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GENE DOSE IMBALANCES IN CHILDREN WITH MENTAL RETARDATION

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ABSTRACT

Submicroscopic chromosome aberrations can cause mental retardation (MR), congenital malformations and miscarriage. It is difficult to estimate the contribution of cytogenetic abnormalities to MR because of relatively low detection sensitivity of conventional cytogenetic methods used in the diagnostic setting. The focus of this thesis has been to improve the detection sensitivity and to study small chromosome aberrations in detail.

Using FISH probes covering the 41 unique subtelomeric regions of the human genome, 111 patients with idiopathic MR and dysmorphic features were screened. Ten cryptic rearrangements (9%) were detected – five *de novo* deletions, one unbalanced *de novo* translocation, three unbalanced inherited translocations and one unbalanced inherited recombinant chromosome. Fifty of the 111 patients were also analyzed by Spectral Karyotyping (SKY) to detect interstitial abnormalities, but no additional rearrangements were found. In one case an unbalanced translocation between chromosome 12q and 17q was detected and this patient together with her family were further investigated. The fragments involved were FISH mapped for accurate size determination and genotype-phenotype correlation was performed**.** Four family members had inherited unbalanced variants – two cases inherited the derivative chromosome 12 and the other two the derivative chromosome 17. This family illustrates that small chromosome imbalances can cause a mild phenotype and normal, or near-normal, cognitive functions.

The robustness and simplicity of different methods for comparative genomic hybridization (CGH) were tested on a set of patients with well-defined sub-microscopic chromosome abnormalities, in order to determine if the technique can be used as a reliable and sensitive diagnostic procedure. We optimized and evaluated CGH performed on metaphase chromosomes and compared the performance to a commercially available high resolution software using a standard dynamic reference interval (HR-CGH). In addition, the performance of CGH on microarrays containing cDNA clones or BAC clones was investigated. From this study, we concluded that the BAC array with an average resolution of approximately 1 Mb is reliable and would add important information in selected cases in a diagnostic setting.

Based on these results, two patient groups were screened for submicroscopic chromosome imbalances by array based CGH using a BAC microarray. The first group of patients consisted of 10 children diagnosed with Kabuki syndrome (KS). This syndrome is a rare multiple congenital anomaly/mental retardation syndrome which was recently reported to be caused by a microduplication on the short arm of chromosome 8. We investigated the implicated region by FISH and microsatellite markers and were unable to confirm the duplication. In addition, the whole genome at a resolution of approximately 1 Mb was screened, without detecting any microdeletions or duplications in this group of patients. Our data demonstrate that the etiology of KS remains unknown. The second group of patients consisted of 49 children with MR and dysmorphism, where G-banding, and in the majority of the cases subtelomeric FISH and SKY, had failed to detect chromosome imbalances. Five abnormalities (10.2%) were detected – four *de novo* interstitial deletions and one *de novo* terminal duplication. In addition, large scale copy number variations (LCVs) were frequently observed.

Array-CGH proved to be a reliable and sensitive tool for the detection of submicroscopic chromosome imbalances and has the advantage over FISH that it enables whole genome screening. Array-CGH will in the future most likely be more widely implemented in the diagnostic setting and the detection of genomic imbalances of clinical significance will increase. Thorough genotype-phenotype correlations needs to be performed for the identification of candidate genes involved in developmental delay.

PUBLICATIONS INCLUDED IN THIS STUDY

This thesis is based on the following publications, which will be referred to by their Roman numerals throughout the text.

I. Anderlid BM, **Schoumans J**, Annerén G, Sahlén S, Kyllerman M, Vujic M, Hagberg B, Blennow E, Nordenskjöld M.
Subtelomeric rearrangements detected in patients with idiopathic mental retardation.
Am J Med Genet 2002 feb 1;107(4):275-84

II. **Schoumans J**, Nielsen K, Jeppesen I, Anderlid BM, Blennow E, Brøndum-Nielsen K, Nordenskjöld M.
A comparison of different metaphase CGH methods for the detection of cryptic chromosome aberrations of defined size.
Eur J Hum Genet 2004; 12: 447-454

III. **Schoumans J**, Anderlid BM, Blennow E, Teh BT, Nordenskjold M.
The performance of CGH array for the detection of cryptic constitutional chromosome imbalances.
J Med Genet 2004; 41: 198-202

IV. **Schoumans J**, Nordgren A, Ruivenkamp C, Brøndum- Nielsen K, Teh BT, Annéren G, Holmberg E, Nordenskjöld M, Anderlid BM.
Genome wide screening using array-CGH does not reveal microdeletions/microduplications in children with Kabuki syndrome.
Eur J Hum Genet 2004 Oct 20; [Epub ahead of print]

V. **Schoumans J**, Sanner G, Nordenskjöld M, Anderlid BM.
Detailed clinical description of four cases with 1.3 and 2.1 Mb chromosome imbalances derived from a familial t(12;17)(q24.33;q25.3).
Am J Med Genet, in press

VI. **Schoumans J**, Ruivenkamp C, Thuresson AC, Holmberg E, Kyllerman M, Anderlid BM, Nordenskjöld M.
Detection of chromosomal imbalances in children with idiopathic mental retardation by array-based comparative genomic hybridization (array-CGH).
Submitted for publication

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1. Houge G, Liehr T, **Schoumans J**, Ness GO, Solland K, Starke H, Claussen U, Stromme P, Akre B, Vermeulen S.
   Ten years follow up of a boy with a complex chromosomal rearrangement: going from a > 5 to 15-breakpoint CCR.
   Am J Med Genet. 2003 Apr 30;118A(3):235-40

2. Anderlid BM, **Schoumans J**, Hallqvist A, Stahl Y, Wallin A, Blennow E, Nordenskjold M.
   Cryptic subtelomeric 6p deletion in a girl with congenital malformations and severe language impairment.
   Eur J Hum Genet. 2003 Jan;11(1):89-92

3. Anderlid BM, **Schoumans J**, Annéren G, Tapia-Paez I, Dumanski J, Blennow E, Nordenskjold M.
   FISH-mapping of a 100-kb terminal 22q13 deletion.
   Hum Genet. 2002 May;110(5):439-43

4. Anderlid BM, Sahlén S, **Schoumans J**, Holmberg E, Ahsgren I, Mortier G, Speleman F, Blennow E.
   Detailed characterization of 12 supernumerary ring chromosomes using micro-FISH and search for uniparental disomy.
   Am J Med Genet. 2001 Mar 15;99(3):223-33

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1. Karpova MB, **Schoumans J**, Ernberg I, Henter JI, Nordenskjold M, Fadeel B. Raji revisited: cytogenetics of the original Burkitt's lymphoma cell line. Leukemia. 2005 Jan;19(1):159-61

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3. Chen J, Lui WO, Vos MD, Clark GJ, Takahashi M, **Schoumans J**, Khoo SK, Petillo D, Lavery T, Sugimura J, Astuti D, Zhang C, Kagawa S, Maher ER, Larsson C, Alberts AS, Kanayama HO, Teh BT.The t(1;3) breakpoint-spanning genes LSAMP and NORE1 are involved in clear cell renal cell carcinomas. Cancer Cell. 2003 Nov;4(5):405-13

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6. Nordgren A, Heyman M, Sahlen S, **Schoumans J**, Soderhall S, Nordenskjold M, Blennow E. Spectral karyotyping and interphase FISH reveal abnormalities not detected by conventional G-banding. Implications for treatment stratification of childhood acute lymphoblastic leukaemia: detailed analysis of 70 cases. Eur J Haematol. 2002 Jan;68(1):31-41

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CONTENTS

LIST OF ABBREVIATIONS

BAC	Bacterial Artificial Chromosome
CdLS	Cornelia de Lange syndrome
cDNA	Complementary DNA
CGH	Comparative Genomic Hybridization
CNS	Central Nervous System
DAPI	4.6-diamino-2-phenylindole
DGS	DiGeorge Syndrome
DNA	Deoxyribonucleic Acid
DOP-PCR	Degenerate Oligonucleotide-Primed PCR
Ensembl	Genomic database from EMBL-EBI and Sanger Institute
FISH	Fluorescent *in situ* hybridization
GTG-banding	G-bands by trypsin using Giemsa
HR-CGH	High Resolution Comparative Genomic Hybridization
IMR	Idiopathic Mental Retardation
IQ	Intelligence Quotient
Kb	Kilo basepairs, 10^3 bp
KS	Kabuki Syndrome
LCR	Low Copy Repeat
LCV	Large Copy number Variants
MCA	Multiple Congenital Anomalies
Mb	Mega basepairs, 10^6 bp
MLPA	Multiplex Ligation-dependent Probe Amplification
MMR	Mild Mental Retardation
MR	Mental Retardation
NAHR	Nonallelic Homologous Recombination
NCBI	National Center of Biotechnology Information
OMIM	Online Mendelian Inheritance in Man
PAC	P1 derived Artificial Chromosome
PCR	Polymerase Chain Reaction
PWS	Prader Willi Syndrome
SKY	Spectral Karyotyping
SMR	Severe Mental Retardation
SMS	Smith Magenis Syndrome
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeat
UCSC	University of California Santa Cruz
ULS	Univeral Linkage System
UPD	Uniparental Disomy
VNTR	Variable Number of Tandem Repeats
VWS	Van der Woude Syndrome
WHS	Wolf Hirshhorn Syndrome

1 INTRODUCTION

The etiology of intellectual and developmental impairment remains unidentified in ~50% of the patients despite extensive clinical examinations and laboratory investigations. This is distressing for both the family and medical professionals. It is making adequate genetic counseling impossible and is hampering the development of strategies for treatment and prevention. Independent lines of evidence point at chromosome imbalance as a major cause of MR and dysmorphism. Constitutional chromosome aberrations are associated with mental retardation (MR), congenital malformations and miscarriage. They often cause specific and complex phenotypes resulting from an imbalance in the normal dosage of genes located in a particular chromosomal segment. A considerable number of cases with mental retardation can be explained by the presence of chromosome abnormalities detected by routine chromosome analysis. However, this method has limited resolution and is unreliable for detection of subtle copy number changes. This strongly implies that screening methods with higher resolution are needed in the diagnostic setting in order to detect subtle chromosome abnormalities.

With the sequence of the human genome assessable, development of new reliable technologies with an increased detection rate of subtle chromosome imbalances has become possible. The focus of this thesis has been to evaluate and improve the detection sensitivity by investigating the robustness and simplicity of several novel screening technologies such as FISH, SKY, HR-CGH and array-CGH in order to improve the detection of chromosome imbalances in patients with MR. Optimization and evaluation was performed in order to determine if these technologies might be introduced as reliable and sensitive diagnostic procedures. In addition, detected submicroscopical chromosome aberrations were studied in detail, in order to get a better understanding of the development of the specific phenotype.

1.1 MENTAL RETARDATION AND THE “CHROMOSOMAL” PHENOTYPE

Mental retardation (MR) is a common disorder which occurs in 2 - 3% of the general population. MR is defined as an intelligence quotient (IQ) below 70 (equals 2 standard deviations below mean IQ = 100). MR is further classified into profound MR (IQ <20), severe MR (IQ 20 – 35), moderate MR (IQ 35 –50)

and mild MR (IQ 50–70). However, most often all three groups scoring IQ <50 are together referred to as “severe mental retardation” (SMR). The prevalence of SMR is 0.3-0.5 % (Roeleveld et al. 1997), while the prevalence of mild mental retardation (MMR) varies between studies due to several factors. Firstly the identification of MMR in younger children is difficult, and secondly, several SMR disorders are associated with malformations and high mortality which makes it more recognizable. Many patients with unexplained MR show dysmorphic facial features, prenatal and/or postnatal growth retardation, malformations and in some cases, a family history of MR is found. These observations indicate the involvement of genetic factors.

