Characterisation of genomic imbalances in patients with mental retardation

Helle Lybæk

Center for Medical Genetics and Molecular Medicine
Haukeland University Hospital
and
Department of Clinical Medicine
University of Bergen

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1. Acknowledgements

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Helle Lybæk

2. Abbreviations

Array-CGH	Microarray-based comparative genomic hybridisation
BAC	Bacterial artificial chromosome
CGH	Comparative genomic hybridisation
CNV	Copy number variation, also known as polymorphism
FISH	Fluorescence *in situ* hybridisation
G-banding	G-bands in the chromosomes produced by the use of Giemsa stain
HR-CGH	High-resolution comparative genomic hybridisation
IQ	Intelligence quotient
kb	Kilo basepairs, 10^3 basepairs
LCR	Low copy repeat, also known as segmental duplication
Mb	Mega basepairs, 10^6 basepairs
MLPA	Multiplex ligation-dependent probe amplification
OMIM	Online Mendelian Inheritance in Man, database of genes and phenotypes (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim)
PAC	P1 derived artificial chromosome
Oligo	Oligonucleotide
SNP	Single-nucleotide polymorphism
UPD	Uniparental disomy
XLMR	X-linked mental retardation

3. Summary

A major cause of mental retardation is chromosomal abnormalities, but due to low sensitivity of conventional chromosomal karyotyping, these abnormalities may stay undetected, and the etiology of the impairment remains unknown. With the development of molecular cytogenetic methods, such as chromosome- and microarray-based comparative genomic hybridisation (HR-CGH and array-CGH), genome-wide detection of submicroscopic chromosomal abnormalities has become possible.

To examine if implementation of such molecular cytogenetic methods would result in improved diagnostics of patients with mental retardation, we applied HR-CGH and array-CGH on patients with mental retardation to investigate the diagnostic utility of the two methods. In addition, the phenotype-genotype correlations in the patients were examined.

By HR-CGH analysis, we found that cryptic genomic imbalances could be detected in five out of 50 examined patients with mental retardation (10%), despite normal findings on conventional karyotyping. Four of the findings were *de novo* and interstitial. Based on a larger population of 554 patients, the HR-CGH detection rate of cryptic imbalances was 7,2%. Of note, 90% of the findings were *de novo*, and only 25% of the findings involved a subtelomeric rearrangement. Subsequent analysis of HR-CGH positive samples by 1 Mb array-CGH demonstrated, as expected, a highly improved mapping accuracy of the array-CGH method compared to the HR-CGH method. Application of 1 Mb array-CGH analysis on 20 selected patients with normal findings on both G-banded karyotyping and HR-CGH analysis gave an additional detection rate of 20%, suggesting a high diagnostic yield of the 1 Mb array-CGH method. Three out of the four findings were *de novo* and interstitial.

The capacity for detecting chromosomal mosaicisms by the CGH methods (both

HR-CGH and array-CGH) was demonstrated by the unique finding of a mosaic combined tetraploidy for chromosomes 8 and 18 in a newborn with developmental delay, despite normal findings on G-banded karyotyping.

The combination of several cytogenetic methods also made it possible to suggest candidate genes that might explain phenotypic features in two different families with mentally retarded individuals. In one family, a ~2,1 Mb sized deletion was located 1,6 Mb distal to a 14q21.1q23.2 paracentric inversion. The deletion involved 16 genes. Among these, *SPTB* causing spherocytosis, and *PLEKHG3*, a guanide nucleotide exchange factor for Rho GTPases, and a candidate gene for causing the phenotype of mild mental retardation. In another family, an 8,9 Mb subterminal 19p13 duplication was detected in a 2 ½-year-old proband with severe mental retardation and extreme precocious puberty. Global gene expression analysis did not reveal candidate gene(s) for the hormonal disturbance. The proband´s mother was shown to have a between-arm insertion of the duplicated 19p13 segment into 19q. The same balanced insertion was found in several other family members, including a maternally uncle who was also severely mentally retarded. Unexpectedly, a 3,9 Mb 2q23.3q24.1-deletion was detected in him. The deletion contained seven annotated genes, and of these, *FMNL2*, a suggested regulator of Rho-GTPases, and *NR4A2*, an essential gene for differentiation of dopaminergic neurons, are possible candidate genes for causing the phenotype of severe mental retardation.

In summary, we have demonstrated the usefulness of HR-CGH and array-CGH as diagnostic tools for patients with mental retardation and have provided detailed genotype-phenotype information on 89 patients. The work also adds knowledge for further understanding of minor or cryptic chromosome imbalances that cause varying degrees of mental impairment, malformations and/or dysmorphism.

4. Introduction

Patients with mental retardation, defined by an intelligence quotient (IQ) below 70, represent a large group of the general population. It is estimated that the prevalence of children having mental retardation with or without dysmorphic features and/or malformations is 1-3% [1-4]. The overall incidence of mental impairment is found to be approximately 30% higher in males than females [5]. The underlying causes of mental retardation remain unidentified in many patients despite extensive investigation. It is estimated that the etiology of mental retardation is unknown in 30-50% of cases with moderate to severe mental retardation (IQ <50) and in 70-80% of cases with mild mental retardation (IQ 50-70) [6-8]. This can be a distressing and emotional situation both for the patient and the parents of an affected child, if an explanation or diagnosis and prognosis can not be given [9]. In addition, knowledge of the recurrence risk in a new pregnancy may also be an important aspect for the parents. If the phenotype-causing abnormality can not be detected then it can not be established if the unbalanced karyotype in the offspring is a *de novo* finding (i.e. not inherited from either parent) or a result of a parental carrier mechanism.

Chromosomal abnormalities are a known cause of mental retardation (with or without dysmorphic features and/or malformations). It is estimated that chromosomal and genetic disorders account for 30–40% of cases with moderate to severe mental retardation, and for 15% of cases with mild mental retardation [7]. Although a considerable number of cases with mental retardation can be explained by the presence of chromosome abnormalities detected by conventional karyotyping, a substantial part of the abnormalities in this patient group are undetectable or overlooked by this method due to limited resolution. Application of screening methods with a higher resolution is therefore needed in the diagnostic setting for an improved detection of submicroscopic abnormalities causing the developmental impairment.

The focus of this thesis has been the characterisation of chromosomal abnormalities in patients with mental retardation, with or without dysmorphic features and/or malformations, for obtaining more knowledge of the genotype-phenotype correlations in these patients. In the thesis we have examined the clinical usefulness of chromosome- and microarray-based comparative genomic hybridisation (HR-CGH and array-CGH) as screening methods for minor or cryptic chromosomal abnormalities (i.e. genomic imbalances) in these patients. In addition, the phenotypic outcome of deletions and duplications in the patients was explored.

4.1. Definition, classification and prevalence of mental retardation

Definition of mental retardation

As described by the World Health Organization (WHO), the American Association on Intellectual and Developmental Disabilities (AAIDD), and the American Psychiatric Association (APA), mental retardation is defined as a significant impairment of intellectual functioning and adaptive behaviour (i.e. the ability to function at age level in an ordinary environment measured by maturation level, learning skills, and social adjustment) with onset before age 18 years [10-12]. According to the WHO published international diagnostic system manual ICD-10 (International Statistical Classification of Diseases and Related Health Problems, 10th ed.) mental retardation is defined as: “a condition of arrested or incomplete development of the mind, which is especially characterized by impairment of skills manifested during the developmental period, skills which contribute to the overall level of intelligence, i.e. cognitive, language, motor, and social abilities.” [11].

Several other terms than “mental retardation” are today used for describing the condition of mental impairment. Among these are “developmental delay”, “delayed psychomotor

development" and "severe learning disability" which, in addition to the term "mental retardation", have been used in the papers presented in this thesis.

Classification of mental retardation

Determination of the degree of mental subnormality is based on the score obtained on a standardised age-dependent intelligence test, such as the Wechsler Intelligence Scales. An intelligence quotient (IQ) below 70, which equals two standard deviations below the mean, is scored as a level of mental impairment [11,12]. In Table 1 is shown the different levels of mental retardation classified by the IQ-score, together with the capacity of functioning, according to the international diagnostic system manual ICD-10 (Blocks F70-F73) [11].

Table 1. Classification of mental retardation (MR) and capacity of functioning based on the intelligence quotient (IQ)-score according to ICD-10

Level of MR	IQ- score	Capacity of functioning
Mild	50 – 69	Likely to result in some learning difficulties in school. Many adults will be able to work and maintain good social relationships and contribute to society.
Moderate	35 – 49	Likely to result in marked developmental delays in childhood but most can learn to develop some degree of independence in self-care and acquire adequate communication and academic skills. Adults will need varying degrees of support to live and work in the community.
Severe	20 - 34	Likely to result in continuous need of support.
Profound	< 20	Results in severe limitation in self-care, continence, communication and mobility.

Most often the groups with an IQ lower than 50 are together referred to as "moderate to severe mental retardation" [6,8].

Since intelligence is not a unitary characteristic, clinicians usually supplement IQ-tests with tests assessing the adaptive behaviour, such as the Vineland Adaptive Behavior Scales, for an

overall measurement of the degree of mental impairment. This is in agreement with the recommendations of APA given in their published manual for psychiatric disorders, DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, 4th ed.), where the combination of an IQ below 70 together with significant limitations in two or more areas of adaptive behaviour (and onset before 18 years) is needed before a person is considered to have a mental impairment [10].

Prevalence of mental retardation

Determination of the prevalence of mental retardation is a challenge and has been a matter of debate [2]. Non-ascertainment, especially of children having mild mental retardation (IQ: 50-69) which can be difficult to recognize, complicates the determination. Children having moderate to severe mental retardation (IQ < 50) are easier recognizable since the mental impairment in this group most often is associated with dysmorphic features and/or malformations. After review of 39 prevalence studies on mental retardation in children, a prevalence of mental retardation on 3% was proposed, however, the reported rates varied greatly between the studies [2]. In prevalence studies made in the Scandinavian countries and in the United States, the prevalence of mental retardation in children was estimated to be around 1% [1,3,4]. Thus the reported prevalence of mental retardation in children seems to be 1-3%. In this group moderate to severe mental retardation accounts for 0,3-0,5 % [2,13].

The etiology of mental retardation remains unknown in 30-50% of cases with moderate to severe mental retardation (IQ <50) and in 70-80% of cases with mild mental retardation (IQ 50-70) [6-8].

4.2. Chromosomal abnormalities in mental retardation

Chromosomal abnormalities causing an alteration of the normal dosage of genes may have

phenotypic consequences. Alteration of the dosage of developmental genes has been shown to be an important cause of mental retardation, and it is estimated that chromosomal and genetic disorders account for 30–40% of cases with moderate to severe mental retardation, but only for 15% of cases with mild mental retardation [7]. Most chromosomal abnormalities are produced by misrepair of broken chromosomes, by improper recombination or by malsegregation of chromosomes during mitosis or meiosis. A chromosomal abnormality may be present in all cells of the body (constitutional abnormality), or may be present in only certain cells or tissues (somatic abnormality, also called mosaic). Chromosomal abnormalities, whether constitutional or somatic, mostly fall into two categories: numerical and structural abnormalities, which will be described in more details in the sections below. An overview of some well-known chromosomal abnormalities associated with mental retardation is given in Table 2 (page 23).

