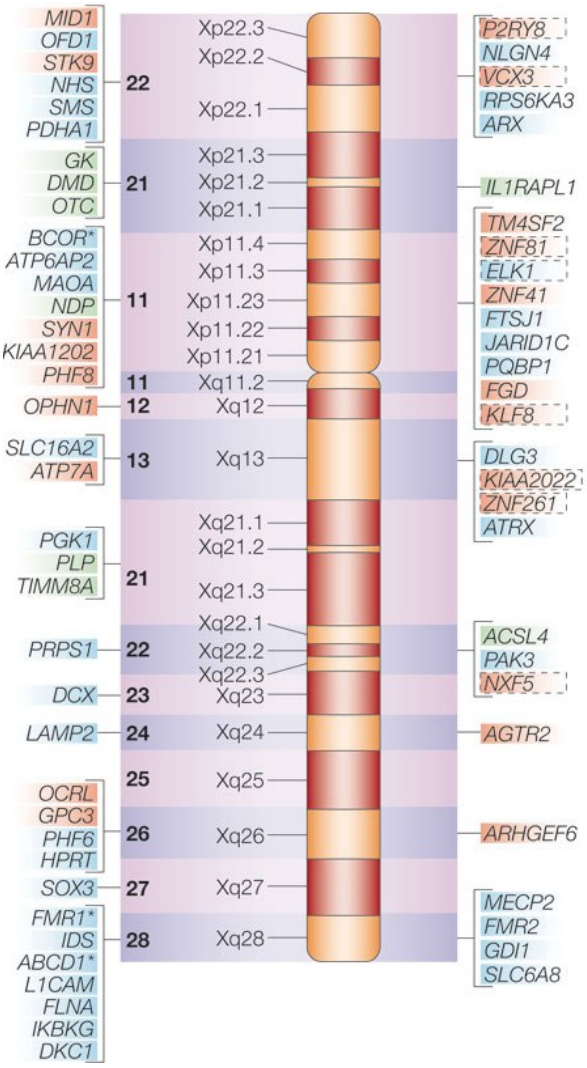


Figure 1.7 – *XLMR genes that are identified by studying balanced X-chromosome rearrangements and deletions (or duplications) are in red and green boxes, respectively; XLMR genes identified by mutation screening are in blue boxes. Boxes that have a dotted outline indicate candidate genes, the status of which is still not confirmed. Genes that are implicated in NS-XLMR are shown on the right. (Taken from [174])*

is implicated in 30% of all families with X-linked mental retardation [180].

Inborn Errors of Metabolism Metabolic disorders are another possible monogenic cause of mental retardation. Over 350 inborn errors of

metabolism have been identified, most of which lead to mental retardation [181]. They usually show autosomal recessive inheritance. In some cases (e.g. phenylketonuria (PKU), galactosemia and congenital hypothyroidism), retardation is preventable with early treatment. All babies in Belgium are tested for these conditions shortly after birth through a simple heel prick. Early treatment can prevent the development of mental retardation in the affected children.

Multifactorial or Complex Inheritance Multifactorial disorders are caused by a combination of multiple factors like genes and environmental factors. It is highly likely that complex inheritance plays an important role in the etiology of mental retardation. In the publication 'The Biology of Mental Defect', Lionel Penrose wrote in 1972: 'the type of inheritance most commonly observed in human genetic material is due to the combined actions of more than one gene. Indeed the number of genes involved can be very large' [182]. In genetically complex disorders, variation at specific gene loci leads to an increased risk for developing these disorders. Identification of genes involved in multi-factorial disorders is very complicated due to the complex inheritance patterns and the influences of environmental parameters. Variable penetrance, overlapping phenotypes and the multitude of possible targets are all factors hampering the identification of the underlying genes. Recent technical developments have facilitated genome wide association studies. The goal of these studies is to link a certain genomic variant at a specific chromosomal position to a certain condition. With the advent of these whole genome wide association techniques (Illumina, Affymetrix), geneticists try to identify loci involved in common and complex diseases like mental retardation [183].

Idiopathic Mental Retardation In up to 50% of patients with mental retardation, no cause can be identified [127, 184]. It is believed that genetic aberrations are the major factor in these patients as well. Probably the underlying mechanism in these patients is multifactorial or polygenic. However also epigenetic modifications such as aberrant methylation can be involved [185]. Mental retardation is often a specific and probably many hundreds or even thousands of genes may lead to mental retardation if inactivated.

Cytogenetic and Molecular Cytogenetic Investigations

Chromosomal aberrations are reported in 4 up to 34% of individuals with mental retardation [122, 127, 186], and hence, chromosome analysis is an essential test in patients with idiopathic mental retardation. When a specific syndrome is suspected, targeted genetic or cytogenetic analysis can be performed (eg. *FMR1* investigations in patients suspected to have fragile-X syndrome, FISH analysis for suspected 22q11.2 deletion syndrome, *MECP2* investigations in patients with suspected Rett syndrome etc).
Chromosomal investigations have been proven to have the highest diagnostic yield in patients with mental retardation and (multiple) congenital abnormalities, hence the term 'chromosomal phenotype' is often used for such patients. As discussed earlier, the coincidence of several disorders (mental retardation and congenital abnormalities) may point to the involvement of several genes and hence to a contiguous gene syndrome. With the advent of high resolution DNA copy number analysis tools as discussed in this thesis, the diagnostic yield of molecular cytogenetic investigations in these patients may improve even significantly.

Research Objectives

Objective 1: The identification of submicroscopic genomic imbalances in patients with unexplained mental retardation and congenital abnormalities

The major aim of this thesis was to identify genomic imbalances in patients with unexplained mental retardation and congenital abnormalities. To this purpose we have introduced the innovative array CGH technology which allows detection of DNA copy number changes with much higher resolution than previous whole genome cytogenetic methods. Several patients with unexplained mental retardation and congenital abnormalities were investigated with array CGH for the identification of small DNA copy number changes (paper 1).

Objective 2: The study of the incidence and genomic distribution of submicroscopic genomic imbalances in patients with idiopathic MR/CA

To achieve this goal, DNA samples from a large cohort of clinically well selected patients with unexplained mental retardation and congenital abnormalities were screened for submicroscopic chromosomal aberrations. The results of this screening, together with review of literature data, lead to the first comprehensive overview of the incidence and genomic distribution of chromosomal imbalances in patients with idiopathic mental retardation (paper 2).

Objective 3: Identification and characterization of a new chromosomal microdeletion syndrome

The identification and registration of new submicroscopic chromosomal aberrations and the detailed clinical characterization of affected patients as indicated in objectives 1 and 2, has lead to the identification of a new recurrent microdeletion syndrome (paper 3). In addition, array CGH analysis has lead to delineation of a 1.8 Mb critical interval on chromosome band 18q12.3 for the known del(18)(q12.1q21) deletion syndrome (paper 4).

Objective 4: Identification of genes involved in mental retardation and/or congenital abnormalities

In the fourth part of this thesis, array CGH in combination with standard molecular methods was used in order to identify genes involved in mental retardation and/or congenital abnormalities. By using array painting on an apparently balanced t(X;9) translocation in a patient with mental retardation and overgrowth, we were able to identify *OPHN1* as the causal gene for the observed phenotypical abnormalities (paper 5).

Objective 5: Development of a versatile analysis platform for high resolution DNA copy number analysis

At the time of implementation of the array CGH technology in our laboratory, only a few tools for array CGH analysis were available. Since all of these tools had important shortcomings, we decided to develop a dedicated analysis platform which met all our needs for data storage, analysis and visualization of high resolution DNA copy number analysis (paper 6).

These research objectives will lead to a better understanding of the causes of mental retardation and human malformation and will contribute to identification of genes involved in development of the brain and other organs.

chapter 2

ArrayCGH in Clinical Diagnostics

Contents

paper 1: Identification of an unbalanced X-autosome translocation by array CGH in a boy with a syndromic form of chondrodysplasia punctata brachytelephalangic type

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Original article

Identification of an unbalanced X-autosome translocation by array CGH in a boy with a syndromic form of chondrodysplasia punctata brachytelephalangic type

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Abstract

Screening of a large series of patients with unexplained mental retardation with a 1 Mb BAC array resulted in the detection of several cryptic chromosomal imbalances. In this paper we present the findings of array CGH screening in a 14-year-old boy with the brachytelephalangic type of chondrodysplasia punctata, mental retardation and obesity. On several occasions, cytogenetic analysis of this boy revealed a normal karyotype. Subsequent screening with array CGH resulted in the detection of a distal 9p trisomy and distal Xp nullisomy caused by an unbalanced X;9 translocation: 46,Y,der(X)t(X;9)(p22.32;p23). The identification of this de novo chromosomal rearrangement not only made accurate genetic counselling possible but also explained most of the phenotypic abnormalities observed in this patient. This study confirms the power of array CGH in the detection of subtle or submicroscopic chromosomal changes.

Keywords: Chondrodysplasia punctata brachytelephalangic type; Obesity; Duplication 9p; Xp deletion; X-autosome translocation; Array CGH

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1. Introduction

Mental retardation occurs in 1–3% of the general population [1]. Determining the cause and establishing the correct diagnosis is usually challenging but essential for accurate genetic counselling [23]. Chromosomal aberrations represent an important cause of mental retardation. In these situations, the mental retardation is often associated with facial dysmorphism and congenital abnormalities. Technological improvements in cytogenetic analysis such as the introduction of fluorescent in situ hybridisation (FISH) and the availability of new molecular methods (marker analysis, Multiplex Ligation-dependent Probe Amplification) have lead to the detection of new chromosomal imbalances including microdeletions and subtelomeric deletions [4,17,21]. Although these molecular approaches increase the resolution of chromosomal investigation, they do not allow a systematic analysis of the whole genome.

Comparative genomic hybridisation (CGH) was introduced as an alternative whole genome screening method to karyotyping [3,11]. Although a high resolution method was developed, CGH still suffered from a limited resolution due to the use of chromosomes as reporters for the detection of the imbalances [12]. This limitation was overcome by applying basically the same technique to arrays of spotted DNA clones (e.g. BAC clones, oligonucleotides or cDNA clones) [5,16,22]. More recently SNP chips, originally introduced for SNP analysis and haplotype mapping, were also shown to be powerful analytical tools for detection of DNA copy number alterations [10]. Array CGH or SNP chips now offer a resolution of ~1 Mb or even higher.

Recent studies have shown that, depending upon the selection criteria, in 10–15% of the patients new submicroscopic deletions or duplications can be detected by array CGH [20,25]. In order to further explore the spectrum of chromosomal defects occurring in patients with "idiopathic" mental retardation, we initiated a collaborative effort to investigate a series of 100 patients. Details of this study will be presented elsewhere (Maas et al., in preparation). Here we report the identification of an unbalanced X-autosome translocation in a boy with mental retardation, obesity, short stature and brachydactyly.

2. Material and methods

2.1. Cytogenetic analysis and FISH

Analysis of G-banded metaphase chromosomes was performed on short-term lymphocyte cultures using standard procedures. FISH was performed as described [24]. Probes used were: subtelomeric probes for chromosome 9 and X, a cosmid clone (34F5) containing the *SHOX* gene, a chromosome X centromere specific probe and painting probe and selected BAC clones for distal 9p (RP11-48M17, RP11-106A1, RP11-352F21, RP11-187K14).

2.2. Array CGH analysis

DNA was isolated from total blood using the Puregene Genomic DNA Purification Kit (Gentra Systems), according to the manufacturer's instructions. Using random prime label-

ling, 500 ng of patient and control female DNA was labelled with Cy3 and Cy5 (BioPrime Array-CGH Genomic Labelling System, Invitrogen). The labelled fragments were suppressed with 150 μg fluorometric QC Cot-1 DNA (Invitrogen) and 400 μg yeast tRNA, and resuspended in 60 μl hybridisation buffer at 37 °C (50% formamide, 10% dextran sulphate, 0.1% Tween 20, 2× SSC, 10 mM Tris pH 7.4). In house produced 1 Mb resolution BAC arrays consisting of 3431 clones spotted in triplicate on CodeLink Activated slides (Amersham Biosciences) were prehybridised at 37 °C during 1 h using 100 μg fluorometric QC Cot-1 DNA (Invitrogen) and 150 μg herring sperm DNA, resuspended in 120 μl hybridisation buffer. After removal of the prehybridisation mixture, patient and control DNA was simultaneously hybridised for 48 h at 37 °C. The slides were washed in 1× PBS/0.05% Tween 20 for 10 min at room temperature, 50% formamide/2× SSC for 30 min at 42 °C and finally 1× PBS/0.05% Tween 20 for 10 min at room temperature. After centrifuge drying, the slides were scanned using a GMS 418 Array Scanner (MWG). The scan images were processed with Imagene software (Biodiscovery) and further analysed with our in house developed and freely available software tool arrayCGHbase (http://medgen.ugent.be/arraycghbase/) [14]. Reporters were excluded from analysis if one of the following criteria were fulfilled: signal to noise ratio < 3; standard deviation of the $\log_2$ transformed ratios between triplicates > 0.2; and only one informative replicate.

3. Case report

The proband was born at term after an uncomplicated pregnancy. Birth weight was 3250 g, length 48 cm and head circumference 34.5 cm. He is the youngest son of healthy, non-consanguineous parents. The family history is unremarkable. The perinatal course was uneventful. At the age of 1 month, cytogenetic analysis was performed because of hypotonia, facial dysmorphism with small, deeply set nose, and short limbs with brachydactyly. A normal male karyotype was found. Because of persistent hypotonia and growth failure, he was re-evaluated at the age of 3 months. Echocardiogram, MRI brain, hearing tests and an ophthalmological evaluation all revealed normal findings. He started walking at 14 months. Excessive weight gain was observed around the age of 3 years. At the age of 5 years he was referred to the genetic outpatient clinic because of short stature, obesity and mental retardation. He was following special education school because of moderate mental retardation (full scale IQ 58). Verbal performance was more impaired than perceptual and motor function. His language development was delayed and his speech was very difficult to understand due to poor language skills and articulation problems. Physical examination at the age of 5 years revealed a weight of 22 kg (P90; BMI = 22.2), length of 99.5 cm (–2.5 S.D.) and head circumference of 52.3 cm (P50–P98). The face was round with flat profile and high forehead (Fig. 1a–c). Remarkably was the short nose with low nasal bridge and anteverted nares. Truncal obesity with inverted nipples was present. The genitalia were male with small scrotum and both testes in the inguinal canals. The hands were short with brachydactyly and bilateral simian crease. Of note were the short distal phalanges of third and fourth fingers (Fig. 1d). Cytogenetic analysis was repeated but found to be normal at the 550 band stage. Prader–Willi syndrome (PWS) was excluded based on DNA-based methylation testing of the PWS region on chromosome 15q11.2-q13. Analysis of the *FMR1*

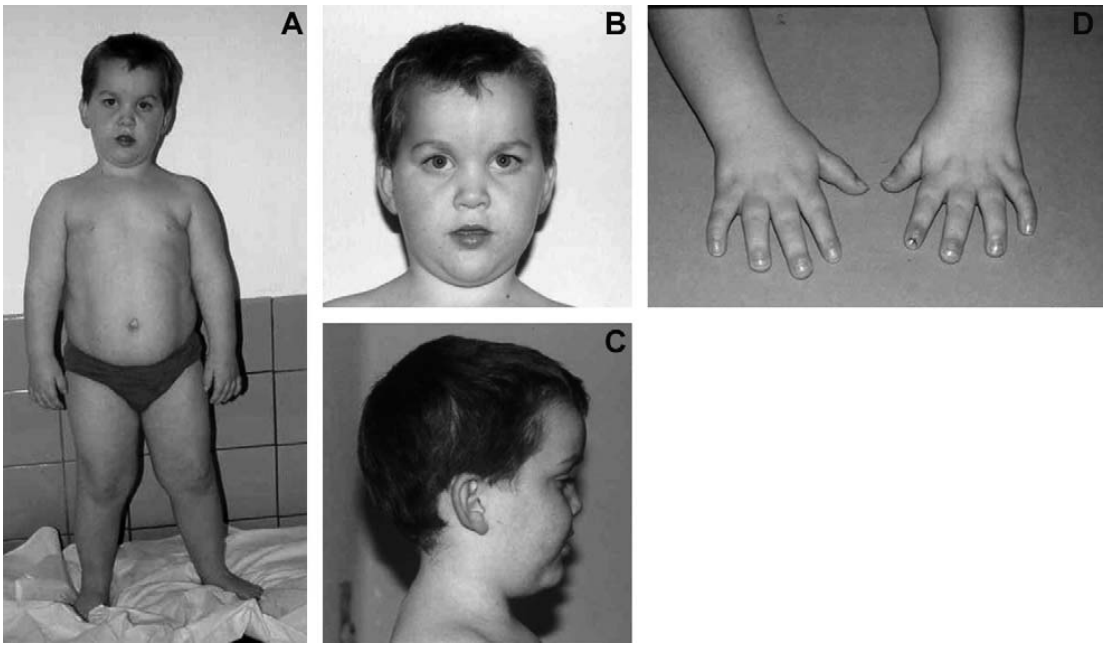

Fig. 1. The proband at the age of 5 years. The boy is short and obese (a). Note the flat facial profile with small, deeply set nose (b, c). The hands are short with small and broad nails on third and fourth fingers (d).

gene revealed a normal methylation pattern and number of CGG trinucleotide repeats. Because of the brachydactyly, radiographs of the hands were taken. These films showed bilaterally short and dysplastic distal phalanges of the third and fourth fingers on both hands (Fig. 2b). Evaluation of earlier hand radiographs did not reveal punctuate calcifications but changes reminiscent for the brachytelephalangic type of chondrodysplasia punctata (MIM 302950) (Fig. 2a). Radiographic evaluation of the remaining parts of the skel-

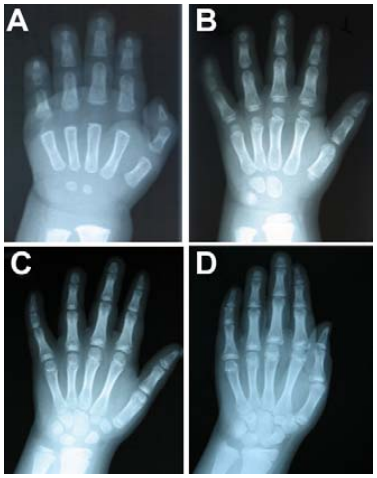

Fig. 2. Radiographs of the left hand at different ages. (a) Radiograph at the age of 1 year shows short distal phalanges in fingers II–V. The shape of the third and fourth distal phalanx resembles an inverted triangle. Punctate calcifications are not seen. (b) At the age of 5 years mainly the distal phalanges of third and fourth finger are short and dysplastic. The epiphysis seems to be attached to the body of the phalanx that still has an inverted triangle shape. (c) The distal phalanges of third and fourth finger remain small at the age of 9 years due to premature closure of the growth plate. There is also mild shortening of the distal phalanx of second finger and middle phalanx of fifth finger. (d) Similar findings are found on the radiograph taken at the age of 12 years.

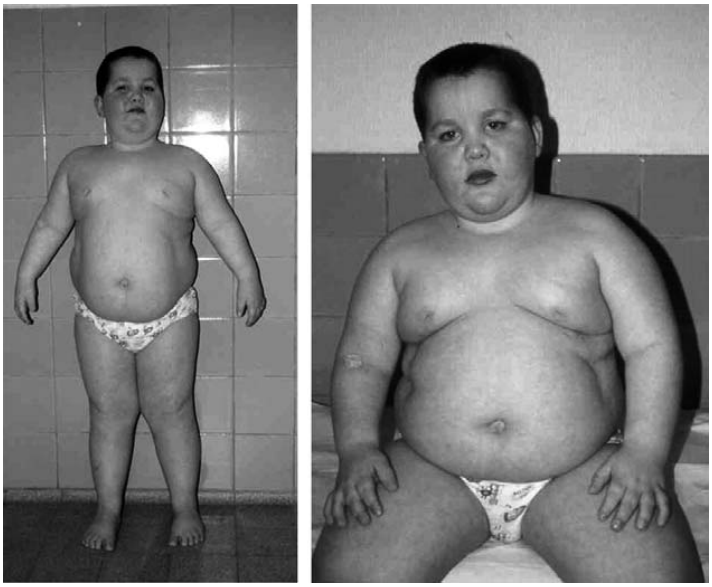

Fig. 3. The proband at the age of 7.5 years showing severe obesity.

eton did not show other abnormalities or areas with punctuate calcifications. The possibility of a microdeletion on the short arm of the X-chromosome, encompassing the *ARSE* gene, was considered. However, molecular analysis at that time failed to detect any abnormalities of the *ARSE* gene. On follow-up the boy developed severe obesity with hyperinsulinism (Fig. 3). Weight and height at the age of 14 years 4 months were 85.8 kg (P97 = 82 kg; BMI = 41.4) and 144 cm (–3.2 S.D.), respectively.

4. Results

Array CGH analysis revealed a gain of distal chromosome 9p material. An array CGH-base representation [14] of the array CGH profile for chromosome 9 is shown in Fig. 4. Evidence for copy number gain was obtained for a total of nine BAC clones representing a region encompassing 12.9 Mb with breakpoints located between clones RP11-187K14 (12872920bp) and RP11-490C5 (15298239bp). This corresponds to a duplication for chromosomal segment 9pter → p23.

Array CGH results were validated by FISH for BAC clones located within the regio showing copy number gain. These hybridisations confirmed an extra copy for each of the investigated clones. For each of the clones the extra signal was located at the distal end of the short arm of the X-chromosome as determined by DAPI counter staining. This was further confirmed using a chromosome X painting probe. FISH with chromosome X and Y subtelomeric probes demonstrated loss of subtelomeric sequences at Xpter. FISH also confirmed deletion of the *SHOX* gene. BAC reporters for the pseudo-autosomal region were excluded from the array CGH analysis for technical reasons and thus could not be assessed. Further array CGH and FISH is ongoing in order to map the distal Xp breakpoint in more detail.

Karyotyping and FISH analysis of both parents yielded normal results indicating that the unbalanced X;9 translocation occurred de novo. The karyotype of the proband is therefore written as: 46,Y,der(X)t(X;9)(p22.32;p23) de novo.