1.2 CYTOGENETICS

Cytogenetics is the study of structure, function and evolution of chromosomes. Chromosomes are named after their ability to take up certain stains (Greek for “stained body”). Only a few years after the identification of the human chromosomes by Flemming in 1882, it was anticipated that chromosomes probably constitute the physical basis of heredity.

After the discovery in 1956 that normal human cells contain 46 chromosomes (Tjio and Levan 1956), studies of chromosomal defects and diseases could be conducted and “cytogenetics” was born. Although the chromosomes could not be analysed in detail, they could be arranged in eight different groups (A-G and the sex chromosomes), based on their size and centromere location. This chromosome classification mainly allowed identification of numerical abnormalities, and it was not until the development of chromosomal banding techniques in 1968 (Caspersson et al. 1968) and applied on human chromosomes in 1970 (Caspersson et al. 1970), that chromosome identification could be facilitated. The chromosome banding techniques allow the identification of microscopic numerical and structural aberrations, including translocations, inversions, deletions and duplications. Conventional chromosome analysis using banding techniques, particularly G-banding (Seabright 1971), are now routine procedures in all cytogenetics laboratories. However, the resolution of this technique is very limited. A routinely prepared metaphase contains ~ 400-500 bands per haploid genome which roughly corresponds to a resolution of 5-10 Mb. High resolution banding techniques (arresting the cell in pro-metaphase) can achieve ~1000 band per haploid genome. However, this analysis is very labor-intensive and not practical for routine analysis. In addition, chromosome banding analysis has limitations that

include the inconsistency with which band resolution can be routinely achieved and the difficulty in visualizing some rearrangements due to staining properties of specific regions of the genome.

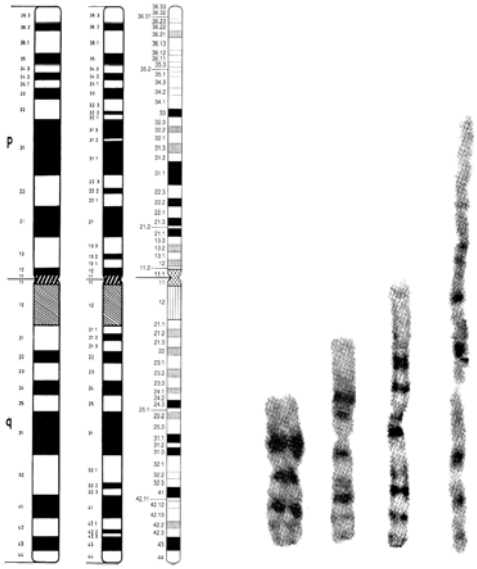

Fig 1 Chromosome banding resolution of chromosome 1 and ideograms at three different levels of resolution with numbering of bands, sub-bands and sub-sub-bands according to the ISCN nomenclature (Mittelman 1995).

1.3 CLINICAL GENETICS

Chromosome mutations involving entire or large parts of chromosomes cause different symptoms, such as MR, Multiple Congenital Anomalies (MCA) and cancer. Shortly after the discovery of the correct number of chromosomes in human cells, several numerical chromosomal aberrations were reported, e.g. trisomy 21 causing Down syndrome (Lejeune et al. 1959), 45,X causing Turner syndrome (Ford et al. 1959), 47, XXY causing Klinefelter syndrome (Jacobs and Strong 1959), trisomy 18 causing Edwards syndrome (Edwards et al. 1960) and trisomy 13 causing Patau syndrome (Patau et al. 1960). The first structural abnormality, the “Philadelphia chromosome” found in bone marrow cells, causing chronic myeloid leukemia was also reported in 1960 (Nowell and Hungerford 1960). However, it took many years before it became clear that the aberration was not a deletion of chromosome 22, but a reciprocal translocation between chromosome 9 and 22.

During the 1980s molecular genetics technologies were rapidly developed. With the introduction of the Polymerase Chain Reaction (PCR) technique it became possible to easily analyze DNA sequences down to the single base pair (bp) level. This became a crucial tool in the diagnostic setting for the

identification of monogenic disorders. During the 1990s the large gap between the molecular genetics and cytogenetic techniques was somewhat reduced by the development of molecular cytogenetic methods such as fluorescent *in situ* hybridization (FISH).

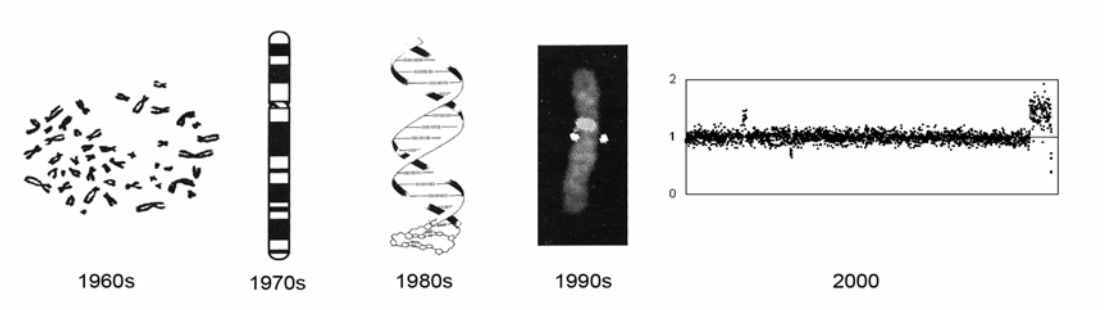

Fig 2 Development of technologies during the past 40 years, Chromosome analysis by solid staining in the 1960s, chromosome identification by G-banding in the 1970s and the development of molecular techniques such as sequencing and PCR during the 1980s. FISH was developed during the 1990s and finally the molecular karyotype by array-CGH during the past few years.

Using relatively large DNA probes labeled with fluorescent dyes specific sequences could be visualized on metaphase chromosomes. FISH was already published in the early 1980s (Langer-Safer et al. 1982; Van Prooijen-Knegt et al. 1982), but became more widely accessible during the 1990s. Applications of FISH include gene mapping and detection of cytogenetic rearrangements.

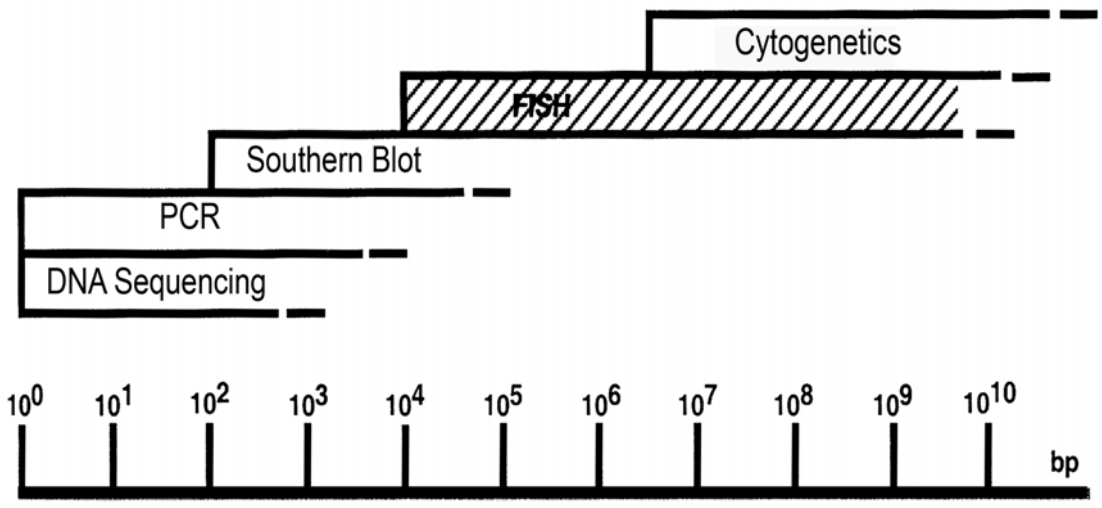

Fig 3 FISH decreasing the gap between molecular genetics and cytogenetics

1.4 GENE DOSE ALTERATIONS ASSOCIATED WITH MR

1.4.1 Chromosome rearrangements

Chromosome rearrangements that result in segmental aneuploidy and alter the dosage of developmental genes have been shown to be an important cause of MR. Chromosome abnormalities have been reported to occur in as many as 40% of individuals with severe MR (Raynham et al. 1996), but only 5-10% of patients with mild MR (Hagberg et al. 1981; Lamont et al. 1986). The majority of chromosome abnormalities detected in severe MR are trisomy 21, 18 and 13. Trisomy 21 (Down syndrome) is the most common and affects one in 700 newborn children. Trisomy 18 and trisomy 13, which are less common result in more severe phenotypes and occurs in 1:6000 and 1:12000 live-born infants, respectively.

Other chromosome abnormalities that are observed in MR patients and cause gene dose alterations are deletions, duplications, unbalanced translocations, inversions, supernumerary marker chromosomes and even more complex rearrangements involving more than two chromosomes. Inheritance of chromosomal rearrangements occurs when a carrier of a balanced chromosomal rearrangement transmits the rearrangements to its offspring in an unbalanced form. In general, individuals carrying constitutional balanced rearrangements are phenotypically normal, but in some cases clinical manifestation can occur if the break affects a transcribed dosage dependent gene. Carriers of balanced chromosome rearrangements have in addition an increased risk of infertility, risk for miscarriages and risk to have children with mental retardation and/or malformations due to the production of gametes with unbalanced chromosome complements. Chromosome rearrangements are most often unique, but chromosome breakage occur more often in certain chromosome regions that have an inherent instability or recombination potential. For example, the reciprocal translocation between chromosome 11 and 22 t(11;22)t(q23;q11) is a common reciprocal translocation reported in many unrelated families (Fraccaro et al. 1980).

1.4.2 Microdeletion syndromes

A number of deletions resulting in a recognizable MR syndrome have been well characterized by phenotype-genotype correlation. Microdeletions can be either

interstitial or terminal and most of them are not detectable by conventional cytogenetics. Deletions in a specific chromosome region can vary greatly in size but still the phenotype is concordant. Examples of microdeletion syndromes are Cri du chat syndrome del(5p), Wolf-Hirshhorn syndrome del(4p), Digeorge syndrome del(22)(q11), Williams syndrome del(7)(q11.2), Smith Magenis syndrome del(17)(p11.2) and Miller Dieker syndrome del(17)(p13.3). The typical phenotype makes clinical diagnosis possible, and when genetic testing is available, the deletion can be detected using a locus specific probe for the region of interest.

The molecular mechanism that is responsible for the formation of many microdeletions and duplications is non-allelic homologous recombination (NAHR) between sequences that have more than 90% homology. These sequences are frequently found across the human genome and are called segmental duplications or Low Copy Repeats (LCRs). Chromosome imbalance is induced by misalignment between segmental duplications during early meiosis. This results in unequal crossing-over and the formation of gametes with either a deletion or duplication. The olfactory receptor-gene (OR) clusters located in large clusters on the short arm of chromosome 4 and 8 are such kind of homologous sequences that have been shown to be prone to genetic rearrangements because of unequal crossovers (Giglio et al. 2001). Hence chromosome imbalances in these regions have frequently been reported. A larger number of microdeletions have been identified compared to microduplications. This might be due to the fact that a loss of gene-function possibly results in a more severe phenotype.