4.2.1. Numerical abnormalities

Aneuploidy, caused by loss or gain of individual chromosomes, can arise through two main mechanisms: Nondisjunction or anaphase lag. Nondisjunction is the failure of paired chromosomes to separate (disjoin) in anaphase of meiosis I, or failure of sister chromatids to disjoin at either meiosis II or at mitosis. Nondisjunction in meiosis produces gametes with 22 or 24 chromosomes, which after fertilization by a normal gamete make a trisomic or monosomic zygote. Nondisjunction in mitosis produces a mosaic (see section 4.2.3.). Anaphase lag is the failure of a chromosome or chromatid to be incorporated into one of the daughter nuclei following cell division, as a result of delayed movement (lagging) during anaphase. Chromosomes that do not enter a daughter cell nucleus are lost.

Autosomal abnormalities

Autosomal monosomies and trisomies are usually embryonic or fetal lethal, with the

exception of trisomies for chromosomes 13 (Patau syndrome [14]), 18 (Edwards syndrome [15]), and 21 (Down syndrome [16]), which are observed in liveborn infants with an approximate frequency of 1:12000, 1:6000 and 1:750, respectively [17]. All the three types of trisomies result in mental impairment, but only trisomy 21 is compatible with long-term survival. Trisomy 21 (Down syndrome) is the most common single known cause of mental retardation and has the highest incidence at birth of any chromosome abnormality [18]. A karyotype of a patient with Down syndrome is shown in Figure 2 (page 25). It should be noted, that numeric autosomal abnormalities which are lethal in constitutional form may be compatible with life when in a mosaic state (see section 4.2.3.).

Sex chromosome abnormalities

Aneuploidy for sex chromosomes is more common in liveborns, and has a milder phenotypic consequence, than aneuploidy for any autosome. The four major sex chromosome abnormalities are: 45, X (Turner/Ullrich-Turner syndrome [19]), 47, XXY (Klinefelter syndrome [20]), 47, XXX (Triple X syndrome [21]), and 47, XYY (XYY syndrome [22]) and they are observed in liveborn infants with an approximate frequency of 1:2700, 1:700. 1:800 and 1:700, respectively [17]. Individuals having these syndromes have a tendency towards learning disability and delayed speech and language skills, with the exception of individuals with Turner syndrome which usually show no mental impairment [17,18].

4.2.2. Structural abnormalities

Structural chromosomal abnormalities are produced by misrepair of chromosome breaks or by recombination (crossover) between homologous - or nonhomologous chromosome- or chromatid segments in the meiotic or mitotic events. Strand exchange, so-called

recombination, can occur between nonhomologous chromosomal segments due to a high similarity in the DNA sequence, causing a “non-allelic homologous recombination”. Sequences with high similarity are frequently found across the human genome and are called low copy repeats (LCRs) or segmental duplications. Non-allelic homologous recombination can occur between homologous chromosomes, between sister chromatids, within the same chromosome arm, or between nonhomologous chromosomes. An unequal meiotic recombination event will produce unbalanced gametes which will result in an increased risk for an unbalanced progeny, as shown in Figure 1. Structural chromosomal abnormalities/ rearrangements are balanced if there is no net gain or loss of chromosomal material, and unbalanced if there is net gain or loss.

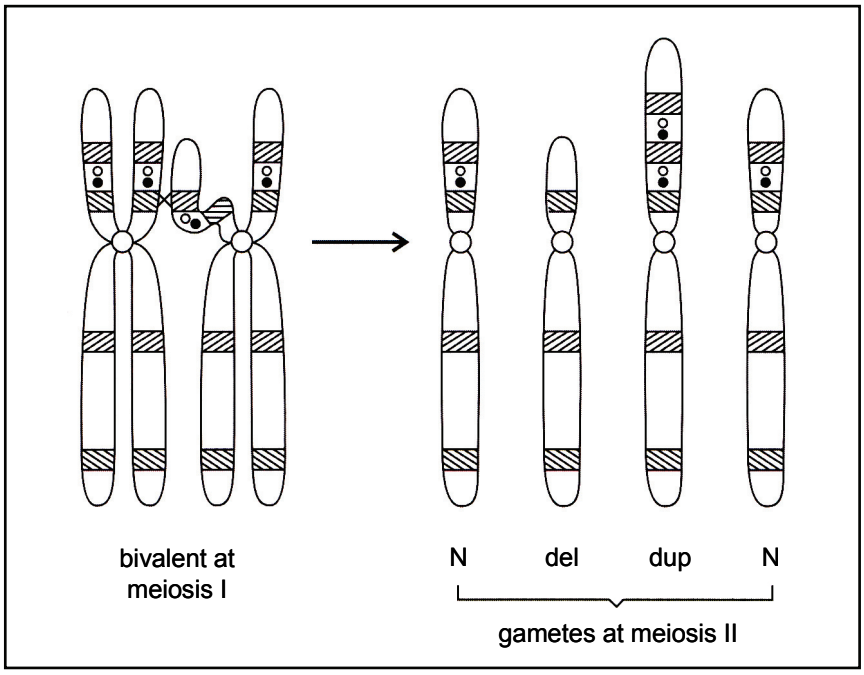

Fig. 1. The mechanism of recombination. *Strand exchange can occur between nonhomologous chromosomal segments due to high similarity in the DNA sequence, they so-called segmental duplications or low copy repeats (shown as hatched boxes). A non-allelic homologous recombination event during meiosis will result in unbalanced gametes. In the figure the recombination occurs between homologous chromosomes producing two gametes with intrachromosomal abnormalities (del = deletion; dup = duplication; N = normal gamete).*
Illustration from Gardner and Sutherland [18].

Balanced structural abnormalities

Balanced abnormalities/rearrangements, as inversions, insertions and balanced translocations, are in general considered phenotypically neutral. However, in some cases a syndromic phenotype can occur if the chromosome break disrupts developmentally important gene(s), or the regulation factors of these. Also the position shift of a chromosome segment, a so-called position effect [23], may result in an altered regulation of gene expression causing abnormal development. Carriers of a balanced rearrangement have an increased risk of infertility, miscarriages and for having children with mental retardation and/or malformations due to the formation of unbalanced gametes.

Unbalanced structural abnormalities

Unbalanced structural abnormalities/rearrangements, such as deletions, duplications, unbalanced translocations, complex rearrangements, which cause gene dose alterations, are often observed in patients with mental retardation (see Table 2, page 23). The abnormalities can be either visible- or not visible by conventional microscopic chromosome analysis (see section 4.5.1.). Not visible abnormalities are called submicroscopic or cryptic.

Submicroscopic structural abnormalities are a major cause of mental retardation/ abnormal development. A large fraction of these is microdeletion syndromes and microduplication syndromes with well characterised genotype-phenotype correlations. They are mostly caused by a non-allelic homologous recombination between LCRs/segmental duplications on homologous chromosomes during meiosis [24-26], as shown in Figure 1. Targeted analysis against genomic regions flanked by directly oriented LCRs have identified novel genomic disorders emphasising the role of LCRs as hotspots for chromosomal rearrangements [27,28]. The majority of the identified syndromes is microdeletion syndromes, possible due to a loss of gene-function most likely results in a more severe

phenotype, and also because microduplications are more technological difficult to detect than microdeletions (see sections 8.1.3. and 8.1.4.).

Subtelomeric abnormalities, visible or submicroscopic, is another major group associated with mental impairment. They are often caused by recombination between nonhomologous chromosomes due to high similarity of repeat sequences located in the telomeres. Subtelomeric abnormalities are found in 5-7% of mentally retarded patients and it appears that the prevalence is higher in patients with a more severe mental retardation than in patients with mild mental retardation [6,29]. Individuals with subtelomeric abnormalities most often have dysmorphic features, growth retardation and congenital anomalies in addition to the mental impairment [30-32]. Also, families with subtelomeric abnormalities have a higher prevalence of mental impairment [29,32]. Since the telomeres are essential for the pairing of homologous chromosomes in meiosis and are critical for the complete replication of chromosome ends, it is not surprising that this type of abnormality has severe consequences. When combining 22 studies with a total of 2585 patients with mental retardation, it was found that approximately half of the subtelomeric abnormalities were caused by a *de novo* deletion, and about half by a balanced translocation segregating in a patient's family [6].

Of all the chromosomes the X-chromosome is extra vulnerable for abnormalities, visible or submicroscopic, since this is the only chromosome which is expressed in a mono allelic manner in the cells. Because of this, a major cause of inherited mental retardation is due to X-linked recessive impairment which is described in more detail in section 4.3.1.

4.2.3. Mosaicism

Chromosomal mosaicism is defined as the presence of two or more populations of cells with different chromosomal content in one individual, which has developed from a single fertilized egg. The major mechanism for mosaicism is mitotic nondisjunction, but also postzygotic

anaphase lag in an initially chromosomally abnormal cell line will result in mosaicism. Many of the mitotically arisen aneuploidies lead to cell death, leaving no trace. But if viable, the earlier in embryogenesis that the mitotic error happens, the greater impact the diverging cell line will have on the phenotype. The phenotypic consequence depends on in which tissue the aneuploid cell line is located (e.g. in the brain) and how large a fraction the diverging cell line represents in the tissue. The mosaicism may be detectable only in blood cells or in fibroblasts (tissue cells). The distribution of chromosomal mosaicism in fibroblasts may show extreme karyotypic variation, also regionally [33]. Constitutional autosomal aneuploidies which nearly always are lethal may be viable when in a mosaic state. Some examples of this are 47,+8/46,N [34] and 47,+9/46,N [18]. In two studies including a total of 6185 patients with mental retardation, the detection of chromosomal mosaicism was 0,4% [35] and 0,5% [36].

4.3. Other genetic factors associated with mental retardation

4.3.1. Monogenic disorders

An altered function of a dosage sensitive single-gene which is important for brain function or development will result in mental impairment. Identification of monogenic causes of mental retardation through searches in the literature and in the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim) revealed 282 mental retardation genes involved in at least 1237 genetic disorders [37]. However, it is reasonable to speculate that the 282 genes represent a substantial underestimate of the correct number of mental retardation related genes, since variations of the terminology used (mental retardation, learning disability, cognitive impairment, developmental delay, etc) and of the clinical descriptions of the reported disorders limit such searches.

The majority of identified genes involved in mental retardation are located on the X-chromosome. Even though the X-chromosome only contains ~4% of all human protein-coding genes, 25,3% of all OMIM entries linked to mental retardation were mapped to the X-chromosome (search done June 2006) [38]. Due to the mode of inheritance, recessive X-linked mental retardation (XLMR) can affect several family members through multiple generations. Over 200 XLMR conditions involving 45 genes have been identified [39]. The most common is the Fragile X mental retardation syndrome caused by a mutation in the gene *FMR1* [40], which is observed with an approximate frequency of 1:5000 and 1:8000 in males and females, respectively [41]. Since males only have one copy of the X-chromosome they are more vulnerable to mutations in the XLMR genes, and it is estimated that about 10% of all male mental retardation is caused by XLMR [42]. The overall incidence of mental impairment is approximately 30% higher in males than females [5], but it is still unknown whether or not all the difference in incidence can be explained by XLMR [42].

Inborn metabolic monogenic disorders may cause mental retardation. The most known example is the autosomal recessive disorder phenyl-ketonuria (PKU/Følling's disease) which is caused by mutations in the gene *PAH* [43], resulting in a defective metabolism of phenylalanine. Untreated PKU will result in irreversible progressive mental impairment. Because of this, all newborns are today screened for PKU. The condition cannot be cured, but if early diagnosed and treated by a special diet the mental impairment can be avoided or limited. Some other examples of autosomal monogenic disorders associated with mental retardation are: Smith-Magenis syndrome caused by mutation in the gene *RAI1* [44,45]; the "9q34 subtelomeric deletion" syndrome caused by mutation in the gene *EHMT1* [46]; and the Miller-Dieker syndrome caused by mutation in the gene *LIS1* [47].