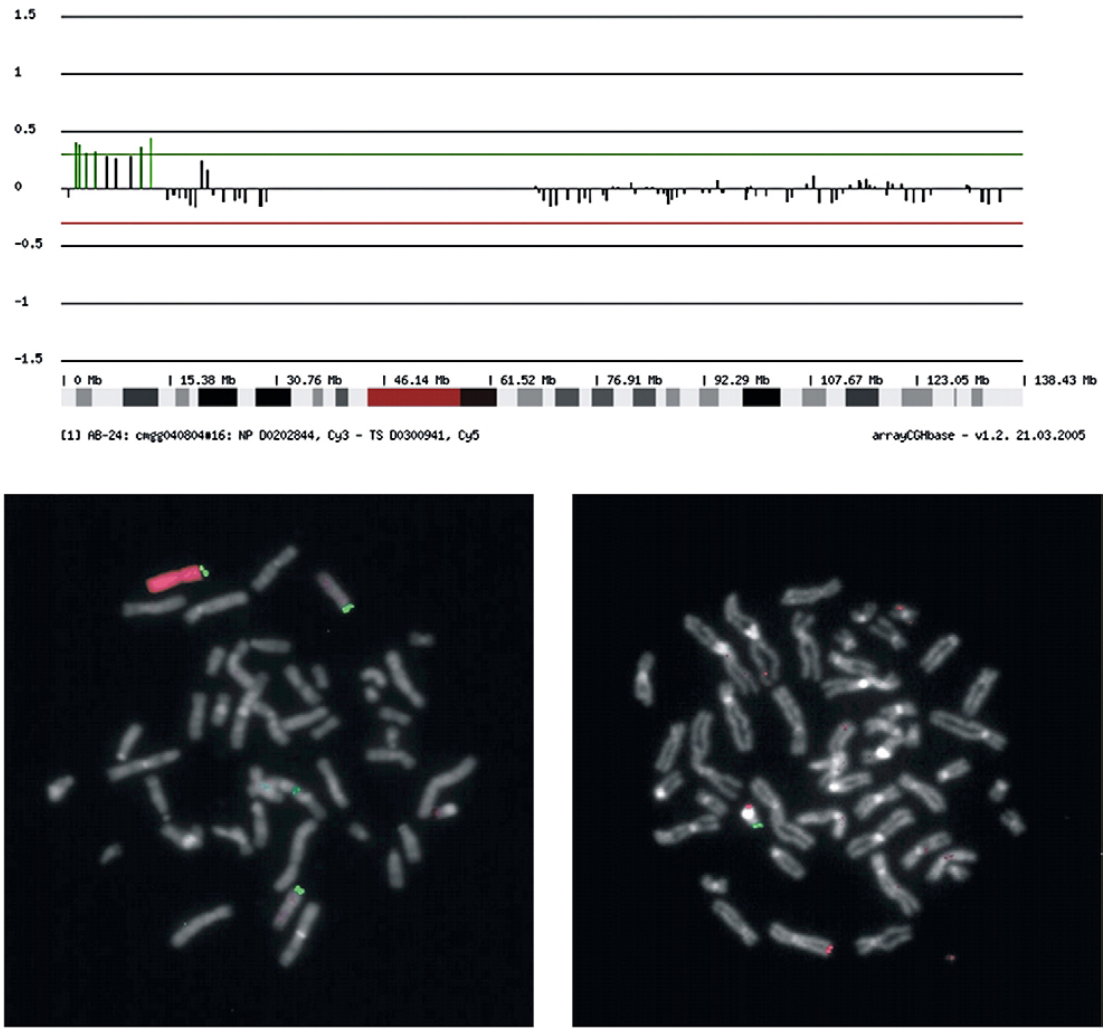

Fig. 4. Top panel shows a chromosome 9 representation of the array-CGH result mapping the relative fluorescence signal (patient versus sex-mismatched control) of all BAC reporters onto their chromosomal position (for details see Section 2). Lower panels show the result of FISH on metaphases from the patient using a subtelomeric 9p probe in combination with an X-chromosome painting probe (right) and telomeric probes for the X- and Y-chromosome (left).

5. Discussion

The proband is the first reported case of a cryptic unbalanced X-autosome translocation, ultimately detected with array CGH. This analysis showed that the distal part of the short arm of chromosome 9 was translocated onto the short arm of the X-chromosome leading to a partial trisomy of the 9pter → p23 segment and to a nullisomy of the Xp22.3-pter segment. On several occasions before, the boy was investigated with cytogenetic analysis that revealed repeatedly a normal male karyotype. The presence of only one X-chromosome in males may hamper the meticulous analysis of this chromosome in the absence of its counterpart. This may explain why the translocation in our case was missed despite the fact that the 9p segment was approximately 12 Mb in size. Molecular tests also failed to unravel the genetic cause of his rather complex phenotype. While the boy had features of the brachytelephalangic type of chondrodysplasia punctata, his overall phenotype did not resemble that

observed in patients with either mutations or microdeletions involving the *ARSE* gene on Xp22.3 [2,6,15,19].

X-autosome translocations occur both in males and females and can be balanced or unbalanced. The consequences of the presence of an X-autosome translocation can be very different for males and females. In females with balanced X-autosome translocations, the normal X-chromosome will be preferentially inactivated whereas in case of an unbalanced translocation the pattern of inactivation will be the one leading to the least functional imbalance. Male carriers of a balanced X-autosome translocation are mostly normal but have an impaired spermatogenesis and infertility. In males with unbalanced X;autosome translocations either in utero lethality or mental retardation with congenital abnormalities can be observed [7,8].

Our patient clearly shows features of the brachytelephalangic type of chondrodysplasia punctata. He has a flat face with very small nose, low nasal bridge and anteverted nares. In addition, he has hypoplasia of some distal phalanges that show at an early age an inverted triangle shape as is typically observed in this type of chondrodysplasia. Puncta are usually restricted to the hands and feet, disappear at an early age and may therefore be missed later on in life. It has been shown that mutations in the *ARSE* gene can cause the brachytelephalangic type of chondrodysplasia punctata [2,6,15,19]. The *ARSE* gene is located in the Xp segment deleted in our patient. We therefore conclude that the chondrodysplasia in this boy is due to the deletion of the distal part of the X-chromosome. The *SHOX* gene is also located in this chromosomal fragment and may therefore be a contributory factor to the growth failure. Both the mental retardation and short stature are most likely the result of a combined effect of the partial trisomy and partial nullisomy. Mental retardation and short stature are constant features of the dup (9p) syndrome [18]. Of interest is the paper by Lewandowski et al. [13] reporting a patient with dup(9)(p24 → p21) who showed mental retardation but with disproportionally defective language as is observed in our proband. This may suggest that at least one gene on 9p is important for proper language development and understandable speech. Obesity has to our knowledge not been reported in patients with a duplication of the short arm of chromosome 9. However, one family (family MRX43) with the association of X-linked mental retardation and obesity has been reported and linked to the region Xp22.31-p21.2, suggesting that at the distal tip of the short arm of the X-chromosome a gene is located that controls body weight [9].

The present report nicely illustrates the new possibilities offered for sensitive screening of submicroscopic chromosomal imbalances. We are now witnessing a more widespread application of this whole genome screening method in the study of unexplained mental retardation, and ultimately a routine based screening can be expected to be developed. The screening of larger series of patients will yield substantial new information on the genetic basis of mental retardation and malformations. This wealth of information will lead to the further description of the so-called morbid genome, a challenge that will need careful collection of genetic and clinical data. To this purpose a new database called DECIPHER has been launched (http://www.sanger.ac.uk/PostGenomics/decipher/). In parallel, an inventory needs to be made of chromosomal regions that are polymorphic and tolerate copy number alterations without phenotypic effect. Finally, these studies may unveil perhaps new classes of hitherto undetected types of chromosomal changes such as the previously detected subtelomeric deletions and duplications due to segmental duplications.

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paper 2: Emerging patterns of cryptic chromosomal imbalance in patients with idiopathic mental retardation and multiple congenital anomalies: a new series of 140 patients and review of published reports

Menten B*, Maas N*, Thienpont B, Buysse K, Vandesompele J, Melotte C, de Ravel T, Van Vooren S, Balikova I, Backx L, Janssens S, De Paepe A, De Moor B, Moreau Y, Marynen P, Fryns J-P, Mortier G, Devriendt K, Speleman F, Vermeesch JR.

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ORIGINAL ARTICLE

Emerging patterns of cryptic chromosomal imbalance in patients with idiopathic mental retardation and multiple congenital anomalies: a new series of 140 patients and review of published reports

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Background: Chromosomal abnormalities are a major cause of mental retardation and multiple congenital anomalies (MCA/MR). Screening for these chromosomal imbalances has mainly been done by standard karyotyping. Previous array CGH studies on selected patients with chromosomal phenotypes and normal karyotypes suggested an incidence of 10–15% of previously unnoticed de novo chromosomal imbalances.
Objective: To report array CGH screening of a series of 140 patients (the largest published so far) with idiopathic MCA/MR but normal karyotype.
Results: Submicroscopic chromosomal imbalances were detected in 28 of the 140 patients (20%) and included 18 deletions, seven duplications, and three unbalanced translocations. Seventeen of 24 imbalances were confirmed de novo and 19 were assumed to be causal. Excluding subtelomeric imbalances, our study identified 11 clinically relevant interstitial submicroscopic imbalances (8%). Taking this and previously reported studies into consideration, array CGH screening with a resolution of at least 1 Mb has been undertaken on 432 patients with MCA/MR. Most imbalances are non-recurrent and spread across the genome. In at least 8.8% of these patients (38 of 432) de novo intrachromosomal alterations have been identified.
Conclusions: Array CGH should be considered an essential aspect of the genetic analysis of patients with MCA/MR. In addition, in the present study three patients were mosaic for a structural chromosome rearrangement. One of these patients had monosomy 7 in as few as 8% of the cells, showing that array CGH allows detection of low grade mosaicisms.

Chromosomal abnormalities are a major cause of mental retardation and congenital malformations. Many chromosomal defects are readily detected by standard or high resolution karyotyping. However, at best, the resolution of cytogenetic analysis is limited to about 5 to 10 Mb. It has long been assumed that a considerable proportion of patients with multiple congenital anomalies and mental retardation (MCA/MR) have submicroscopic chromosomal imbalances, not detectable by routine karyotyping. Such hidden abnormalities have been detected at the subtelomeric regions in around 5% of these patients.[1–4] Following the introduction of the principle of array comparative genomic hybridisation (CGH),[5 6] genome-wide high resolution analysis for DNA copy number alterations became feasible. In analogy with karyotyping, genome-wide array CGH has been termed molecular karyotyping.[7–9] The first papers by Vissers *et al*[10] and Shaw-Smith *et al*[11] reported as much as 15–24% of segmental aneusomies in patients with idiopathic mental retardation and dysmorphism. A few additional studies reported detection rates between 10% and 25%.[12–14] To evaluate the clinical relevance of a chromosomal imbalance, there is a need to collect genotype and phenotype information in a large number of patients. This will allow the determination of the incidence and the genomic distribution of disease causing imbalances and may reveal the underlying mechanisms causing chromosomal imbalances.

In this study we report array CGH data on a new series of 140 patients and review the findings of 292 previously reported patients in order to determine the overall incidence and clinical relevance of each of these chromosomal imbalances. In addition, we provide the first evidence that array CGH screening allows the detection of low grade mosaicism for chromosomal aberrations.

METHODS

Selection of patients

This was a collaborative study between the genetic teams of Leuven and Gent. Patients were selected for the study by clinical geneticists from both teams. The study was approved by the institutional review board and appropriate informed consent was obtained from human subjects. Subjects had mental handicap without known aetiology, but a chromosomal aberration was suspected because of the association with one or more major congenital malformation (such as congenital heart defect, cleft palate, brain malformation, and so on), or dysmorphism (three or more minor anomalies), or both. Ages varied between one and 62 years, with a mean age of 13.1 years. The number of males and females was about equal. All patients had a normal karyotype on G banding analysis at ISCN +550. The presence of a

Abbreviations: BAC, bacterial artificial chromosome; CGH, comparative genomic hybridisation; CNV, copy number variation; MCA/MR, mental retardation and multiple congenital anomalies; PAC, P1 derived artificial chromosome; RTQ-PCR, real time quantitative polymerase chain reaction

subtelomeric abnormality was excluded by fluorescence in situ hybridisation (FISH) or multiplex ligation-dependent probe amplification (MLPA) in 31 of 140 patients. Genomic DNA from each patient was isolated either from blood lymphocytes or from cultured fibroblasts. When consent could be obtained, full phenotypic descriptions of patients with anomalies were submitted to DECIPHER (database of chromosomal imbalance and phenotype in humans using ensembl resources: http://www.sanger.ac.uk/PostGenomics/decipher/).

Array CGH

Bacterial artificial chromosome (BAC) arrays were developed from the 1 Mb clone set of the Sanger Institute which contains 3431 BAC and PAC clones, as previously described.[8 15 16] In short, BAC and PAC (P1 derived artificial chromosome) DNA was isolated from 1 ml bacterial cultures and amplified by two rounds of degenerate oligonucleotide primer polymerase chain reaction (DOP-PCR) using an amino linked primer in the second PCR,[15] and purified on Multiscreen purification plates (Millipore Inc, Bedford, Massachusetts, USA). Purified aminolinked PCR products were spotted in duplicate or triplicate at a concentration of 250 ng/µl on three dimensional CodeLink Bioarray System slides (Amersham Biosciences, Piscataway, New Jersey, USA) with a Lucidea spotter (Amersham Biosciences) or a QArrayMini spotter (Genetix). DNA (300 ng) was labelled by a random prime labelling system (BioPrime Array CGH genomic labelling system, Invitrogen, San Diego, California, USA) using Cy3 and Cy5 labelled dCTPs (Amersham Biosciences). Probe concentration and labelling efficiencies were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, Delaware, USA). Following labelling, hybridisation, and washing of the slides, arrays were scanned at 532 nm and 635 nm using a GenePix 4000B scanner (Molecular Devices) or a GMS 418 scanner (MWG).

Image and data analysis

The scan images were processed with Imagene software (Biodiscovery, El Segundo, California, USA) and further analysed with an in-house developed and freely available software tool, ''arrayCGHbase'' (http://medgen.ugent.be/arrayCGHbase/).[16] In brief, spot intensities were corrected for local background and only spots with signal intensities at least 1.5 times above background were included in the analysis. Where useful, further normalisation of the data was achieved by two dimensional Lowess normalisation using Bioconductor software.[17] Following this normalisation, the values of the duplicates/triplicates on the array and the duplicate experiments were averaged and a $\log_2$ value was calculated. If signal intensity ratios among replicate spots deviated by more than twice the overall standard deviation of all intensity ratios, the spot was not analysed further. At least 95% of the spotted clones fulfilled these quality criteria. The experiment was only scored successful if the standard deviation of the $\log_2$ of the overall spot intensity ratios was less than 0.096. Typically, this SD value for a combined experiment is between 0.035 and 0.06. Clones that have been identified in previous control hybridisations and other studies as being polymorphic were excluded from the analysis.[8 18] Of the 3431 targets on the array, 57 autosomal and eight X chromosomal clones are considered to be polymorphic.

Two or more flanking targets exceeding a value of the mean $\pm$ four times the SD of the $\log_2$ of all intensity ratios for that hybridisation experiment were further investigated to confirm the presence or absence of a genomic imbalance. Single targets showing hybridisation intensity ratios exceeding a value of $\pm$ [$\log_2$(3/2)–2*SD] were also further validated. Validation was undertaken by metaphase FISH for all potential deletions and both metaphase and interphase FISH analysis for all potential duplications larger than 2 Mb in size. Real time quantitative PCR was used to confirm duplications smaller than 2 Mb in size. If in two or more flanking clones the $\log_2$ of the combined intensity ratios exceeded the threshold value of 4×SD, FISH or real time quantitative PCR experiments always confirmed the presence of a chromosomal imbalance. If the intensity ratio exceeded $\pm$ [$\log_2$(3/2)–2*SD] at only one isolated clone in both experiments, a false positive rate of one every seven patients is observed.

FISH

Labelling of the DOP amplified BAC DNA that was used for spotting the arrays was carried out by DOP-PCR on a thermocycler (GeneAmp9700, Applera, Nieuwekerk a/d Ijzer, Netherlands). The reactions were done in a total volume of 50 µl containing 5 µl of 15 µM DOP 1, 2, 3 primermix, 5 µl of 10× PCR buffer w/o $MgCl_2$, which is specially designed for use with Platinum® *Taq* DNA polymerase (Invitrogen), and 5 µl of 50 mM $MgCl_2$. For the dNTPs we used 1 µl of 10 mM dATP, dCTP, dGTP each, 0.7 µl of 10 mM dTTP, 1 µl of 1 mM SpectrumGreen™ or SpectrumOrange™ dUTP (Vysis, Abbott Laboratories, Abbott Park, Illinois, USA) or 5 µl of 10× dNTP mixture containing 1 mM biotin-14-dCTP, 1 mM dCTP, 2 mM dATP, 2 mM dGTP, 2 mM dTTP in 10 mM Tris-HCl (pH 7.5), 1 mM Na_2 EDTA (Bioprime DNA labelling system, Invitrogen). Platinum® *Taq* DNA polymerase (Invitrogen) (0.5 µl), 2 µl of the DOP amplified BAC DNA and H_2O to 50 µl were added. After initial denaturation at 95°C for 10 minutes, the reaction was as follows: 35 cycles of 95°C for one minute, 60°C for one minute, 72°C for one minute, and a final extension step of 72°C for 10 minutes.

Purification of the PCR product was carried out with the Qiaquick 8 PCR purification kit (Qiagen NV, Venlo, Netherlands) using QIAvac 6S vacuum according to the suppliers' instructions.

In addition to the region-specific BAC clones used for validation of array CGH results in patients with suspected imbalance, a chromosome 7 centromere specific probe was used for analysis of patient 19 with suspected monosomy 7 mosaicism (see Results). In all, 200 cells were screened for this patient and a control sample by two independent observers.

Before FISH, cells were air dried on slides and pretreated with pepsin followed by fixation with a 1% free formaldehyde solution and subsequent dehydration with ethanol. After hybridisation O/N at 37°C, the slides were washed for one minute in 0.4× SSC/0.3% NP40 solution at 72°C, one minute at 2× SSC/0.1% NP40 solution at RT, and one minute at 2× SSC. The cells were counterstained with DAPI and the slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, California, USA). The signal was visualised by digital imaging microscopy with Cytovision capturing software (Applied Imaging, Santa Clara, California, USA). FISH was done as described.[19]

Real time quantitative PCR (RTQ-PCR)

The oligonucleotides were selected by using PrimerExpress 2.0.0 ABI Prism oligo design software (Applied Biosystems, Lennik, Belgium). A penalty score less than 150 was used to analyse the selected oligonucleotides further. The primers and amplicon were separately checked to exclude any repetitive sequences by using the BLAST program from the NCBI browser (http://www.ncbi.nlm.nih.gov/BLAST/) and

the repeatmasker program (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker).

RTQ-PCR was carried out using the qPCR mastermix Plus for SYBR Green I without UNG (Eurogentec, Liege, Belgium) according to the manufacturer's instructions. The final volume of 25 µl contained 0.5 mM of both forward and reverse primers, 12.5 µl of 2× reaction buffer and 5 µl of DNA solution in the range of 2 to 50 ng per reaction. Total genomic DNA from human blood was purified by using an automated version of the purification protocol using Chemagic Magnetic Separation (Chemagen Biopolymer Technologie AG, Baesweiler, Germany).

PCR was carried out in triplicate from each fraction using 50℃ for two minutes and 95℃ for 10 minutes, followed by 40 cycles of 95℃ for 15 seconds and 60℃ for 60 seconds. An 81 base pair DNA fragment within the p53 gene (forward: 5′-CCC AAG CAA TGG ATG ATT TGA-3′ and reverse: 5′-GAG CTT CAT CTG GAC CTG GGT-3′) was used as a control amplicon (Eurogentec). Serial fivefold dilutions of this target ranging from 100 ng to 0.16 ng per experiment served as a standard quantitation curve.

RTQ-PCR was done with the locus-specific oligonucleotides of interest on an ABI PRISM 7000 Sequence Detection System (SDS) according to the manufacturer's instruction manual (Applied Biosystems, Lennik, Belgium). The amplification results and the melting curve were analysed with the ABI Prism 7000 SDS software version 1.1 (Applied Biosystems). The DNA levels were normalised to the gene p53 and relative differences were calculated according to the relative quantitation method.[20]

RESULTS

Array CGH findings in 140 patients with unexplained MCA/MR

One hundred and forty patients with unexplained mental retardation and features suggestive of a chromosomal anomaly (for example, a major malformation or multiple minor anomalies) were analysed on a 1 Mb BAC array. The DNA from each patient was labelled and hybridised with label swap versus the DNA of two other MCA/MR patients, rather than using a ''normal'' reference sample. Dye swap hybridisations for three patients in three hybridisations reduces by half the number of experiments and the cost per patient sample. This approach may be counterintuitive and seem inappropriate in a diagnostic setting. However, *the* ideal reference genome is non-existent owing to large scale copy number variations between the genomes of different ''normal'' individuals.[21 22] To mask benign copy number variation (CNVs), other groups have used pooled DNA of from seven to 10 different male or female subjects as reference material.[11–14] For frequently occurring CNVs, intensity ratios will be reduced. If a CNV were present in 50% of the population, the intensity ratio difference at this locus would be reduced by half. Rather than improving the outcome, this result complicates data interpretation. One disadvantage of using patients as reference in three hybridisations could be that similar imbalances in two or three of the patients would result in equal intensity ratios for the affected region and potentially mask imbalances. However, the finding that the recurrence of a similar chromosomal imbalance in two patients with idiopathic MCA/MR is less than 1% (see below) makes the risk that a similar imbalance would occur in two and three independent patients smaller than, respectively, $1/10^4$ and $1/10^6$.

A chromosomal imbalance was detected in 28 patients (20.1%). An overview of all imbalances is shown in fig 1, and array CGH profiles for aberrant chromosomes are presented as supplementary information. Table 1 summarises the genotype and phenotype of these 28 patients. For eight patients the imbalance spanned more than five clones (>5 Mb in size), for 10 patients between two and five clones (1–8 Mb in size), and for 10 patients the imbalance was only a single clone (<3 Mb). In two patients there was evidence of mosaicism for a structural chromosomal aberration and in one patient a low grade mosaicism for chromosome 7 monosomy was detected (see below). In 17 of 24 patients in whom the parents could be investigated the chromosomal imbalance was de novo by either FISH (deletions or duplications larger than 3 Mb) or quantitative PCR (qPCR) (small duplications). While none of the imbalances smaller than 5 Mb could be detected by high resolution karyotyping, three large deletions (in patients 7, 12, and 15) and two mosaics (in patients 14 and 18) became apparent after retrospective analysis of the karyotype. Eight imbalances (5.7%) involved a subtelomeric region.

All de novo alterations can be considered causal for the MCA/MR phenotype observed in the patients. For four of the 28 patients with a chromosomal imbalance, the parents were not available for genotyping. One of these (patient 1) had a large deletion on 1p36.2 spanning multiple clones. As the observed phenotype in this patient resembles that of patients with known 1p terminal deletions, this imbalance was considered causal. For patients 3, 19, and 20 only one or two clones were abnormal making the causal relation between genotype and phenotype difficult to determine.

For seven of the 28 patients the imbalance (three duplications and four deletions) was inherited from one of the parents. These parents were phenotypically normal with the exception of the father of patient 27, who had mild learning disabilities, and the mother of patient 7, who was similarly affected as the daughter. Patient 27 presented with cleft lip and palate, mild learning difficulties, and a truncus arteriosus. A duplication on chromosome 22q11.2 was detected in this girl and her father. In view of previous reports describing 22q11.2 duplications (including those inherited from normal parents), we assume a direct relation between the 22q11.2 duplication and the observed phenotype in this patient. Patient 10 and one of two imbalances in patient 7 have been listed as polymorphic in the Toronto polymorphism database.[21] In patient 7, the larger deletion on chromosome 5 spanning between 6.8 and 11.8 Mb was also present in the similarly affected mother. Hence this deletion is likely to be causal for the phenotype. In patient 5, the duplicated region in the healthy father and son contains only a single gene, the glycogen branching enzyme (GBE1); dosage effect for this gene seems a rather unlikely cause. In patients 6, 17, and 28, single clone imbalances are inherited and the causal relation between genotype and phenotype remain to be determined.

In summary, we consider that at least 19 of the 28 observed imbalances are causal for the MCA/MR in the patients.

Cytogenetic features of (low grade) mosaic chromosomal imbalances

A further interesting observation in this study was the finding of three mosaics. In patient 16, array CGH revealed increased average intensity ratios for a 12 Mb region compatible with a duplication spanning the long arm of chromosome 13 from band 13q31.3 to 13q33.1 (fig 2A). The average $\log_2$ of the intensity ratio values of the abnormal clones was 0.38. As the theoretical intensity ratio of a duplication is $\log_2$ (3/2) or 0.58, the estimated mosaicism level is 0.38/0.58 or 65%. FISH analysis confirmed the duplication to be present in 60% of cultured lymphocytes (fig 2B).