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1.4.3. Subtelomeric aberrations

Chromosomal rearrangements affecting telomeric regions have in the past few years turned out to be an important etiological contributor to mental retardation. There are several facts that suggest that cryptic rearrangements of these regions is the cause of many cases of idiopathic MR. Firstly, chromosome pairing and homology searching is initiated at the telomeres (Barlow and Hulten 1996; Scherthan et al. 1996). Secondly, the subtelomeric regions are rich in pseudogenes and repeat sequences that share homology between non-homologous chromosomes resulting in mispairing at early meiotic prophase (Brown et al. 1990; Flint et al. 1996; Flint et al. 1997). Finally, increased recombination rates have been observed at the telomeres (Blouin et al. 1995).

The chromosome ends do not stain well with Giemsa, and appear as light bands with G-banding. Small abnormalities in the subtelomeric regions can thus easily escape detection by routine chromosome analysis. The subtelomeric regions are also known to be relatively gene rich and often involved in chromosomal rearrangements (Saccone et al. 1992).

Human telomeres contain complex repetitive DNA sequences. The chromosome end is composed of short tandem repeats with the base sequence $(TTAGGG)_{n,}$ which is highly conserved and is identical among vertebrates. Telomeres protect chromosome ends from degradation, recombination and fusion events. Internal of the essential telomeric repeats, eukaryotic chromosomes also have a more complex set of repeats called the subtelomeric repeats. These sequences are not conserved in eukaryotes and their function is unknown.

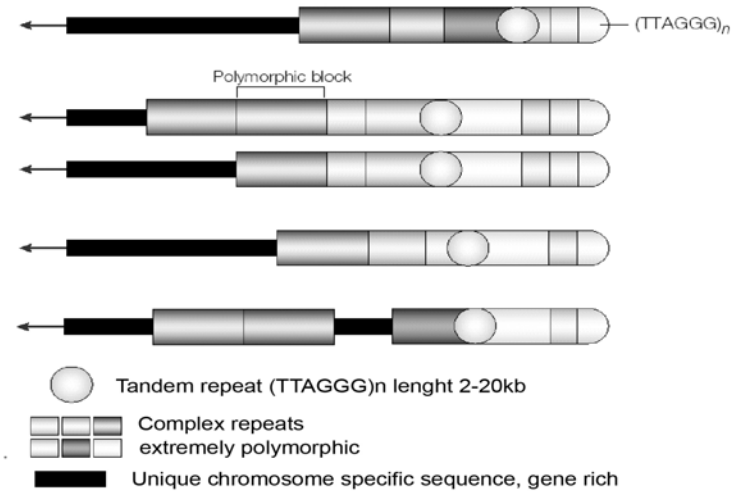

Fig 4 Examples of telomere structure in humans

A complete set of subtelomeric FISH probes was presented in 1996 and updated in 2000 (Knight and Flint 2000). Since then, numerous studies of subtelomeric aberrations have been reported that differ in patient selection, number of patients, and testing methodology. More than 2500 patients have been screened and reported, resulting in a wide range of detection rates (from 0 % to 23.5%). The results from a number of subtelomeric screening studies are summarized in Table 1. Screening for subtelomeric abnormalities has become a diagnostic test that is offered by diagnostic laboratories. Many different screening approaches have been developed to make the method less costly and less labor-intensive, such as automated fluorescent

genotyping (Rio et al. 2002), M-TEL (Popp et al. 2002) S-cobra –FISH (Engels et al. 2003), MLPA (Rooms et al. 2004) and microarray (Veltman et al. 2002).

Table 1 Summary of subtelomeric screening studies

Method	Number of patients	Level of MR	Number of abnormalities	Frequency	Reference
FISH	17	Unspecified	4	23.5	(Viot 1998)
FISH	209	Unspecified	8	3.8	(Vosanova 1998)
FISH	43	Unspecified	1	2.3	(Lamb 1999)
Genotypes	27	Unspecified	2	7.4	(Slavotinek et al. 1999)
FISH	284 182 75	Mod/severe Mild Controls	21 1 0	7.4 0.6 0	(Knight et al. 1999)
FISH	154	Unspecified	4	2.6	(Ballif et al. 2000)
Genotypes	24	Mod/severe	2	8.3	(Colleaux et al. 2001)
FISH/MAPH	28 42	Mod/severe Mild	1 0	3.6 0	(Sismani et al. 2001)
FISH	82 68 controls	Mod/severe Mild 150	0 0 0	0 0 0	(Joyce et al. 2001)
Genotypes	120	Unspecified	5	4.2	(Rosenberg et al. 2001)
FISH	254	Mod/severe	13	5.1	(Riegel et al. 2001)
FISH	150	Mod/severe	6	4.0	(Fan et al. 2001)
CGH/FISH	17	Unspecified	5	29.4	(Joly et al. 2001)
FISH	117 44	Mod/severe Mild	13 0	11.1 0	(Rossi et al. 2001)
Prins	65	Unspecified	2	3.1	(Bonifacio et al. 2001)
Genotypes	30	Mod/severe	2	6.7	(Borgione et al. 2001)
FISH/SKY	50	Unspecified	2	4.0	(Clarkson et al. 2002)
FISH/SKY	82 29	Mod/severe Mild	7 3	8.5 10.3	(paper I)
FISH	250	Unspecified	10	4	(Baker et al. 2002)
Genotypes	150	Mod/severe	14	9.3	(Rio et al. 2002)
M-TEL	30	Unspecified	4	13.3	(Popp et al. 2002)
FISH	81 103	Mod/severe Mild	0 1	0 1	(van Karnebeek et al. 2002)
FISH	21 11	Mod/severe Mild	3 0	14.3 0	(Helias-Rodzewicz et al. 2002)
FISH	40	Unspecified	3	7.5	(Dawson et al. 2002)
FISH	372	Unspecified	25	6.8	(Jalal et al. 2003)
MLPA	75	Unspecified	4	5.3	(Rooms et al. 2004)

To determine which patients to select for testing, de Vries *et al*. (de Vries et al. 2001) developed a five-item checklist that included a family history of mental retardation, prenatal and/or postnatal growth abnormalities, two or more dysmorphic facial features, and non-facial dysmorphisms or congenital anomalies. Prenatal onset of growth retardation and a family history of MR were the best indicators of an abnormal result in their study. Baker *et al*. (Baker et al. 2002) applied the same checklist to nine patients identified with subtelomeric abnormalities in their study, and found that two of nine patients had scores of 2 points and would have been missed if a score of 3 points or more was required for entry. They stressed that the diversity of clinical phenotypes in the positive cases, along with the variability in intellectual disability that ranged from an isolated language disorder to severe MR, would make it difficult to apply strict criteria for testing. Rio *et al* (Rio et al. 2002) found that the presence of congenital anomalies, behavioral problems and postnatal growth retardation were the most frequent findings in their series of mentally retarded children with abnormalities, and not intrauterine growth retardation or a family history of mental retardation. Although the exact criteria for offering testing varies in all the reports, most investigators include one or more features in addition to mental retardation or developmental delay. In summary, the importance of chromosomal anomalies as a cause of IMR is now well recognized and rearrangements involving the chromosome ends are found in approximately 5% of the cases (de Vries et al. 2003; Flint and Knight 2003).

1.5 OTHER GENETIC MECHANISMS ASSOCIATED WITH MR

1.5.1 MR and MCA caused by single gene defect

MR and MCA disorders are also caused by mutations within a single gene. Various kinds of pathogenic mutations can be observed, such as nonsense mutations, missense mutations, frameshift mutations, mutations affecting the splicing sites of exons and expansion of trinucleotide-repeats. Mutations in genes expressed in the brain or genes that are crucial for brain development result in MR. Mutations that destroy or alter the function of a gene that has a pivotal role during early embryonic development will cause malformations of different organs and tissues and result in MCA.

Fifty percent of all genes are thought to be expressed in the brain and some genes are only expressed in the CNS. The database of genes and phenotypes Online Mendelian Inheritance in Man (OMIM) displays 1233 entries for MR (November 2004), which means that a large number of single gene disorders include MR in their phenotype.

A large number of patients with MCA/MR syndrome can only be diagnosed by a characteristic recognizable phenotype, because the cause of the syndrome is still unknown. The characteristics of many of these syndromes are variable, a fact that complicates the diagnosis. Several MCA/MR syndromes such as Kabuki syndrome (KS), CHARGE syndrome and Cornelia de Lange syndrome (CdLS), have a “chromosomal” phenotype indicating that various genes might be responsible for the phenotypes. For all three above mentioned syndromes, several patients with chromosome abnormalities have been reported, but without pointing towards a common candidate chromosome region. The only exception is the 3q26-27 region found in several CdLS patients with a mild phenotype. Investigation of this region was carried out by using a patient with the classical CdLS phenotype that carried a *de novo* balanced translocation between chromosome 3 and 17 with its breakpoint in 3q26.3 (Ireland et al. 1991). However, this investigation did not lead to the identification of a candidate region or gene(s) with a direct involvement in the pathogenesis of CdLS (Tonkin et al. 2004a).

Because of the complex phenotypes in these syndromes, it has been speculated that they might be caused by microdeletions/microduplications involving more than one gene. However, two recent reports document that mutations in the *NIPBL* gene cause CdLS (Krantz et al. 2004; Tonkin et al. 2004b). The types of mutations identified in *NIPBL* included missense, splice site, nonsense, and frameshift. Severe protein-truncating mutations likely lead to haploinsufficiency of the NIPBL protein. Haploinsufficiency of NIPBL was documented as a disease mechanism in the report of a child with classic features of CdLS who was stillborn but was found prenatally to have a large, cytogenetically visible deletion of chromosome 5p13.1-14.2 (Hulinsky 2003).
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Another syndrome where a causative gene was recently identified is CHARGE syndrome. By screening 17 patients with this syndrome using array-CGH with a 1 Mb resolution, a deletion on 8q12 was found in one of the patients. This finding finally led to the identification of the candidate gene *CHD7* which was found to be mutated in more than half of the remaining CHARGE patients

(Vissers et al. 2003). The identification of candidate genes in these two syndromes demonstrates that one single gene which plays a pivotal role during early embryonic development can cause a complex phenotype involving many different tissues. Array-CGH has proved to be very helpful to identify candidate genes involved in the development of malformations and cognitive dysfunctions and hopefully we will be able to unmask more congenital malformation disorders with this method.

1.5.2 Imprinting

Chromosome sequences may appear normal, but can still be pathogenic if they have the wrong parental origin. Genomic imprinting is an epigenetic mechanism of non-Mendelian inheritance that is unique to mammals. A small number of genes are imprinted and are expressed differently according to their maternal or paternal origin. As chromosomes pass through the male and female germlines they acquire an imprint to signal a difference between paternal and maternal alleles in the developing organism. Even if the sequence of the gene is not altered, gene-malfunction will occur if two imprinted allels are inherited from the same parent. An individual with both homologs derived from the same parent (uniparental disomy, UPD) may show symptoms if the chromosome contains imprinted genes. Several human genetic diseases that exhibit non-Mendelian inheritance, such as Prader-Willi syndrome (PWS), Angelman syndrome (AS) and Beckwith-Wiedemann syndrome (BWS) have been linked to UPD and several imprinted genes have been identified. UPD can be detected by investigation of the parental origin of chromosomes using microsatellite markers.

1.6 VARIATIONS IN THE HUMAN GENOME

Variations in the human genome are present in many forms, including single-nucleotide polymorphism (SNP's), small insertion-deletions polymorphisms, Variable Number of Repeat Sequences (VNTRs) and Low Copy Repeats (LCRs). LCRs, also called segmental duplications, are located along the entire genome and consist of regions, 5 to 400 kb in size, flanked by short repeated sequences that share more than 90% homology. These regions usually contain genes, pseudogenes or regulatory elements and are thought to have a key role in human genomic evolution. The availability of the human genome sequence has allowed the identification of genome regions rich in

LCRs (Cheung et al. 2003). It has been shown that unstable regions, susceptible to chromosomal rearrangements are often closely located or surrounded by such sequences (Scherer and Green 2004). Segmental duplications may be responsible for many chromosome rearrangements through NAHR (Bailey et al. 2002).