4.3.2. Genomic imprinting

Genomic imprinting is a non-Mendelian mechanism of inheritance in which differences in gene function depend on whether the allele was inherited from the mother or the father. The effect of imprinting is a differential silencing and tight gene expression regulation of the genes located in the imprinted chromosome segment. It is estimated that ≤ 1% of the total human genes are regulated by this mechanism [48]. Many of the imprinted genes are expressed in the brain, and there is increasing evidence that these genes influence brain function and behaviour by affecting neurodevelopmental processes [48,49]. Imprinted genes or imprinted gene domains are vulnerable to mutations or abnormalities, as deletions or duplications, causing an altered gene expression. Also, even though the sequences of the genes are unaltered, a distribution of two imprinted alleles from the same parent (uniparental disomy; UPD) may have a phenotypic consequence due to the altered gene dosage. Six imprinting domains have been identified on the autosomes [50] and one well known is located on chromosome 15 at 15q11-q13. Depending on the parental origin of the affected alleles, deletions, mutations or UPD in this region will give rise to Prader-Willi syndrome if loss of expression of paternally imprinted genes or Angelman syndrome if loss of expression of maternally imprinted genes. Today, nine such non-Mendelian inheritance imprinting developmental syndromes have been identified [50]. Genetic marker analysis of the affected child and the parents can establish if an UPD is present.

4.4. Various etiological causes of mental retardation

It is difficult to give an accurate estimate of the distribution of etiological causes of mental retardation or to give an estimation of the proportional contribution of cytogenetic abnormalities in mental retardation because reports vary in parameters such as clinical criterias of patient selection and of the examination methods used. But as an overview some

well-known chromosome abnormalities and syndromes associated with mental retardation are given in Table 2.

Table 2. Selected well-known chromosomal abnormalities and syndromes associated with mental retardation Table from Xu *et al*., [51].

Trisomy 21/Down syndrome	
Fragile X syndrome	
Unbalanced translocations	
Duplications	
Deletions (interstitial, terminal)	
Extra structurally abnormal chromosomes (markers)	
Diploid/triploid mosaicism[a]	
Submicroscopic aberrations at breakpoints in apparently balanced rearrangements	
Subtelomere rearrangements	
Cryptic deletions	
del(1)(p36.3)	Monosomy 1p
del(4)(p16)	Wolf-Hirschhorn
del(5)(p15)	Cri du chat
del(7)(q11.23q11.23)	William syndrome
del(8)(q24.1q24.1)	Langer-Giedion syndrome
del(11)(p13p13)	WAGR[b] syndrome
del(15)(q11q13)pat	Prader-Willi syndrome
del(15)(q11q13)mat	Angelman syndrome
del(16)(p13.3)	Rubinstein-Taybi syndrome
del(17)(p11.2p11.2)	Smith-Magenis syndrome
del(17)(p13.3)	Miller-Dieker syndrome
del(20)(p11.23p11.23)	Alagille syndrome
del(22)(q11.2q11.2)	VCF[b]/DiGeorge
Uniparental disomy	
UPD(14)mat	IUGR[b], developmental delay, precocious puberty, short stature, small hands and feet
UPD(14)pat	Polyhydramnios, facial anomalies, severe neurologic involvement, skeletal anomalies and growth retardation
UPD(15)mat	Prader-Willi syndrome
UPD(15)pat	Angelman syndrome

[a]Most of the reported cases with a normal karyotype in blood but diploid/triploid mosaicism in the cultured fibroblasts [van der Laar et al., 2002].
[b]WAGR: Wilm's tumor, aniridia, genitourinary malformations and retardation of growth and development; VCF: velocardiofacial; IUGR: intrauterine growth retardation.

4.5. Detection of chromosomal abnormalities and genomic imbalances

Cytogenetic karyotyping aims at the identification of chromosomal abnormalities which can explain the phenotype, or specific phenotypic features, of the patient. In addition, the identification of a chromosomal aberration in specific patients has proven to be a successful way to identify the implicated genes and gain insight in the phenotypic consequences of a given gene malfunction. Several strategies can be used for examination of patients with mental retardation (with or without dysmorphic features and/or congenital abnormalities). The methods can either be directly targeted against regions or loci known to have phenotypic consequences or they can be genome-wide. In the sections below are described four widely used methods which all have been extensively used in the papers presented in this thesis. However it should be noted, that other methods like multiplex ligation-dependent probe amplification (MLPA)[52], and multiplex amplifiable probe hybridization (MAPH)[53], also have been proven to be useful for characterisation of patients with mental retardation [54-57].

4.5.1. Conventional microscopic G-banded karyotyping

Although already discovered in 1955 that normal human cells contained 46 chromosomes [58], identification of the individual chromosomes and chromosome bands was not possible until the development of chromosome banding techniques during the 1970s [59,60]. Since then, the most used method for chromosome analysis has been conventional microscopic karyotyping by G-banding. The method utilises the ability of Giemsa stain to bind differently to AT-basepairs versus GC-basepairs in DNA, and thereby constructing a dark and light chromosome band pattern which is distinctive for each chromosome. The resolution of the method, at a 400-550 band level in routinely prepared metaphases, is estimated to be 5-10 Mb depending on the density of the chromosome band pattern. A G-banded karyotype from a

patient with Down syndrome is shown in Figure 2. The method is especially useful for the detection of numerical chromosomal abnormalities or structural chromosomal abnormalities involving larger chromosomal segments, whereas smaller aberrations may be overlooked or are undetectable by the method. G-banded karyotyping has been applied in all the papers (papers I – V) presented in this thesis.

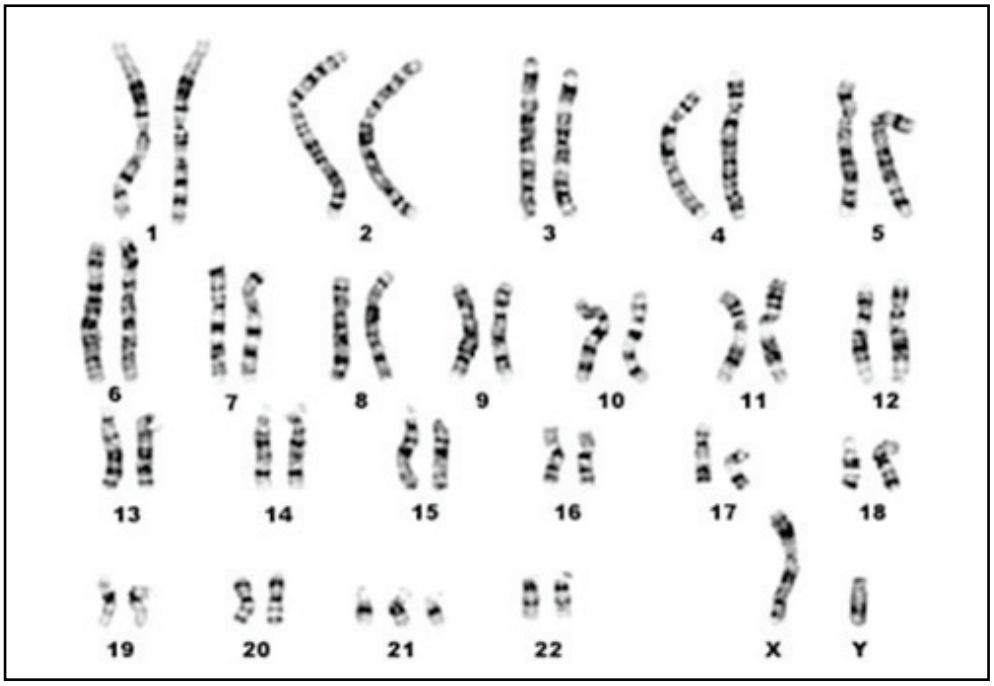

Fig. 2. G-banded karyotype of trisomy 21 (Down syndrome). *The presence of Down syndrome in a male was identified by an extra copy of chromosome 21 (47,XY,+21).*
Illustration by Helle Lybæk.

4.5.2. Fluorescence *in situ* hybridisation (FISH)

Development of the fluorescence *in situ* hybridisation (FISH) method during the 1980s and 1990s [61-66] made it possible to detect abnormalities which were undetectable (i.e. submicroscopic) or difficult to classify by G-banded karyotyping. The method utilises the ability of single-strand DNA to hybridise to complementary DNA sequences. When the DNA strands in the chromosome become separated, a targeted fluorescence-visible DNA sequence (also called probe) can hybridise onto them. Commercial available labelled probes targeted against specific loci (e.g. microdeletion syndrome probes), specific sequences (e.g.

subtelomere probes), or chromosomes (e.g. paint or multicolour probes), have facilitated the detection of cryptic chromosomal rearrangements, and minor or submicroscopic abnormalities. In recent years, probes made from bacterial artificial chromosomes (BACs) or P1 derived artificial chromosomes (PACs) have become widely used, enabling FISH analysis at a higher resolution. The resolution of the method depends on the probe, FISH-technique and target DNA used, ranging from 3 Mb down to a few kilobases [67,68]. Some examples of different types of FISH-analysis are shown in Figure 3. The FISH-method has proven to be a powerful tool in clinical cytogenetics. However, the method has some major limitations: 1) When analysing for submicroscopic abnormalities one must have a hypothesis about the possible affected region (e.g. based on the phenotype of the patient), since only one or a few loci can be investigated at the same time, and 2) When using multicolour FISH-probes for genome-wide analysis (as in multiplex FISH or spectral karyotyping (SKY)), flaring of fluorescence at the interface of translocated segments may lead to misinterpretation [69]. The FISH method has been applied in all the papers (papers I-V) presented in this thesis.

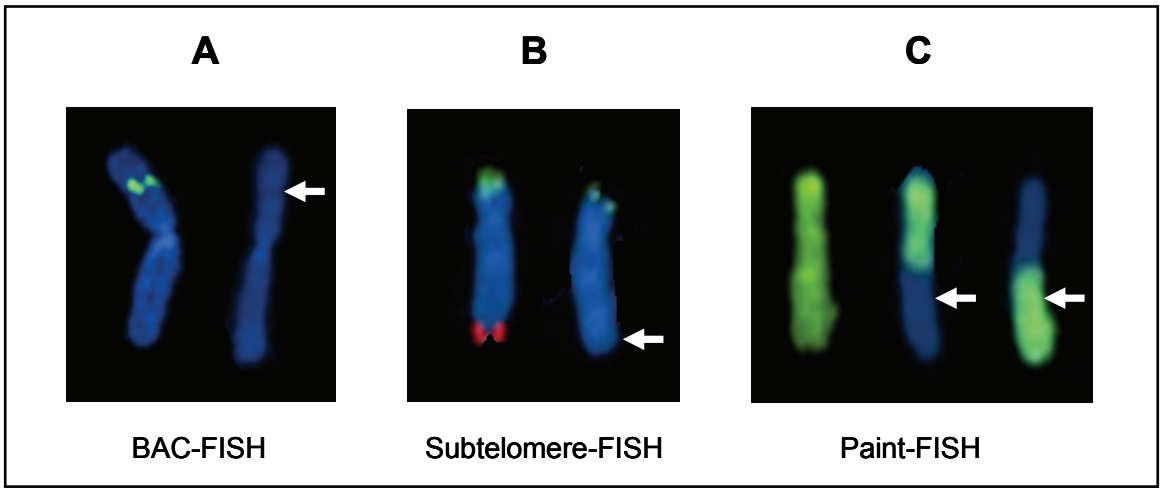

Fig. 3. Examples of different types of FISH-analyses. *A) BAC-FISH analysis detected a p21p21-deletion on chromosome 3 (marked by an arrow); B) Subtelomere-FISH analysis detected a subtelomeric deletion of the q-subtelomere on chromosome 19 (marked by an arrow); C) Paint-FISH analysis of chromosome 4 (painted green) revealed a balanced translocation between chromosome 4 and chromosome 6 (marked by arrows).*
Illustration by Helle Lybæk