In patient 14, standard array CGH revealed a 5 Mb deletion at 11q22.1–23.1. FISH with clone RP11-87N22 confirmed the deletion at the 11q22.1 locus in all cells. Forty clones flanking

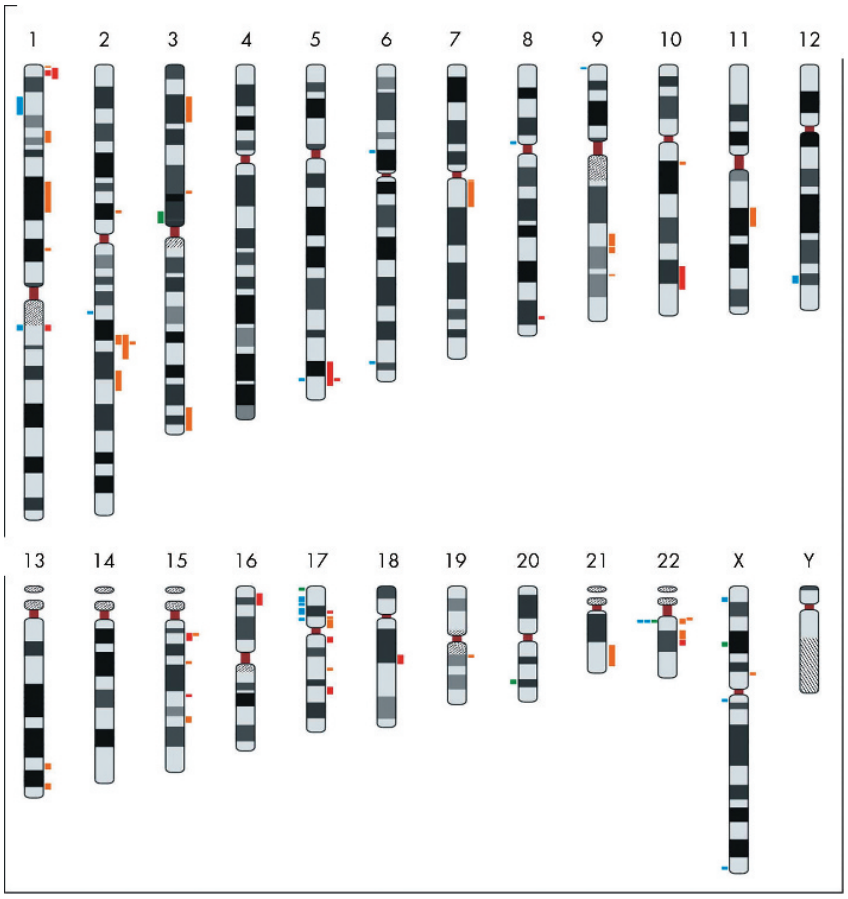

Figure 1 Overview of all published interstitial submicroscopic imbalances detected by array-CGH in patients with mental retardation and multiple congenital anomalies (MCA/MR). Microdeletions and duplications identified in this study are represented by, respectively, red and green bars. Microdeletions and duplications identified by previous array CGH studies[10–14] are indicated by, respectively, the orange and the blue bars. Polymorphic variants from de Vries *et al*[12] are not shown.

this deletion (14 proximal and 26 distal to the deleted segment) showed a mean intensity ratio of 0.21, suggesting a duplication of the adjacent region at 11q21-qter in approximately 35% of the cells (fig 2C). FISH with clone RP11-744N12 located within this presumed duplicated region showed a translocation of 11q21-qter onto chromosome 9 in 6% of the cells, in contrast to the estimated 35% (fig 2D). As this FISH analysis was performed on lymphocytes following stimulation with phytohaemagglutinin, and DNA used for array CGH was extracted from uncultured lymphocytes, we assumed that culturing resulted in clonal selection of the normal cells. FISH on uncultured lymphocytes confirmed this hypothesis and showed three signals of RP11-744N12 in as many as 25% of the nuclei of uncultured lymphocytes.

Array CGH analysis on patient 9 revealed an average intensity ratio of −0.0496 for the clones from chromosome 7 (fig 2E). The level of mosaicism is calculated to be 5%. Interphase FISH analysis by two independent observers using a centromere 7 specific probe revealed a single signal in 10.5% of the nuclei of peripheral white blood cells of the patient while in a control sample a single signal was observed in only 3.5% of the nuclei. The difference between these two proportions was significant ($p<0.01$), thus confirming the presence of the monosomy in approximately 8% of the patient's white blood cells. This finding can probably be explained by the presence of a (pre)malignant clone in this patient.

Review of published reports on MCA/MR patients with submicroscopic imbalances

To obtain insight into the incidence, characteristics, and genomic distribution of imbalances detected by array CGH in MCA/MR patients, all published genomic imbalances were reviewed (fig 1 and table 2).[10–14] From a total of 192 patients screened by arrays at ~1 Mb resolution, 41 imbalances were detected (21%), of which at least 20 (10%) were de novo. Of the 192 patients, 113 were screened for subtelomeric imbalances before array CGH. The number of interstitial imbalances was 35 (18%), of which at least 17 were de novo (8.8%). In addition, de Vries *et al* analysed 100 patients previously shown not to carry subtelomeric imbalances using an array covering the full genome and detected de novo alterations in 10 patients.[12] Five imbalances were likely to be causal, but parents were not available for analysis. Of these 15 imbalances, five were smaller than 1 Mb.

Figure 1 shows that the imbalances were more or less scattered across the genome and appeared mostly randomly distributed over all chromosomes. Some chromosomal regions appeared non-randomly involved. Interstitial aberrations at chromosome 1p36 were detected in two patients in the present study and in three published array CGH cases. Hence, in addition to the 1p36 terminal deletion syndrome—considered to be the most common subtelomeric microdeletion syndrome[23]—interstitial subtelomeric deletions also appear to be common. At two loci (1q21.1 and 5q35.1), both

Table 1 Summary of copy number changes detected by array CGH, short clinical description and parental analysis

Case	Clinical details*	Molecular karyotype	Parents	No of clones	Size (MB)†	Flanking clones
1	Retinal dystrophy, growth retardation, short fingers, low set ears, epicanthic folds	46,XY.arr cgh del(1)(p36.23p36.32)	Not determined	5	4.6–8.1	RP4-785P20, RP11-338N10
2	Microcephaly, ventricular septal defect, large corneae, midface hypoplasia, presacral groove	46,XY.arr cgh del(1)(p36.31p36.32)	De novo	3	2.8–6.0	RP4-785P20, RP11-49J3
3	Short stature, microcephaly, strabismus, unilateral renal agenesis, simple ears	46,XX.arr cgh del(1)(q21.1q21.1)	Not determined	2	0.9–4.0	RP11-533N14, RP11-301M17
4	Epilepsy, brachydactyly type E, scoliosis, absence of some toenails, synophrys	46,XX.arr cgh der(2)t(2;22)(q37;q13)	De novo	chr 2: 4 chr 22: 3	4.0–4.7 1.6–2.9	chr2: RP11-556H17, RP11-15L18 chr22: cN75H12, RP5-925J7
5	Seizures, spasticity, hypotonia, hypoplastic cerebellum and brain stem, Dandy-Walker malformation	46,XY.arr cgh dup(3)(p12.2p12.2)	Inherited (pat)	3	0.5–2.1	RP11-425D6, RP11-359D24
6	Coarse facial features, Dandy-Walker malformation, wide pontine cisterns, right cerebellar lobe atrophy, hirsutism, pigmented nevi	46,XX.arr cgh del(3)(p12.1)	Inherited (pat)	1	0.1–2.5	RP11-474M18
7	Pectus excavatum, sacral dimples, recurrent infections, sparse eyebrows, small high nasal bridge	46,XY.arr cgh del(5)(q34q35.1)del (15)(q13.1)	Inherited (mat)¶ inherited (pat)‡	chr 5: 8 chr 15: 2	6.9–11.8 0.8–3.5	chr5:RP11-505G12, RP11-420L4, chr15:RP11-408F10, RP11-38E12
8	Tetralogy of Fallot, double outlet right ventricle, hypertelorism, high and broad forehead, brachycephaly	46,XX.arr cgh del(5)(q35.1q35.1)	De novo	1	0.2–2.8	RP11-20O22
9	VSD, absent thumbs, growth retardation, hydronephrosis, preductal coarctation of aorta	46,XX.arr cgh del(7)(pterqter) .ish 46,XX[92]/45,XX.−7[8]	De novo	212	158	CTB-164D18,RP4-764O12
10	Axial hypotonia, short stature, stereotypic movements, hypertelorism, strabismus	46,XY.arr cgh del(8)(q24.23q24.23)	Inherited (pat)‡	1	0.2–1.5	RP11-17M8
11	Chondrodysplasia punctata brachytelephalangic type, obesity, short stature, small deeply set nose, hypotonia	46,Y.arr cgh der(X)t(X;9)(p22.32;p23)	De novo	chr 9: 16 chr X: 5	chr 9:13.0–13.9 chr X: 5.4–6.9	chr 9: RP11-187K14, GS1-77L23 chr X: RP11-60N3, CTB-98C4
12	Short stature, microcephaly, VSD, preductal coarctation of aorta, midface hypoplasia	46,XX.arr cgh der(9)t(9;20)(q34.3;q13.33)	De novo	chr 9: 4 chr 20: 7	chr 9: 3.1–4.7 chr 20: 3.5–4.8	del:RP11-399H11, GS1-135I17, dup:RP5-836E13, CTB-81F12
13	Hypotonia, spasticity, abdominal muscle hypoplasia, fine hair, macroglossia	46,XX.arr cgh del(9)(q34q34)	De novo	1	0.1–0.6	GS1-135I17
14	Valvar pulmonary stenosis, cleft uvula, epilepsy, hypoplastic corpus callosum, hypoplastic genitalia	46,XX.arr cgh del(11)(q22.3q23.3) [66]/ der(9)t(9;11)(qter;q21),del(11)(q22.3q23.3)[33]	De novo	chr 9: 6 chr 11: 50	del: 8.5–10.2 dup: 40.4–41.1	del:RP11-531F16, RP11-114K7, dup:RP11-685N10, RP11-469N6
15	Carpal synostosis, macrocephaly, strabismus, oral frenulae, autistic behaviour	46,XX.arr cgh del(10)(q25.1q26.11)	De novo	10	8.2–10.3	RP11-271I13, RP11-355F22
16	Broad thumbs, nasal speech, strabismus, deep hoarse voice, trigonocephaly	46,XX.arr cgh dup(13)(q31.3q33.1) .ish 46,XX[40]/46,XX dup(13)(q31.3q33.1)[60]	De novo	14	12.2–13.9	RP11-388D4, RP11-564N10
17	Microbrachycephaly, almond shaped eyes, wide nasal bridge, large mouth, synophrys	46,XX.arr cgh del(15)(q22.2q22.2)	Inherited (mat)	1	0.2–3.2	RP11-231A23
18	Dysplastic ears, median cleft palate, small penis, brachycephaly, unilateral preauricular fistula	46,XY.arr cgh dup(16)(p13.2p13.3) .ish der(22)t(16;22)(p13.2p13.3;p21)	De novo	9	7.4–8.3	RP11-433P17, RP11-148F10
19	Generalised hypotonia, scoliosis, congenital heart disease, short stature, brachycephaly	46,XY.arr cgh dup(17)(p13.3p13.3)	Not determined	1	0.1–1.7	RP11-135N5
20	Camptodactyly, ectropion, hypoplastic cerebellar hemispheres and vermis, hypertelorism, genital hypoplasia	46,XY.arr cgh del(17)(p12p12)	Not determined	1	0.1–2.3	RP1-27J12
21	Microcephaly, long eyelashes, long columella, deep presacral groove, lacrimal duct stenosis	46,XX.arr cgh del(17)(q11.2q11.2)	De novo	1	0.1–1.9	RP11-474K4
22	Psychiatric disorder, macrocephaly	46,XX.arr cgh del(17)(q23.2q24.1)	De novo	3	1.1–4.2	RP11-115N5, RP11-74H8
23	Small stature, narrow thorax, macrocephaly, downslanting palpebral fissures, prominent maxillary incisors	46,XY.arr cgh del(18)(q12.3q12.3)	De novo	2	1.4–4.6	RP11-486C18, RP11-463D17

Table 1 Continued

Case	Clinical details*	Molecular karyotype	Parents	No of clones	Size (MB)†	Flanking clones
24	Joint laxity, scoliosis, hyperelastic skin, webbed neck, beaked nose	46,XX.arr cgh dup(20)(q13.13q13.2)	De novo	2	0.7–2.7	RP5-1071L10, RP5-994O24
25	Myopia, nasal speech, cleft uvula, pulmonary stenosis, strabismus	46,XX.arr cgh del(22)(q12.2q12.2)	De novo	2	0.7–2.2	CTA-57G9, RP1-76B20
26	Hypotonia, adduction of thumbs, claw toes, syndactyly fingers 3/4, dorsiflexion of the wrists	46,XX.arr cgh del(22)(q13.33q13.33)	De novo	3	1.4–1.9	CTA-722E9, CTB-99K24
27	Cleft lip and palate, truncus arteriosus type I, short neck, ptosis, uteronephrosis,	46,XX.arr cgh dup(22)(q11.21 q11.21)	Inherited (pat)	1	0.1–4.2	XX-91c
28	Epilepsy, microcephaly, abdominal situs inversus, VSD, hypotonia	46,XY.arr cgh dup(X)(p21.3p21.3)	Inherited (mat)	2	0.3–1.2	RP11-37E19, RP6-27C10

*All patients presented with mental retardation. Only the five most relevant dysmorphic features are retained in the table. Full phenotypic descriptions together with the genotype data can be viewed in Ensembl (www.ensembl.org/index.html) through the Decipher DAS server.
†Sizes of the aberrations are shown from a minimal to maximal size in megabases.
‡This imbalance has already proven to be polymorphism.
¶Mother has the same phenotype including mental retardation. Further family could not be investigated.
chr, chromosome; mat, maternal; pat, paternal; VSD, ventricular septal defect.

a duplication and a deletion were observed. Possibly these sites may mark novel microdeletion syndromes caused by recurrent non-homologous recombination in low copy repeats. Of particular interest is the finding of a familial duplication on 22q11.2 in this study, as well as in three previous reported cases (two de novo and one case of unknown origin), further suggesting the recurrent nature of this duplication and the variable phenotypic effect.

DISCUSSION

This study is the largest series of patients reported who have been screened for chromosomal imbalances with a 1 Mb resolution BAC array. In a total of 140 patients, 28 chromosomal imbalances were detected (20%). These included seven duplications, 18 deletions, and three unbalanced translocations. To determine the causal role of these chromosomal aberrations, parents were investigated in 24 of 28 patients. In addition, the Toronto database of normal variants was consulted. About three quarters (17/24) of the observed chromosomal aberrations were de novo and not reported before as a normal variant. In one patient for whom the parents could not be tested, available phenotypic data for similar published cases indicated that the genotype could explain the observed phenotype, and in one patient with inherited deletion the mother was equally affected. This brings the total of clinically relevant imbalances to 19. Taking into account these data and excluding those subtelomeric imbalances that could have been detected by FISH or MLPA/MAPH analysis, our study has identified 11 clinically relevant imbalances (8%) undetectable by karyotyping and subtelomeric screening. This is in accordance with previous findings of 10–15% causal interstitial submicroscopic imbalances in patients with MCA/MR.[10–14] Imbalances identified thus far in MCA/MR patients have been positioned on the human genome map in order to assess their genomic distribution and to detect overlapping regions. This map further confirms that most imbalances are scattered across the genome.

From our data and data from other published reports it has become clear that the clinical application of array CGH poses new challenges. While it is assumed that de novo alterations result in the observed phenotype, only the recurrent association of imbalances with specific phenotypic features will reinforce this causal relation. Hence, it will be essential to collect genotypic and phenotypic information on a large number of MCA/MR patients. In contrast to de novo alterations, many chromosomal imbalances are inherited. Although it is likely that frequently occurring genomic CNVs may not have major disease causing phenotypic effects, rare variants, such as the six familial inherited imbalances detected in this study, should be evaluated with care. In particular, imbalances of regions which are recurrently involved in familial transmission from a normal parent to affected children will pose specific problems for genetic counselling, as illustrated by the 22q11.2 duplication. This is in line with previous observations that 22q11 duplications result in diverse phenotypes from normal to mild to severe, and sharing a tendency for velopharyngeal insufficiency with DiGeorge/VCFS (velo-cardio-facial syndrome) but with other distinctive characteristics as well.[24 25] The 22q11 duplication syndrome may hallmark a novel paradox encountered by molecular karyotyping, as the causal relation between a chromosomal anomaly and an associated phenotype becomes blurred. Hence, imbalances inherited from phenotypical normal parents may contribute to the phenotype through variable penetrance or expressivity, or both, through epigenetic effects, or by uncovering a recessive mutation on the non-deleted allele. To understand the involvement of these variations in the observed phenotypes, it will be necessary not

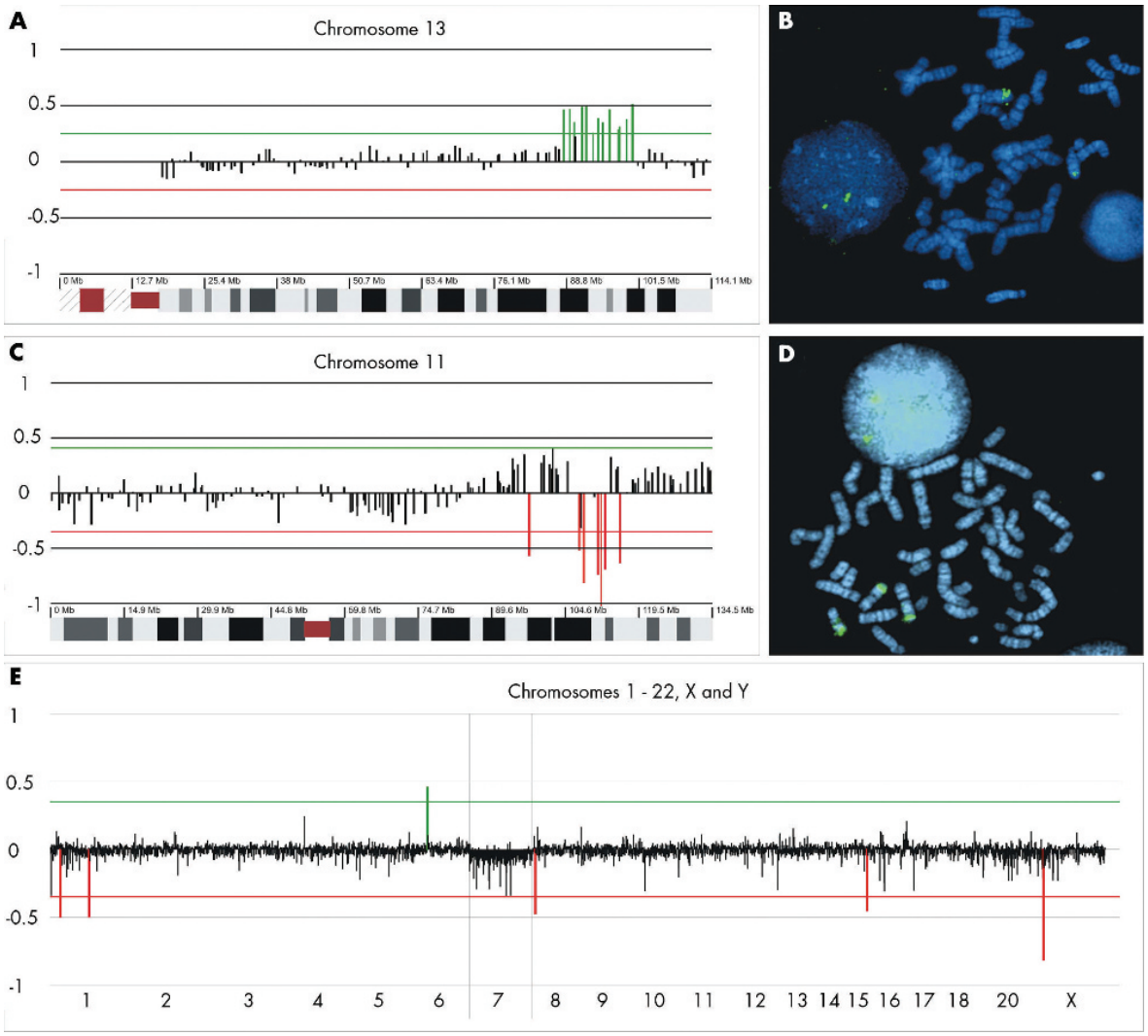

Figure 2 Cytogenetic analysis of patient 16 (panels A and B), patient 14 (panels C and D) with segmental chromosomal mosaicisms, and patient 9 (panel E) with a mosiacism monosomy of chromosome 7. (A) Partial molecular karyotype enlarging the ratio profiles for chromosome 13; in the x axis clones are ordered from the centromere to the q-arm telomere, and the y axis shows the $\log_2$ transformed intensity ratios at each locus. Red lines indicate the threshold for clone deletion or duplication ($\pm$4*SD). (B) Fluorescence in situ hybridisation (FISH) with PAC 1091O16 confirmed that the duplication at 13q32The duplication was present in 60% of the cultured lymphocytes. (C) Partial molecular karyotype enlarging the ratio profiles for chromosome 11. On the x axis clones are ordered from the p-arm telomere to the q-arm telomere and the y axis shows the $\log_2$ transformed intensity ratios at each locus. Red lines indicate the threshold for clone deletion or duplication ($\pm$4*SD). (D) The duplication at 11q24.3 was confirmed with clone BAC 744N12 and was the result of a translocation between 11q and 9q. FISH on cultured and uncultured lymphocytes showed the duplication to be present in, respectively, 6% and 25% of the cells. (E) Molecular karyotype showing the ratio profiles for the chromosomes 1 to 22, X, and Y. Chromosome 7 is positioned between the two vertical lines, and shows $\log_2$ transformed intensity ratios with an average of -0.05.

only to collect benign variation in the genome and information on de novo imbalances associated with disease phenotypes, but also to collect both genotype and phenotype information from patients with familial inherited imbalances and phenotypically normal parents. To start this data collection, both genotype and phenotype data from all patients who consented was submitted at the DECIPHER database (http://www.sanger.ac.uk/Postgenomics/decipher/).

Segmental chromosomal imbalances in mosaic state are causal in several MCA/MR syndromes.[26] The present study illustrates that array CGH may detect segmental chromosomal imbalances which may be overlooked in standard karyotyping when a small number of cells is analysed or when the abnormality is too small to arouse suspicion. A remarkable observation in one of the mosaics was that phytohaemagglutinin stimulation of lymphocytes and subsequent short culture apparently induced a selective growth advantage for the normal cells. Clearly, such culture effects can bias the final cytogenetic observations, as was observed in patient 14. Presently a theoretical model is being developed which should enhance the sensitivity for the detection of low grade mosaicism. Clearly, the presence of a large deletion present in as few as 5% of cells can easily be detected. The ability to detect low grade mosaics will allow the detection of chromosomal aneuploidies in highly contaminated specimens such as aborted fetuses[27] and in the analysis of tumours and leukaemias.[28]

In all reports, including this study, the number of deletions (57) was greater than the number of duplications (24). This may have both a technical and a biological component.

Table 2 Published reports: summary of intrachromosomal copy number changes detected by array CGH

Paper	No of patients*	Intrachromosomal			No of targets on array
		De novo	Familial	Unknown	
Vissers L *et al*[10]	20 (0)	2	2	1	3569
Shaw-Smith *et al*[11]	50 (41)	7	5	0	~3500
Rosenberg *et al*[13]	81(0)	4	7	3	~3500
Schoumans *et al*[14]	41(41)	4	0	0	2600
This study	140 (31)	11	7	3	~3500
Total	**332**	**28**	**21**	**7**	
De Vries *et al*[12]	100	10	0	5	32447

*Number on which subtelomeric imbalances have been excluded before array CGH was carried out.
CGH, comparative genomic hybridisation.