In addition, two recent studies have shown that large-scale copy number polymorphisms (LCVs), that involve deletions or duplications of several kb to several hundred kb, are present in the human genome sequence and contribute to genomic variation between individuals without clinical phenotype (Iafrate et al. 2004; Sebat et al. 2004). Both studies identified about 200 loci that are susceptible to copy number variation in our genome. Surprisingly, a large number of LCVs contain coding genes, hence LCVs may contribute to phenotypic variation and disease susceptibility.

2 METHODS

2.1 Chromosome analysis

Metaphase slides were prepared from phytohaemagglutinin-stimulated, thymidine-synchronised lymphocytes (in a few cases cultured fibroblasts or amniocytes were used). The cells were harvested after mitotic arrest using colcemid and chromosome analysis was performed according to standard staining procedures using trypsin treatment followed by Giemsa staining. Routine chromosome classification was performed at a resolution of 400-550 bands per haploid genome.

2.2 Fluorescence *in situ* hybridization (FISH)

FISH is a technique that allows visualization of genetic alterations directly on interphase nuclei and metaphase chromosomes. A fluorescent labeled DNA probe is hybridized onto cells that are fixed and immobilized on a glass slide and detection is performed using a fluorescent microscope. The choice of FISH probes is dependant on the biological question in mind and the locus of interest must be at least approximately known. Locus specific probes can be made from BAC, PAC, cosmid or fosmid clones or from PCR products. The resolution depends on the probe size (>3kb) and the target DNA used. FISH is a powerful technique that allows detection of deletions, duplications, rearrangements and mapping of translocation breakpoints. However, it is relatively labor intensive because each locus has to be analyzed separately. Even when combining different fluorescent dyes, the number of different loci that can be visualized simultaneously and reliably distinguished is limited. The coverage of all 41 different unique chromosome specific near-telomeric sequences in a single subtelomeric FISH assay has therefore been developed into a multi-hybridization protocol where a single slide is divided into 24 small hybridization squares for each specific chromosome. The telomeres of the short arm and the long arm of each chromosome are labeled with a different dye and hybridized on metaphase chromosomes in the 24 different squares on the slide.

For FISH-mapping of translocation breakpoints, BAC and PAC clones can be selected from the public free accessible genome browsers from NCBI (http://www.ncbi.nlm.gov), Ensembl (http://www.ensembl.org) or UCSC

(http://genome.ucsc.edu), and based on their location on the physical map. They can be acquired at a low cost from Resources for Molecular Cytogenetics (Bari, Italy), The Wellcome Trust Sanger Institute (Cambridge, UK) and BACPAC Resource Center Children's Hospital (Oakland Research Institute, Oakland,CA), and investigated within a short period of time. In addition there are a large number of commercially available probes.

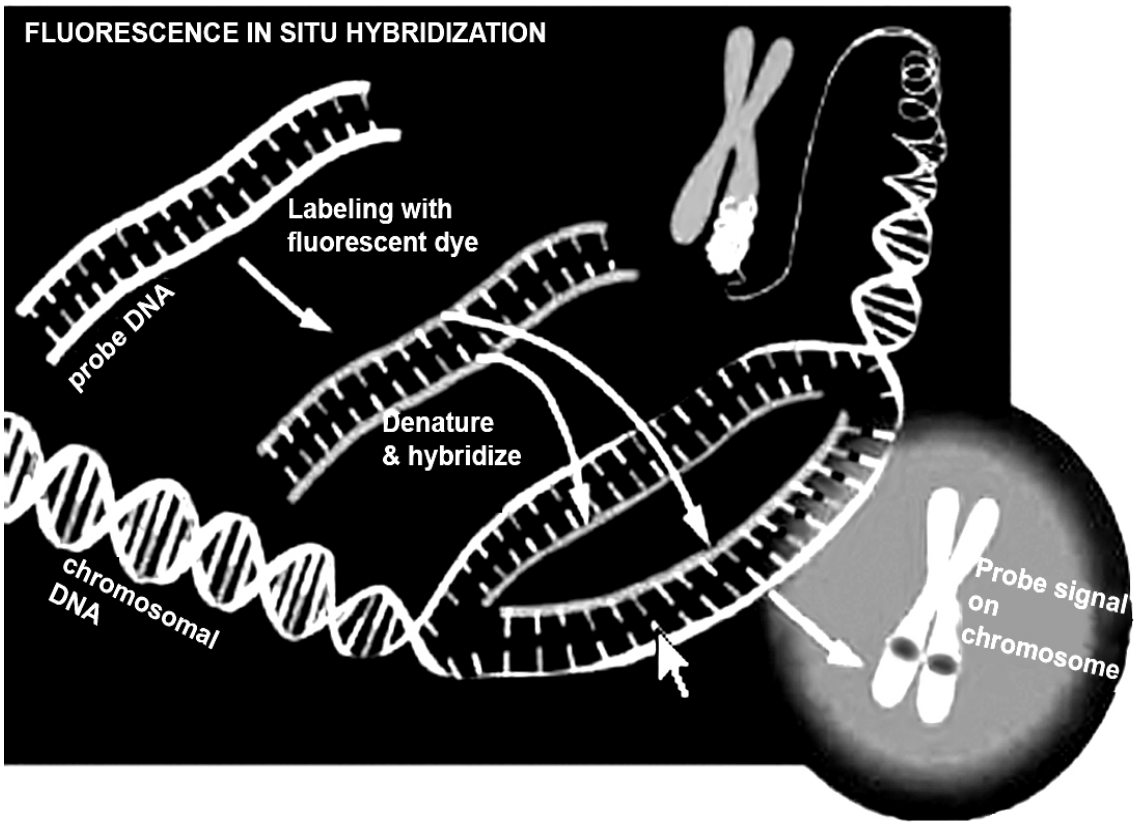

Fig 5 FISH : A DNA-probe is labeled with a fluorescent dye, denatured and hybridized on metaphase chromosomes.

2.3 Spectral karyotyping (SKY)

For the SKY technique various combinations of fluorescent dyes are used to make it possible to give every single chromosome a different color. This method is of particular use when dealing with complex aberrations. A probe cocktail is prepared from flow-sorted chromosomes, in which each chromosome is labeled with a combination of one to four different fluorochromes. Using five different fluorochromes (Spectrum Orange, Texas Red, FITC, Cy5 and Cy5.5) in 24 different combinations, each chromosome is stained by a specific color. Spectral Karyotyping (SKY) combines interferometry, CCD imaging and optical microscopy to simultaneously

measure all points in the sample emission spectrum (Schrock et al. 1996). SKY is able to detect translocations, but not inversions or small duplications and deletions.

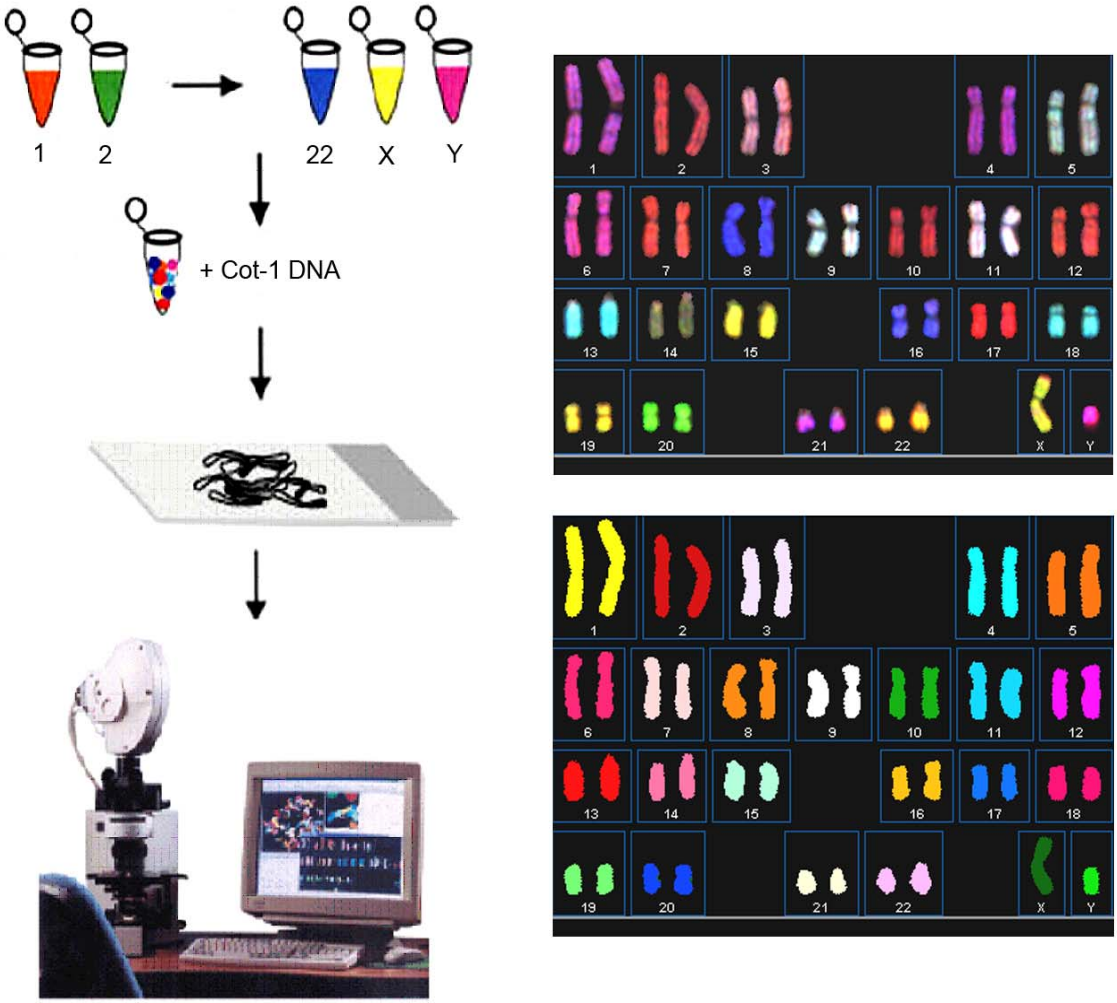

Fig 6 SKY: Flow-sorted chromosomes are DOP-PCR amplified and labeled using 5 fluorochromes. A probe cocktail containing 24 specific colors for each chromosome (derived from a combination of 1-4 fluorochromes) is hybridized on metaphase chromosomes. Cot-1 DNA is added for blocking of repetitive sequences. For image capturing a fluorescence microscope equipped with a CCD camera and Spectracube is used. Computer software allows chromosome identification and classification by each chromosome specific spectral color.

2.4 Comparative genomic hybridization (CGH)

The application of CGH to metaphase spreads enables genome-wide analysis of gross DNA copy number imbalances. This method has been widely used for analysis of tumor genomes and constitutional chromosome aberrations since it was first reported in the early 1990s (Kallioniemi et al. 1992). CGH is based on hybridization of differentially labeled test and

reference DNA, which are competitively hybridized to normal metaphase chromosomes on a glass slide. Hybridization of repetitive sequences is blocked by the addition of Cot-1 DNA. The fluorescence ratio of the test versus reference hybridization signal intensity is determined along the chromosome, indicating the relative DNA copy number compared to a normal diploid genome. The main disadvantage of metaphase CGH is its low resolution. In general the resolution of conventional CGH has been reported to be 5-10 Mb. However, the resolution of CGH analysis is dependent on the slide quality as well as the labeling- and hybridization efficiency (Kallioniemi et al. 1994). The DNA is usually labeled by nick translation, which is an enzyme mediated labeling method based on the incorporation of reporter molecules covalently attached to nucleotide triphosphate substrates in polymerization. Non enzyme mediated labeling methods, such as ULS™ labeling are based on a marker/platinum complex that links to the purines of the DNA (Wiegant et al. 1999; Heetebrij et al. 2003).