4.5.3. Chromosome-based high-resolution comparative genomic hybridisation (HR-CGH)

Development of the comparative genomic hybridisation (CGH) method during the early 1990s made genome-wide screening for chromosomal imbalances possible without the need for living cells from the investigated patient [70]. In addition, only a small amount (≤1 μg) of patient sample is needed for the analysis. The method utilises competitive hybridisation of differentially fluorescence labelled patient DNA and normal reference DNA towards normal human metaphase chromosomes. Hybridisation of highly repetitive sequences is blocked by the addition of unlabeled Cot-1 DNA [61,63]. The ratio of fluorescence signal intensity of hybridised patient DNA versus reference DNA is calculated along the chromosomes and compared to a dynamic standard reference interval based on normal-to-normal hybridisations [71]. A deviation of the ratio-profile from the standard reference interval indicates a copy number change in the patient. The HR-CGH result is scored at a 99,5% confidence interval, and the location and size of a detected genomic imbalance is based on the position of the "imbalance-bar" on the respective chromosome ideogram generated by the analysis software. An outline of the HR-CGH method is shown in Figure 4 panel A. The resolution of the HR-CGH method is 2-3 Mb for deletions [72,73], but is less sensitive for duplications. The HR-CGH method has proven to be a powerful tool in clinical cytogenetics. The method has however a major limitation since only unbalanced chromosomal abnormalities (i.e. genomic imbalances) can be detected. Balanced structural chromosome aberrations, such as balanced reciprocal translocations or inversions, can not be detected as they do not change the DNA copy number. The HR-CGH method has been applied in all the papers (papers I-V) presented in this thesis.

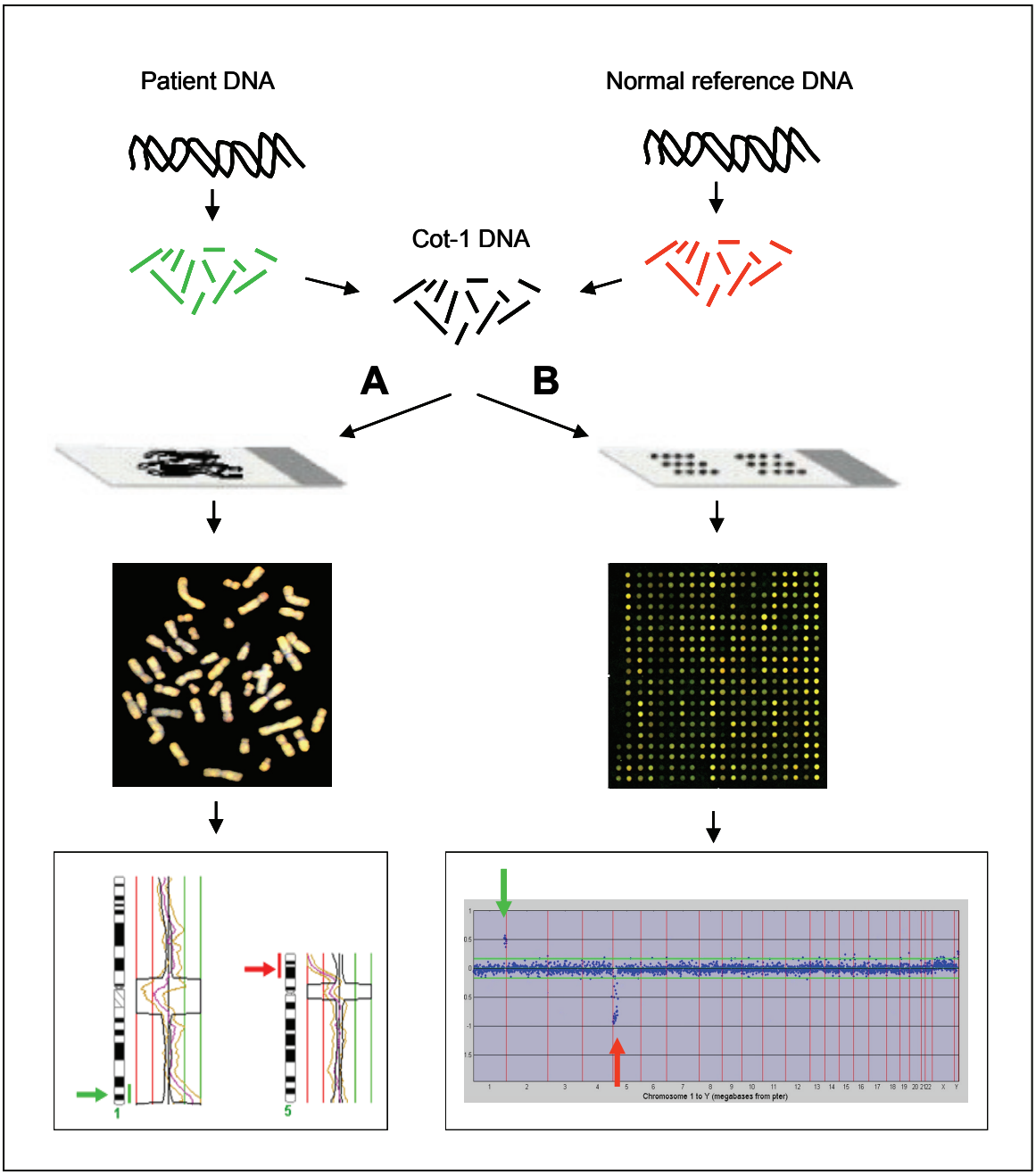

Fig. 4. Outline of the CGH-methods: Chromosome-based high-resolution CGH (HR-CGH) in panel A, and microarray-based CGH (array-CGH) in panel B. *Patient DNA (labelled green) and normal reference DNA (labelled red) is hybridised either onto chromosomes (HR-CGH) or onto immobilized DNA on a slide (array-CGH). Signals are detected using either a fluorescence microscope or a laser scanner and the ratio values between patient and reference are quantified and analysed with computer programs. An imbalance is detected as: A) A ratio plot (orange lines) deviation from the dynamic standard reference interval (black lines) in HR-CGH analysis, or B) A ratio plot (blue dots) deviation from the defined thresholds for normal DNA copy numbers (green lines) in array-CGH analysis. The same patient sample was analysed by both CGH methods, showing a duplication on chromosome 1 (marked with green arrows) and a deletion on chromosome 5 (marked with red arrows).*
Illustration by Helle Lybæk.

4.5.4. Microarray-based comparative genomic hybridisation (array-CGH)

During the late 1990s, chromosome-based CGH was further developed into microarray-based CGH (array-CGH) [74-76]. The array-CGH method is based on the same principle of competitive hybridisation as chromosome-based CGH, but the targeted normal metaphase chromosomes have been substituted with normal DNA-sequences spotted onto a glass slide (also known as a microarray or shortly an array). When using arrays, the sensitivity of the method is no longer limited by the quality of the metaphase spreads, but by the size and density of the spotted genomic DNA-fragments, which improves the sensitivity of the CGH method significantly. An outline of the array-CGH method is shown in Figure 4 panel B. In array-CGH analysis, the ratio of fluorescence signal intensity of hybridised patient DNA versus reference DNA is calculated for each DNA fragment spotted on the array, and a ratio deviation from the defined thresholds for normal DNA copy numbers indicates a copy number change in the patient. The thresholds for normal DNA copy numbers can either be manually determined or determined by the analysis software algorithm. Information of the genomic location of the DNA fragments spotted onto the array can be found in genomic databases, as the Ensembl database (http://www.ensembl.org). Therefore, the size and location of an array-CGH detected genomic imbalance can be mapped by the genomic location of the detected DNA-fragment(s) showing a deviating signal intensity ratio. Different types of DNA can be spotted onto the array, as cDNA, PCR-products, genomic clone-insert DNA or oligos. The predominant type of array-platform has been arrays based on large-insert DNA from BAC or PAC clones, and arrays consisting of DNA from ~3500 BAC/PAC clones, enabling array-CGH analysis at an average resolution of 1 Mb, have become widely used for genome-wide screening of genomic imbalances [77-82]. An example of a 1 Mb array-CGH analysis detecting both a duplication and deletion on chromosome 7 is shown in Figure 5.

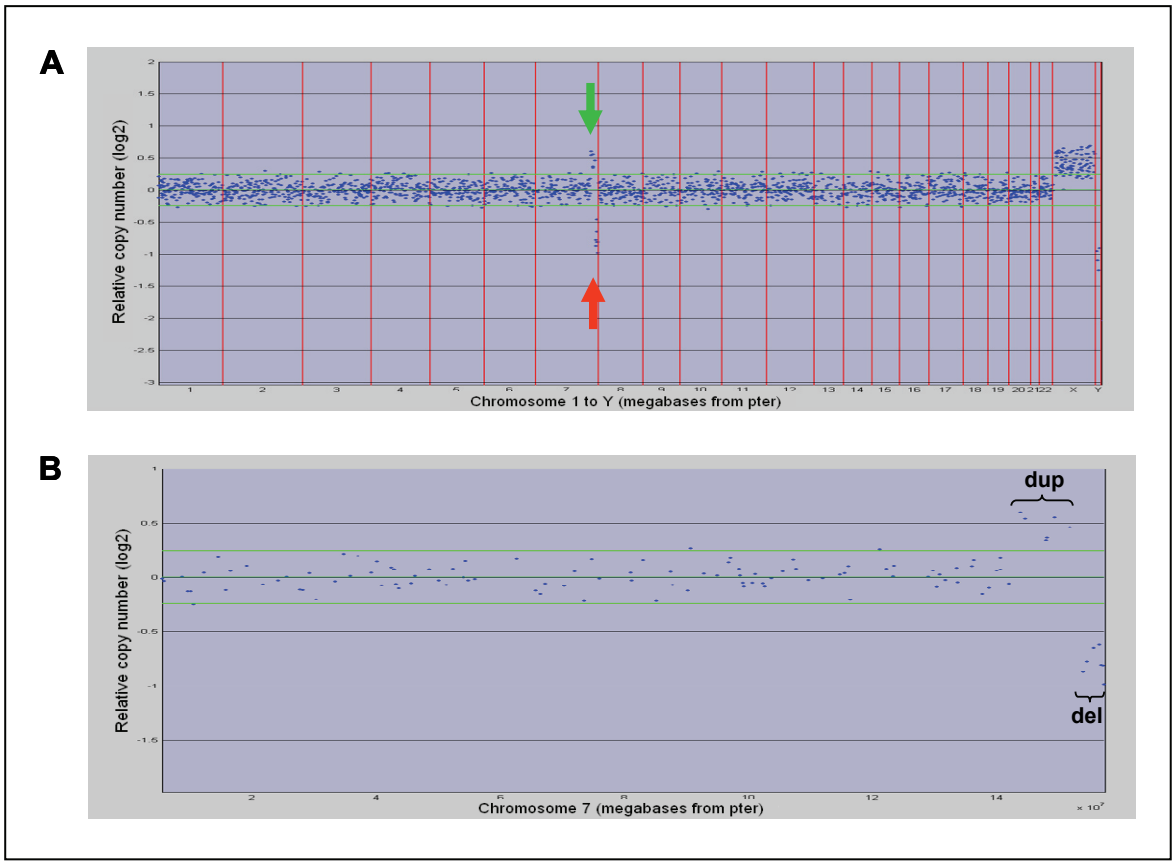

Fig. 5. Array-CGH analysis performed on a 1 Mb resolution BAC-array. *The 1 Mb array-CGH profile of the patient sample is shown for all the chromosomes in panel A, and for chromosome 7 only in panel B. A deviation of the clone signal intensity ratios (blue dots) from the algorithm defined thresholds for normal DNA copy numbers (green lines) indicates a genomic imbalance. In the analysis, a sex mis-matched hybridisation between patient DNA (female) and reference DNA (male) was carried out, giving the expected deviation in clone signal intensity ratios on chromosome X and chromosome Y. A) Both a duplication (marked by a green arrow) and a duplication (marked by a red arrow) were detected on chromosome 7. B) The duplication segment (marked by "dup") was mapped to 7q35q36.1 and the deletion segment (marked by "del") to 7q36.2qter.*
Illustration by Helle Lybæk.