Technically, most threshold algorithms may favour more false negatives for duplication events as compared with deletion events. Most threshold algorithms determine cut offs for both deletions and duplications at equal distance from the mean of all intensity ratios. As the intensity ratios for chromosomal deletions are more distant from the mean (ratio of 1/2) as compared with the intensity ratios observed for duplications (ratio of 3/2), inevitably there is a greater chance that some duplications may be missed. Second, there may be a biological bias. Duplications generally result in a milder phenotype; therefore there may be a selection bias in this patient population. In addition, the frequency of random duplication events in the human genome may be lower than the frequency of deletion events. Van Ommen[29] estimated the frequency of deletion events to be one in every eight births, and the duplication frequency one in every 50 births. This suggests that the number of deletion events is about sixfold greater than the number of duplication events. In patients with MCA/MR, deletions outnumber duplications by approximately twofold.

In conclusion, we confirm that a high percentage of MCA/MR cases hitherto considered idiopathic is caused by submicroscopic chromosomal imbalances. Consequently, screening of selected patients with normal karyotypes seems desirable and feasible. The availability of commercial platforms and improved hybridisation schemes resulting in reduction of costs for these analyses opens the way for implementing array CGH in routine diagnostic analysis. At present it remains unclear what resolution of the array will be optimal for screening MCA/MR patients. Higher resolution arrays may reveal larger numbers of small chromosomal imbalances. However, the finding of only 10% of de novo imbalances in a cohort of 100 patients by a full coverage array may indicate that higher resolution does not necessarily increase the diagnostic yield. More studies using high resolution arrays are needed to compare the incidence of small imbalances in different patient populations. Nevertheless, using a 1 Mb resolution array, some imbalances smaller than 1 Mb are being missed. In addition, the false positive rate may be lowered, especially if the identification of imbalances is based on intensity alterations of three or more aberrant flanking clones.[12] Considering the large percentage of inherited chromosomal imbalances, establishing both benign copy number variations in the human genome as well as developing a comprehensive morbid map of the human genome will be of major importance for understanding which imbalances are causative.

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Conflicts of interest: none declared

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paper 3: Osteopoikilosis, short stature and mental retardation as key features of a new microdeletion syndrome on 12q14

Menten B, Buysse K, Hamilton SJ, Hellemans J, Costa T, Fagerstrom C, Anadiotis G, Kingsbury D, McGillivray BC, Marra MA, Friedman JM, Speleman F, Mortier G.
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SHORT REPORT

Osteopoikilosis, short stature and mental retardation as key features of a new microdeletion syndrome on 12q14

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This report presents the detection of a heterozygous deletion at chromosome 12q14 in three unrelated patients with a similar phenotype consisting of mild mental retardation, failure to thrive in infancy, proportionate short stature and osteopoikilosis as the most characteristic features. In each case, this interstitial deletion was found using molecular karyotyping. The deletion occurred as a de novo event and varied between 3.44 and 6 megabases (Mb) in size with a 3.44 Mb common deleted region. The deleted interval was not flanked by low-copy repeats or segmental duplications. It contains 13 RefSeq genes, including *LEMD3*, which was previously shown to be the causal gene for osteopoikilosis. The observation of osteopoikilosis lesions should facilitate recognition of this new microdeletion syndrome among children with failure to thrive, short stature and learning disabilities.

Classical cytogenetic analysis has played an essential role in the discovery of recurrent segmental deletions in patients with clinically recognisable mental retardation such as Prader–Willi, Miller–Dieker, Langer–Giedion and velocardiofacial syndromes.[1] The subsequent delineation of commonly deleted segments and mapping of small atypical deletions have allowed the identification of genes responsible for the major clinical features of these contiguous gene deletion syndromes.[2] Recently, molecular karyotyping was proven to be a more powerful tool in detecting submicroscopic deletions or duplications in patients with so-called idiopathic mental retardation with or without congenital malformations. Molecular karyotyping studies have shown that in about 10% of these cases segmental imbalances can be found.[3–9] Using this new genome-wide screening technology, new disease genes can be identified, as illustrated for the *CHD7* gene in CHARGE syndrome.[10] Very recently, some new microdeletion syndromes were identified using molecular karyotyping.[11–13]

Here, we report on three unrelated patients with de novo 12q14 microdeletions. They share osteopoikilosis, short stature and learning disabilities as common phenotypic features. In two cases the deletion was approximately 6 megabases (Mb) in size whereas in the third patient a 3.44 Mb deletion was detected.

The first proband (03g1858) is a girl born at term with a birth weight of 2060 g. Pregnancy and delivery were uneventful. At the age of 6 months, she presented with failure to thrive and by the age of 1 year, length, weight and head circumference were all far below the third centile. Work-up for this failure to thrive only revealed hypertension, for which she received medication. The diagnosis of Russell-Silver syndrome was considered at that time. The girl also showed delayed neuromotor development and experienced learning difficulties requiring an individualised programme at school. Clinical evaluation at the age of 16 years revealed proportionate short stature with a weight of 31.8 kg (−4 SDs), height of 131.5 cm (−6.2 SDs), span of 131 cm and head circumference of 49 cm (−4.4 SDs). The face was mildly dysmorphic with synophrys, mild hypertelorism, broad and high nasal bridge, micrognathia and maxillary overbite. These clinical features were not reminiscent of Russell-Silver syndrome. Imaging studies revealed the presence of ectopic kidneys, and an aortogram showed on each side two renal arteries with an aberrant origin. In addition, malrotation of the small bowel, a medially positioned spleen and an unusually shaped (rectangular) liver were found. Radiographic evaluation showed multiple osteopoikilosis lesions in the pelvis, shoulders, wrists, hands and feet (fig 1A).

The second female proband (D0502619, figure 1B,C) was born at term with a weight of 2300 g. The pregnancy was complicated by oligohydramnios. The postnatal course was uneventful. Early in infancy very poor growth and development became apparent. At the age of 4 years she was diagnosed with scoliosis, type 1 Arnold-Chiari malformation and ultimately syringomelia requiring a shunt. She also had a release for a tethered spinal cord. Reflux nephropathy with small kidneys, mild hypertension and diabetes mellitus were diagnosed in childhood. In school, mild learning problems became apparent, and therefore she was put on an individualised education plan. At the age of 14 years she presented with complaints of tingling pain in the medial part of her right foot. Clinical evaluation revealed a weight of 51.3 kg (mean for age), height of 142.3 cm (−3.5 SDs) and head circumference of 53.3 cm (−0.66 SDs). Her face was round with rather deep-set eyes, bushy eyebrows and thin lips. (fig 1B,C). A thoracolumbar scoliosis was noted. The skin showed several areas of increased pigmentation. Mild swelling without other inflammatory signs was present on the right foot. The gait pattern was somewhat antalgic. Radiographic evaluation revealed numerous osteopoikilosis lesions in the distal part of the tibia and fibula as well as in the right foot. In addition, the second right metatarsal showed a thickened and irregular cortical lesion suggestive of melorheostosis at the diaphysis. At the age of nearly 16 years, she is now functioning at the level of a 10-year-old child. She is quite sociable and tends to be very articulate and repetitive.

The third patient (#4818) is the male product of the first pregnancy of an unrelated 22-year-old mother and 35-year-old father. The pregnancy was complicated by hyperemesis. There were no adverse exposures. The family history was non-contributory. Delivery was at term with weight at the third centile and length at the tenth centile. The boy failed to thrive during the first year. At age three years six months, all growth measurements were below the third centile, and development was a year delayed. Growth hormone levels and bone age were normal. At age four, he was found to have delays in fine motor

Abbreviations: FISH, fluorescence in situ hybridisation; Mb, megabase

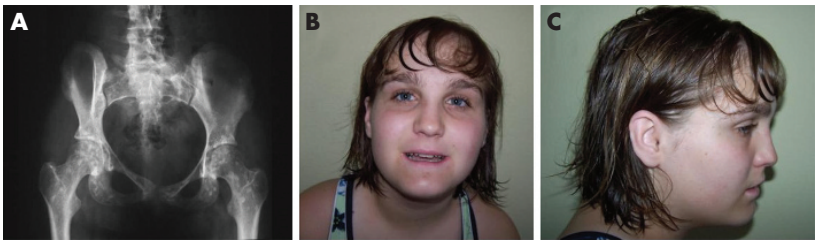

Figure 1 Clinical and radiographic features of the microdeletion syndrome. (A) Radiograph of the pelvis in patient 03g1858 showing multiple osteopoikilosis lesions in the proximal parts of the femurs and pubic bones. Facial phenotype of patient D0502619 with anteroposterior (B) and lateral (C) view (patients 03g1858 and #4818 refused clinical photographs). Parental/guardian informed consent was obtained for publication of this figure.

skills and speech. Six secondary teeth were missing on dental radiographs. After sustaining a fracture of the right tibia, he developed clawing of the toes and electromyogram findings suggestive of sciatic nerve injury. Radiographs documented osteopoikilosis lesions within multiple bones. When seen at 12 years of age, he was noted to have mild developmental delay, with difficulties in spelling and reading. He was tiny, with all growth measurements below the third centile. His face was triangular with widely spaced eyes. There were yellowish raised areas on the skin overlying the upper chest and flank. Trichothiodystrophy was considered as a diagnosis, but his hair was normal. He was reported to have a tremor that increased with writing. The patient was seen again at 18 years of age. His final height of 152 cm and weight of 41 kg were both below the third centile. He described a slow increase in tremor, most marked upon arising or intention. His overall health had been good, and he had achieved a normal puberty. He was entering grade 12, taking applied mathematics, and having problems with English.

The microdeletion in the first proband (03g1858) was identified during the course of mapping the gene for osteopoikilosis.[14] The patient showed loss of heterozygosity for a stretch of markers in the linkage interval, which resulted in a considerable reduction of the critical region and finally led (through a candidate gene approach) to the identification of *LEMD3* as the causal gene for osteopoikilosis. The deletion was confirmed with fluorescence in situ hybridisation (FISH) using BAC clone RP11-305O6, as described by Van Roy *et al*.[15] Breakpoints were further delineated using array CGH as described by Menten *et al*[16] with a custom tiling path array for chromosomal bands 12q14–12q15. BAC clones were selected based upon the May 2004 human genome project assembly (http://genome.ucsc.edu/) (table 1, appendix).

After publication of the first patient, a second patient with a similar phenotype was identified. FISH with BAC clones RP11-305O6 and RP11-361O1 confirmed the presence of a microdeletion encompassing *LEMD3* on chromosome 12. As in the first proband, the size of the deletion and the position of the breakpoints were determined by array CGH using a custom tiling path array with overlapping BAC clones (fig 2). The size of the deletion in the first two cases was similar (about 6 Mb) with an overlap of about 5.3 Mb. The genomic position of the deletion in patient 1 was slightly more telomeric than that in patient 2. Karyotyping and FISH of the parents of patients 1 and 2 yielded normal results, indicating that both deletions occurred de novo.

The microdeletion in the third patient (#4818) was identified during a study of 100 children with idiopathic mental retardation and normal standard chromosomal analysis, using Affymetrix GeneChip® Human Mapping 100K arrays.[9] Breakpoints were mapped to SNP rs10506536 (63342649 base pairs) and SNP rs10492198 (66780095 base pairs), indicating a 3.44 Mb deletion. Affymetrix Genechip® analysis of the parents yielded normal results, indicating that this deletion also occurred de novo. The deletion in the child was confirmed by FISH using BAC RP11-91K23. This deletion is smaller but lies entirely within the 5.3 Mb region that was deleted in both patients 1 and 2 (fig 2).

Segmental duplications or low copy repeats have been shown to play an important role in the formation of recurrent microdeletion syndromes by non-allelic homologous recombination.[17–20] However, no evidence of segmental duplications or low-copy repeats was found near any of the six breakpoints in these three patients. Microdeletions may also occur without involvement of low copy repeats.[12 21–23] Recent reviews estimate that only 25–50% of copy-number variants are associated with segmental duplications.[24] Recurrent microdeletions that are not associated with low-copy repeats usually have different breakpoints in each case. They most likely result from breakage with subsequent nonhomologous endjoining. Given the apparent absence of low copy repeats near the 12q breakpoints described here, we suspect that a mechanism of nonhomologous endjoining may be responsible for occurrence of the microdeletions in our three cases.

In 1995, Jurenka and Van Allen reported a patient with mental retardation, short stature and a mixed sclerosing bone dysplasia reminiscent of melorheostosis.[25] We suspect that this patient may have the same microdeletion as found in our three probands. Unfortunately, no DNA from this patient was available to test this hypothesis.

The similar phenotype in our three probands is remarkable. All three patients had a low birth weight and presented in infancy with failure to thrive. They subsequently showed delayed neuromotor development and finally mild mental retardation. They do not show a remarkable facial dysmorphism but all have a proportionate short stature with osteopoikilosis lesions on skeletal radiographs. One patient (#D0502619) developed a melorheostosis lesion in the foot. We have shown in previous studies that loss-of-function mutations in the *LEMD3* gene can result in osteopoikilosis and/or melorheostosis lesions.[14 26] However, failure to thrive, short stature and mental retardation are not observed in patients with either osteopoikilosis or melorheostosis. These findings must therefore be the result of haploinsufficiency for other contiguous genes in the microdeletion interval. In the common deleted region two interesting genes (*HMGA2* and *GRIP1*) reside, which may account for these additional clinical problems in our patients (fig 2).

HMGA2 codes for an architectural factor belonging to the high-mobility group (HMG) of proteins. It is characterised by three conserved DNA-binding domains, AT hooks and an acidic C-terminal tail. This gene product is involved in DNA packaging and plays an important role as a transcription factor in gene regulation.[27] Recently, *HMGA2* was described as the putative causal gene in a patient with overgrowth, lipomas and a de novo pericentric inversion of chromosome 12.[28] Battista *et al* reported a murine model with a constitutively expressed truncated form of *Hmga2*, which led to gigantism associated with lipomatosis. The authors proposed that disruption of the *Hmga2* gene led to upregulated expression.[29] Disruptions and

Table 1 BAC clones used for breakpoint detection in patients 1 and 2, with their name, sanger name, chromosome, start position and end position according to the NCBI 36 genome assembly

Name	Sanger name	Chromosome	Start (base pairs)	End (base pairs)	Patient 1	Patient 2
RP11-103L8	bA103L8	12	60326906	60482957	+	+
RP11-96F13	bA96F13	12	60399378	60556846	+	+
RP11-402I16	bA402I16	12	60470226	60639036	+	+
RP11-196A13	bA196A13	12	60516544	60670139	+	+
RP11-15P10	bA15P10	12	60592183	60768715	+	+
RP11-542G14	bA542G14	12	60637601	60804185	+	+
RP11-155D5	bA155D5	12	60695975	60861269	+	+
RP11-120M24	bA120M24	12	60829050	60980979	+	+
RP11-97A6	bA97A6	12	60917552	61052698	+	+
RP11-169M9	bA169M9	12	60920103	60948010	+	+
RP11-467D14	bA467D14	12	60966290	61162052	+	−
RP11-142E5	bA142E5	12	61085454	61271298	+	−
RP11-151H22	bA151H22	12	61221704	61367847	+	−
RP11-570O18	bA570O18	12	61280315	61431623	+	−
RP11-131G23	bA131G23	12	61343791	61525936	+	−
RP11-538D3	bA538D3	12	61529122	61684800	+	−
RP11-24L23	bA24L23	12	61634279	61805538	−	−
RP11-263K23	bA263K23	12	61706479	61872677	−	−
RP11-61L21	bA61L21	12	61883235	61951183	−	−
RP11-105I10	bA105I10	12	61907586	62065780	−	−
RP11-52P5	bA52P5	12	62021250	62183689	−	−
RP11-267F23	bA267F23	12	62033781	62189805	−	−
RP11-134D22	bA134D22	12	62196683	62347713	−	−
RP11-272A21	bA272A21	12	62218641	62371090	−	−
RP11-415I12	bA415I12	12	62349962	62538439	−	−
RP11-367H3	bA367H3	12	62470168	62644125	−	−
RP11-274J7	bA274J7	12	62508196	62670790	−	−
RP11-456A6	bA456A6	12	62577649	62745265	−	−
RP11-196H14	bA196H14	12	62738094	62928731	−	−
RP11-290I21	bA290I21	12	62854244	62854997	−	−
RP11-444B24	bA444B24	12	66740902	66931728	−	−
RP11-71J4	bA71J4	12	66926693	67077364	−	+
RP11-81H14	bA81H14	12	67059831	67208966	−	+
RP11-185H13	bA185H13	12	67160267	67368737	−	+
RP11-254B13	bA254B13	12	67191207	67347334	−	+
RP11-531F4	bA531F4	12	67255471	67462087	−	+
RP11-450G15	bA450G15	12	67366710	67543065	−	+
RP11-410I6	bA410I6	12	67507773	67668188	−	+
RP11-249J13	bA249J13	12	67574683	67726286	−	+
RP11-43A22	bA43A22	12	67681180	67830736	+	+
RP11-324P9	bA324P9	12	67816914	68000647	+	+
RP11-73J11	bA73J11	12	67862072	68020699	+	+
RP11-426B12	bA426B12	12	67926203	68135266	+	+
RP11-159A18	bA159A18	12	68032940	68179706	+	+
RP11-23C15	bA23C15	12	68135291	68315468	+	+
RP11-15L3	bA15L3	12	68217524	68403913	+	+
RP11-161M18	bA161M18	12	68369051	68562065	+	+
RP11-21I1	bA21I1	12	68442353	68627676	+	+
RP11-21C8	bA21C8	12	68508458	68666217	+	+
RP11-384F11	bA384F11	12	68610540	68808607	+	+
RP11-60E14	bA60E14	12	68706960	68858931	+	+

+, Presence of two copies; −, the BAC clone is deleted according to the array CGH profile.

rearrangements of *HGMA2* leading to aberrant gene expression are a frequent observation in lipomas and other benign mesenchymal tumours.[30–37] Overexpression has also been reported in malignant tumours.[38] Interestingly, Zhou *et al*. reported a ''pygmy'' phenotype in *Hmga2*$^{-/-}$ murine models,[39] with heterozygous mice displaying a milder phenotype (80% of the weight of wild-type mice).[40] Taken together, these data are consistent with an important role for *HMGA2* in growth. Hence, haploinsufficiency of this gene may result in short stature as observed in our patients. To test this hypothesis, a series of patients with idiopathic proportionate short stature will be tested for loss-of-function mutations in the *HMGA2* gene.

Glutamate receptor interacting protein 1 (*GRIP1*) is a good candidate gene for mental retardation. *GRIP1* is highly expressed in adult human and fetal brain as well as in other organ systems. The gene produces three different transcripts by alternative splicing and contains seven highly conserved domains. All *GRIP1* products contain the PDZ domain, which is important in synaptic function.[41] *GRIP1* proteins localise through their PDZ domains to α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors in cultured rat hippocampal neurons.[42 43] These AMPA receptors mediate synaptic transmission through glutamate, the major excitatory neurotransmitter in the central nervous system. *GRIP1* is implicated in targeting AMPA receptors to the synapse.[42 44 45] *GRIP1* is also involved in the induction of long-term potentiation in rat hippocampal mossy fibres.[46] In addition to their role in glutamergic synaptic transmission, *GRIP1* products also localise to GABAergic synapses in rat hippocampal cultures and intact rat brain.[47 48] Homozygous Grip1 knockout mice die as embryos.[49] The heterozygous *Grip1* knockout mouse phenotype has not yet been reported. The observations that *GRIP1* codes for a non-redundant protein that it is highly expressed in fetal and adult human brain, and involved in glutamergic synaptic transmission, support the possibility that *GRIP1* haploinsufficiency caused the learning problems in our patients.

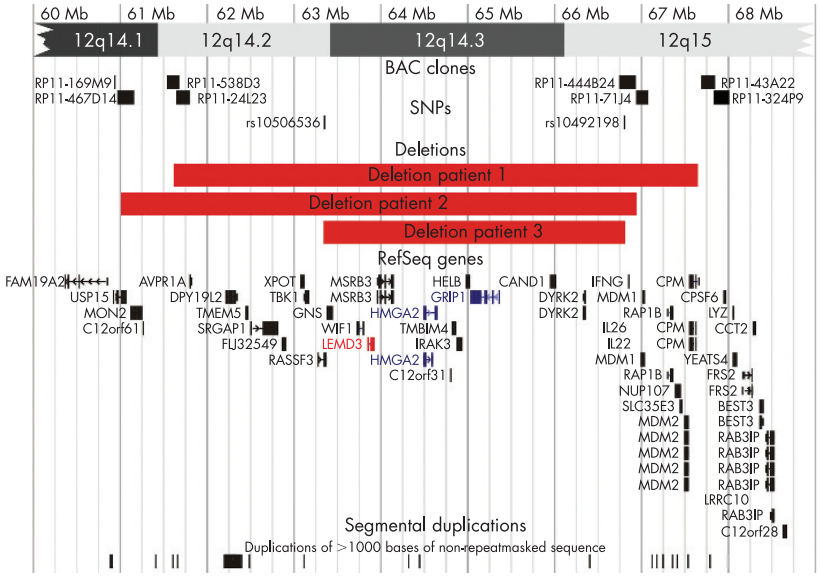

Figure 2 Chromosomal map of the deleted region with breakpoint flanking BAC clones for patients 1 and 2, breakpoint flanking SNP markers for patient 3, RefSeq genes (UCSC Genome Browser on Human Mar. 2006 Assembly) and segmental duplications. Karyotypes are written as: 46,XX,del(12)(q14.2q15).ish del(12)(q14.3)(RP11-305O6-) arr cgh 12q14.2q15(RP11-24L23RP11-249J13)x1 de novo for patient 1, 46,XX,del(12)(q14.1q15).ish del(12)(q14.3)(RP11-305O6-,RP11-361O1-) arr cgh 12q14.1q15(RP11-467D14 RP11-444B24)x1 de novo for patient 2 and 46,XX,del(12)(q14.2q15). arr cgh 12q14.2q15(rs10506536 rs10492198)x1 de novo for patient 3.

We conclude that we have identified a newly recognisable microdeletion syndrome involving chromosome 12q14. The clinical phenotype is mainly characterised by mild mental retardation, low birth weight with failure to thrive in early infancy and proportionate short stature. The osteopoikilosis lesions on skeletal radiographs are the most distinguishing feature of this microdeletion syndrome that should facilitate recognition of this peculiar disorder among children with mental retardation and growth failure.

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paper 4: Delineation of a critical region on chromosome 18 for the del(18)(q12.1q21.1) syndrome

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Delineation of a critical region on chromosome 18 for the del(18)(q12.1q21.1) syndrome

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ABSTRACT

Deletions involving the long arm of chromosome 18 have been reported in many patients. Most of these deletions are localized in the telomeric region and are detectable by standard cytogenetic analysis. However, smaller and interstitial deletions leading to a recognisable phenotype and residing in the region on chromosome 18q12.3 have also been identified. Using array CGH, we detected an interstitial deletion of only 1.8 Mb in size in chromosomal band 18q12.3. The phenotype of this proband corresponded very well with that observed in other reported cases with a structural monosomy of 18q12.3. Further characterization of the deletion allowed us to determine a critical region for the del(18)(q12.1q21.1) syndrome.

1 INTRODUCTION

ArrayCGH has been proven to be very helpful in (1) the identification of submicroscopic chromosomal aberrations leading to mental retardation and congenital abnormalities (MR/MCA) [1-5],(2) the characterization of new microdeletion syndromes [6-9] and (3) the identification of genes responsible for MR/MCA syndromes 10. To the best of our knowledge, only one critical region in a recurrent deletion syndrome has thusfar been delineated using arrayCGH [11]. Most recurrent microdeletions are flanked by Low Copy Repeats (LCRs), leading to similar breakpoints in all affected patients and hampering in this way the identification of the responsible critical genes. Recurrent interstitial deletions not mediated by LCRs are less common. Deletions involving the long arm of chromosome 18 have been reported in many patients. Most of these deletions are telomeric deletions and are detectable by standard cytogenetic analysis. However, smaller, interstitial deletions, encompassing chromosome band 18q12.3, and parts of neighbouring bands, have also been identified [12-23]. All these cases with microscopic detectable interstitial deletions share similar clinical features, including mental retardation, mild facial dysmorphism and abnormal behaviour. Here we describe a boy with typical features of the del(18)(q12.1q21.1) syndrome due to a 1.8 Mb deletion within chromosomal band 18q12.3.