2.5 High resolution metaphase CGH (HR-CGH)

Kirchhoff *et al*. optimized the CGH technique by developing an algorithm for automated correction of the interfering effect of unsuppressed interspersed repetitive sequences (Kirchhoff et al. 1997). For refined sensitivity of the CGH analysis, CGH profiles are evaluated by the use of a dynamic standard reference interval based on hybridization of normal samples instead of fixed thresholds. The method called “high resolution CGH” (HR-CGH) has permitted detection of chromosomal deletions as small as 3 Mb (Kirchhoff et al. 1999).

2.6 Array based CGH (array-CGH)

Array-CGH is based on the same principles as metaphase CGH, except that the hybridization is performed on immobilized large genomic clones on a glass surface instead of chromosomes. Solinas-Toldo *et al.* developed a CGH protocol with target DNAs arrayed in small spots on a glass slide (Solinas-Toldo et al. 1997), and the first arrays that were used for array-CGH contained 3360 human cDNA clones as hybdridization targets to map gene amplifications and deletions in breast cancer (Pollack et al. 1999). One of the major limitations of using cDNA clones for array-CGH is the fact that the clones are unevenly distributed across the genome. Analysis enables only the

detection of aberrations in known genes, while gene poor regions or gene deserts are completely excluded from the analysis.

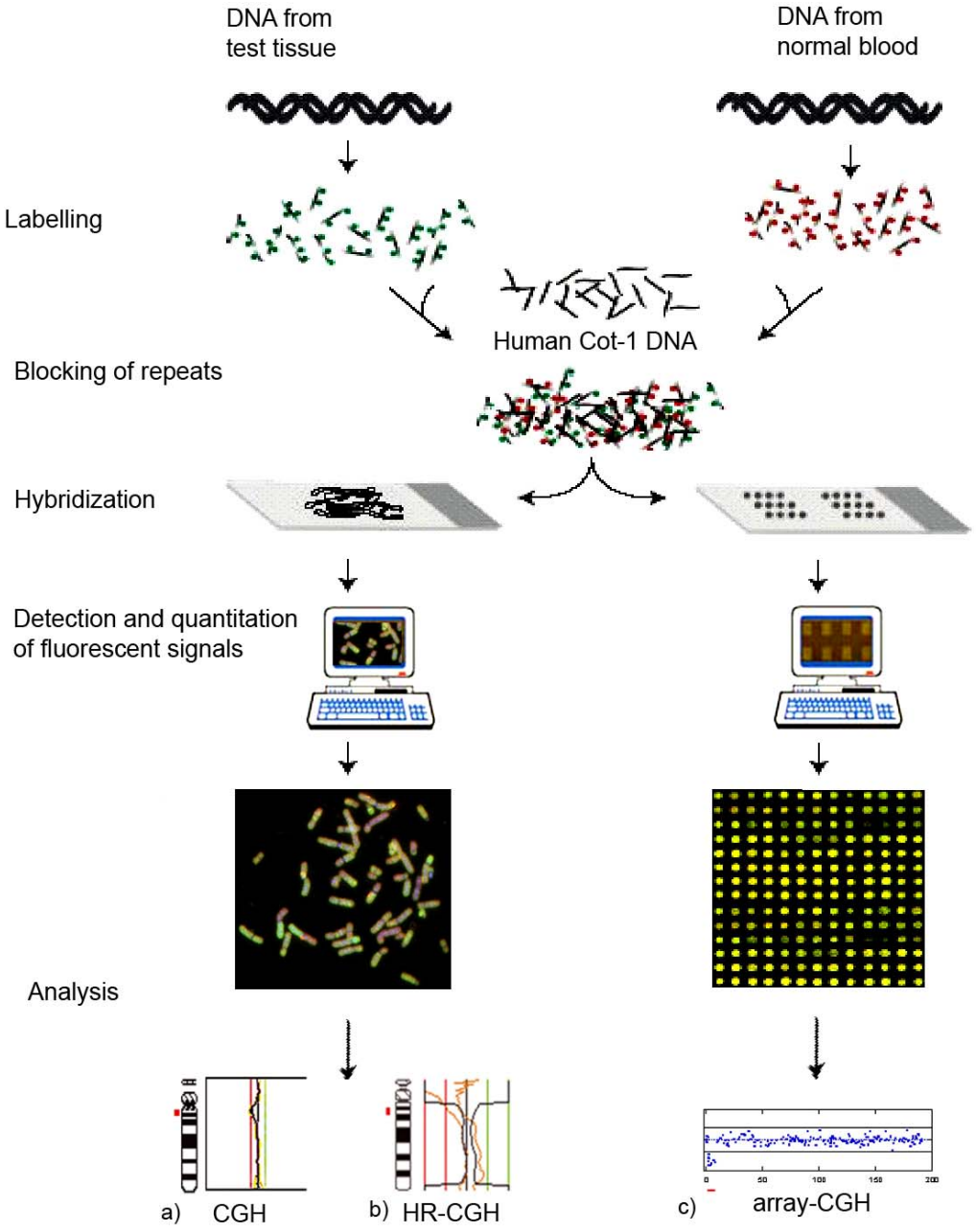

Fig 7 Comparative Genomic Hybridization (CGH): Differentially labeled DNA in red and green is hybridized either on chromosomes (conventional CGH and HR-CGH) or on immobilized clones on a slide (array-CGH). Signals are detected using either a fluorescence microscope or a laser scanner and ratio values between test and reference are quantified and analyzed with computer programs. a) CGH ratio plot (black lines) and fixed threshold (red and green line), b) HR-CGH ratio plot (orange lines) and dynamic standard reference interval (black lines) c) array-CGH ratio plot (blue dots) and threshold (black lines).

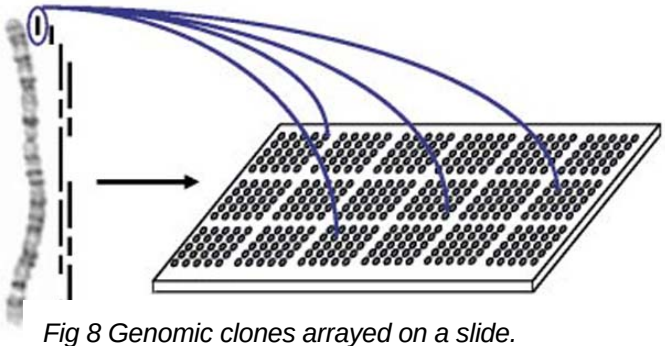

Fig 8 Genomic clones arrayed on a slide.

The current methodological approach for the construction of genomic arrays is now dominated by the use of genomic BAC and PAC clones which can be selected across the whole genome. Whole BAC isolation was initially done from large bacterial cultures because BACs are single-copy vectors and yield a low amount of DNA (Solinas-Toldo et al. 1997; Pinkel et al. 1998) Several methods were since then developed to circumvent this labor intensive approach e.g. ligation-mediated PCR (Snijders et al. 2001) and degenerate oligonucleotide-primed PCR (DOP-PCR) (Telenius et al. 1992; Veltman et al. 2002).

Whether using cDNA or BAC clones, array-CGH was initially mainly demonstrated to be very useful for detection of cancer-associated DNA copy number aberrations (Heiskanen et al. 2000; Lucito et al. 2000; Hodgson et al. 2001). It has been a challenge to achieve the adequate performance needed for the reliable detection of very small constitutional-associated gene dose alterations. During the past few years, several different strategies have been developed to improve the specific signal-to-noise ratio in order to increase the sensitivity. A few examples are the repeat-free and non-redundant strategy (Buckley et al. 2002), the use of specifically designed DOP-PCR primers for amplification of the BAC clones (Fiegler et al. 2003) and the attachment of DNA on to uncoated glass surface by chemical coupling (Cai et al. 2002).

The array format for CGH has many potential advantages over the use of chromosomes. It offers rapid genome-wide analysis at high resolution. The resolution is determined by the genomic distance between the clones as well as their sizes and the information it provides is directly linked to the physical and genetic map of the human genome.

2.7 Multiplex ligation-dependent probe amplification (MLPA)

MLPA is a method that detects copy number changes of genomic DNA. In order to investigate different DNA targets simultaneously, MLPA tests are

designed in such way that the length of each amplification product has an unique size between 130-480 bp and the fragments are separated by electrophoresis (Schouten et al. 2002). Amplification is performed by hybridization of two probes that are designed to bind adjacently to the target specific sequences in such way that they can be joined by the use of ligase. Comparison of the relative peak area of each amplification product to a normal control reflects the relative copy number of the target sequence. To avoid false positive and false negative results, several normalizations are applied (e.g. normalized by the mean value of all peaks and normalization by the peak areas of a normal control that are run together with the samples). MLPA is thus a fast, sensitive, relatively cheap and easy to perform technique. However, to develop a probe mix for each sequence of interest is time consuming. Each probe requires the design and preparation of a M13 clone and the purification and restriction enzyme digestion of that clone. MLPA reactions are also more sensitive to contamination of PCR inhibitors compared to ordinary PCR reactions and it is not suitable for detection of new mutations, since the probe has to be designed at a known locus of interest.

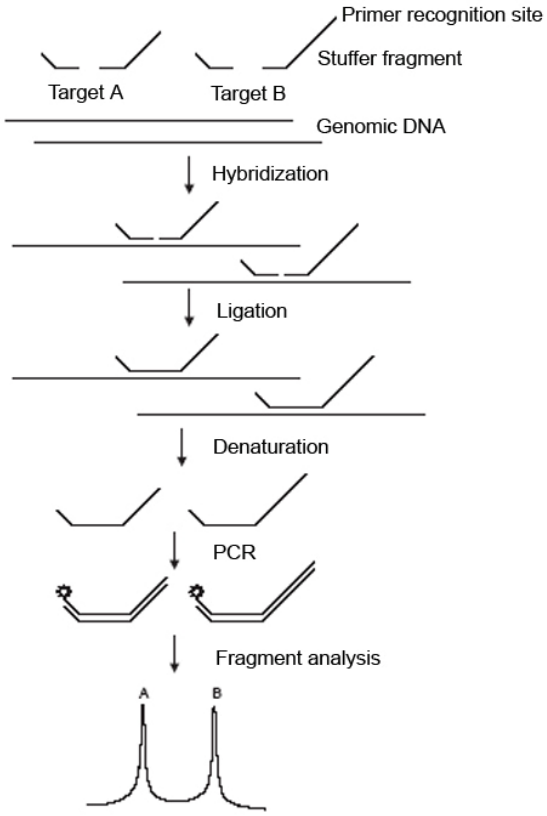

Fig 9 MLPA technique

2.8 Quantitative fluorescence polymerase chain reaction (QF-PCR)

QF–PCR involves the amplification of chromosome-specific, microsatellite DNA sequences that consist of small arrays of tandem repeats known as short tandem repeats (STRs). STRs are stable and polymorphic, that is, they vary in length between subjects, depending on the number of times the di- tri-, or tetra-nucleotides are repeated. The sample DNA is amplified by PCR using fluorescent primers so that products can be visualized and quantified as peak areas of the respective repeat lengths using an automated DNA sequencer.