Only genomic imbalances covered by the DNA-fragments spotted on the array can be detected by the array-CGH analysis. Therefore, the higher density of DNA-fragments spotted on the array, the higher sensitivity of the array-CGH method. As a consequence of this, high resolution BAC-arrays with overlapping BAC clones (so called tiling-arrays) have been developed for specific syndrome regions [83], for single chromosomes [38,84] or for genome-

wide coverage [85]. To further improve the sensitivity of the array-CGH method, arrays with oligonucleotide-fragments (so called oligos) being ~2500 times smaller than BAC DNA have been developed [86]. The accession and use of arrays have until recently been limited to non-commercial "in house-made" arrays, but lately also a huge collection of commercial arrays have been available at a reasonable low cost. The commercial arrays are mainly oligo-arrays, but also "cytogenetic-focused" BAC-arrays targeted against regions associated with constitutional genetic disorders are available. Also oligo-arrays based on single-nucleotide polymorphisms (SNPs) are commercial available, enabling both allelotyping (i.e. the ability to detect uniparental disomies) and examination for DNA copy numbers in the same analysis. Oligo-arrays facilitate genome-wide array-CGH analysis at a much higher resolution than BAC-arrays [87]. At the moment (September 2008) an array-CGH resolution as high as ~6 kb is possible by the use of commercial available oligo-arrays. The improved detection capacity of the array-CGH method towards submicroscopic abnormalities (i.e. genomic imbalances) indicates that the method in the future will be an important tool in clinical cytogenetics. But it should be noted that, as for the HR-CGH method, only unbalanced chromosome abnormalities changing the DNA copy number can be detected by the array-CGH method. The array-CGH method has been applied in four of the papers (papers II-V) presented in this thesis.

5. Aims of the study

The overall aim of this study was to screen for chromosomal imbalances and obtain more knowledge of the genotype-phenotype correlations in patients with varying degrees of mental retardation with or without dysmorphism and/or malformations. The study has focused on the following topics:

1) Evaluation of chromosome- and microarray-based comparative genomic hybridisation (HR-CGH and array-CGH) as diagnostic screening methods for detecting minor or cryptic chromosomal imbalances in patients with mental retardation.

2) Exploration of the phenotypic outcome of deletions and duplications in patients with mental retardation in order to obtain genotype-phenotype information.

3) Detection of occult chromosomal mosaicisms in patients with abnormal development.

4) Evaluation of candidate genes that might explain phenotypic features in families with mentally retarded individuals.

6. List of papers

Paper I

Ness GO, Lybæk H, Houge G. Usefulness of high-resolution comparative genomic hybridization (CGH) for detecting and characterizing constitutional chromosome abnormalities.

Am J Med Genet. 2002 Nov; 113(2): 125-36.

Paper II

Lybæk H, Meza-Zepeda L, Kresse SH, Høysæter T, Steen VM, Houge G. Array-CGH fine mapping of minor and cryptic HR-CGH detected genomic imbalances in 80 out of 590 patients with abnormal development.

Eur J Hum Genet. 2008 Nov; 16(11): 1318-28.

Paper III

Lybæk H, Øyen N, Fauske L, Houge G. A 2.1 Mb deletion adjacent but distal to a 14q21q23 paracentric inversion in a family with spherocytosis and severe learning difficulties.

Clin Genet. 2008 Dec; 74(6): 553-9.

Paper IV

Houge G, Lybæk H, Gulati S. Mosaicism for combined tetrasomy 8 and 18 in a dysmorphic child: A result of failed tetraploidy correction?

Submitted for publication.

Paper V

Lybæk H, Ørstavik KH, Prescott T, Hovland R, Breilid H, Stansberg C, Steen VM, Houge G. An 8,9 Mb 19p13 duplication associated with precocious puberty and a sporadic 3,9 Mb 2q23.3-2q24.1 deletion containing *NR4A2* in mentally retarded members of a family with an intrachromosomal 19p-into-19q between-arm insertion.

Revised manuscript published: Eur J Hum Genet. 2009 Jan 21. [Epub ahead of print].

7. Summary of results

Paper I

In this study we investigated the usefulness of the HR-CGH method for the detection and characterisation of minor or cryptic chromosomal imbalances. A total of 66 patients with mental retardation and most also having dysmorphic features and/or malformations were examined. After conventional G-banded karyotyping, blood samples were selected for HR-CGH analysis, either because the observed chromosomal abnormality was small and doubtful or difficult to classify (16 patients), or because clinical features were suggestive of a chromosomal abnormality but the karyotype was found normal by G-banding (50 patients). In the first group, HR-CGH identified the origin of additional chromosome material in nine patients, of which two patients had marker chromosomes in mosaicism. In addition, an apparently balanced *de novo* translocation was in one patient identified to be unbalanced. In the last group, HR-CGH detected a chromosomal imbalance in five patients, of which four patients had an intrachromosomal imbalance, giving a detection rate of 10% in patients having an apparently normal karyotype.

Paper II

As an extension of the study described in paper I, and for more precise phenotype-genotype information, we applied 1 Mb resolution array-CGH for investigation of all HR-CGH findings done in our diagnostic laboratory during a 6-year period. A total of 590 patients, all except three being mentally retarded, and most also having dysmorphic features and/or malformations, had been examined by HR-CGH analysis. Of these, 36 patients had minor but visible findings on G-banded karyotyping, whereas 554 patients had normal finding on G-banded karyotyping. In the last group, a genomic imbalance was detected by HR-CGH in 40

patients (7,2%): 29 deletions, 3 duplications, 4 unbalanced translocations, and 4 occult trisomy mosaicisms. When genomic BAC-arrays became available from the Norwegian Microarray Consortium, 1 Mb array-CGH was applied on all HR-CGH positive samples for more precise mapping, and thus more accurate phenotype-genotype information. To examine the diagnostic sensitivity of 1 Mb array-CGH in the remaining 514 patients with normal findings on both G-banded karyotyping and HR-CGH analysis, a subset of 20 patients with particularly high suspicion of having a chromosomal imbalance as the cause of abnormal development was selected. In four of the patients (20%) an imbalance was detected: three deletions and one duplication. Of note, 73 out of the 80 array-CGH mapped patients had a *de novo* finding (91%). Taken together, the work provided detailed phenotype-genotype information on 80 patients with minor and cryptic chromosomal imbalances.

Paper III

In this study, a familial 14q21.1q23.2-inversion that co-segregated with spherocytosis and severe learning difficulties or mild mental retardation was investigated by BAC-FISH and oligo-based array-CGH. As expected, a deletion of the beta-spectrin gene *SPTB*, a known cause of spherocytosis, was found. More unexpectedly, this deletion was ~1.6 Mb distal to the 14q23.2 inversion breakpoint. The deletion spanned ~2.1 Mb and contained 15 annotated genes in addition to *SPTB*, among them *PLEKHG3,* a guanide nucleotide exchange factor for Rho GTPases. *PLEKHG3* is highly expressed in the brain and our best candidate gene for causing the mild mental retardation.

Paper IV

In this study, the cause of marked dysmorphic features, malformations and developmental delay in a newborn was examined. G-banded karyotyping gave normal findings, whereas a

50% DNA increase of both chromosome 8 and 18 was detected in the patient by HR-CGH and 1 Mb resolution array-CGH. Most unexpectedly, FISH analyses of meta- and interphases identified the DNA increase to be caused by a mosaic combined tetrasomy of chromosomes 8 and 18. Allele analyses showed an equal allele distribution from the parents, showing that the double tetrasomy was a result of mitotic malsegregation of all four chromatids of both chromosome pairs. At age 20 months, double tetrasomic leukocytes could no longer be detected by CGH in the child. Pairing of homolog chromosomes is occasionally observed in metaphase spreads from fetal cell cultures. That such pairing may have a function is indirectly suggested by our findings in the patient. We suggested that the possible origin of the double tetrasomy is incomplete correction of a tetraploid state resulting from failed cytokinesis or mitotic slippage during early embryonic development. If so, this suggests that normal cells may have a mechanism for tetraploidy correction that involves pairing of homolog chromosomes that could be important for genomic maintenance.

Paper V

In this study, the cause of puberty onset before age 5 months, short stature, hand anomalies and severe mental retardation in a 2 ½ year old girl was examined. Normal findings were made on G-banded karyotyping and HR-CGH analysis, whereas an 8,9 Mb subterminal 19p13 duplication containing 215 predicted genes was detected by 1 Mb array-CGH analysis. It was initially assumed that the duplication contained the kisspeptin receptor gene *GPR54*, known to stimulate induction of puberty, but more refined duplication mapping by high-resolution oligo array-CGH excluded this possibility. In an attempt to understand the genotype-phenotype correlation, global gene expression was measured in skin fibroblasts. The overall gene expression pattern was quite similar to controls, and only about 25% of the duplicated genes had an expression level that was increased by more than 1,3-fold, with no obvious

changes that could explain the extreme precocious puberty. G-banded karyotyping of the proband´s mother showed a balanced between-arm insertion of the duplicated segment that resembled a pericentric inversion. The same balanced insertion was found in several other family members, including one who had lost a daughter with severe mental retardation and menarche at age 10. Another insertion carrier was severely mentally retarded and short statured, but not dysmorphic. His phenotype was initially ascribed to a presumed cryptic chromosome 19 imbalance caused by the 19p-into19q insertion, but subsequent array-CGH detected a 3,9 Mb deletion of 2q23.3-2q24.1. This novel microdeletion involved seven genes, of which, *FMNL2*, a suggested regulator of Rho-GTPases, and *NR4A2*, an essential gene for differentiation of dopaminergic neurons, may be critical genes for the proposed 2q23q24 microdeletion syndrome.

8. Discussion

8.1. Methodological considerations

8.1.1. G-banded karyotyping

In the study, we have used the G-banding method for initial screening of chromosomal abnormalities. This method is a very useful tool but has some limitations one should bear in mind when interpreting the result. First, the resolution of the method depends on the quality of the metaphase spreads and the density of the chromosome bands. Despite continous efforts to achieve the best possible chromosome quality for the analysis, we sometimes observed reduced quality of the chromosomes in patient samples, resulting in a lowered resolution. Also, chromosomal rearrangements between segments with similar chromosome band pattern or chromosomal abnormalities in regions with a low chromosome band density may remain undetected although the size of the imbalance is above the usual resolution of the G-banding method. Finally, phytohemagglutinin (PHA)-stimulation during culturing of blood leukocytes may favour growth of normal cells, leading to a selection against cells bearing (growth limiting) imbalances. This phenomenon has previously been described for patients having chromosomal abnormalities in mosaicism [35,77]. Thus the harvested cells may not always represent the "true chromosomal content" in the patient. Because of the issues described above, patients having normal findings on G-banded karyotyping were, if recommended by experienced clinicians at our department, followed up by FISH, HR-CGH, or recently, array-CGH analysis, for further examination.