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2 METHODS

2.1 Cytogenetic analysis and FISH

Analysis of G-banded metaphase chromosomes was performed on short-term lymphocyte cultures using standard procedures. FISH was performed as described [24].

2.2 Array CGH analysis

DNA was isolated from total blood using the Puregene Genomic DNA Purification Kit (Gentra Systems), according to the manufacturer's instructions. Using random prime labelling, 500 ng of patient and control female DNA was labelled with Cy3 and Cy5 (BioPrime Array CGH Genomic Labelling System, Invitrogen) and subsequently purified (Microspin G-50 columns, Amersham Biosciences). The labelled fragments were suppressed with 150 μg fluorometric QC Cot1-DNA (Invitrogen) and 400 μg yeast tRNA, and resuspended in 60 μl hybridisation buffer at 37°C (50% formamide, 10% dextran sulphate, 0.1% Tween 20, 2x SSC, 10 mM Tris pH 7.4). In-house produced 1 Mb resolution BAC arrays consisting of 4048 clones spotted in triplicate on CodeLink Activated slides (Amersham Biosciences) were prehybridised at 37°C during 1h using 100 μg fluorometric QC Cot1-DNA (Invitrogen) and 150 μg herring sperm DNA, resuspended in 120 μl hybridisation buffer. After removal of the prehybridisation mixture, patient and control DNA was simultaneously hybridised for 48h at 37 °C. The slides were washed in 1x PBS/0.05% Tween 20 for 10 min at room temperature, 50% formamide/2x SSC for 30 min at 42°C and finally 1x PBS/0.05% Tween 20 for 10 min at room temperature. After centrifuge drying, the slides were scanned using a GMS 418 Array Scanner (MWG). The scan images were processed with Imagene software (Biodiscovery) and further analysed with our in-house developed and freely available software tool arrayCGHbase (http://medgen.ugent.be/arraycghbase/) [25]. Reporters were excluded from analysis if one of the following criteria were fulfilled: signal to noise ratio < 3; standard deviation of the log2 transformed ratios between triplicates > 0.2; and only one informative replicate.

3 RESULTS

3.1 Clinical findings

The proband was referred to the genetic outpatient clinic at the age of 3 years because of psychomotor retardation. He was the oldest son of healthy, non-consanguineous Caucasian parents. The pregnancy was unremarkable. Because of advanced maternal age, an amniocentesis was performed which revealed a normal male karyotype. He was born at 8 months gestation by cesarean section because of solutio placentae. His birth weight was 2110 g and length 42 cm. There were no signs of respiratory distress or asphyxia after birth. However feeding problems were present due to poor suck reflex.

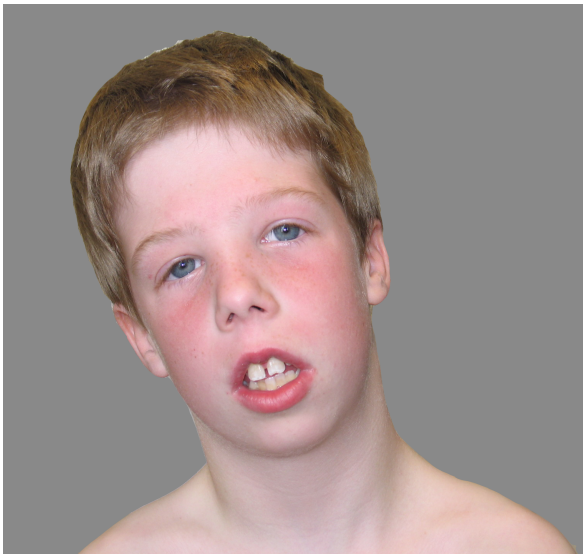

Fig. 1. Facial features of the proband at the age of 10 years 9 months. Note the downslanting palpebral fissures, broad nasal tip, everted lower lip and prominent maxillary incisors

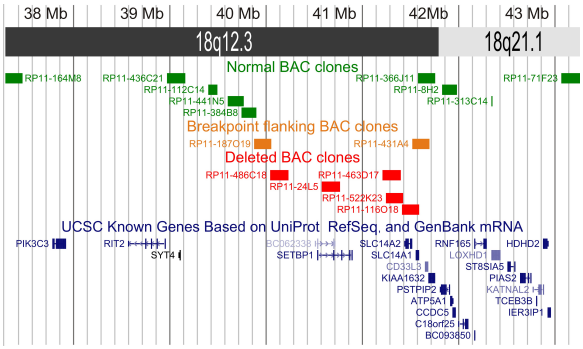

Fig. 2. Overview of the deleted region, BAC colons used in FISH analysis (green=not deleted, red=deleted, orange=breakpoint spanning), and known genes in region. The karyotype can be written as 46,XY.arr cgh 18q12.3q12.3(RP11-486C18'RP11-463D17)x1

Later on, starting around the age of 6 months, chronic and recurrent upper airway and gastrointestinal infections resulted in failure to thrive. His motor development was delayed with sitting at the age of 10 months and walking at the age of 22 months. Psychometric testing, performed at the age of 31 months, revealed a developmental age of 18 months (Bayley developmental screening test). Neurological evaluation showed hypotonia with poor muscle tone. Physical examination at the age of 34 months revealed a weight of 13 kg (-1.5 sd), length of 89,5 cm (-1.5 sd) and head circumference of 50,8 cm (+1 sd). The head was relatively macrocephalic with a prominent occiput and a flat forehead. His facial dysmorphism was characterized by small palpebral fissures, bilateral epicanthal folds, small nares, broad nasal tip, small but normally placed ears, small chin, high-arched palate and small teeth. Other congenital anomalies were absent. His hands were relatively small. CT scan of the brain, electroencephalogram, echocardiogram and skeletal radiographs failed in revealing any abnormalities. On further follow-up severe speech and language delay became apparent. At the age of 4 years he started in special education school. His limited communication skills and low self-esteem often resulted in frustration with aggressive behaviour towards his parents, younger brother and other younger children. Last evaluation was done at the age of 10 years 9 months. He still had a relatively large skull (head circumference + 1sd) in comparison with weight (-2.5 sd) and height (-2.2 sd). New clinical findings included downslanting palpebral fissures, everted lower lip with drooling, prominent maxillary incisors, small shoulders and thorax (figure 1).

3.2 Molecular cytogenetic findings

Chromosome analysis revealed a normal, male karyotype; 46,XY. Subsequent array CGH analysis revealed a small interstitial deletion of 2 consecutive BAC-clones in chromosome band 18q12.3. The proximal breakpoint was located between clones RP11-164M8 (37482575 bp) and RP11-486C18 (40044271 bp). The distal breakpoint was situated between RP11-463D17 (41408713 bp) and RP11-8H2 (41851567 bp) at the boundary of band 18q21.1. This corresponded to an interstitial deletion of 1.4 to 4.4 Mb. The deletion was confirmed with FISH and breakpoints were further fine-mapped. The BAC clone RP11-187O19 (39888100 bp - 40067033 bp) encompassed the proximal breakpoint and the BAC clone RP11-431A4 (41528702 bp - 41701608 bp) the distal breakpoint. These findings reduced the size of the deleted interval to a 1.8 Mb region (Figure 2). Karyotyping and FISH analysis of both parents yielded normal results, indicating that the deletion occurred de novo.

4 DISCUSSION

We present the first patient with a submicroscopic interstitial deletion in the proximal part of the long arm of chromosome 18, del(18)(q12.3q12.3). The deletion was detected by array CGH as part of a screen of 140 individuals with mental retardation and multiple congenital anomalies (MR/MCA), as reported elsewhere [2]. Several other patients have been described with deletions encompassing band 18q12.3. Tinkle et al. has emphasized the consistent pattern of phenotypic abnormalities observed in children with the del(18)(q12.2q21.1) syndrome [20]. An overview of the phenotypical characteristics of all previously described patients is provided by Kotzot et al. [15]. Affected individuals have moderate to severe mental retardation with only minor dysmorphic features. They are usually short and obese and have relative macrocephaly with high forehead. Other reported facial dysmorphic features include deep-set eyes with epicanthic folds, ptosis of upper eyelids, strabismus, abnormal ears with prominent antihelices and large lobules, hypoplastic midface with small nose and flat/wide nasal bridge, wide/hypoplastic philtrum and a high arched palate [15,16,20]. The dermatoglyphics in patients with del(18)(q12.2q21.1) are often reported to be abnormal. Most patients present with hypotonia or seizures. Behavioural problems such as autism spectrum disorder, hyperactivity and aggression are not uncommon [26]. Interestingly, most of these features are present in our patient who carries only a small deletion of 1.8 Mb in chromosomal band 18q12.3, suggesting that the critical region for the del(18)(q12.2q21.1) syndrome is residing in this part of chromosome 18. Krasikov et al. postulated that the critical region for the interstitial 18q phenotype was either located at q12.2 or q12.3 and does not require deletion of q21.1 [16]. Our findings confirm that only a small critical region on the long arm of chromosome 18 is responsible for most, if not all, of the observed features. According to the UCSC genome browser, this critical region contains only 5 genes (SLC14A1, SLC14A2, SETBP1, CD33L3 and KIAA1632). SLC14A1 codes for a urea transporter in human erythrocytes and endothelial cells of the vasa recta of the kidney and is involved in the KIDD (Jk) blood group system [27]. Although Jk-null red blood cells have reduced urea permeability, the Jk deficiency is not associated with

any obvious clinical phenotype except for a urine concentration defect [28]. SLC14A2 encodes a homologous urea transporter in the kidney [29]. Genetic variation in the human urea transporter-2 is associated with variation in blood pressure [30], whereas homozygous-null mice appear normal and are fertile. However, after water restriction, the inner medullary tissue shows a marked depletion in urea [31]. SET binding protein 1 (SETBP1) has been described as a fusion gene with NUP98 (Nucleoporin, 98-KD) in a case of paediatric acute T-cell lymphoblastic leukaemia with an 11;18 translocation [t(11;18)(p15;q12)] [32,33]. No further relevant functional information was available for SETBP1, CD33L3 and KIAA1632. Besides direct effects, gene expression can be perturbed by position effects due to the separation of regulatory sequences from the core gene sequence. As these position effects have been described for distances up to 900 kb [34], information was collected on all genes in a 1 Mb interval flanking the critical region. At least two other genes were selected because of their potential involvement in the pathogenesis of the disorder observed in our patient. RIT2 (RIC-like protein without CAAX motif 2), better known as RIN (RAS-like protein expressed in neurons), belongs to the RAS superfamily of small GTPases [35]. Small GTPases regulate a wide variety of cell processes, including growth, cell differentiation and cell movement. In contrast to other family members, RIT2 is only expressed in neurons 35 and Rit2 induces neurite outgrowth in rat pheochromocytoma PC12 cells [36]. In addition, Rit2 binds calmodulin in a Ca2+ dependent manner, suggesting an important role in the calcium/calmodulin mediated signalling pathways 35,36. Moreover, several other members of the RAS superfamily of small GTPases have been shown to be implicated in mental retardation syndromes. Mutations in HRAS have been described in patients with Costello syndrome, loss-of-function mutations in KRAS are implicated in Noonan syndrome and LEOPARD syndrome, while gain-of-function mutations in KRAS have been described in cardiofaciocutaneous syndrome 37. The second candidate gene is SYT4 (synaptotagmin-4), a brain specific member of a large family of synaptic vesicle proteins. Different hypotheses have been postulated concerning its molecular function. Several studies [38-40] ascribe a protective function to Syt4 upregulation in which neural activity is reduced by inhibiting neurotransmitter release. However, others postulate that Syt4 rather promotes than inhibits synaptic transmission [41]. Other functional evidence comes from the characterisation of Syt4 null mice [42,43]. Learning and memory tests in these amorphic mice suggest that Syt4 is critical for brain function and affects hippocampal-dependent learning and memory. Furthermore, the mutant mice show impaired motor coordination. Array CGH screening of patients with mental retardation and similar phenotypic features could reveal more microdeletions in this region and help us further in the confirmation and precise delineation of the critical region on 18q12.3. This in turn might lead to the identification of the responsible gene(s) for the del(18)(q12.2q21.1) phenotype.

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paper 5: Report of female patient with mental retardation and overgrowth due to a chromosomal rearrangement disrupting the *OPHN1* gene on Xq12

Menten B, Buysse K, Vermeulen S, Vandesompele J, Ng B, Carter N, Mortier G, Speleman F. (submitted)

Report of a female patient with mental retardation and overgrowth due to a chromosomal rearrangement disrupting the OPHN1 gene on Xq12

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ABSTRACT

We report on a patient with mental retardation, seizures and macrosomia with advanced bone age in whom a de novo apparently balanced chromosomal rearrangement 46,XX,t(X;9)(q12;p13.3) was identified. Using array CGH on flow-sorted derivative chromosomes (array painting) and subsequent FISH and qPCR analysis, we mapped and sequenced both breakpoints. The Xq12 breakpoint was located within the gene encoding for oligophrenin 1 (OPHN1) whereas the 9p13.3 breakpoint was assigned to a non coding segment within a gene dense region. Disruption of OPHN1 by the Xq12 breakpoint was considered the major cause of the abnormal phenotype observed in the proband.

1 INTRODUCTION

A small subset of patients with mental retardation and/or congenital abnormalities present with an apparently balanced de novo chromosomal rearrangement. In most patients it is assumed that the observed phenotypic anomalies are the result of submicroscopic deletions or duplications or alternatively disruption, activation or inactivation of a gene or genes located at or near the breakpoints. Until now, only a limited number of such apparently balanced de novo rearrangements have been investigated to the basepair level. In some instances detailed analysis has led to the identification of disease related genes [1]. Here we describe the combined application of array painting [2], FISH and real-time quantitative PCR which enabled us to map and sequence the breakpoints of a balanced reciprocal translocation t(X;9)(q12;p13.3) in a girl with mental retardation and overgrowth.

2 METHODS

2.1 G-Banding

Karyotyping was performed on short term lymphocyte cultures from peripheral blood with G-banding. Karyotypes were described according to the guidelines of the ISCN 2005.

2.2 Chromosome flowsorting

Purification of the translocated chromosomes, derivative 9 and X, was carried out using a flow cytometer (MoFlo®, DAKO) as described previously [2, 3]. DNA from the flow sorted chromosomes was used as template for rolling-circle amplification (RCA) with Repli-G (Molecular Staging). The amplified DNA was subsequently used as template DNA for array painting,, qPCR and sequencing [2].

2.3 Array painting

Using random prime labeling, 500 ng of amplified derivative chromosome 9 and derivative chromosome X DNA was labeled with Cy3 and Cy5 respectively (BioPrime Array CGH Genomic Labeling System, Invitrogen). Repetitive sequences were suppressed with 100 µg Cot-1 DNA (Invitrogen) and 400 µg yeast tRNA. The labelled fragments were resuspended in 60 60 µl hybridisation buffer at 37°C (50% formamide, 10% dextran sulphate, 0.1% Tween 20, 2x SSC, 10 mM Tris pH 7.4). In-house produced 1Mb BAC arrays were prehybridized at 37°C during 1h using 50 µg Cot-1 DNA (Invitrogen) and 150 µg herring sperm DNA, resuspended in 120 µg hybridisation buffer. After removal of the prehybridization mixture, DNA from both derivatives was simultaneously hybridized for 48h at 37°C. The slides were washed in 1x PBS/0.05% Tween 20 for 10 min at room temperature, 50% formamide/2x SSC for 30 min at 42°C and finally 1x PBS/0.05% Tween 20 for 10 min at room temperature. After centrifuge drying, the slides were scanned using a GMS 418 Array Scanner (MWG). The scan images were processed with Imagene software (Biodiscovery) and further analyzed with our in-house developed and freely available software tool arrayCGHbase (http://medgen.ugent.be/arraycghbase/) [4]. Data points were excluded from analysis if one of the following criteria were fulfilled: signal to noise ratio < 5; standard deviation of the log2 transformed ratios between triplicates > 0.2; only one informative replicate.

2.4 FISH with region specific probes

Fluorescence in situ hybridisation (FISH) was performed as described [5]. Locus specific RPCI-BAC probes for chromosome X and 9 were obtained by screening several assemblies (http://genome.ucsc.edu/) of the human genome project. All probes were relocated to the March 2006 Genome assembly (Table 1). BAC probes were labeled either with digoxigenin or biotin and hybridized on patients' metaphase chromosomes.

2.5 Fiber-FISH

Fiber-FISH analysis was performed according to Speleman et al. [6]. Fiber-FISH slides were prepared from a lymphoblastoid cell line of the patient (EBV689) and a normal male control cell line (EBV99) and hybridized with probes RP11-331F9, RP11-112J3 on chromosome 9 and probe RP3-360E18 on chromosome X, respectively flanking and spanning the breakpoint.

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2.6 PCR analysis

Primers for qPCR were designed to amplify products along the sequence interval of interest determined by fiber-FISH. qPCR was performed as described previously [7]. Once the breakpoints were found to lie between two primers, further primers were chosen to amplify fragments in between. Junction fragments were generated by using forward and reverse primers from the different chromosome sequences either side of the breakpoints.

2.7 Sequencing

The junction fragments were cleaned with exo-SAP (GE Healthcare) sequenced by using the di-deoxy chain terminator method [8] with the BigDye v3.1 ET terminator cycle sequencing kit from Applied Biosystems. After the sequencing reactions, the products were electrophoresed on a 3100 Genetic Analyzer and analyzed with Sequence Analysis and SeqScape (Applied Biosystems).

3 RESULTS

3.1 Clinical findings

The proband was born at 38 weeks gestation after an uncomplicated pregnancy. Birth weight was 2580 g (P10), length 45,5 cm (P10=47cm) and head circumference 33 cm (P10-P25). The postnatal course was uneventful. The girl had a delayed neuromotor development with sitting at 11 months and walking at 23 months. She developed a first epileptic insult at the age of 10 months for which she was treated with phenobarbital. The seizures were characterized by upward deviation of the eyes, anteflexion of the head and flexion of the legs. A first evaluation at the age of 14.5 months revealed a weight of 13.9 kg (P90=12kg), length of 82 cm (P90=81cm) and head circumference of 47.5 cm (P50-P90). There was no clear facial dysmorphism at that age. Just intermittent strabismus, a receding frontal hairline and mild hypotelorism were noted. Diagnostic work-up because of the seizures revealed a de novo balanced reciprocal translocation between the long arm of the X chromosome and the short arm of chromosome 9 (46,XX,t(X;9)(q12;p13). An MRI of the brain showed only mild dilatation of the ventricular system. Psychometric evaluation at the age of 18 months revealed an IQ score of 60. Around the age of 3 years the antiepileptic treatment was switched from phenobarbital to valproate. At the age of 5 years she started in special education school. On follow-up she developed an accelerated growth with shift of all parameters at or above the 90th centile. According to the bone age atlas of Greulich and Pyle, her bone age at the age of 8 years was between 11 and 12 years. On the last physical examination she was 8 years old. Weight was 69.4 kg (+4.6 sd) (BMI: 28.5), length 155.7 cm (+1.38 sd) and head circumference 54.2 cm (+2 sd). She had a friendly and affectionate personality. Her speech was not so easy to understand. When talking, she tended to hold her head in an oblique position, thus looking mainly with the left eye. Dysmorphic facial features included prominent incisors and relatively large ears (figure 1).

On the trunk a supernumerary nipple on both sides was observed. A mild extension deficit was present in the elbows and knees. In the upright position, she was holding her knees in mild flexion. Her gait was slow and unsteady. Her feet were small and flat.

3.2 Molecular cytogenetic findings

Partial G-banded karyotypes of the apparently balanced chromosomal rearrangement are shown in figure 2A. The karyotype could be described as 46,XX,t(X;9)(q12;p13). Following chromosome sorting and rolling circle amplification, array painting was performed (Figure 2B). Together with FISH analysis using region specific probes (Table 1), the breakpoint could be localized in BAC RP11-360E18 on chromosome Xq12 and in between BACs RP11-331F9 and RP11-112J3 on chromosome 9p13.3 (Figure 2B). The breakpoints were fine mapped using fiber FISH and could be further localised in RP11-360E18 and RP11-331F9 to an accuracy of ˜20kb (Figure 2C).

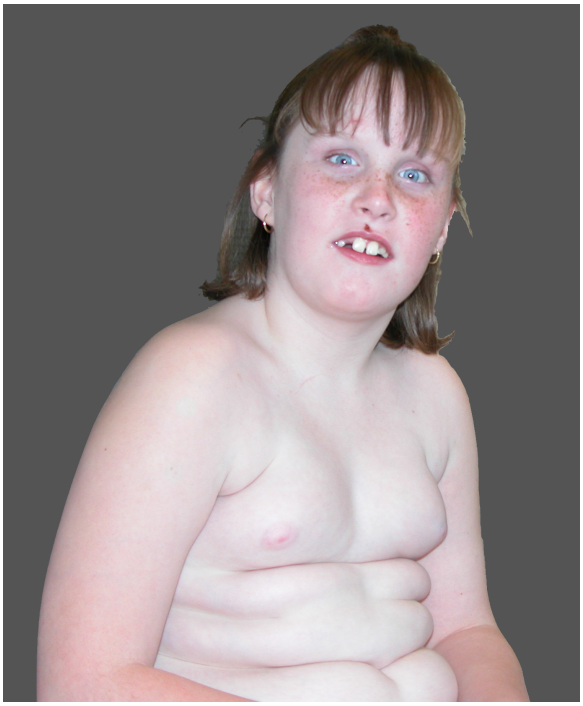

Fig. 1. Clinical photograph of the proband at the age of 8 years showing obesity and mild facial dysmorphism with prominent maxillary incisors, relatively large ears and high forehead.

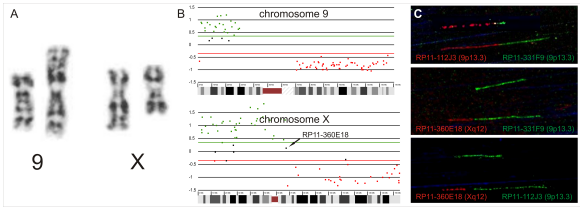

Fig. 2. Derivative chromosome 9 and derivative chromosome X after G-banding (A) array CGH profile after array painting for chromosome X and chromosome 9 (B) Fiber-FISH results with BAC clones RP11-112J3, RP11-360E18 (red) and BAC clones RP11-331F9 and RP11-112J3 (green) (C)

Consecutive qPCRs allowed us to narrow down the breakpoint on chromosome X between 67404215 bp and 67407331 bp (3116 bp) and on chromosome 9 between 35656338 bp and 35656776 bp (438 bp). Subsequently, using appropriate primers on both derivatives, both breakpoints were amplified and sequenced. (Figure 3).

4 DISCUSSION

We report on a girl with mental retardation (IQ=60) ,seizures and macrosomia with advanced bone age. Congenital abnormalities of the internal organs were not observed. Only a mild craniofacial dysmorphism

A agccagcagaaggcaacaaatagtt…acagagacacaaaaaatgctt
caaagaaatcaatgaatcc(a)**(a)cacactggcagcattcacaaac**
acacccatcaccaactgc…**ccttc**cttcatccaagcccttcaagtta

B **tggttggtaagctattaattattgcctcaatt**…**cggtctatcagttt**
tgttgatcttttcaaaaaaccagctccgttagtgctttgggtgggtg
gaagaggggtggggaatagag…ctg**gaacagctgctaaactccgctc**

Fig. 3. Sequence of both breakpoints. A for derivative 9 and B for derivative X. The nucleotides in bold represent chromosome 9 sequence, the underlined nucleotides represent primer sequence used for cloning, the adenine between brackets indicates one basepair which is lost either from chromosome 9 or chromosome X.