DNA amplified from normal subjects who are heterozygous (have alleles of different lengths) is expected to show two peaks with the same area. DNA amplified from subjects who are trisomic will exhibit either an extra peak (being triallelic) with the same area, or only two peaks (being diallelic), one of them twice as large as the other. Subjects who are monosomic will exhibit only one peak.

3 AIMS OF THE PRESENT STUDY

1) To develop and evaluate molecular cytogenetic methods in order to improve the detection rate of submicroscopic chromosomal imbalances.

2) To investigate if submicroscopic chromosomal imbalances are involved in the etiology of certain malformation syndromes.

3) To outline gene dose alterations in patients in order to establish phenotype-genotype correlations and to pinpoint candidate genes causing developmental disorders.

4 SUBJECTS

4.1 PATIENT SELECTION

The patients reported in **paper I** were selected for genetic testing with subtelomeric FISH because of a strong suspicion of chromosome aberration. They all had unexplained MR, had been extensively examined, and they showed a normal karyotype by routine chromosome analysis (>400-450 bands). In addition, microdeletion syndromes and Fragile X syndrome were excluded in patients with a phenotype suggestive for these disorders. Thirty six patients were recruited from Clinical Genetics Departments in Sweden (Uppsala, Gothenburg and Stockholm) and the remaining patients were referred by pediatric neurologists. Almost all patients were young children at the time of investigation and only four patients were older than 20 years. The majority of the patients (74%) were classified as SMR and 26% of the cases had MMR. All MMR patients had in addition to MR at least one of the following criteria: dysmophism, a family history, or malformation. Of the whole investigated group, 69% showed dysmorphic features, 23% had malformations, 50% had a behavioral disorder and 36% of the cases had a family history of MR.

The unbalanced inherited translocation in the index patient described in **paper V** was identified in the subtelomeric study and reported in paper I. Fine mapping of the sizes of the unbalances and genotype-phenotype correlation was performed on all affected family members.

Seventeen cases with known cryptic aberrations were selected for the CGH study (**paper II)**: Twelve cases with subtelomeric rearrangements, two cases with an interstitial deletion and four cases with a microdeletion syndrome; i.e. DiGeorge syndrome, Prader Willi syndrome, Wolf Hirschhorn syndrome and a mosaic case with Smith Magenis syndrome, Table 2. The 17 cases encompassed altogether 26 rearrangements (21 terminal aberrations and 5 interstitial aberrations). Their size ranged from 1.3 to 20.5 Mb (42% of the aberrations were smaller than 5 Mb), and 19 different chromosome arms were involved. All cases had previously been identified by subtelomeric FISH (paper I), microdeletion FISH, SKY or HR-CGH analysis at the Karolinska Institute or at the J.F.Kennedy Institute in Glostrup, Denmark. The cases identified at the two different institutes were exchanged for completely blinded testing.

The evaluation of array-CGH (**paper III)** was performed by using ten out of the seventeen cases from paper II. These ten cases were all identified and size mapped at Karolinska Institutet and consisted of 16 chromosome imbalances.

For the study on Kabuki syndrome (**paper IV)**, we collected DNA samples from children diagnosed with this disorder from Sweden and Denmark (Department of Clinical Genetics in Uppsala, Gothenburg, Umeå, Stockholm and the J.F. Kennedy Institute in Glostrup). Kabuki syndrome is a rare disorder affecting about 1/30 000 and the diagnosis can be difficult. Therefore only a small number of patients are diagnosed in Sweden with this disorder. Blood samples were obtained from only seven Swedish patients and three Danish patients. All patients had been evaluated by an experienced clinical geneticist prior to inclusion in the study. In addition to DNA samples we obtained fixed metaphase cell suspensions from five of the patients.

The patient group studied by array-CGH (**paper VI)** consisted of 49 patients and all cases fulfilled the same criteria as the patients in the subtelomeric study, with a majority of SMR cases. All patients were children aged between 6 months and 16 years at the time of investigation. The 49 patients consisted of 20 patients that had previously been included in the subtelomeric FISH/SKY screening study, where no abnormalities were detected, 8 patients diagnosed with Cornelia de Lange syndrome (CdLS) and 21 IMR patients referred to the Department of Clinical Genetics in Stockholm. Ten of these 21 patients had in addition been screened by subtelomeric FISH prior to array-CGH analysis. Recent reports have shown that mutations in the *NIPBL* gene cause the CdLS phenotype in about half of the investigated cases. This gene had not yet been identified at the time that our eight CdLS patients were screened by array-CGH.

5 RESULTS AND DISCUSSION

5.1 Screening patients with IMR by subtelomeric FISH (paper I)

In order to detect subtle subtelomeric abnormalities in patients with idiopathic MR, 41 FISH probes were used to screen all chromosome specific subtelomeric regions (excluding the short-arm of acrocentric chromosomes). Rearrangements were detected in 9% of the patients (10 out of 111) and the subtelomeric FISH results are summarized in Table 2. A *de novo* deletion was found in five patients (cases 1, 3, 4, 6 & 10), three patients had an unbalanced inherited translocation (cases 2, 7 & 8), one case had an unbalanced *de novo* translocation (case 9) and two siblings had an unbalanced recombinant chromosome inherited from a balanced pericentric inversion in their father (case 5a and b). Fourty percent of the cases inherited the chromosome abnormalities from one of their parents and prenatal diagnosis was offered to the translocation carriers.

Table 2 Summary of subtelomeric FISH results

Case	G	MR	DF	MF	GR	FH	SKY	G-band	FISH results	Origin
1	F	MMR	+	-	-	+*		-	2q monosomy	*de novo*
2	F	SMR	+	+	-	+	Pos	+	2q trisomy 4q monosomy	paternal
3	F	SMR	+	-	Postnatal	-		-	4p monosomy	*de novo*
4	M	SMR	+	+	Prenatal	+*		-	6p monosomy	*de novo*
5a,b	M	SMR	+	-	Postnatal	+		+	6p monosomy 6q monosomy	paternal
6	F	SMR	+	-	-	-		-	9q monosomy	*de novo*
7	M	SMR	+	+	Postnatal	-	Pos	+	9q trisomy 21q monosomy	maternal
8	F	MMR	+	+	Postnatal	+	Neg	-	12q trisomy 17q monosomy	paternal
9	F	SMR	+	-	-	-	Pos	-	20q triomy 22q monosomy	*de novo*
10	F	MMR	+	-	-	-		-	22q monosomy	? #

*G=Gender, DF=Dysmorphic features, MF=Malformations, GR=Growth retardation, FH=Family history of MR, # father not available for testing, * affected siblings did not share the chromosome abnormality*

None of the aberrations were detected by routine chromosome analysis. However, three rearrangements (cases 2, 5 & 7) were retrospectively

detectable by G-banding at 400-500 band resolution. The other seven aberrations were not detectable by pro-metaphase analysis.

In order to screen for interstitial translocations that could have escaped detection by G-banding, SKY was performed on 50 of the patients that showed no abnormalities in the subtelomeric screening. Subtle intra-chromosomal aberrations, such as small deletions, duplications and inversions can not be revealed by SKY. No additional abnormalities were found. However, three of the four unbalanced translocations (cases 2, 7 & 9) could also be identified by SKY.

In the majority of the cases genotype-phenotype correlation was established, except for two of the cases (cases 1 and 4) and the effect of the terminal deletion on their phenotype is not clear. Case 1 had a deletion on 2q, both parents had normal chromosomes and no abnormalities were found in her sister, who was more severely mentally retarded. Furthermore, the subtelomeric region of 2q has been reported to be polymorphic and deletions have frequently been observed without clinical significance. In all other cases, the aberration detected is most likely the underlying cause of their phenotype. Case 3 had a small terminal 4p deletion and showed many features recognizable in the Wolf Hirshhorn syndrome phenotype. Case 5a and b shared many features described in patients with a duplication of 6q and case 6 showed an overlapping phenotype with other cases reported with a loss of the 9q telomere (Stewart et al. 2004). Case 7 clearly shared features with two other patients with a duplication of terminal 9q that were identified at the Department of Clinical Genetics in Stockholm (unpublished data). In two cases (Cases 2 & 9) the chromosome fragments involved in the imbalance were so large that a phenotypic expression is most probable and case 8 is discussed in chapter 5.2. The size of the deletion on 22q in case 10 was only 100 kb and her phenotype was concordant with previously described cases with terminal deletions on 22q (Anderlid et al. 2002b). At the time of investigation the Multiprobe™ screening kit was used which contained the cosmid probe n85a3. When the deletion was size mapped clone 99k24 was shown to span the breakpoint. This clone is included in the more recent subtelomeric screening kit. It would therefore have been more difficult to detect the deletion when using the recent kit, since only a reduced signal intensity was observed with this clone.

During the past years some clones have been replaced in the subtelomeric FISH screening kits (Multiprobe™ and ToTel Vysion™) in order to improve the performance, e.g. clones that hybridized on multiple locations in the genome,

clones that were reported to be polymorphic and clones that gave weak signals because they contained too many repeats. Some of the replaced clones are located slightly more distant from the chromosome ends, because these regions contain more unique sequences and less repeats. However, if the clones are located too distant from the telomeres, very small terminal aberrations might escape detection.

In our subtelomeric screening study (paper I), we investigated 81 patients with moderate/severe MR and found abnormalities in 7 patients (8.5%). The remaining 29 patients were classified as mild MR and we found abnormalities in 3 (10.3%) of these patients. These findings give a higher detection rate in MMR compared to other subtelomeric studies. In general, abnormalities are found in about 5% of mentally retarded patients when investigated by subtelomeric screening methods (Biesecker 2002; Flint and Knight 2003). In addition, the detection of subtelomeric aberrations has led to the recognition of novel microdeletion syndromes such as monosomy 1p36 (Heilstedt et al. 2003a; Heilstedt et al. 2003b) monosomy 9q34 (Stewart et al. 2004) and monosomy 22q13.3 (Wilson et al. 2003).

5.2 Genotype-phenotype correlation of gene dose alterations (paper V)

A submicroscopic reciprocal unbalanced translocation between chromosome 12q and 17q was identified in a girl born in 1982 in the subtelomeric FISH screening (Case 8). When the family was further investigated we found that the father carried a balanced translocation as well as his half sister, his mother (who was not investigated because she was no longer alive at the time of investigation, but must have carried the translocation) and the girl's brother and half brother (Fig 10). In addition to the index patient, three family members inherited unbalanced variants: the girl's half sister, her cousin and her uncle.

The fragments involved were FISH mapped for accurate size determination. Using 19 BAC clones covering the distal part of chromosome 12q and 17q the imbalances were mapped to 2.1 Mb and 1.3 Mb respectively. Two cases (III 1 and IV 8) inherited the derivative chromosome 17 and the other two (IV 3 and IV 6) had the derivative chromosome 12.

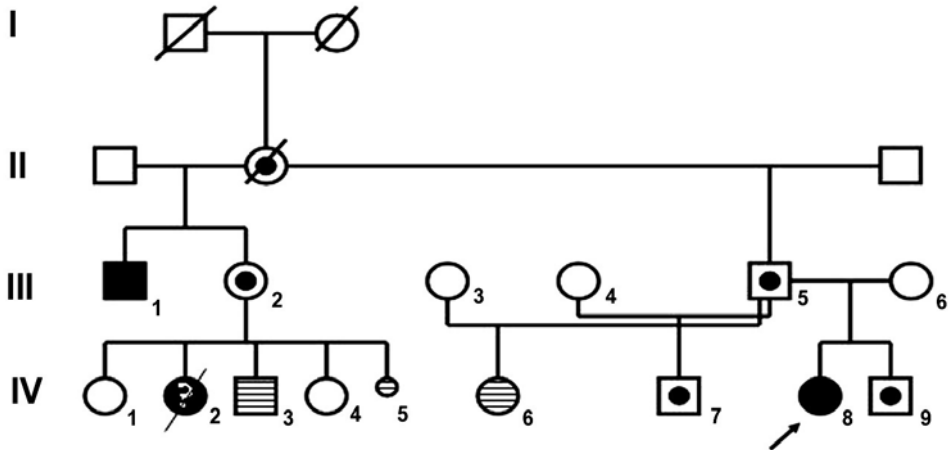

Fig 10 Pedigree of the t(12;17)(qter;qter) family. Case III 1 and IV 8 (index patient) carry the derivative chromosome 17 (dup12qter, del 17qter) and are illustrated by black symbols. Case IV 3 and IV 6 carry the derivate chromosome 12 (del12qter;dup17qter) and are illustrated by striped symbols. Black represents carriers of der(17), striped represents carriers of der(12) and black dot represents balanced carrier of t(12;17).