8.1.2. FISH

We have used the FISH method for targeted chromosomal examination or for verification of results obtained by other methods. The FISH method enables high-resolution analysis but has

some possible short comings which may lead to a false result. As described above, obtaining good quality chromosomes from a patient sample can be a challenge. Limited probe resolution may also bias the result. This is especially relevant when analysing for intrachromosomal duplications since two closely located FISH signals may be overlapping or can not be distinguished in the fluorescence microscope, resulting in a false-negative result. Because of this, repeated HR-CGH analysis was done if an initially HR-CGH-detected cryptic duplication could not be verified by FISH analysis. More recently, we have applied oligo array-CGH for detection and verification of cryptic duplications. Finally, when using BAC/PAC clones as probes, it should be kept in mind that the information of the genomic clone location reported in the databases may be incorrect. As a consequence of this, we have, if available in the examined region, used verified sequenced BAC clones in the FISH-analyses.

8.1.3. HR-CGH

In the initial period of this study, we used HR-CGH for more extensive genome-wide screening of genomic imbalances. The method enables screening at a higher resolution than the G-banding method, but has some limitations or possible biases, which one should bear in mind. As for the methods above, the output of an HR-CGH analysis is highly dependable on the chromosome quality. Because of this, chromosomes from the same normal donor were used in the analyses. Furthermore, slides with the metaphase spreads were produced in large batches and each slide was manually checked in a light microscope for ensuring a similar and good chromosome quality. When using HR-CGH analysis it should be kept in mind, that a normal finding only shows that the DNA copy number in the karyotype is balanced and *not* that the karyotype is normal. Therefore, the HR-CGH method has in the study been used in combination with the G-banding method for visualisation of the patients´ chromosomes. To

reduce the level of possible false-positive HR-CGH findings, imbalances were scored as a deviation from the standard reference interval of CGH ratio profiles at 99,5% confidence intervals [71]. However, small imbalances or imbalances in mosaicism may only be visible at lower confidence intervals (e.g. 95% or 99%), therefore these intervals were also inspected for interpretation of the result. It should also be noticed that duplications are more difficult to detect than deletions by HR-CGH analysis, since duplications "only" cause a 50% change in patient DNA content compared to the normal DNA ratio (ratio of 3/2) while deletions lead to a 100% change in DNA content (ratio of 1/2). Therefore, the risk for false-negative findings by this method is higher for duplications than deletions, especially when the imbalances are small. In mosaicism, the CGH-detected (both HR-CGH and array-CGH) level of change in patient DNA content may represent an even higher level of DNA change in the affected cells. Therefore, and because the patient chromosomes are not visible by the HR-CGH method, all HR-CGH findings were re-examined by G-banded karyotyping on high quality chromosomes (band level above 600), by BAC-FISH analysis, or recently by array-CGH analysis, for examination of the cause for the detected change in patient DNA content and for verification of the HR-CGH finding.

8.1.4. Array-CGH

More recently, we have introduced array-CGH as a new method for genome-wide examination of genomic imbalances. The method enables screening at a higher resolution than the HR-CGH method, although with some limitations or possible biases. First, the quality of an array-CGH analysis is highly dependable on the performance of the array used. The majority of the array-CGH analyses described in this thesis were done on 1 Mb BAC-arrays which were produced in-house by the Norwegian Microarray Consortium. For achieving best possible quality, arrays were printed in batches of 25 slides, and at least one

array from the batch was test hybridised before the array-batch was "released" for further use. For ensuring comparable quality of the array-CGH analyses from batch to batch, each new batch was always tested on two samples with known minor imbalances and on a normal-to-normal hybridisation. If these control assays were not satisfactory, the array batch was not further used. For reduction of the background noise (i.e. noise-to-signal ratio) in the array-CGH analyses, only Cot-1 DNA batches which had been thoroughly tested in several normal-to-normal hybridisations, showing a satisfying blocking ability of repetitive sequences, were used in the array-CGH experiments. To reduce the detection of normally occuring copy number variations (CNVs) (see section 8.3.), a commercial reference DNA consisting of a pool of 10 normal individuals (either females or males) was used in the analysis. As for HR-CGH, a normal finding by array-CGH only shows that the DNA copy number in the karyotype is balanced and *not* that the karyotype is normal. Also, only imbalances covered by the array-spotted DNA-sequences can be detected by the method. This is particular a problem when using "low-resolution" arrays like the 1 Mb BAC-array. Commercial high-resolution oligo-arrays, which have been used in papers III and V in this thesis, have smaller "inter-sequence-gaps", enabling array-CGH analysis down to a resolution of ~6kb. However, the shorter oligo-sequences (25-mer or 60-mer) compared to the larger size of BAC DNA (~150 kb), make the oligo-arrays more vulnerable for unspecific hybridisation causing a higher level of background noise. Also the commercial oligo-arrays do not have oligo-duplicates present in the array, whereas in the BAC-arrays, which we have used for 1 Mb array-CGH analyses, each BAC DNA is spotted four times on the array, making the array-CGH data more robust. Thus both types of arrays have their forces and limitations, which should be borne in mind. In the array-CGH analyses, or DNA copy number analyses if a SNP-based oligo-array has been used, we have used the analysis software and available algorithms as recommended by the respective supplier. It should be noted, that the array-CGH threshold algorithms favour

detection of deletions compared to duplications since the threshold cut offs for both deletions and duplications are determined at an equal distance from the mean of all intensity ratios. As the intensity ratios for deletions are more distant from the mean (ratio of 1/2: log2 = -1,0) compared to the intensity ratios for duplications (ratio of 3/2: log2 = 0,59), there is a higher risk that some duplications may be missed. For visualisation of the patients´ chromosomes, array-CGH analysis was always used in combination with G-banded karyotyping. Array-CGH findings were verified by re-examination of G-banded high quality chromosomes (band level above 600), by BAC-FISH analysis, or by array-CGH analysis on another type of array.

8.1.5. Microarray-based gene expression analysis

In one of the papers, we have used microarray-based gene expression analysis for global profiling of the transcriptome of patient fibroblasts compared to fibroblast from control persons. A commercial single-channel oligo array-platform was used in the analyses. It is important to note that genes expressed in the brain causing a certain phenotype of mental impairment, may not be expressed in the examined fibroblasts, and vice versa, genes expressed in the examined fibroblasts may not be active in the brain. Also, the use of expression data from a single patient calls for cautious procedures. We therefore only included genes with a high signal-to-noise ratio to filter out most genes with unreliable expression levels. Since no obvious candidate genes were identified in the analyses, real-time PCR verification and validation of single-genes was not performed.

8.2. Clinical usefulness of HR-CGH and array-CGH

As discussed in the Introduction, the etiology remains unknown for the majority of patients with mental retardation. This situation can be explained in part by the limited resolution of the chromosomal analysis methods previously used for routine examination of these patients. The

development of molecular cytogenetic methods, as HR-CGH and array-CGH, has enabled chromosomal analysis at a higher resolution than the traditional cytogenetic method. For this reason, we have in this thesis examined the clinical usefulness of HR-CGH and array-CGH, for the detection and characterisation of minor and cryptic chromosomal imbalances in patients with mental retardation. In addition, we have explored the phenotypic outcome of the CGH-detected duplications and deletions to obtain phenotype-genotype information of patients with varying degrees of mental impairment, malformations and dysmorphism.

This thesis also illustrates the impressive development that the CGH method has undergone during the last decade. Firstly, the implementation of microarray technology has made genomic screening at a resolution below 3 Mb possible. Moreover, the recent developments of high density oligo-arrays have tremendously increased the sensitivity of the array-CGH method and made detection of genomic imbalances down to a size of few kilobases possible. The years to come will show the limit in diagnosic resolution of the array-CGH method when examining patients with mental retardation.

8.2.1. Detection of cryptic imbalances by HR-CGH (papers I and II)

In patients with mental retardation and/or dysmorphic features and malformations, an unbalanced karyotype can be found by G-banded karyotyping in 10% [88] to 16% [8] of the individuals. In patients having normal G-banded karyotypes, subtelomeric aberrations can be detected in 5-7% of the individuals by subtelomere FISH-screening [31,62,89,90]. As described in the Introduction, the prevalence of subtelomeric abnormalities appears to be higher in patients with a more severe mental retardation than in patients with mild mental retardation [6,29], and addition, families with subtelomeric abnormalities have a higher prevalence of mental impairment [29,32]. Subtelomere FISH-screening has been an important

diagnostic tool for examination of patients with mental retardation, however, cryptic interstitial abnormalities remain undetected by this method. Because of this, we decided to examine the capacity of the HR-CGH method for the genome-wide detection of cryptic chromosomal imbalances -both interstitial and subtelomeric- in patients with mental retardation. A summary of the results presented in papers I and II is shown in Table 3.

Table 3. Detection of cryptic chromosomal imbalances in patients with mental retardation by HR-CGH analysis All patients (cases) had normal findings on G-banded karyotyping.

Study	Number of cases	Abnormal findings		
		Total number (%)	Interstitial imbal.	*de novo* events
Paper I	50	5 (10,0)	4	4
Paper II	554	40 (7,2)	30[a]	36

[a]Based on fine-mapping results by 1 Mb array-CGH analysis.

Based on 50 patients, the HR-CGH detection rate of chromosomal imbalances was 10% in patients having an apparently normal karyotype (Table 3, paper I). Of note, four of the patients had an interstitial abnormality and the majority of the HR-CGH findings were *de novo* (Table 3). Thus, our findings showed that the HR-CGH method was very useful for identification of novel cryptic abnormalities which are undetectable by G-banding and FISH subtelomere-screening. In addition, based on 16 patients, the method was found useful for additional characterisation of visible findings which were difficult to classify based on the G-banding result (paper I).

Based on these findings, the HR-CGH method was implemented in our cytogenetic diagnostic laboratory for examination of selected patients with mental retardation. Paper II summarises the HR-CGH detections made in these patients during a 6-year-period. A total of 590 subjects had been examined (all except three being mentally retarded), of which 554 patients had

normal findings on G-banded karyotyping. In this larger sample, the HR-CGH detection rate of cryptic imbalances was 7,2% (Table 3). Others studies have reported a HR-CGH detection rate of 10% and 12% based on 144 and 424 patients, respectively [91,92]. The lower detection rate in our study may be due to different criteria of patient selection for HR-CGH analysis. Of note, 90% of the patients had a *de novo* finding, and only 25% of the findings involved a subtelomeric rearrangement (Table 3). HR-CGH analysis was also applied on 36 patients having a minor but visible finding on G-banded karyotyping for more precise characterisation (paper II). 94% of these patients had a *de novo* finding.

HR-CGH findings were made in 76 patients, and, except for the Y-chromosome, genomic imbalances were found on all chromosomes. The highest numbers of aberrations, with deletions being as common as duplications, were seen on chromosomes 7 and 8. On chromosome 19, our most gene-dense chromosome, only duplications were observed. Another interesting observation was that in the group of 40 patients having cryptic abnormalities (i.e. normal G-banded karyotypes), deletions were ten times more common than duplications, and the six non-mosaic patients known to have severe mental retardation all had deletions. In contrast, in the group of 36 patients having minor but visible abnormalities, deletions- and duplications were equally common findings, and the six patients with severe mental retardation all had duplications. One explanation may be that small deletions are easier to detect than duplications. It is also possible that small duplications have milder phenotypic consequences which reduce the chance of patient ascertainment.