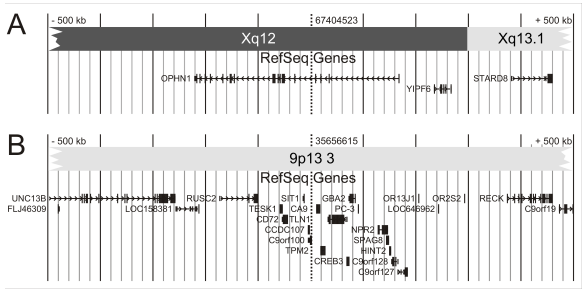

Fig. 4. Genomic context of the breakpoints on chromosome 9 (A) en chromosome X (B) with the RefSeq genes (UCSC Genome Browser on March 2006 Assembly) in a 500kb interval upstream and downstream of the breakpoint. The dotted line indicates the breakpoint

with strabismus and a receding frontal hairline was noted. On the trunk, a supernumerary nipple on both sides was present. Brain MRI revealed a mild dilatation of the ventricular system. Karyotyping was performed and revealed an apparently balanced translocation: 46,XX,t(X;9)(q12;p13). Breakpoints were sequenced after flow-sorting and array painting in order to determine their genomic position. On Xq12, the breakpoint interrupts the 390kb large oligophrenin 1 gene (*OPHN1*) (Figure 4). *OPHN1* has 25 exons and encodes a Rho-GTPase-activating protein. The Rho proteins are important mediators of intracellular signal transduction affecting cell migration, cell morphogenesis and synapse maturation [9]. The oligophrenin protein regulates cytoskeletal dynamics through Rho-GTPase modulation and is specifically involved in dendritic spine morphogenesis [10]. Of all the genes involved in X-linked mental retardation, six encode regulators or effectors of Rho-GTPase proteins, suggesting an important role of the Rho signalling pathway in cognitive functions [11]. Oligophrenin expression is highest in the developing central nervous system, and more precisely in the neuronal and glial cells [12]. In adult life, oligophrenin expression is enriched in the hippocampus, the olfactory bulb, and the Purkinje cell layer of the cerebellum. Although *OPHN1* mutations were first described to cause a rather nonspecific form of mental retardation [9], several publications have now highlighted the recognizable phenotype with neurological abnormalities and mild facial dysmorphism [11, 13, 14]. Cerebellar hypoplasia with vermian dysplasia is frequently observed on brain imaging studies and seem to result in dysmetria, adiadochokinesia, and oculomotor problems (nystagmus, strabismus, external ophthalmoplegia) in the affected individuals. [15, 16]. Ataxia is rarely observed. Seizures are commonly reported. The expression of oligophrenin 1 in the craniofacial skeleton, especially at the level of the mandible [11] may explain the facial dysmorphism seen in some patients with the 'oligophrenin syndrome'. The craniofacial dysmorphism is more pronounced in older patients and includes a long face with prominent chin, hypotelorism, deep-set eyes with prominent supraorbital ridges, long tubular nose, short philtrum, and thin upper lip. Macrocephaly and tall stature have also been reported in several families [11, 17], In accordance with its X-linked recessive inheritance pattern, the 'oligophrenin 1 syndrome' phenotype is usually encountered in affected males, with female carriers only showing a rather mild and nonspecific phenotype (learning difficulties and strabismus) unless they carry an X;autosome translocation as is illustrated by our patient. In females with an X;autosome translocation, X inactivation initially occurs at random but is followed by cellular selection, favouring the cells without a partial autosome inactivation. Accordingly, nearly 95% of females with a balanced X;autosome translocation show a skewed X-inactivation pattern with the normal X chromosome inactivated in almost all cells [18]. This explains the more pronounced phenotype observed in females with an X;autosome translocation. The chromosome 9p13 breakpoint is localised within a noncoding segment on chromosome 9. A position effect on the expression of the flanking genes can not be ruled out. Position effects due to chromosomal aberrations, leading to altered expression of genes have been described up to 1.3 Mb of the breakpoint [19, 20]. This possibility was not further investigated because the major part of the abnormal phenotype in our patient could be explained by the disruption of the *OPHN1* gene. So far, only a few constitutional, apparently balanced, translocations have been mapped at the sequence level, mostly due to the huge amount of experimental laboratory. However, the information on responsible genes obtained through above described efforts is of the utmost importance. In fact, the *OPHN1* gene was identified through the breakpoint analysis of a female patient with a t(X;12) translocation [21]. The strategy followed in this study is an example of a clear cut way to facilitate the analysis of balanced translocations, enabling to narrow down the translocation breakpoints in only a limited number of experiments. Although technically challenging, this study further illustrates the feasibility for rapid breakpoint mapping and sequencing, using a combined approach of chromosome flow sorting (or alternatively microdissection), linear DNA amplification and array CGH. It can be anticipated that similar studies on larger series of de novo apparently balanced translocations in mentally retarded patients will lead to the discovery of several new genes as a cause for mental retardation.

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chapter 3

ArrayCGH Data Analysis

Contents

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arrayCGHbase: an analysis platform for comparative genomic hybridization microarrays

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Abstract

Background: The availability of the human genome sequence as well as the large number of physically accessible oligonucleotides, cDNA, and BAC clones across the entire genome has triggered and accelerated the use of several platforms for analysis of DNA copy number changes, amongst others microarray comparative genomic hybridization (arrayCGH). One of the challenges inherent to this new technology is the management and analysis of large numbers of data points generated in each individual experiment.

Results: We have developed *arrayCGHbase*, a comprehensive analysis platform for arrayCGH experiments consisting of a MIAME (Minimal Information About a Microarray Experiment) supportive database using MySQL underlying a data mining web tool, to store, analyze, interpret, compare, and visualize arrayCGH results in a uniform and user-friendly format. Following its flexible design, *arrayCGHbase* is compatible with all existing and forthcoming arrayCGH platforms. Data can be exported in a multitude of formats, including BED files to map copy number information on the genome using the Ensembl or UCSC genome browser.

Conclusion: *ArrayCGHbase* is a web based and platform independent arrayCGH data analysis tool, that allows users to access the analysis suite through the internet or a local intranet after installation on a private server. *ArrayCGHbase* is available at http://medgen.ugent.be/arrayCGHbase/ .

Background

The introduction of a microarray based comparative genomic hybridization method (arrayCGH) in 1997 paved the way for higher resolution detection of DNA copy number aberrations [1]. ArrayCGH is founded on the same principles as metaphase CGH, but uses mapped reporters instead of whole chromosomes. One of the major challenges in arrayCGH studies remains the accessibility, management, and interpretation of the vast amount of data generated in single experiments, and parallel comparison of multiple experiments. Typically, these arrays contain 3,000 to 30,000 reporters, each of which has multiple biological annotations (chromosomal position, sequence information, gene name, biological and molecular function,...) as well as physical (grid layout) and quality control (sequence verification, FISH mapping information,...) annotations. In addition, the description of the DNA samples under investigation and the applied lab protocols should be easily accessible. For classical CGH, several commercial software packages are available to analyze and interpret the data of a CGH experiment. Also for arrayCGH there are a number of separate software systems that individually address some of the needs, such as databases for data storage (BASE [2]), applications for clustering and visualization of microarray data (seeGH [3], M-CGH [4], CGHAnalyzer [5], aCGH-smooth [6] and CGH-Miner [7]), public genome databases that contain reporter information, commercially available Laboratory Information Management Systems (LIMS), and various storage methods for recording biomaterial annotations. However, none of these software packages or databases combine all these features (see Supplemental Table). In this paper, we present the development of a web based open source arrayCGH analysis platform, *arrayCGHbase*, that combines all these features and on top provides additional unique aspects making the analysis and sharing of arrayCGH data easily implementable for both research and routine purposes.

Implementation

MIAME compliant database

arrayCGHbase runs in Windows, Linux, Macintosh, and Unix environments. Particular attention was paid to the use of open source software for the development of *arrayCGHbase*. The software was developed in the PHP scripting language, with all data being stored in a relational, MIAME [8] (Minimal Information About a Microarray Experiment) supportive, MySQL database and communicated to the user through an Apache Web server (Figure 1). After installation on a private server, experiments can be shared by different users over the internet or a local intranet. *ArrayCGHbase* integrates DNA sample information, lab protocols, extracted data, and contains a plug-in architecture for data transformation, analysis, and graphical display, allowing users to develop their own modules. Reporters can be directly linked to the Ensembl [9] or UCSC [10] genome browsers, providing additional up-to-date information on each reporter. Reporters can also be manually imported into the MySQL database with the ability to update all linked experiments. The structure of *arrayCGHbase* was designed to follow the laboratory workflow and is compatible with all types of arrayCGH experiments and data formats (dual colour genomic clone, cDNA [11], or oligonucleotide [12] arrays spotted on any substrate, physical layout, type of array, as well as single channel hybridizations such as the Affymetrix SNP chips [13]). With a personal account and administrated access levels, a user can enter new DNA samples, annotate these, and append all relevant sample information such as quantity, quality, and applied lab protocols at each step. Each user can group experiments together into projects and, in a uniform and streamlined fashion, apply filters and transformations and run analyses. Data is exportable in several formats for offline analysis using other (dedicated) software tools, for publication or for sharing data with the research community. For advanced users, an SQL query window allows interrogation of the underlying MySQL database.

Data processing and visualization routines

A first and important step in data analysis of arrayCGH experiments is the processing of large, possibly noisy data sets to identify the specific reporters that are differentially hybridized and hence show an aberrant copy number. Data processing is performed in a streamlined four-step manner: (1) the local noise or background associated with the experiments is removed, (2) the quality of the experiment is assessed and poor quality features are removed, (3) ratios are calculated, transformed to $\log_2$ scaled ratios, and normalized, and finally (4) reporters that show altered ratios are identified and hence, reporters with aberrant copy number are identified. In the past, this normally required the sequential processing of data by different, often incompatible programs. Using established and widely used microarray (CGH) data processing procedures, *arrayCGHbase* will automatically correct the signal intensities, filter out unwanted poor quality features (based on signal to noise ratio, image processing software related flags, or other user defined filters), normalize the fluorescence intensity ratios, score levels of differential hybridization, combine the results of replicate experiments and assess the quality of individual and replicate experiments. All these steps are user adjustable.

Input data and local background correction

The experimental input data for *arrayCGHbase* consists of export files generated by image analysis software. Currently, the program recognizes files from GenePix Pro versions 2.0–4.0, Scanalyze version 2.0, UCSF SPOT version 2.0, Imagene versions 4.0 – 5.5 and the Affymetrix

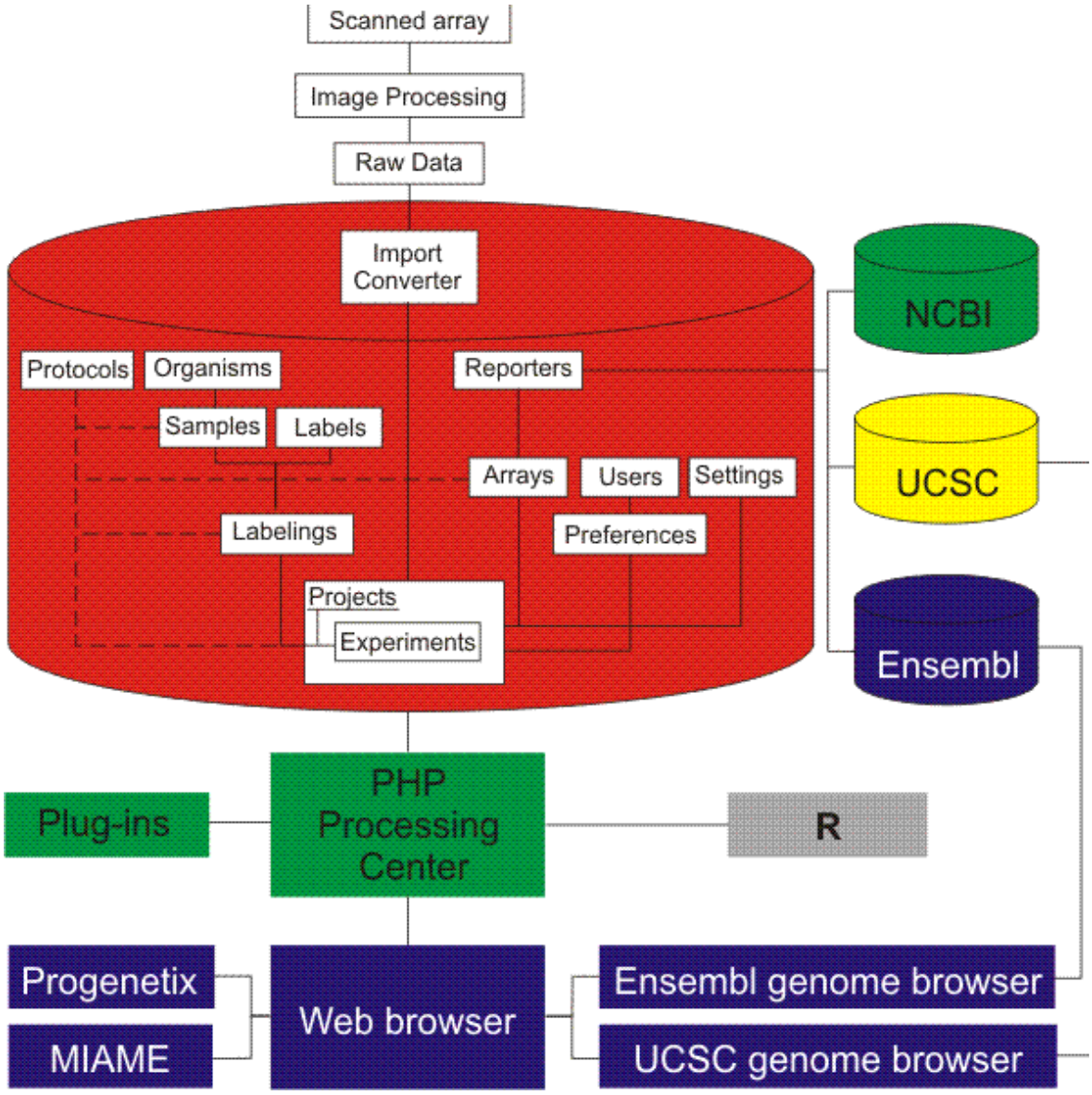

Figure 1
***arrayCGHbase* scheme**. The data is stored in a MIAME supportive MySQL database (red) and reporter info is updated using the NCBI, Ensembl and UCSC genome database. Data and results are presented to the user through a web browser via the PHP scripting language. Data-normalization and other analysis or result visualization methods can be integrated using the plug-in architecture. Further data processing using the R statistical scripting language will be implemented in the near future. Results can be exported to a Progenetix or MIAME compatible format, or visualized on the genome using the Ensemble or UCSC genome browser.

Chromosome Copy Number Tool. The program can easily be updated for the recognition of other data input formats upon request. Moreover, *arrayCGHbase* has an interactive import wizard, which makes it possible to import data at your own desire. The processing steps may be changed by altering the parameters at the input stage. By default, the results for each feature are defined as the median foreground minus background intensities for each dye (as determined by the image processing software). The ratio of each feature is determined as the relative background corrected signal between the two dyes or in the case of single color experiments as the corrected signal intensity.

Poor quality flagging

Nearly every experiment contains features of poor quality, comprising features that have unusual morphology (e.g. doughnut patterns), exhibit uneven hybridization, or have saturated signal intensity. After background corrections, *arrayCGHbase* can automatically flag features of inferior quality using different criterions (e.g., the standard deviation between replicates), by a manually set signal or signal-to-noise threshold, or using image processing generated flag annotations.

Normalization

Following calculation of the corrected signal intensities and filtering for good quality features, the relative contributions of the fluorescence intensities are compared. To go from a multiplicative space to an additive space, ratios are $\log_2$ transformed. Ideally, the signals of the two dyes should be equal for nucleic acid reporters that have equal amounts in the test and reference samples (i.e., the $\log_2$ transformed ratios of the two corrected signals should approach zero for reporters hybridizing to an equal degree in both fluorescence channels). However, in practice the ratio of the corrected signal intensities deviates from the expected ratio due to the different molecular and physical characteristics of the dyes, the different amounts of DNA used for labeling with the different dyes, the spatial heterogeneity in the hybridization conditions across the slide, and many other factors. Normalization compensates these effects by applying a data transformation such that ratios of reporters with unchanged copy-number are close to zero. In the normalization step, an appropriate term is added or subtracted from the $\log_2$ transformed ratio for each feature. The program allows normalization in several ways, either by global normalization or subgrid (or pin) normalization, or by a combination of different normalization procedures.

A major issue in microarray normalization is the definition of the set of constant probes to which the data are normalized. The most widely accepted method employs the 'constant majority' method, which assumes that the majority of reporters do not change in ratio. This method, which is implemented in *arrayCGHbase*, is generally applicable to most experiments as it is valid even in cases where up to 50% of reporters have altered ratios, it does not require prior knowledge of which features remain constant, and allows for intensity and spatial variation. Hence, this method calculates a scaling term from the median of all ratios, excluding all outliers. In this way the distribution of all ratios is transformed so that it centers around zero.

Quality control

Percentage of good quality spots

This first quality assessment is a basic calculation of the number of reporters (or features) that are not flagged based on quality measures (user defined parameters and thresholds, see above).

Intra- and inter-array hybridization quality

Three other major quality parameters can be determined with *arrayCGHbase* for each experiment. The first assesses the variation between reporters present in replicates on the array (typically duplicates or triplicates). An increased variation typically reflects lower quality hybridizations resulting in less reliable ratios. A second quality parameter is the standard variation between the different reporters on the array that show a normal (unaltered) copy number. This quality measure is only applicable in experiments with few reporters with aberrant copy number. The third quality measure is the average ratio for reporters with aberrant copy number. This ratio should significantly differ from zero to allow identification of differentially hybridized reporters. This last quality measure is only applicable in experiments where DNA copy number aberrations are known or validated. These parameters provide an objective quality measure and can also be helpful to compare different experiments.

In addition to these parameters, different graphical displays, such as ratio-intensity plots (usually referred to as MA plots), dual channel intensity scatter plots, and ratio histograms give an idea of the quality of an individual experiment or series of experiments (Figure 2). In all these visualizations, thresholds for gains and losses are displayed and can be adjusted. The slide viewer generates a virtual spatial view of all features on the array using the ratio, or signal and background intensities; this viewer allows the identification of problematic regions or artifacts on the slide surface. Clicking on an individual feature shows specific data associated with this feature (e.g., reporter name, signal intensities, and data quality flags).

Scoring chromosomal regions with aberrant copy number

The final step in arrayCGH data processing is the identification of reporters that exhibit differential hybridization, corresponding to chromosomal regions that have altered

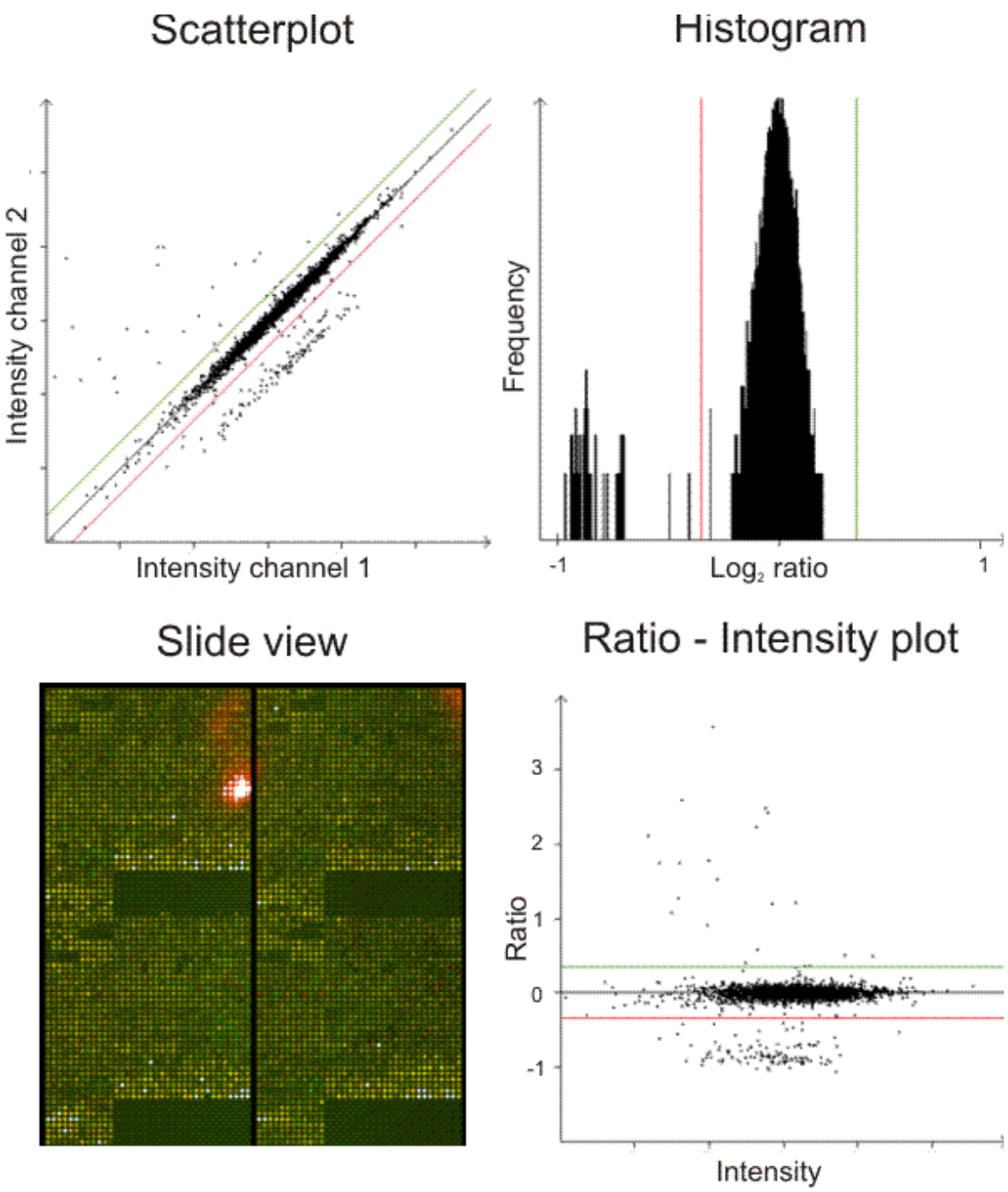

Figure 2
Quality control graphs. Graphical displays to assess the quality of an experiment, such as a dual channel intensity scatter plot, ratio histogram, ratio-intensity plot and a virtual slide view.

copy number. The major issue is to identify those reporters whose relative ratios stand out from the experimental noise with sufficient statistical significance. *arrayCGHbase* currently incorporates two scoring methods. The most widely used approach is to define a ratio threshold and identify the probes that exhibit ratios greater or smaller than this threshold. Another, statistically more sound approach, is to use a floating threshold based on the standard deviation of all reporters in a given experiment. Reporters that exhibit ratios greater than this threshold will be defined as differential [14]. Both methods are implemented in *arrayCGHbase* and can be applied on each individual feature, or on the mean value of replicates. Besides the aberrant feature scoring methods, two other algorithms are available: a universal data smoothing algorithm, as well as a breakpoint-identification algorithm, which both consist of a moving window along the chromosomes and hence make use of the spatial "along the chromosome" distribution of the reporters. With these algorithms, chromosomal breakpoints can be easily identified in more noisy datasets. By writing custom plugins (in PHP or R), sophisticated algorithms that use segmentation methods (e.g. Cluster Along Chromosomes, CLAC [7]) or others, can be implemented by any user in a straightforward way.