Reports of constitutional chromosome aberrations involving 12qter and 17qter are scarce, and the majority of cases are results of malsegregation of balanced translocations. The phenotype of patients with a partial duplication of terminal 12q is recognizable as a clinically identifiable syndrome (de Muelenaere et al. 1980; Harrod et al. 1980; Tajara et al. 1985; Tengstrom et al. 1985; McCorquodale et al. 1986). Partial duplication of 17q has also been described in individuals with a recognizable syndrome. However, in both the 12q and 17q duplications, all reported cases with a recognizable phenotype had been cytogenetically detected, the chromosome fragments involved were thus of a much larger size compared to our patients. Small terminal deletions of 12q has to our knowledge not been previously detected and only one case with a terminal 17q deletion has been reported. Phenotype correlation using reports from the literature was therefore complicated.

The two individuals with derivative chromosome 17 showed a distinct phenotype with mild mental retardation, early motoric development delay, postnatal growth retardation, orofacial cleft and repeated ear infections, and they both developed a bilateral hearing impairment. The breakpoint of the translocation was located approximately 300 kb telomeric from the *ACTG1* gene. This gene located at 17q25.3 has been reported to cause autosomal dominant hearing loss (van Wijk et al. 2003). In our two patients the gene is thus not deleted but it can not be excluded that the gene expression is affected by a heterochromatin-induced positional effect.

The phenotype in the cases with derivative chromosome 12 was milder and only partly concordant. The deceased girl IV:*2* had some malformations in common with cases 1 and 3, and it is most probable that her malformations were the result of a chromosome imbalance caused by the familial translocation. To our knowledge, our two cases carrying the der(12)t(12;17)(q24.33;q25.3) are the first patients reported with such a small terminal deletion of chromosome 12.

This family illustrates that small chromosome imbalances can be detected in individuals with a mild phenotype and normal, or near-normal, cognitive functions.

5.3 Evaluation of comparative genomic hybridization (papers II & III)

Molecular cytogenetic methods, such as FISH, CGH and multicolor karyotyping have made it possible to increase the resolution and to identify very small chromosome rearrangements as the cause of mental retardation. However, even more sensitive methods are required to detect the smallest rearrangements. Before the development of HR-CGH only a few groups have reported conventional CGH used for detection of imbalances in MR patients (Joly et al. 2001; Sanlaville et al. 2002). Based on its technical limitations and low resolution, there is little reason to apply CGH to chromosomes of metaphase cells in routine diagnostic settings. An increased resolution of about 3 Mb have been reported using a standard dynamic reference interval (Kirchhoff et al. 1999). This standard dynamic reference interval has been incorporated into the commercially available HR-CGH software from Applied Imaging. Consequently, CGH analysis has been introduced into the diagnostic setting, although a 3 Mb resolution is unattainable by most clinical cytogenetics laboratories. So far only two studies have been reporting the usefulness of this method with a 10-12% detection rate when applied on IMR patients ((Kirchhoff et al. 2001; Ness et al. 2002; Kirchhoff et al. 2004). However, the resolution of the technique is not clear from the results of these studies since the size of the abnormalities detected are not mapped. We therefore tested different CGH approaches in order to determine if they might be introduced as reliable and sensitive diagnostic procedures. Firstly, we investigated the performance of different CGH approaches on metaphase chromosomes, and later, when we had access to microarrays, we also tested the robustness and simplicity of different array-CGH platforms on a set of patients with well-defined sub-

microscopic chromosome abnormalities. The results of the CGH testing are summarized in Table 3.

We modified the CGH performed on metaphase chromosomes in our laboratory by using chemical labeling strategies and increasing the chromosome quality on the slides used. We then compared its performance to a commercially available high resolution software (HR-CGH) using a dynamic standard reference interval. The test material we used for validation of the methods consisted of 17 cases that contained 26 constitutional rearrangements not detected by standard karyotyping, but previously identified by subtelomeric or microdeletion FISH probes, Spectral Karyotyping (SKY) or HR-CGH. For an accurate validation of the different CGH methods, all aberrations were first size mapped using BAC and PAC clones that were selected from Ensembl and UCSC genomic browsers. The aberrations ranged in size from 1.3 to 20.5 Mb and 11 of the aberrations were smaller than 5 Mb. The chromosome abnormalities that had been identified in the two different institutes were exchanged and normal samples were added for completely blinded testing. The samples that were tested and identified at the same institute were also analyzed blindly. However, taking into consideration the fact that some knowledge of the type of aberrations possibly would bias the analysis, the testing was considered “semi blinded”,

The HR-CGH method detected all aberrations >6Mb and a few smaller, while the modified CGH method was able to detect all but three aberrations >1.8 Mb. The modified CGH method was superior in the detection of terminal imbalances, while the HR-CGH software was more successful in the detection of imbalances located very close to the centromeric regions. The resolution of both metaphase CGH methods were not evenly along the chromosomes and most likely dependent on chromosome structure and condensation. In general, metaphase CGH has limitations in sensitivity and robustness, and it is not very accurate in the delineation of the size or location of the chromosome imbalance.

For the evaluation of array-based CGH, an array containing 21632 cDNA clones and three arrays containing 1000, 1400 and 2500 BAC and PAC clones respectively, were used. In this study, ten out of seventeen cases were tested. These cases contained 16 chromosome abnormalities ranging in size from 1.3-20 Mb.

The cDNA array did not detect small heterozygous deletions or duplications. This is most likely due to the fact that cDNA microarrays were primarily developed for expression profiling. The cDNA sequences are therefore mostly selected based on gene function, not on chromosome location, which results in poor coverage of some chromosomal loci.

Table 3 CGH test results

Case	Chromosome aberration	Size Mb	MM-CGH	HR-CGH	Array-CGH 21632	1000	1400	2500
1	Monosomy 7qter	13.2	+	+	+	+	+	+
	Trisomy 10qter	19.1	+	+	+	+	+	+
2	Monosomy 18pter	5.8	+	+	-	-		+
	Trisomy 13qter	5.9	+	-	-	-		+
3	Monosomy 21qter	2.5	+	-	-	-		+
	Trisomy 9qter	11.2	+	+	+	-		+
4	Monosomy 12qter	2.1	+	-	-	-	+	+
	Trisomy 17qter	1.3	-	-	-	-	+	+
5	Monosomy 4qter	8.7	+	+	+	+		+
	Trisomy 2qter	20.5	+	+	+	+		+
6	Monosomy 6qter	3.5	+	-	-	+	+	+
	Trisomy 6pter	14.7	+	+	+	+	+	+
7	Deletion 15q24	10.3	+	+	+	-	-	+
8	Deletion 6pter	1.8	+	-	-	-	-	+
9	Deletion 22q11(DGS)	2.5	-	-		-	+	+
10	Deletion 17p11 (SMS)	3.6	+	+		-	+	+
11	Deletion 10q25	8.1	+	+				
12	Deletion 4pter (WHS)	6.9	+	+				
13	Deletion 15q11-13 (PWS)	~ 4	+	+				
14	Monosomy 9qter	4.1	+	-				
	Trisomy 22qter	5.7	-	-				
15	Deletion 1pter	8	+	+				
16	Monosomy 13qter	3.9	+	-				
	Trisomy 5qter	6.5	+	+				
17	Monosomy 13qter	19.6	+	+				
	Trisomy 2pter	3.5	-	-				

MM-CGH= modified metaphase CGH, HR-CGH=high resolution CGH, array-CGH containing 21632 cDNA clones, 1000-, 1400 - and 2500-BAC clones respectively.

The BAC microarray clearly performed better for the detection of small aberrations since the clones were evenly distributed along the genome. However, the resolution is determined by the genomic distance between the clones spotted on the array and at least 2500 clones are needed to cover the whole genome at approximately 1 Mb intervals. The array that contained 1000

clones did not perform well. False positive and false negative results were observed as well as undetectable large aberrations. This was a first generation array manufactured in 2001 and it contained a large number of clones that were not FISH verified. This resulted in a large number of errors in the physical mapping of the clones, since the physical map at the time of investigation was not as accurate as it currently is. Thereafter, an improved array containing 1400 FISH verified BACs was developed, including extra clones covering the microdeletion syndromes and subtelomeric regions. This array was tested on five samples (unpublished data, table 3) and was able to correctly detect all abnormalities.

Finally, an array consisting of 2500 FISH verified and end sequenced clones was produced with an average resolution of approximately 1 Mb across the genome. Using this array we were able to detect all aberrations studied (16) and determine the size to 1 Mb accuracy. As a result of the sequencing of the human genome, most BAC and PAC clones are currently correctly mapped, making it possible to develop reliable BAC arrays. The 2500 clone array, now contains 132 additional clones and more clones will be added in the future in order to further increase the resolution. From this study, we concluded that the BAC array containing 2632 clones would become a reliable and important tool in the clinical evaluation of mental retardation.

5.4 Genome-wide screening of children with MR by array-CGH (papers IV & VI)

Two groups of patients were selected for genome-wide screening at 1 Mb resolution. The first group consisted of ten Caucasian patients diagnosed with Kabuki syndrome (KS). This is a rare multiple congenital anomaly/MR syndrome and it is characterized by a distinct facial appearance, mental retardation, postnatal growth retardation, skeletal anomalies and unusual dermatoglyphics and fetal fingertip pads. This syndrome was first described in 1981 (Kuroki et al. 1981; Niikawa et al. 1981) and was first thought to be fairly uncommon outside of Japan. It is now well recognized all over the world and at least 350 cases have been reported. The male-to-female ratio among reported patients is nearly equal. Most patients are sporadic, but a few families are known with possible parent-child transmission of the disorder (Halal et al. 1989; Say et al. 1993; Kobayashi and Sakuragawa 1996; Silengo et al. 1996; Tsukahara et al. 1997; Frediani et al. 2001). Several individuals diagnosed with KS have been reported with chromosomal abnormalities

suggesting candidate genomic regions harbouring the causative gene(s). Except for several abnormalities involving the sex chromosomes, abnormalities were found on chromosome 6 (Jardine et al. 1993), chromosome 4 (Fryns et al. 1994), chromosome 13 (Lynch et al. 1995) chromosome 1 (Lo et al. 1998) and translocations involving chromosome 15 and 17 (Galan-Gomez et al. 1995) and chromosome 3 and 10 (Digilio et al. 2001) in patients with KS. However thus far, there have been no autosomal cytogenetic aberrations in common. A few patients were reported with clinical manifestation of both Kabuki syndrome and van der Woude syndrome (VWS), suggesting a common cause. The VWS1 critical region is located at 1q32-q41 and a deletion of that region was excluded in patients with KS (Makita et al. 1999).