8.2.2. Comparison of mapping by HR-CGH versus array-CGH (paper II)

The recent implementation of microarray technology was expected to increase the sensitivty of the CGH method. Because of this, when affordable BAC-arrays became available from the Norwegian Microarray Consortium, all 76 HR-CGH-positive samples in paper II were re-

examined by 1 Mb array-CGH analysis. This was done for deletion and duplication fine-mapping, and thus more precise genotype-phenotype information. This effort also made it possible to compare the mapping accuracy of genomic imbalances detected by HR-CGH and by 1 Mb array-CGH. In general, both deletion and duplication sizes were overestimated by the HR-CGH software. In three out of the 40 patients with cryptic imbalances, HR-CGH indicated the location of a deletion or duplication that actually missed its true position. In three of the 36 patients with minor but visible imbalances, the HR-CGH detection in the subtelomeric regions was found to be misleading (i.e. no detection or inaccurate mapping of subtelomeric imbalances), as shown in Figure 6 where an incomplete subtelomeric HR-CGH detection was observed (case 58 in paper II).

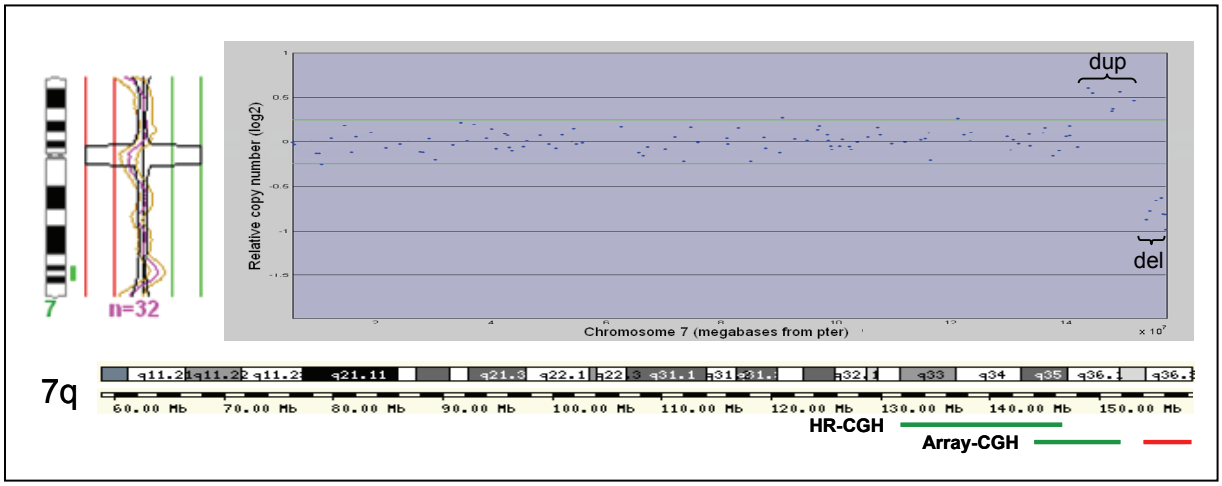

Fig. 6. Incomplete HR-CGH detection of a subtelomeric imbalance. *The 99.5% confidence interval HR-CGH ratio profile together with the 1 Mb array-CGH profile of the case is shown in the figure. The imbalances mapped by HR-CGH analysis and array-CGH analysis are shown as green bars (gains) or red bars (losses) beneath an 850-band ideogram of the q-arm of chromosome 7. HR-CGH analysis indicated a 7q-duplication from 7q33 to 7q35, while 1 Mb array-CGH analysis showed a duplication [dup(7)(q35q36.1)] with a terminal deletion [del(7)(q36.2qter)].*
Illustration by Helle Lybæk.

The comparison also demonstrated that the HR-CGH software may overlook even visible subtelomeric imbalances. A possible explanation of this problem could be that the dynamic standard reference intervals used in the HR-CGH software [71] corrects for regions with

variable signal intensities which especially is found in the heterochromatin regions, the centromeres and the telomeres, which may lead to a lowered HR-CGH detection in the subtelomeric regions. Based on our mapping results, additional specific subtelomere screening should be applied (e.g. by FISH) for improved detection, if using HR-CGH analysis for genome-wide screening of chromosomal imbalances, as is also found by others [73,91]. The mapping results demonstrated an improved performance of 1 Mb array-CGH compared to HR-CGH.

It should also be noted that 1 Mb array-CGH analysis of the three samples with a HR-CGH-detected cryptic imbalance in paper I which were not included in paper II (cases 1, 4 and 5), showed that the HR-CGH findings in the cases 4 and 5 were false-positives. This was unexpected since both HR-CGH findings were verified by either FISH-analysis (case 4) or by re-examination of G-banded high quality chromosomes (case 5). This emphasises the importance of critical interpretation when additional methods are applied for verification of CGH findings, and that results obtained on both G-banded karyotyping and FISH-analysis may lead to a false verification of a CGH finding. The 1 Mb array-CGH identification of false-positive HR-CGH findings confirms an improved performance of the array-CGH method.

8.2.3. Detection of cryptic imbalances by 1 Mb array-CGH (paper II)

For examination of the clinical usefulness of 1 Mb array-CGH analysis in the remaining 514 patients with normal findings on both G-banded karyotyping and HR-CGH analysis, a subset of 20 patients with phenotypes highly suggestive of a genomic imbalance was selected. The results are shown in Table 4.

Table 4. Detection of cryptic chromosomal imbalances in patients with mental retardation by array-CGH only All patients had normal findings on G-banded karyotyping and HR-CGH analysis.

Study	Number of cases	Abnormal findings		
		Total number (%)	Interstitial imbal.	*de novo* events
Paper II	20	4 (20,0)	4	3

Four new findings were made: three deletions (size range 3,1-3,3 Mb) and one duplication (size 8,7 Mb), all being interstitial (Table 4). The results were verified by array-CGH analysis done on a commercial 1 Mb BAC-array (CytoChip). Our results demonstrated a 1 Mb array-CGH detection-rate of cryptic imbalances on 20% (Table 4). However, the sample is too small to conclude if this high detection rate is a chance finding or due to skilled patient selection. The results though indicated an improved detection capacity of cryptic imbalances by 1 Mb array-CGH compared to HR-CGH.

The diagnostic potential of 1 Mb array-CGH analysis was illustrated on a larger sample of patients when combining data from five different European studies [77-81] including 332 patients, which gave a 1 Mb array-CGH diagnostic detection rate of 8,4% [77]. This indicated only a slightly improved diagnostic yield of the 1 Mb array-CGH method as compared to the HR-CGH method (i.e. 7,2%). But it is important to keep in mind, that the yield of different diagnostic approaches is dependent on patient ascertainment. Also, in the five European studies summarised above, the 1 Mb array-CGH diagnostic detection rates varied from 8% [77] to 16% [78]. Of note, 34% of the patients in these studies had been pre-screened for subtelomeric imbalances, and elimination of a similar fraction of patients with subtelomeric findings from our cohort would have reduced our diagnostic HR-CGH detection rate to 6,7% (paper II).

The largest cohort of patients who have been screened for genomic imbalances by array-CGH

analysis includes 8789 individuals with a variety of developmental problems [93]. Most of them had normal findings on initial chromosome- and DNA analyses. By the use of an array with a lower resolution than 1 Mb but targeted against chromosomal regions of known clinical relevance, a diagnostic yield of 6,9% was found [93]. Interesting, full coverage BAC array-CGH analysis (~100 kb resolution) of 100 patients with normal findings on both G-banded karyotyping and MLPA subtelomere screening gave a diagnostic pick-up rate of 10% [85]. Thus an increase in analysis resolution does not necessarily result in a similar fold increase in diagnostic yield. At least not when using BAC-arrays.

Our findings of a higher detection capacity of cryptic imbalances and an improved mapping accuracy demonstrated an improved diagnostic utility of the array-CGH method when compared to the HR-CGH method.

8.2.4. Detection of occult mosaicisms by CGH (papers II and IV)

Unlike meiotic non-disjunctions, mitotic non-disjunctions are rarely observed in humans with the exception of mosaicism for trisomy 8, 9 or 20 [18,34,94]. In the papers II and IV, we describe five occult chromosomal mosaicisms that were detected by CGH (both HR-CGH and array-CGH), despite normal findings on G-banded karyotyping. The results are shown in Table 5.

Table 5. Detection of occult chromosomal mosaicisms by both HR-CGH and array-CGH
All cases had normal findings on G-banded karyotyping.

Study	Abnormal cases	Cases with mosaicism	Cases with mosaicism in %	Percentage of mosaicism[a]	Type of chromosomal abnormality
Paper II	40 of 554	4 of 40	10,0	15,0 – 36,0	Three trisomy 9 One trisomy 14
Paper IV	1 of 1	1 of 1		15,0	Combined tetraploidy 8+18

[a]Determined by interphase FISH analysis of 100 nuclei (paper II) and 200 nuclei (paper IV).

The CGH-detection of chromosomal mosaicism in our cohort of 554 patients was 0,7%, and accounted for 10% of the cryptic findings made (Table 5, paper II). These results demonstrated the strength of the CGH methods in detecting mosaicisms which may be overlooked by G-banded karyotyping when a small number of metaphases is analysed. Also interesting, recent studies have shown that phytohemagglutinin (PHA)-stimulation during culturing of blood leukocytes may lead to a selection against cells in a mosaic state [35,77]. This possible bias is avoided when analysing unstimulated leukocyte DNA by the CGH-methods, which ensures a more precise examination of the "true chromosomal content" in the patient.

The importance of this latter precaution was illustrated in paper IV, where a mosaic combined tetraploidy of chromosomes 8 and 18 was detected by both HR-CGH and 1 Mb array-CGH in blood DNA from a newborn child with markedly dysmorphic features, malformations and developmental delay, despite normal findings on G-banded karyotyping (Table 5, paper IV). Of note, in contrast to mosaic trisomies, the finding of mosaic whole-chromosome tetrasomy is without precedence in patients with mental retardation. Thus, our finding of a mosaic combined tetrasomy of two chromosomes is very unique. The double tetrasomy was caused by mitotic malsegregation of all four chromatids of both chromosome pairs, indirecty suggesting that a mechanism for tetraploidy correction involving pairing of homologues, which could be important for genome maintenance, may be present in normal cells. Interestingly, interphase FISH analysis of fibroblasts at age 4 months and of a buccal smear at age 14 months did no longer detect double tetrasomic cells. Also, CGH- and interphase FISH analyses of a new blood sample at age 20 months only displayed normal findings, indicating an early selection against the tetrasomic cells in the bone marrow.

The reason for this unique tetraploidy finding is either a very rare chromosomal aberration, or that somatic malsegregation of chromosomes is a more common cause of

abnormal development than previously appreciated but is not found due to negative selection against such cells during embryonic development and during culturing of blood cells for routine karyotyping. We may have been fortunate to observe this event because we performed CGH on "unbiased" (unstimulated) leukocyte DNA from a newborn. A mosaicism grade as low as 8% has been detected by 1 Mb array-CGH analysis [77], again showing the important capacity of the CGH methods in detecting this type of chromosomal abnormality.