Chromosome visualization

A wide variety of result viewers are available. The results can be mapped upon standard ISCN (International Standard on Cytogenetic Nomenclature) ideograms in an electronic karyotype, or visualized per chromosome or zoomed in on a region of interest (Figure 3). Moreover, various CGH profile views provide the user with a tool to compare different experiments and to identify regions with relevant copy number alterations. Views are returned to the user either as PNG (Portable Network Graphic) or as SVG (Scalable Vector Graphic) files, with the ability to scale images according to screen width.

Data export

Processed data can be exported as MIAME compliant text files and figures; these include the original feature signal and background intensities, the normalized ratio value, a list of reporters that are differentially hybridized, and the data quality parameters. Additionally, a file can be generated for submission of arrayCGH results directly into Progenetix [15], a comprehensive collection of published cytogenetic abnormalities in human neoplasms. Lastly, BED files can be created to map results and visualize the experiment from within the Ensembl or UCSC genome browser.

ArrayCGHbase at work

In several publications from our research group, *arrayCGHbase* has been successfully used to analyse arrayCGH data to identify and delineate copy number aberrations [16-19]).

At the demo site, users can explore the data published in Hellemans et al. [16], a small ~5 Mb deletion in chromosome 12q identified using SNP chips), the results of a case report of the identification of an unbalanced X-autosome translocation by arrayCGH in a boy with a syndromic form of chondrodysplasia punctata brachytelephalangic type [17], a distal 9p trisomy and distal Xp nullisomy caused by an unbalanced X;9 translocation: 46, Y, der(X)t(X;9)(p22.32;p23) detected with a 1 Mb BAC array), and the copy number profile of a cancer cell line NGP.1A.TR [18]). It is possible to look at the raw data of these hybridizations and more importantly, test the performance of the program using different settings.

Conclusion

We present *arrayCGHbase*, a versatile web based, platform independent data storage and analysis tool for processing microarray CGH data. Routines were implemented for feature flagging, data normalization, data quality assessment and the identification of chromosomal regions with aberrant copy number. A zoomable graphical interface allows immediate identification of altered genomic regions and the underlying gene content by several database links. A multitude of export functions allow the user to further process the results. The easy plug-in architecture makes it possible for each user to add custom algorithms for data analysis and visualization and share these with the user community. This webtool and database will enable investigators to interpret single experiments and compare large data sets efficiently throughout different array platforms and provides all of the essential features and links for further investigation of the genomic regions of interest.

Future developments

arrayCGHbase will continually be updated to incorporate new processing methods that will be developed both within and outside our laboratory. Immediate plans include the addition of export and import functions to R [20] or Bioconductor [21] to be able to apply several available mathematical algorithms such as two-dimensional LOWESS normalization [22]. Immediate export functions to the DECIPHER web site [23] to link phenotypical data to actual experiments will also be included. The *arrayCGHbase* source code is freely available under a Creative Commons License, to encourage others to develop new analysis methods and utilities that will further improve its capabilities.

Availability and requirements

An *arrayCGHbase* demo site is available at http://medgen.ugent.be/arrayCGHbase/. At this site, all quality

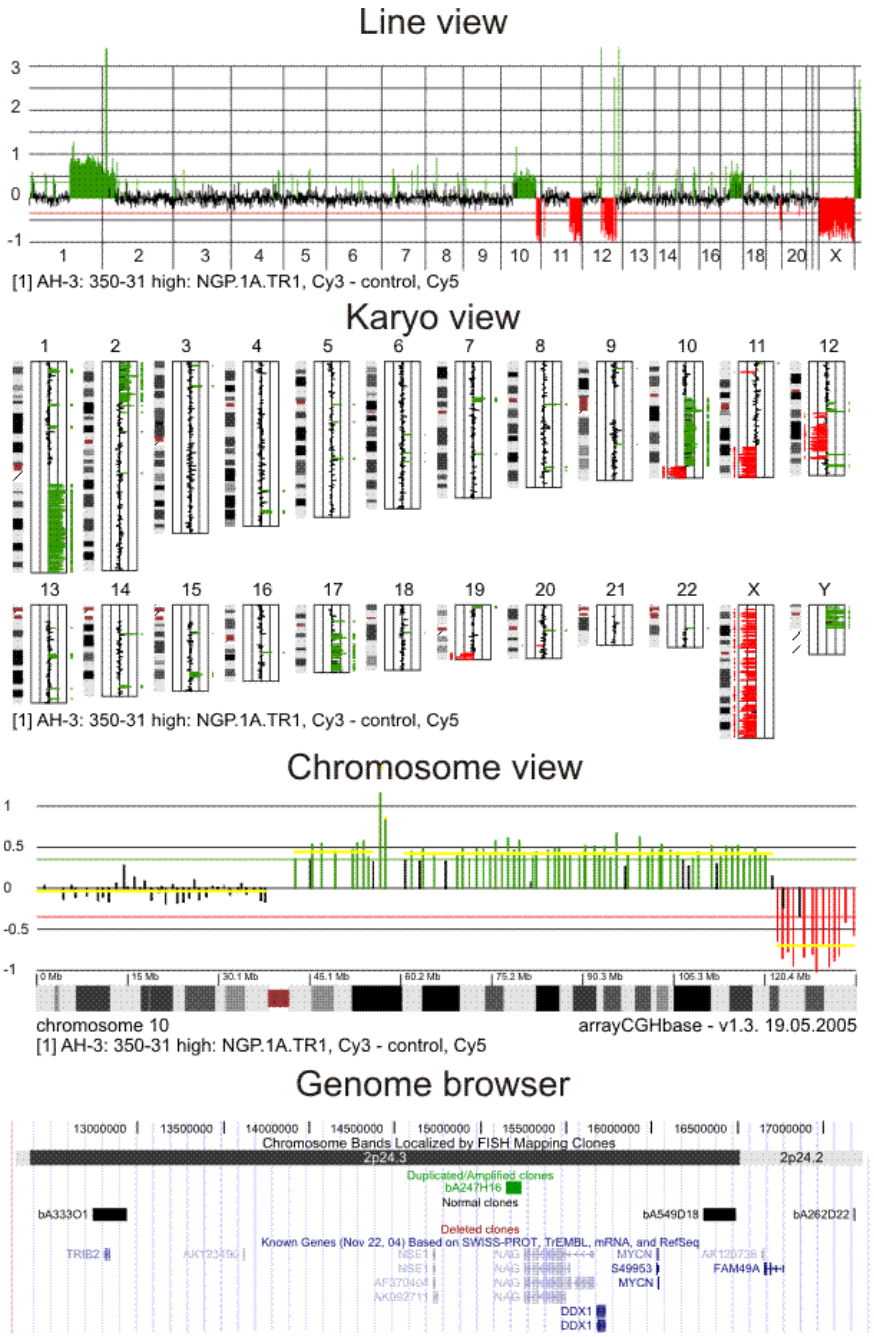

Figure 3
Selected result viewers. Graphical displays of *arrayCGH* results of neuroblastoma cell line NGP.1A.TR1: line view (all reporters ordered by chromosome and chromosomal position on one line), karyo view (al reporters mapped on their chromosomal position on a standard ISCN ideogram), chromosome view (zoom on one chromosome or chromosomal region) with breakpoint identification algorithm, and genome browser view (neuroblastoma cell line IMR32), with all reporters and their copy number status displayed in the UCSC genome browser.

control features and other features can be tested for several experiments with BAC arrays as well as SNP chips (see '*arrayCGHbase* at work'). At the same site, the complete package can be freely downloaded for local installation on a private hosted web server. For local use, additional software is required such as the MySQL database [24], a web server (e.g. Apache [25]), and PHP hypertext preprocessor [26]. These software packages are freely available and are key parts of LAMP (Linux, Apache, MySQL, PHP), an open source web platform. Enquiries for *arrayCGHbase* should be made to arrayCGHbase@medgen.ugent.be.

Glossary

Reporter: any DNA fragment (BAC, PAC, cosmid, fosmid, cDNA clone, oligonucleotide, genomic PCR product) used for hybridization

Feature: physical reporter spotted, printed, or otherwise linked to a substrate at a specific location

PHP: Hypertext PreProcessor (server-side scripting language)

MIAME: Minimal Information About a Microarray Experiment

MySQL: My Structured Query Language

ISCN: International System for human Cytogenetic Nomenclature

BED: Browser Extendable Data

Authors' contributions

BM was the principle programmer of arrayCGHbase. FP, KDP, PR and SVV contributed ideas for different features and display requirements. JV oversaw the project; all other authors have reviewed the manuscript and FS and JV were the final editors of the manuscript.

Additional material

Additional File 1

Comparison between different already available arrayCGH software programs and arrayCGHbase for the analysis and visualization of arrayCGH data.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1471-2105-6-124-S1.xls]

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26. **PHP Hypertext Preprocessor** [http://www.php.net/]

chapter 4

General Discussion and Future Perspectives

This thesis is part of a wave of new findings emerging from array CGH investigations that herald a new era in cytogenetics. After the successful implementation of array CGH in our lab, a large cohort of ~200 well selected patients with mental retardation and/or congenital abnormalities (MR/CA) was screened for submicroscopic aberrations. The present work highlights a number of important aspects of array CGH investigations in the study of mental retardation and congenital abnormalities (MR/CA).

The Implementation of High Resolution DNA Copy Number Analysis in the Investigation of Patients with Mental Retardation and/or Congenital Abnormalities

In a close collaboration with The Center for Human Genetics, Leuven, Belgium and The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK, a large insert clone array to cover the entire genome with an average 1 Mb resolution was developed. Protocols were optimized and quality criteria were defined and reported [60, 61]. Screening for submicroscopic aberrations in patients with a normal karyotype, and mental retardation and/or congenital abnormalities resulted in the identification of an unbalanced t(X;9) translocation in a boy with the brachytelephalangic type of chondrodysplasia punctata, mental retardation and obesity [187]. The identification of this *de novo* chromosomal rearrangement not only made accurate genetic counseling possible but also explained most of the phenotypic abnormalities observed in this patient. This paper nicely illustrated the new possibilities offered for sensitive screening of submicroscopic chromosomal imbalances by array CGH.

The Incidence of Copy Number Variations in Patients with MR/CA

This and other pioneering array CGH analyses [83, 188–192] on a total of 432 investigated patients with idiopathic mental retardation and/or congenital abnormalities (MR/CA) showed that in about 20% of cases chromosomal imbalances (DNA copy number variations, CNVs) were detected. This clearly reflects the high resolution of array CGH, which can detect CNVs of 1 Mb or less depending on the density of reporters spotted on the arrays, in contrast to standard karyotyping which has a resolution limited to 5-10 Mb. CNVs detected with array CGH include deletions, duplications and in some in-

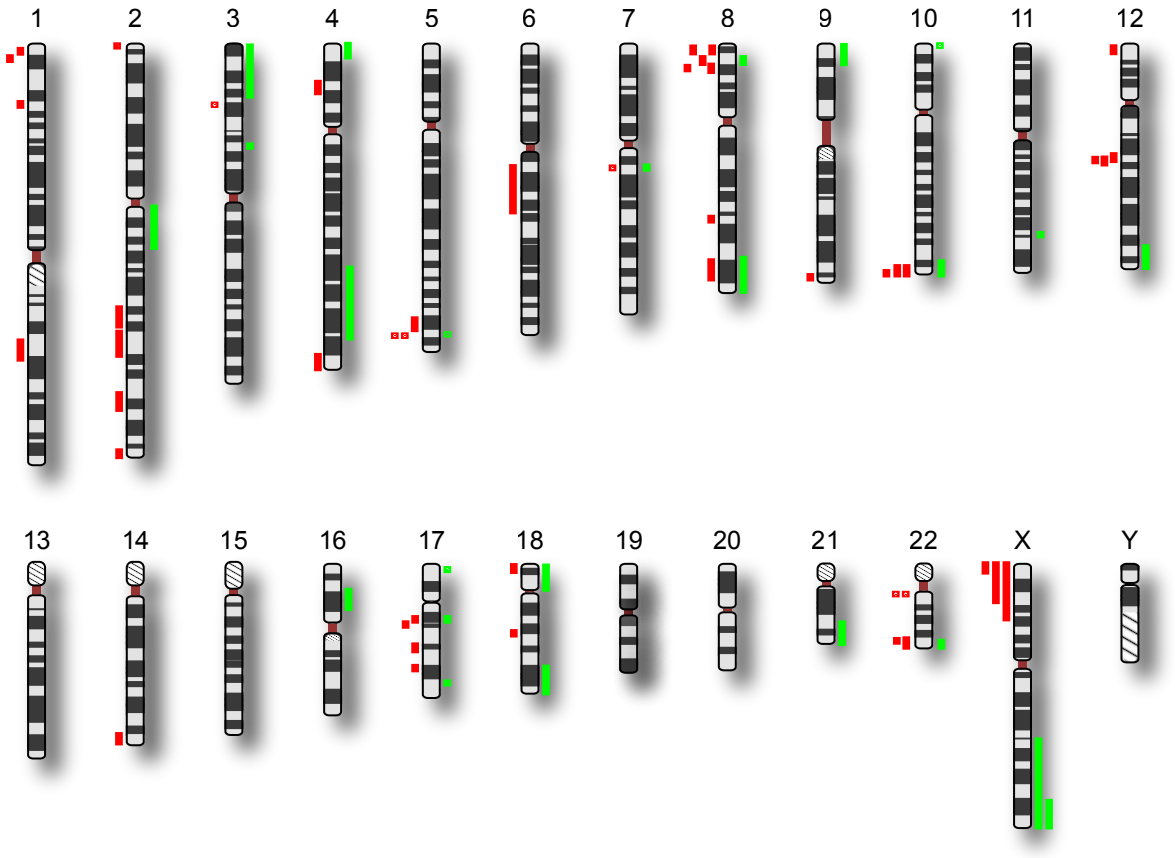

Figure 4.1 – *overview of all chromosomal imbalances detected with array CGH in patients with mental retardation and multiple congenital abnormalities (MR/MCA). In some of these patients a chromosomal aberration was detected with conventional karyotyping, but array CGH was used to unambiguously identify and delineate the genomic defect.*

stances cryptic unbalanced translocations. Our study and review of literature data allowed for the first time to obtain a reliable assessment of the incidence of *de novo* CNVs occurring in this patient group, a figure which was determined to be in the range of ˜8% (excluding patients with subtelomeric imbalances).

Genomic Distribution of Chromosomal Aberrations in MR/CA Patients and Genomic Causes for Chromosomal Rearrangements

A second important observation which we could make based upon our array CGH screening and review of available data was the fact that most of the CNVs seem to be scattered throughout the entire genome. Figure 4.1 gives an overview of all chromosomal imbalances in patients with mental retardation and multiple congenital abnormalities detected with array CGH in our center. In contrast to most of the recurrent deletion/duplication syndromes, for the great majority of the newly identified CNVs no direct evidence was found for an underlying role of the genomic structure in the formation of these CNVs. Although further high resolution mapping of many of these breakpoints is still ongoing, there is no evidence for the implication of segmental duplications (also termed Low Copy Repeats, LCRs) through non allelic ho-

mologous recombination. One of the exceptions in this regard is the newly discovered recurrent microdeletion syndrome mediated by LCRs on chromosomal band 17q21.31 [149–151]. Nevertheless, for some chromosomal regions evidence is accumulating that some form of genomic instability (or other mechanism) seems to be implicated. One series of examples are the frequently occurring 1p36 deletions which occur with an incidence of 1/5000 - 1/10000 and which seem to target a whole range of different breakpoints located within chromosomal band 1p36 [162, 164]. In our patient cohort we also identified in two patients an interstitial 1p36 deletion with distinct breakpoints for each of the two cases, further corroborating previous data.

The Long Way Towards the Description of the Human Morbid Map and Functional Annotation of the Human Genome

Determining the genotype/phenotype relationship in contiguous gene syndromes is a critical step in localizing the genes involved in the different characteristics of the phenotype and in understanding the underlying molecular basis of the different aspects of the condition. The major challenge in defining new microdeletion syndromes is to collect patients with overlapping deletions (or duplications) and to try to delineate common clinical features. An important milestone of this work has been the description of a new microdeletion syndrome on chromosome band 12q14 [165]. In the case of the 12q14 deletion the collection of additional patients was facilitated through the presence of a very peculiar clinical feature, i.e. osteopoikilosis. For many patients with a given CNV such a characteristic feature may not be present which may hamper or slow down collection of similar patients. As indicated in the Introduction section several initiatives have been taken in order to coordinate the daunting task of collecting all these data (Decipher, Ecaruca). This approach has been proven to be productive as illustrated by e.g. the detection of the new 17q21.31 deletion syndrome.

Another important aspect of the ongoing array CGH screening of MR/CA patients is the collection of patients which may allow further refined clinical description of the various CNVs. Systematic screening will allow the description of some of the known microdeletion syndromes in children at younger age than the age when patients are more typically referred. Also, some of the more rare deletion/duplication syndromes will now be described in much greater detail as an increasing number of patients is being detected. As an illustration, in our study we identified a patient with the reciprocal of the 7q11.23 deletion (ie the duplication) in Williams-Beuren syndrome. Because the girl is still very young, it is hard to compare her phenotypical characteristics with the recently published cases of 7q11.23 duplications [159], but as indicated, such clinical information is important as part of the emerging syndrome description. The finding of CNVs in relation to clinical disorders also opens, as mentioned previously, the possibility for identifying genes that contribute to specific aspects of the phenotype and are integral part of the functional annotation of the human genome. Together with the clinical relevant information array CGH has provided, another currently less defined 'product' of array CGH analyses has come to the surface, the so called normal copy number variation.

Normal Copy Number Variation

The study of the human genome of normal individuals using high resolution array CGH has

unveiled a new level of large scale copy number polymorphisms, i.e. segmental duplications or Low Copy Repeats (LCRs) [108–110]. A number of studies using different methodologies have uncovered this important source of normal variation [193, 194]. More recently, an in depth study was conducted using both BAC and SNP arrays, providing a detailed inventory of this new type of variation in the human [106]. A stunning total of 1447 copy number variable regions (CNVRs), covering 360 Mb and encompassing 12% of the entire human genome, were identified. These CNVRs represent more nucleotides than SNPs identified thus far, underscoring the importance of CNV in genetic diversity and evolution [106]. When applying array CGH in the study of patients' genomes this normal variation may complicate straightforward interpretation of deletions or duplications, in particular for chromosomal regions for which genotype-phenotype correlations have not been thoroughly studied. Typically, such CNVs are assumed to be causal when not present in both parents. When one of the parents also carries the CNV, its relation to the patients' phenotype becomes more difficult to interpret. To facilitate the interpretation of such CNVs, a database has been established which collects all data on such CNVs (http://projects.tcag.ca/variation/). If CNVs have been not been reported before, the clinical geneticist will not be able to make firm conclusions, and even when such CNVs have been reported as variants, care should be taken in interpreting the data. Hemizygosity of genes due to chromosomal deletions will not always lead to haploinsufficiency, but a deletion may unravel a nonsense mutation on the remaining allele resulting in an affected child with an inherited deletion from one parent and a recessive mutation from the other parent [195]. In addition, epigenetic factors can have an effect through aberrant methylation etc. [180, 185]. Moreover, it is known that some microdeletion syndromes (and other genetic diseases) can show a variable phenotypic spectrum and penetrance, ranging from normal to severely affected, as exemplified by the 22q11.2 deletion syndrome [152].

Identification of Genes Leading to MR/MCA

In the past, chromosomal aberrations have been very helpful in the identification of disease genes and genes involved in particular malformations when perturbed. Such examples are the *TBX1* gene within the 22q11.2 deletion interval implicated in the characteristic aortic arch malformations, the *EXT1* and *TRPS1* gene leading to the pathognomonic skeletal features of the Langer-Giedion syndrome and the elastin gene (*ELN*) in Williams-Beuren syndrome [19]. In some instances, the genes leading to mental retardation have been unmasked from the relatively large series of genes for which hemizygosity results from the recurrent deletion, e.g. the lissencephaly gene (*LIS1*) in Miller-Dieker syndrome [144] and the *RAI* gene in Smith-Magenis syndrome [196]. However, in most instances, the genes contributing to the specific phenotypic components of the deletion syndromes are difficult to identify. For the recurrent deletion syndromes this is due to the fact that breakpoints are almost always at the same locus and the relatively large number of genes implicated. For non recurrent deletions (or duplications) genotype-phenotype correlation is hampered by the lack of patient series which allow to assess the phenotypic variability. Despite this limitations, array CGH has also contributed to the identification of new disease genes. The gene responsible for CHARGE syndrome was identified by screening several patients with this condition and identi-

fying two patients with a 2.7 Mb de novo overlapping microdeletion on 8q12. A subsequent candidate gene approach - sequencing the candidate genes in the deleted interval in patients with CHARGE syndrome without a microdeletion - lead to the identification of *CHD7* (chromodomain helicase DNA-binding protein 7) as the causal gene [129]. As illustrated by the present thesis, high resolution array CGH analysis of a 12q14.3 deletion has helped in the identification of *LEMD3* as the causal gene for osteopoikilosis, Buschke-Ollendorff syndrome (BOS) and melorheostosis by diminishing a candidate region identified by linkage analysis [197].

Array CGH analysis of apparently balanced chromosomal translocations, detected a high frequency of cryptic deletions and duplications at the breakpoints [198]. Although array CGH cannot provide information on truly balanced structural rearrangements, the combination of flow cytometry of derivative chromosomes and array CGH (array painting) has led to the characterization of these breakpoints [199, 200]. This technique helped us in the identification of oligophrenin 1 (*OPHN1*) as the causal gene for the observed mental retardation and overgrowth in a patient with a t(X;9) translocation [166]. The present work also illustrates the possible contribution of array CGH in the delineation of the critical region for recurrent deletion syndromes. In this study we identified a small interstitial deletion on chromosome 18q12.3 in a patient with clinical features of the del(18)(q12.1q21.1) syndrome. We were able to delineate the critical region for this syndrome to an interval of 1.8 Mb, enabling hereby the determination of the crucial genes for this microdeletion syndrome [201].

Although currently there is no cure for mental retardation, a recent study on the Down syndrome mouse model has indicated that attenuating the severity of mental retardation is not so unrealistic as it may seem at first sight [202]. Identifying the underlying causal genes and their function, may help in the development of a proper treatment in patients with mental retardation.

Array CGH: Reflections Regarding Future Applications and the Relation versus Classical Cytogenetics

From the results of this thesis and concurrent studies performed worldwide it cannot be ignored that array CGH is most likely to become the method of choice for the search for genome wide deletions and duplications in the human genome. At present, karyotyping requires in most instances the culture of living cells and the skill full preparation and interpretation of banded chromosomes. This requires highly trained technicians and implicates a time consuming procedure. Array CGH will become a standard molecular technique. DNA isolation, simple DNA labeling using commercial kits and well controlled denaturation and subsequent hybridization, are steps amenable for automation, which can be performed within less than 2 days. Data handling is facilitated through dedicated software and interpretation of (technical) results is simple and straightforward, as specifically illustrated in this thesis by the development of our own dedicated web based software tool arrayCGHbase [203]. Although prices for slides and consumables are at present still relatively high, the above mentioned advantages are so important that array CGH will soon become the first choice of investigation in patients with mental retardation and/or multiple congenital abnormalities. Conventional karyotyping will take second position in all cases. In positive cases, karyotyping can shed light on the nature of the

chromosomal aberration (array CGH only produces information on which regions are gained or lossed, but does not provide any information on the underlying rearrangement). For negative cases, karyotyping will be performed to exclude balanced rearrangements.