It has previously been speculated that Kabuki syndrome was caused by a microdeletion or duplication (Matsumoto and Niikawa 2003) too small to detect by conventional cytogenetics. In our screening study, we detected some single clone polymorphisms but we were not able to identify an etiological cause for Kabuki syndrome. In a recent report, an interstitial microduplication of 8p22-23.1 was presented in six cases with this disorder (Milunsky and Huang 2003). Using three FISH clones (RP11-92C1, RP11-31B7, RP11-112G9) that had previously been reported to be duplicated on chromosome 8p, we investigated five of our patients by performing FISH on metaphases and interphase nuclei. From the remaining five patients no cell suspensions were available for FISH analysis, therefore we investigated them by QF-PCR using three microsatellite markers (D8S550, D8S552, D8S520) located in the same region. In addition, three clones on the 1 Mb array covered 8p22-23.1, and no gains were detected. We therefore concluded that none of our Kabuki patients had the 8p duplication. Other studies have recently reported the failure to confirm the 8p duplication in Kabuki patients (Miyake et al. 2004; Hoffman 2005) as well as unpublished investigations performed by several other laboratories (personal communication).

Moreover, multiple patients without the KS phenotype, but with rearrangements of the short arm of chromosome 8 involving region 8p23.1 have been reported, including inverted and tandem duplications of 8p, deletions of 8p23, pericentric inversions (p23q22), and isolated duplications of 8p23. The clinical significance of duplication of 8p23.1 remains controversial (Barber et al. 1998; Tsai et al. 2002). It is difficult to ascertain the frequency of interstitial dup 8p23.1 in the population because cases are often difficult to detect without high resolution analysis, and the true incidence may therefore be under-reported. Although, an inversion polymorphism in this region between two 8p olfactory receptor gene

clusters has been found in 26% of a population of European descent (Giglio et al. 2001) and 27% of a population of Japanese descent (Sugawara et al. 2003). The candidate duplicated region suggested by Milunsky and Huang is located between the two complex low-copy repeats REPD and REPP (Sugawara et al. 2003) and two of the mothers of the investigated KS patients were carriers of an inversion at 8p23. Milunsky and Huang concluded that since the duplication found in their six unrelated KS patients included 8p22, (which has been reported to result in phenotypic abnormalities) the duplications found in their patients were responsible for the KS phenotype (Milunsky and Huang 2003). Further studies to determine genotype-phenotype correlations with potentially altered gene expression patterns of the genes located in that region have not yet been performed. It is difficult to dissect the relationship between the chromosomal anomaly from other genetic and environmental factors, and the question of why some patients with dup 8p display pathological features while others are phenotypically normal remains unanswered.

The second group of patients consisted of 49 children with mental retardation that all fulfilled the same clinical criteria as was used in paper I. Twentytwo of the cases had already been tested in the subtelomeric study (paper I) and 11 of these 22 had also been investigated by SKY. We added 19 patients that had been referred to the Department of Clinical Genetics in Stockholm for genetic evaluation and 8 of them had been subtelomeric screened prior to array-CGH investigation. The remaining 8 patients were diagnosed with Cornelia de Lange syndrome (CdLS) which is a congenital multiple malformation disorder.

We detected abnormalities in 5 cases (10.2%) with sizes ranging from 700 kb to 14 Mb. In two cases (cases 1 and 2) we found a large deletion on chromosome 2q, which was not detected by conventional cytogenetics despite their size. This illustrates the need for more sensitive and reliable methods for detection of chromosome abnormalities in the diagnostic setting since not only small aberrations escape detection by G-band analysis. The deletions detected are very likely the cause of the phenotype in these two patients since their phenotypes are partly concordant with other reported cases with deletions in the same chromosome regions (Boles et al. 1995; Nixon et al. 1997; Koolen et al. 2004).

Two cases (cases 4 and 5) had a deletion that partly covered regions of well characterized microdeletion syndromes (SMS and WHS). Case 4 had an interstitial deletion that spans both the *WHSC1* and *WHSC2* genes, associated

with Wolf Hirshhorn syndrome. The child showed the classical characteristic facial features of this syndrome. The *LETM1* gene, which is deleted in the majority of WHS patients has been reported to be associated with seizures which is included in the WHS phenotype (Rauch et al. 2001; Zollino et al. 2003; Van Buggenhout et al. 2004). Despite the fact that this gene was not deleted in our patient, the child had late onset epilepsy. The breakpoint of the deletion is very closely located to this gene and altered gene expression can not be excluded. Case 5 had an interstitial deletion located close to the SMS critical region, therefore the child was further investigated with the specific microdeletion FISH probe for SMS. No deletion was detected using this probe but the patient's phenotype showed many overlapping features with SMS.

Table 4 Summary of array-CGH results

Case	Chromosome Imbalance	Size In Mb	Array results	Phenotype	MR	Age	Gender	Origin
1	del(2)(q24.3q31.1)	10	Deletion of 12 clones	DF, GR, MF	Severe	2	Female	*de novo*
2	del(2)(q22.3q24.1)	14	Deletion of 14 clones	DF, GR, MF	Severe	13	Female	*de novo*
3	dup(9)(pter)	0.7	Duplication of 3 clones	CDLS-like	Mild	7	Female	*de novo*
4	del(4)(p16.2p16.3)	6	Deletion of 11 clones	WHS-like	Severe	11	Female	*de novo*
5	del(17)(p11.2	2	Deletion of 2 clones	SMS-like	Moderate	10	Male	*de novo*

DF= Dysmorphic features, GR= growth retardation, MF= malformations, T-FISH=subtelomeric FISH, G-band= Giemsa banding analysis > 400

Case 3 had a small *de novo* terminal 9p duplication and was clinically diagnosed with CdLS. It is not clear if this duplication is the cause of the phenotype in this patient. In a recent report, mutation in the *NIPBL* gene have been detected in 47% of CdLS patients (Gillis et al. 2004). Furthermore, polymorphisms have been reported in the terminal end of the short arm of chromosome 9. A mutation in this gene has not yet been ruled out in our eight patients diagnosed with CdLS, but CdLS patients reported with a mutation in this gene showed a more severe phenotype than case 3. Further investigation will be needed to determine the significance of this finding.

Previous studies reported that, along with chromosome imbalances of clinical importance, array-CGH detects so called LCVs (large scale copy number variations (Iafrate et al. 2004; Sebat et al. 2004)). These polymorphisms were also frequently observed in our study.

6 CONCLUDING REMARKS

Examination of the chromosome ends by subtelomeric specific probes has clearly increased the resolution in these regions. Various publications have reported detection rates for telomeric abnormalities among patients with MR between 0 and 23% depending of the mode of ascertainment and study inclusion criteria. So far, over 2500 subjects with mental retardation have been tested and reported, ~ 5% of whom appeared to have subtelomeric rearrangements. In our study we reported a detection rate of 9%, and subtelomeric screening was subsequently introduced into the clinic as a diagnostic procedure. By the end of 2003, more than 300 patients had been screened and rearrangements have been identified in ~ 6% of the cases (unpublished data).

One striking and recurring observation is cases with relatively large chromosome aberrations that escape detection in regular banded karyotypes. The average band size at 300 band resolution is 10 Mb/band. Although patients have been analyzed with at least 400 band resolution several detected rearrangements of 10-15 Mb were first identified with molecular tools (paper I & paper VI). When the precise location was known, it could also in retrospect be confirmed with G-banding. This also speaks in favor of applying molecular tools for detection of chromosome aberrations.

The detection rate of array-CGH in patients with IMR is still unclear. There are so far only three reports (including our study), and the detection rate ranges between 10-24%. However, after exclusion of possible polymorphisms all three studies detect abnormalities close to 10%. The same variable results will probably be found, as was the case when subtelomeric screening was first widely applied. Investigations of larger sample sizes are needed to truly estimate how frequent subtle chromosome anomalies are in IMR patients.

It is very important to make the collected data from genotype-phenotype correlations accessible for clinical geneticists and other medical professionals for the recognition of “chromosomal phenotypes”. The online databases to access phenotypes correlated to gene dose alterations, such as DECIPHER at http://www.sanger.ac.uk/PostGenomics/decipher/ and ECARUCA at http://www.ecaruca.net/, will in the future become tremendously helpful for interpreting chromosome abnormalities. In the past, reports of chromosomal

abnormalities correlated to phenotypes were only possible on patients with large chromosomal abnormalities detectable by conventional cytogenetics. Accurate localizaton and size mapping of aberrations is crucial for identification of candidate genes. Despite the relatively small size of the chromosome fragment involved in the translocation of our family described in paper V, many genes were involved in the imbalance and candidate genes responsible for the phenotypes were difficult to pinpoint. It is however very important to report imbalances in gene rich chromosome regions that only cause a mild phenotype in order to possibly identify genes that are not dosage dependant.

We performed a thorough investigation of different CGH methods in order to determine if the sensitivity and simplicity of these methods would be sufficient for use in the diagnostic setting. Even after consideration of the improvements that could be made to the method's sensitivity, we found CGH performed on metaphase chromosomes not reliable enough for accurate detection and size determination of abnormalities.

On the other hand, array based CGH has in the past few years undergone a tremendous development. This method has proven to be a powerful tool for detection and delineation of subtle chromosome imbalances and recently the *CHD7* gene causing CHARGE syndrome was identified using array-CGH.

Our investigation of Kabuki patients did not reveal an etiological cause of the syndrome. Our results suggest that gene dose alterations in 8p are unlikely to be the cause in our series of KS cases. The wide spectrum of characteristics of KS complicates the diagnosis and it might therefore be possible that KS is clinically and genetically heterogeneous. However, it seems unlikely that KS syndrome is caused by 8p duplication since it is only found in one study of six cases while world-wide investigations of about 100 patients originating from different populations did not lead to the confirmation of the initial finding (personal communication).

7 FUTURE PERSPECTIVES

Genome-wide screening of patients with a chromosomal phenotype by array-CGH has already given us more knowledge and understanding of IMR. High resolution genomic screening of IMR patients might in addition unravel the cause of other syndromes that are not yet characterized today, as was the case when characterizing the CHARGE syndrome.

At this point, BAC arrays have mostly been used but so called SNP-arrays have successfully been applied for the detection of cancer associated chromosome imbalances. This kind of array does not only enable the detection of copy number changes, but also loss of heterozygosity (LOH), resulting from the duplication of one allel and hence without copy number change. The main drawback of SNP-arrays was their resolution across the genome, which is determined by the location of SNPs. Recently, the resolution of SNP-arrays has drastically improved by the increased number of SNPs. They may in the future be used for screening of IMR patients because they have the advantage over BAC arrays that they can identify UPD and chromosome regions that contain imprinted genes. Due to the large number of polymorphisms that will be detected we will however also meet difficulties in the interpretation of results when screening IMR patients with high resolution methods. Regions containing coding genes can also be present in variable copy numbers without causing obvious clinical manifestations which makes it very challenging to determine whether a subtle abnormality has clinical significance.

The array-CGH resolution has already been 10 fold increased by using a clone set of ~32500 overlapping clones which covers the whole genome. High resolution whole genome screening (independent of what platform will be used in the future) will generate a large amount of data that will raise the question “how much is too much and how little is too little”. It will not be possible to interpret the large amount of data that will be generated in the near future by array-CGH until we learn more about polymorphisms. Structural polymorphisms may play an analogous role in determining genetic diversity within the human population and there might be relationships between large-scale copy-number variations and susceptibility to genetic rearrangements and development of disorders.

It is of crucial importance to systematically catalog frequencies of the polymorphisms and clinical significance in a publicly accessible matter such as

the database of genomic variants. However, the polymorphic sites need to be followed up with a detailed analysis using higher resolution assays, to more accurately map how large they are and what genes they contain. The characterization of polymorphisms will give more insight into the plasticity of the human genome.

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