8.2.5. Genotype-phenotype correlation of gene dose alterations (papers III and V)

The combination of several cytogenetic methods made it possible to identify candidate genes that might explain phenotypic features in two different families with mentally retarded individuals (papers III and V). In addition, the studies showed that such a combination of methods may be crucial for a correct characterisation of the chromosomal abnormalities being present in the patient.

In paper III, G-banded karyotyping of a six-year-old boy with spherocytosis and mild mental retardation identified a paracentric inversion on chromosome 14. As expected, based on the patients' phenotype of spherocytosis [95], BAC-FISH analysis against the beta-spectrin gene *SPTB* showed a deletion. But quite unexpectedly, the deletion was located outside the BAC-FISH mapped telomeric inversion breakpoint. Oligo array-CGH analysis located the deletion to be ~1,6 Mb distal of the inversion breakpoint, spanning ~2,1 Mb, as summarised in Figure 7. The deletion contained 16 annotated genes, including *SPTB* causing spherocytosis, and *PLEKHG3,* a guanide nucleotide exchange factor for Rho GTPases. Rho GTPases are key regulators of the actin- and microtubule cytoskeletons and their possible involvement in mental retardation is well known [96,97]. For this reason, and because *PLEKHG3* is highly

expressed in the brain, it is a possible candidate gene for causing the mild mental retardation.

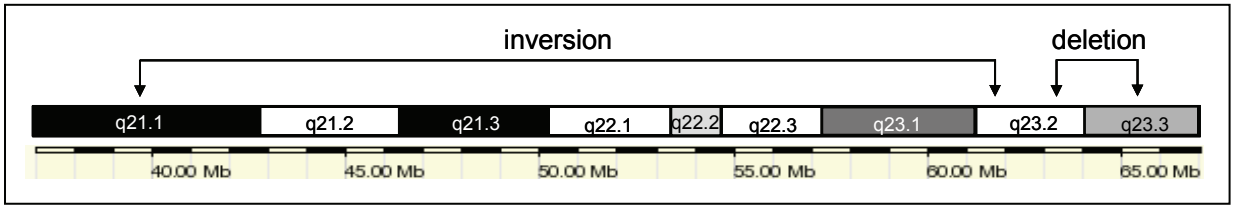

Fig.7. A ~2,1 Mb deletion located ~1,6 Mb distal to a 14q21.1q23.2 paracentric inversion.
A schematic figure showing the positions of the paracentric inversion and downstream deletion on chromosome 14. An 850-band ideogram of chromosome 14 covering the region from 37 Mb to 67 Mb from 14pter is used for the illustration.
Illustration by Helle Lybæk.

Beside the patient, six other relatives, through three generations, were also known to have spherocytosis and a least two of them also had severe learning difficulties. Analysis of these two latter individuals gave the same chromosomal findings as identified in the proband. Due to limited access of samples from additional family members, we were not able to establish if the deletion in the family arose independently of the familiar inversion or as part of a single meiotic event.

The recognizable phenotype of spherocytosis and the combination of several cytogenetic methods was crucial for the complete characterisation of the patient. Our results demonstrated that inversions can be associated with microdeletions close to but not including one of the inversion breakpoints, and that one should look for causative genes for a given phenotype not only in the breakpoint region of a *de novo* inversion or translocation – the real cause may be a *de novo* rearrangement nearby - or even elsewhere in the genome.

In paper V, a 2 ½-year-old girl with onset of puberty before age 5 months, malformations and severe mental retardation was examined. An 8,9 Mb subterminal 19p13 duplication was detected by 1 Mb array-CGH, despite normal findings on G-banded karyotyping and

HR-CGH. Based on the phenotype of precocious puberty, we initially assumed that the duplication contained the kisspeptin receptor gene *GPR54*, known to stimulate induction of puberty [98,99]. However, mapping by high-resolution array-CGH excluded this possibility. Of note, the duplication proved to be difficult to detect in some of the array-CGH software at standard settings, even with a good data quality, making manual adjustments necessary for the detection. Measuring of the global gene expression in skin fibroblasts from the patient did not give leads for the hormonal disturbance or the mental impairment, illustrating that severe mental retardation and malformation can occur with only subtle changes in gene expression.

The proband´s mother was, unexpectedly, showed to have a between-arm insertion of the duplicated segment that resembled a pericentric inversion. The same balanced insertion in several other family members, including one who had lost a daughter with severe mental retardation and puberty from age 10. Another carrier of the insertion, a maternal uncle, had also severe mental retardation, and we initially ascribed his phenotype to a presumed cryptic chromosome 19 imbalance caused by the 19p-into19q insertion. Instead, most unexpectedly, a novel 3,9 Mb deletion of 2q23.3-2q24.1 was found. The deletion contained seven annotated genes, among them the formin-like 2 gene *FMNL2*, and *NR4A2*, a member of the steroid-thyroid hormone and retinoid receptor superfamily. *FMNL2* has similarity to genes involved in Rho-dependent signal transduction [100], and as mentioned above, the involvement of Rho-dependent GTPases in mental retardation is well known [96,97]. *NR4A2*, is a transcription factor essential for the differentiation of dopaminergic neurons in substantia nigra, and mutations of the gene have been linked to familial parkinsonism [101]. Thus, *FMNL2* and *NR4A2* are likely candidate genes for causing the severe mental impairment in this individual. The presence of a 2q23q24 microdeletion syndrome has been suggested based on an overview of nine patients with deletions that included 2q23.3-2q24.1 [102]. The phenotypes, as well as the deletion sizes, are quite variable, but epilepsy and mental

retardation are recurrent features, as seen in this individual. For this reason, we proposed that the 3,9 Mb region may be critical for this suggested syndrome.

During the examination of this family several unexpected results were found, and the study showed how challenging it may be to determine phenotype-genotype correlations, even in patients with recognizable phenotypes. Our results demonstrated that between-arm insertions are high-risk chromosomal rearrangements that can easily be misinterpreted as pericentric inversions, and that duplications can be difficult to detect, even by array-based copy number analyses. We were not able to determine a cause for the extreme precocious puberty in one individual, and this patient underscores the need for careful mapping of chromosomal aberrations before making assumptions about genotype-phenotype correlations. Furthermore, the study reminded us that the etiology of severe mental retardation is not necessarily the same in closely related individuals, and that array-CGH is very useful for detecting alternate explanations for mental retardation phenotypes in the same family.

8.3. The challenge of interpreting detected chromosomal abnormalities

The use of 1 Mb array-CGH analysis for genome-wide screening of chromosomal imbalances in phenotypic normal humans lead to the discovery of extensive genomic rearrangements ranging in size from kilobases to megabases, which were not detectable by high-resolution G-banded karyotyping [103,104]. These normally occuring genomic variants have been called copy number variations (CNVs) or polymorphisms [105]. For measuring the extent of CNVs in the human genome, a recent study examined 270 normal individuals by tiling-BAC array-CGH analysis and high-resolution SNP-based DNA copy number analysis [106]. A total of 1447 CNVs were identified, covering ~12% (360 Mb) of the human genome [106]. The median sizes of the CNVs were 228 kb (tiling BAC-array) and 81 kb (SNP-array), and the

mean sizes were 341 kb and 206 kb, respectively. Very interestingly, the CNVs included hundreds of genes in deletions, duplications, insertions and complex multi-site variants in addition to disease loci, functional elements and segmental duplications/LCRs [106]. This indicates, that although the CNVs do not have (major) phenotypic consequences themselves (i.e. as they were detected in normal individuals), they can predispose for genomic rearrangements which in turn can result in a clinical phenotype, as described by others [24,25,27,28,105,107-109].

The presence of CNVs in the human genome complicates the clinical interpretation of genomic imbalances detected in phenotypically abnormal individuals, since a finding of an imbalance does not automatically indicate a pathogenic effect. If the detected imbalance in a patient involves a known deletion/duplication syndrome, or if the imbalance has occurred *de novo* in the patient, and especially if it contains genes with effects compatible with the clinical findings of the patient, then the finding is considered as pathogenic. If the imbalance is familial but previously unclassified, then it is difficult to determine its phenotypic consequence.

The full understanding of the function and significance of CNVs associated with mental impairment is still to be solved. But as the copy number variation databases expand, as the Database of Genomic Variants (DGV; http://projects.tcag.ca/variation/), and genomic gains and losses are better associated with specific phenotypes as in the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER; https://decipher.sanger.ac.uk), clinical interpretation will be more reliable by comparisons of patient findings with CNVs in such databases.

Also, as the genotype-phenotype databases, such as DECIPHER, expand, it will be easier to determine the genotype-phenotype correlations in the patients. However, since many of the

(non-) published rapports on patients are never reported to these databases, a lot of useful information is missing in the public domain. As a consequence of this, a new genotype-phenotype correlation-tool has recently been developed, in which biomedical concepts (e.g. microcephaly) are mapped onto the human genome at a cytogenetic band level by mining literature (i.e. MEDLINE abstracts) on chromosomal aberrations [110,111].

9. Concluding remarks

Mapping of genomic imbalances with distinct phenotypes may be useful not only for evaluation of the clinical importance of a *de novo* imbalance, but also for narrowing down regions of particular interest when searching for genes whose dosage is critical for normal development.

In this study, we have reported the usefulness of HR-CGH and array-CGH as screening methods for cryptic chromosomal imbalances in patients with mental retardation. The finding of a unique mosaic combined tetraploidy for chromosomes 8 and 18 by CGH in newborn blood DNA may indicate that somatic malsegregation of chromosomes is a more common cause of abnormal development than previously appreciated. The bias introduced by cell culturing is avoided by using CGH as the screening method.

In a study of 590 patients, the HR-CGH diagnostic detection rate of cryptic imbalances was 7,2 %. Of note, the findings were mainly interstitial and 90% were *de novo*. An improved diagnostic utility was demonstrated when 1 Mb array-CGH was applied on HR-CGH positive samples. In 20 patients with normal HR-CGH findings, the 1 Mb array-CGH diagnostic detection rate of cryptic imbalances was 20%. Evidently, the more recent array-CGH method is an even better diagnostic screening tool than HR-CGH in patients with mental retardation.

Detailed molecular cytogenetic studies of selected cases made it possible to find candidate genes that may be important for normal brain development and function.

10. Future perspectives

During the last few years, commercially high-resolution oligo-arrays have been developed and become available in an impressive rate. And, maybe more important, the costs of these arrays have dropped markedly, making them more affordable for more laboratories that now get the opportunity of extensive analysis for submicroscopic chromosomal abnormalities. Also many array-systems have become highly automated which enable analysis of many patients in a short time period. High-resolution analyses of a large number of patients with mental retardation will enhance the likelihood of detecting dosage sensitive single-genes (e.g. "knock-out patients" or "knock-in patients"), or chromosome-regions, which are crucial for normal brain development and function. Also, high throughput of such analyses will provide more knowledge of the occurrence and function of the CNVs. However, it is crucial that data obtained worldwide on patients with mental retardation are reported to- and collected in public accessible databases for a larger collection of genotypic-phenotypic correlations in this patient group, which in turn will facilitate the interpretation of genomic findings made. Today, there are several such databases, some examples are DECIPHER (Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources; https://decipher.sanger.ac.uk/), ECARUCA (European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations; http://ecaruca.net), and DGV (Database of Genome variation; http://projects.tcag.ca/variation/).

It is realistic to hope in the years to come that the combination of high-resolution detection of submicroscopic abnormalities with more knowledge on the phenotypic consequences of deletions, duplications and chromosomal rearrangements in patients with mental retardation, will result in substantionally more patients – and their relatives – getting an explanation of the causes of their impairment.

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