Recent developments in whole genome amplification protocols are opening the way for new applications for array CGH, i.e. single-cell analysis [204, 205]. These single-cell techniques can be employed in certain tumor samples, but also offer new possibilities for the reliable single-cell analysis in preimplantation genetic diagnosis (PGD) [206]. Several laboratories have used FISH to screen for the most common aneuploidies leading to failed implantation or abortion (trisomy 13, 16, 18, 21, 22, X and Y) after in vitro fertilization in older patients. However, aneuploidy of other chromosomes (although not viable) is not excluded. Moreover FISH screening is very labor intensive and often hard to interpret. With array CGH it becomes feasible to screen the whole genome of a single blastocyst for aneuploidy, and even structural aberrations [204, 205]. Despite all these advantages, classical karyotyping can be expected to remain with us for some time as this is currently the only method which allows the unequivocal screening for balanced chromosomal rearrangements (e.g. in case of sub- or infertility or *de novo* translocations in children with mental retardation). In time, other approaches, perhaps entire genome sequencing, may also make this aspect of karyotyping obsolete.

Future Opportunities for the Search for Genetic Causes for Idiopathic Mental Retardation

Although the detection rate of these new high resolution DNA copy number analysis techniques is high (˜11%), the underlying (genetic) cause for MR/MCA in many patients remains unknown. Mental retardation is often a specific and probably many hundreds or even thousands of genes may lead to mental retardation if inactivated. Single nucleotide changes in a gene can be causal but beyond doubt in many cases the underlying mechanism in these patients will be the result of subtle interplay between different genes. Furthermore, the role of epigenetic modifications remains to be investigated. Large association studies might be helpful in the identification of multifactorial or multigenic causes, but as it is assumed that many genes might be involved in mental retardation, also these studies will have many difficulties detecting the causal genes. One alternative possibility to study genetic causes for mental retardation could be through gene expression profiling of brain tissue, but for obvious reasons such studies are precluded in patients. Although mouse models could offer a realistic alternative for such studies, assessing the phenotypes of mental retardation in mice represents another daunting challenge.

Initial arrays were constructed with a 1 Mb resolution, already improving the resolution of conventional karyotyping 5 to 10 times [83, 84]. Although very labor intensive, tiling path large insert clone arrays were constructed with resolutions up to ˜75 kb [63, 64]. Nowadays, BAC and PAC microarrays are replaced by oligonucleotide arrays enabling very high resolutions (up to 6 kb) both in research and diagnostics [207]. Genomic copy number scanning of all 250 000 exons in the human genome will enable immediate disease gene identification in cases exhibiting single exon duplications and/or deletions, further improving the diagnostic yield of array CGH [208]. For some custom applications, tiling path arrays have been developed, enabling the resequencing of parts of the/a

genome [66]. How much further these technical improvements will go, is hard to predict, but many outstanding scientists predict, that it will become feasible to (re)sequence everyone's genome within a couple of years. Although the scientific information this will provide is beyond imagination, it will also put us for some very difficult (ethical) issues and choices.

In conclusion, this thesis illustrates several important applications of array CGH in the field of clinical cytogenetics.

Important consequences of this new performant methodology are improved genetic diagnosis in patients with unexplained mental retardation, progress towards genotype-phenotype correlation in such patients and identification of candidate disease genes. Functional studies of these genes will contribute to our understanding in their involvement in morphogenesis and embryogenesis of the brain and other organs.

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Summary

About one to three percent of the human population is afflicted by mild to severe mental retardation, often in association with congenital abnormalities (MR/CA). These abnormalities in normal human morphogenesis may express themselves as subtle dysmorphic signs not causing any harm or present as severe disabling and life-threatening malformations such as congenital heart defects. It is well established that constitutional chromosomal aberrations are an important cause for MR/CA. The screening for such chromosomal rearrangements is done by widely used routine analysis of banded metaphase chromosomes (karyotyping). Given the limited resolution of such analyses (5-10 Mb), it was anticipated that a significant number of submicroscopic deletions or duplications (DNA copy number variations, CNV) were overlooked in patients with idiopathic mental retardation with or without congenital anomalies. This thesis represents one of the first exhaustive studies of this patient group using a new and more sensitive method for detection of CNVs. This technique, termed array comparative genomic hybridization (array CGH), allows the genome wide screening for submicroscopic aberrations in one single experiment. Array CGH uses reporter DNA molecules more or less evenly spread throughout the entire genome which are spotted or synthesized in an array on a glass slide. Each reporter is used to interrogate the DNA copy number of a specific genomic region through the competitive hybridization of differentially fluorescent labeled patient and control DNA. Together with the tedious optimalization of the technique, also a web based open source (MySQL) database platform was developed for the analysis and visualization of large amount of array CGH data (medgen.ugent.be/arrayCGHbase) (paper 6). A total of 140 carefully clinically selected patients with mental retardation and/or congenital abnormalities were analyzed for hidden chromosomal aberrations in a collaborative effort with the Center for Medical Genetics Leuven (KUL). This initial study together with a review of other published investigations, allowed for the first time to establish a reliable figure of the number of submicroscopic CNVs in this patient population. When excluding patients with subtelomeric imbalances which could be identified through FISH or MLPA analyses, array CGH still allowed to detect CNVs in an additional ~8% of patients (paper

2). A major challenge resulting from this new flow of information is the search and description of new microdeletion/microduplication syndromes. Although most CNVs seemed to be scattered across the entire genome we were able to describe a new microdeletion syndrome characterized by osteopoikilosis, mental retardation and short stature. This observation was facilitated through the identification of *LEMD3* as the causal gene for osteopoikilosis, Buschke-Ollendorff syndrome (BOS) and melorheostosis in the 12q14.3 deleted interval and subsequent, the finding of two additional patients with a 12q14.3 microdeletion (paper 3). The present work also illustrates the possible contribution of array CGH in the delineation of the critical region for recurrent deletion syndromes. In this study we identified a small interstitial deletion on chromosome 18q12.3 in a patient with clinical features of the del(18)(q12.1q21.1) syndrome. We were able to delineate the critical region for this syndrome to an interval of 1.8 Mb, enabling hereby the determination of the crucial genes for this microdeletion syndrome (paper 4). This thesis also further illustrates the power of combined flow cytometry and array CGH for rapid identification of translocation breakpoints. Using this approach we were able to identify *OPHN1* as the causal gene for the observed mental retardation and overgrowth in a girl with an apparent balanced t(X;9) translocation (paper 5).

In conclusion, the presented work clearly illustrates several important applications of array CGH in the field of clinical cytogenetics. The use of this new performant methodology will greatly improve the diagnostic yield in patients with unexplained mental retardation, provide more insights into genotype-phenotype correlations and ultimately lead to the identification of the causal genes. Functional studies of these gene products will enhance our understanding of the genetic regulation in normal human morphogenesis, embryogenesis and brain functioning. Finally, it is my believe that implementation of array CGH will represent a major and perhaps last wave of innovation in cytogenetics, as the latter may become largely redundant. Ultimately and perhaps earlier than we can anticipate, sequencing of the whole genome of a patient may eventually emerge as the method of choice.

Samenvatting

Ongeveer twee tot drie percent van de bevolking is getroffen door milde tot ernstige mentale retardatie, vaak in associatie met congenitale afwijkingen (MR/CA). Deze afwijkingen in de normale humane morfogenese kunnen zich presenteren als milde dysmorfe kenmerken zonder ernstige defecten, of als ernstige levensbedreigende malformaties zoals congenitale hartafwijkingen. Het is algemeen gekend dat constitutionele chromosomale afwijkingen een belangrijke oorzaak vormen van MR/CA. Het onderzoek van dergelijke patiënten voor chromosomale afwijkingen gebeurt door de wijdverspreide routine analyse van gebandeerde metafase chromosomen. Door de lage resolutie van dergelijk onderzoek (5-10 Mb), werd verwacht dat een belangrijke proportie van alle submicroscopische deleties en duplicaties (DNA kopie aantal veranderingen) werden gemist in patiënten met mentale retardatie met of zonder congenitale afwijkingen. Deze thesis is één van de eerste studies op deze patiëntengroep, waarbij gebruik werd gemaakt van een nieuwe en meer sensitieve methode voor de detectie van submicroscopische afwijkingen. Microarray-gebaseerde vergelijkende genoom hybridisatie (array CGH), laat de genoomwijde detectie toe van submicroscopische afwijkingen in één enkele experiment. Array CGH maakt gebruik van DNA molecules die min of meer gelijkmatig over het genoom verspreid zijn en geïmmobiliseerd of gesynthetiseerd worden op een microscoop draagglaasje. Elke DNA molecule (reporter) wordt gebruikt om de DNA status te onderzoeken van een specifieke genomische regio door de competitieve hybridisatie van het differentiëel fluorescent gemerkte patiënt en controle DNA. Samen met de nauwgezette optimalisatie van de techniek, werd een web gebaseerd 'open source' (MySQL) platform ontwikkeld voor de analyse en visualisatie van array CGH data. (medgen.ugent.be/arrayCGHbase) (paper 6). Een totaal van 140 klinisch geselecteerde patiënten met mentale retardatie en/of congenitale afwijkingen werden onderzocht voor submicroscopische chromosomale afwijkingen in een gezamenlijke studie met het Centrum voor Menselijke Erfelijkheid Leuven (KUL). Deze initiële studie, samen met een overzicht van de reeds gepubliceerde onderzoeken, maakte het voor de eerste maal mogelijk een betrouwbare incidentie van deze submicroscopische chromosomale afwijkingen in de patiëntenpopulatie te schatten. Wanneer subtelomerische deleties, die eveneens met FISH of MLPA kunnen worden opgespoord, buiten beschouwing werden gelaten, werd er in ~ 8% van de patiënten een submiscroscopische afwijking

gedetecteerd (paper 2). Een enorme uitdaging voortvloeiend uit deze initiële studie was de zoektocht naar, en de beschrijving van, nieuwe microdeletie/microduplicatie syndromen. Hoewel de meeste kopie-aantal veranderingen verspreid over het genoom lagen, waren we toch in staat een nieuwe recurrente microdeletie, gekarakteriseerd door osteopoikilose, mentale retardatie en kleine gestalte te beschrijven. Een eerste patiënt met een microdeletie van chromosoomband 12q14.3 maakte het mogelijk *LEMD3* als causaal gen voor osteopoikilose, Buschke-Ollendorff syndroom (BOS) en melorheostosis te identificeren. Vervolgens werd in twee andere patiënten met osteopoikilose, mentale retardatie en kleine gestalte eveneens een deletie van deze regio gedetecteerd (paper 3). Dit werk illustreert eveneens de bijdrage van array CGH in de afbakening van de kritische regio van recurrente microdeletie syndromen. In deze studie identificeerden we een kleine interstitiële deletie op chromosoom 18q12.3 in een patiënt met de klinische kenmerken van het del(18)(q12.1q21.1) syndroom. We waren in staat de kritische regio voor dit syndroom af te bakenen tot een 1,8 Mb regio, waarbij we eveneens de mogelijks causale genen konden identificeren (paper 4). Deze thesis illustreert eveneens de kracht van het combineren van flow cytometrie en array CGH voor de snelle identificatie van translocatie breukpunten. Met deze nieuwe aanpak, was het mogelijk om *OPHN1* als causaal gen te identificeren in een patiënte met mentale retardatie en overgroei en een schijnbaar gebalanceerde t(X;9) translocatie (paper 5).

Als conclusie, kunnen we stellen dat deze thesis verschillende belangrijke applicaties van array CGH in de klinische (cyto)genetica illustreert. Het gebruik van deze performante methodologie zal leiden tot een hogere detectieratio van de ethiopathogenese in patiënten met idiopathische mentale retardatie. Bovendien zal deze technologie verdere inzichten verschaffen in genotype-fenotype correlaties en uiteindelijk leiden tot de identificatie van genen verantwoordelijk voor mentale retardatie en aangeboren afwijkingen. Functioneel onderzoek van deze genen zal ons inzichten verschaffen in de normale humane ontwikkeling, embryogenese en werking van onze hersenen. Ten slotte, ben ik ervan overtuigd, dat array CGH de volgende en misschien wel laatste golf van innovatie is in de cytogenetica. Uiteindelijk, en misschien sneller dan we verwachten, zal sequenering van het volledige genoom van de patiënt de techniek bij uitstek worden in de genetische diagnostiek.

Résumé

Les aberrations chromosomiques constitutionnelles constituent une cause importante de retard mental et d'anomalies congénitales. Ces anomalies peuvent varier de signes dysmorphiques discrets à de multiples malformations congénitales comme des malformations cardiaques, des malformations des membres, etc. L'analyse des bandes chromosomiques est la technique la plus utilisée en cytogénétique classique mais a une résolution limitée (5 à 10 Mb). L'étude des chromosomes et les analyses génétiques en général ont largement contribué à l'identification des causes de retard mental et/ou de malformations congénitales. La mise en évidence d'une cause génétique est d'une importance cruciale dans la prise en charge de ces patients et pour le conseil génétique familial. En raison d'une résolution limitée du caryotype conventionnel, l'éthiopathogenèse des anomalies congénitales et du retard mental reste cependant inconnue dans un tiers des patients. L'advenue d'une nouvelle technique d'hybridation comparative du génome à haute résolution sur puce à ADN (CGH array) permet une analyse globale du génome révélant précisément des aberrations chromosomiques infra-microscopiques chez ces patients atteints d'un retard mental ou de malformations congénitales. Son principe consiste à cohybrider une même quantité d'ADN d'un malade et d'un témoin, marqué chacun par un fluorochrome différent, sur des sondes d'ADN fixées sur une lame de verre. Après l'hybridation, la fluorescence est mesurée et le rapport de l'intensité de fluorescence de l'ADN du malade sur celle de l'ADN normal (= ratio) est calculé pour chaque clone. Ce ratio est directement corrélé au nombre de copies d'ADN du segment étudié. Une puce à chromosome artificiel bactérien (BAC-array) pangénomique d'une résolution moyenne de 1 Mb a pu être produite en collaboration étroite avec le centre de Génétique Humaine de Leuven, Belgique et le Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK. Plusieurs paramètres ont été optimalisés et des critères de qualité pour les résultats de CGH array ont été établis. Une étape importante dans la procédure de CGH array est l'analyse finale des données et la visualisation des résultats. Une base de données publique et figurant sur le réseau fut développée, permettant ainsi la sauvegarde, l'analyse et la visualisation d'un grand nombre de données typiquement générées lors d'analyses en CGH array

(medgen.ugent.be/arrayCGHbase) (paper 6). Suite à la réussite de la mise en uvre de la CGH array, une large cohorte d'environs 140 patients présentant un retard mental et/ou des anomalies congénitales fut analysée à la recherche d'aberrations infra-microscopiques. Une aberration chromosomique fut détectée dans ~ 8% des cas elle fut présumée être la cause du phénotype observé (paper 2). Dans une seconde partie de cette étude, l'utilisation de la CGH array à haute résolution pour l'analyse de la délétion 12q14.3 a contribué à l'identification du gène *LEMD3*, gène causal de l'ostéopoikilose, du syndrome de Buschke-Ollendorff (BOS) et de la mélorhéostose. De plus, plusieurs autres patients présentant une délétion au niveau de cette bande chromosomique ont été identifiés. Ces données sont suggestives d'un nouveau syndrome microdélétionnel récurrent sur la bande chromosomique 12q14.3 caractérisé par une ostéopoikilose, un retard mental ainsi qu'une petite taille (paper 3). En outre, la découverte par CGH array d'une petite délétion interstitielle sur le chromosome 18q12.3 nous a permis de délimiter une région critique pour le syndrome de del(18)(q12.1q21.1). Ce fut un des premiers syndromes délétionnels récurrents pour lesquels la CGH array a pu contribuer à la délimitation d'une région critique. De tels résultats constituent une ouverture vers l'identification de gènes causaux (paper 4). Ce travail démontre également l'utilité de la CGH array et plus en particulier de l'array painting pour l'étude de translocations chromosomiques balancées apparentes. Cette approche a permis l'identification de *OPHN1* comme gène causal du phénotype associant un retard mental, un surpoids et une croissance accélérée chez un patient présentant une translocation t(X;9) (paper 5).

En conclusion, cette thèse illustre de nombreuses applications importantes de la CGH array en cytogénétique clinique. Cette nouvelle méthodologie permet une amélioration du diagnostic génétique pour des patients atteints d'un retard mental inexpliqué, un progrès dans la corrélation génotype-phénotype ainsi que l'identification de gènes candidats de la maladie. L'étude fonctionnelle de ces gènes contribuera à une meilleure compréhension de l'implication de ces gènes dans la morphogenèse et dans l'embryogenèse du cerveau et d'autres organes. Finalement, j'ai l'intime conviction que la CGH array sera une étape majeure et peut-être la dernière étape innovatrice en cytogénétique, avant que celle-ci ne disparaisse définitivement. On peut penser qu'à court terme le séquençage du génome du patient devienne la méthode de choix.

Abbreviations

AAIDD *American Association on Intellectual and Developmental Disabilities (formerly AAMR)*
AAMR *American Association on Mental Retardation*
ADHD *attention-deficit hyperactivity disorder (ADHD)*
APP *amyloid beta (A4) precursor protein*

BAC *bacterial artificial chromosome*

CATCH 22 *cardiac defects, abnormal facies, thymic hypoplasia/aplasia, cleft palate, hypocalcemia, and 22q11 deletion*
cDNA *copy deoxyribonucleic acid*
CGH *comparative genomic hybridization*
CHARGE *Coloboma of the eye, central nervous system anomalies, Heart defects, Atresia of the choanae, Retardation of growth and development, Genital and/or urinary defects, Ear anomalies and/or deafness*
COBRA *combined binary ratio labelling*
CREBBP *CREB-Binding Protein*
CT *computed tomography*
CYLN2 *cytoplasmic linker 2*

DNA *deoxyribonucleic acid*
DOP-PCR *degenerate oligonucleotide primer polymerase chain reaction*
DSM-IV *diagnostic and statistical manual of mental disorders, 4th edition*

EEG *electroencephalogram*
ELN *elastin*
EST *expressed sequence tag*
ETS2 *Erythroblastosis Virus E26 Oncogene Homolog 2*
EXT1 *exostoses (multiple) 1*

FISH *fluorescence in situ hybridisation*
FMR1 *fragile X mental retardation 1*

GATA4 *GATA binding protein 4*
GTF2I *general transcription factor II, i*
GTF2IRD1 *GTF2I repeat domain containing 1*

HGD *homogentisate 1,2-dioxygenase*
HMGA2 *high mobility group AT-hook 2*
HNPP *hereditary neuropathy with liability to pressure palsies*

ICD-10 *international classification of diseases 10th edition*
IQ *intelligence quotient*

JAG1 *jagged 1*

Kb *kilobase (1000 basepairs)*

LCR *low copy repeats*
LIMK1 *LIM domain kinase 1*
LINE *long interspersed element*
LIS1 *lissencephaly 1*
LOH *loss of heterozygosity*

MAPH *multiplex amplifiable probe hybridization*
MAPT *microtubule-associated protein tau*
Mb *megabase (1000000 basepairs)*
MECP2 *methyl-CpG-binding protein-2*
M-FISH *multiplex FISH or multicolour FISH*
MLPA *multiplex ligation-dependant probe amplification*
MR *mental retardation*
MR/MCA *mental retardation/multiple congenital abnormalities*
MRI *magnetic resonance imaging*
mRNA *messenger ribonucleic acid*
MSX1 *MSH Homeobox 1*

NAHR *non allelic homologous recombination*
NDN *necdin homolog (mouse)*
NF1 *neurofibromin 1*
NHEJ *nonhomologous end-joining*

NIH *National Institutes of Health*
NMR *nuclear magnetic resonance*

OPHN1 *oligophrenin 1*

PAC *P1-plasmid derived artificial chromosome*
PAGE *polyacrylamide gel electrophoresis*
PAX6 *paired box gene 6 (aniridia, keratitis*
PCR *polymerase chain reaction*
Perl *Practical Extraction and Report Language*
PHP *php hypertext prepocessor*
PKU *phenylketonuria*

RAI1 *retinoic acid-induced 1*
RNA *ribonucleic acid*
RPCI-11 *Roswell Park Center Institute-11 BAC library*

SINE *short interspesed element*
SKY *spectral karyotyping*
SMS *Smith-Magenis syndrome*
SNP *single nucleotide polymorphism*
SNRPN *small nuclear ribonucleoprotein polypetide N*
SRO *shortest region of overlap*

TBX1 *T-box transcription factor*
TRPS1 *trichorhinophalangeal syndrome 1*

UBE3A *ubiquitin protein ligase E3A*

VCFS *velocardiofacial syndrome*
VDJ *variable, diversity and joining gene segments*
VEGF *vascular endothelial growth factor*
VNTR *variable number of tandem repeats*

WAIS *Wechsler adult intelligence scale*
WHSC1 *Wolf-Hirschhorn syndrome candidate 1*
WT1 *Wilms tumor 1*

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Congresses, courses, workshops and meetings

1st Dutch Belgium Molecular Cytogenetics Meeting, 19th September 2002, Erasmus MC, Rotterdam, The Netherlands

2nd VIB MicroArray Users Group Meeting, 22nd November 2002, UZ Gasthuisberg, Leuven, Belgium

Wetenschapsdag Universitair Ziekenhuis Gent, 17th January 2003, Ghent, Belgium (poster presentation)

Identification of Genetic Defects in Cancer, Truncus Communis voor de doctoraatsopleiding, 28th February 2003, Ghent, Belgium

Introductory course in Bio-informatics 2003, Institute for Continuing Education in Science, Faculty of Science, University Ghent, Belgium

3th annual meeting BeSHG 2003, 7th February 2003, Leuven, Belgium (poster presentation)

2nd Dutch Belgium Molecular Cytogenetics Meeting, 18th September 2003, Erasmus MC, Rotterdam, The Netherlands

Wetenschapsdag Universitair Ziekenhuis Gent, 22nd Januari 2004, Ghent, Belgium (poster presentation)

4th annual meeting BeSHG 2004, 19th March 2004, Gent, Belgium (poster presentation)

COST B19 Meeting, 29th April - 2nd May 2004, Hindsgavl Casle, Middelfart, Denemark (oral presentation)

LOC/LOD/LOG meeting 'Molecular Cytogenetics', 17th June 2004, Amsterdam, The Netherlands (oral presentation)

arrayCGH-MC: Marie Curie Conferences and Training Courses on arrayCGH and Molecular Cytogenetics 2004, 29th September - 2nd October 2004, The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK (oral presentation)

Bioinformatics in Microarray Research 6th October 2004, Center for Biomedical Systems Biology, Amsterdam, The Netherlands (invited speaker)

ACLF-Microarray meeting, 15th December 2004, Paris, France (oral presentation)

5th annual meeting BeSHG 2005, 28th January 2005, Liege, Belgium (poster presentation)

European Human Genetics Conference 2005, 7th - 10th May 2005, Prague, Tsjech Republic (poster presentation)

Introduction to Bioinformatics, Truncus Communis voor de doctoraatsopleiding, 29th May 2005, Ghent, Belgium (invited speaker)

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6th annual meeting BeSHG 2006, 17th February 2006, Antwerp, Belgium (poster presentation)

2nd DECIPHER meeting, The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK (oral presentation)

arrayCGH-MC: Marie Curie Conferences and Training Courses on arrayCGH and Molecular Cytogenetics 2006, 13th - 16th September 2006, Leuven, Belgium (poster presentation)

European School of Genetic Medicine, 8th Course in Molecular Cytogenetics and DNA Microarrays, 24th - 28th September 2006, Bertinoro di Romagna, Italy (invited speaker)

American Society for Human Genetics Conference 2006, 9th - 13th October 2006, New Orleans, USA (poster presentation)

arrayCGH-MC: Marie Curie Conferences and Training Courses on arrayCGH and Molecular Cytogenetics 2006, 2nd workshop, 16th - 21st October 2006, Ghent, Belgium

Genomic Disorders 2007, 21st - 24th March 2007, The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK (oral presentation)

7th annual meeting BeSHG 2007, 20th April 2007, Gosselies, Belgium (oral presentation)