IDENTIFICATION OF GENETIC DEFECTS INVOLVED IN X-LINKED MENTAL RETARDATION

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THESIS

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Revised Thesis
IDENTIFICATION OF GENETIC DEFECTS IN X-LINKED MENTAL RETARDATION

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DECLARATION

I hereby declare that this submission is my own work and that to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement is made in the text.

Semarang, August 2010

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ABBREVIATIONS

ACSL4	Acyl-CoA synthetase long chain family member 4
AGTR2	Angiotensin II receptor type 2
AP1S2	Adaptor-related protein complex 1. sigma 2 subunit
AR	Androgen Receptor gene
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor 6
ARX	Aristaless-related homeobox
ATRX	α-thalassemia-mental retardation, X-linked
BRWD3	Bromodomain and WD repeat domain containing 3
CASK	Calcium/calmodulin-dependent serine protein kinase
CNV	Copy Number Variations
CUL4B	Cullin 4B
DLG3	Dics, large homolog 3
DNA	Deoxyribo Nucleic Acid
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-IV
FGD1	FYVE, RhoGEF and PH-domain-containing 1
FISH	Fluorescence In Situ Hybridization
FMR1	Fragile X Mental Retardation 1
FMR2	Fragile X Mental Retardation 2
FTSJ1	Ftsj homolog 1
GDI1	GDP dissociation inhibitor 1
GRIA3	Glutamine receptor, ionotrophic, AMPA3
HUWE1	HECT, UBA-and WWE-domain containing 1
IL1RAPL1	Interleukin 1 receptor accessory protein-like 1
IQ	Intelligence Quotient
JARID1C	Jumonji, AT rich interact domain 1C
LOD	Logarithm of Odds
MAGT1	Magnesium transporter 1
MECP2	Methyl-CpG binding protein 2
MLPA	Multiplex Ligation-Dependent Probe Amplification
MR	Mental Retardation
MRX	Nonsyndromic X-linked mental retardation
MRXS	Syndromic X-linked mental retardation
NLGN3	Neuroligin 3
NLGN4X	Neuroligin 4, X-linked
OMIM	Online Mendelian Inheritance in Man
OPHN1	Oligophrenin 1
PAK3	p21 protein (Cdc42/Rsc)-activated kinase 3
PQBP1	Polyglutamine-binding protein 1
RPL10	Ribosomal protein L10
RPS6KA3	Ribosomal protein S6 kinase, 90kDa, polypeptida 3
RUNMC	Radboud University Nijmegen Medical Center
SHROOM4	Shroom family member 4
SLC6A8	Solute carrier family 6 (creatine), member 8

SLC9A6	Solute carrier family 9 (sodium-hydrogen exchanger)
STR	Short Tandem Repeats
SYP	Synaptophysin
TM4SF2	Transmembrane 4 superfamily member 2
UPF3B	UPF3 regulator of nonsense transcripts homolog B
XCI	X-Chromosome Inactivation
XLMR	X-linked mental retardation
ZDHHC9	Zinc finger, DHHC-type containing 9
ZNF41	Zinc finger protein 41
ZNF674	Zinc finger family member 674
ZNF711	Zinc finger protein 711
ZNF81	Zinc finger protein 81

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2007	Frequency of Hepatitis B vaccination in Neonatals 0-2 months old on Primary Health Care Region of Balapulang 2007-2008. Clerkship thesis. Faculty of Medicine Diponegoro University

IDENTIFIKASI DEFEK GENETIK PADA
X-LINKED MENTAL RETARDATION

Latar Belakang: *X-linked mental retardation* (XLMR) berperan pada 40% pria penderita retardasi mental (RM). Defek genetik berperan pada 50% kasus MR. Terdapat 56 *loci* XLMR non-sindromik (MRX) dan 35 loci XLMR-sindromik (MRXS) yang belum diketahui gen penyebabnya.

Tujuan: Identifikasi defek genetik pada keluarga XLMR.

Metode: Pemeriksaan klinis dan analisis sitogenetik konvensional dilakukan pada 4 keluarga MRXS dan 6 keluarga MRX, dilanjutkan analisis pengulangan CGG pada regio promoter *FMR1*. Analisis *linkage* dilakukan dengan *STR-markers* polimorfik pada kromosom X dilanjutkan perhitungan skor LOD. Dilakukan pemeriksaan status inaktivasi kromosom X wanita pembawa dengan metode *FMR1* dilanjutkan metode *AR* bila tidak informatif untuk *FMR1*. Dilakukan pemilihan kandidat gen dalam *linkage interval* dan analisis mutasi.

Hasil: Tidak dijumpai adanya kelainan kromosom numerikal dan Fragile-X pada 10 keluarga ini. Terdapat variasi *linkage interval* antara 20 Mb hingga 121 Mb. Tidak terdapat mutasi *HSD17B10, UBQLN2, SYP, ARGHEF* pada keluarga W92-053 (XLMR dan hipomielinasi). Tidak terdapat mutasi *SLITRK2* dan *SLITRK4* pada keluarga P03-0452 dan 13753/HC (XLMR dan hidrosefalus). Tidak terdapat mutasi *GPC3* pada keluarga DF27004 (XLMR dan pertumbuhan berlebih). Keluarga W092-053, PO3-0452, DF27004, dan W08-2152 menunjukkan penyimpangan inaktivasi kromosom-X pada wanita pembawa.

Kesimpulan: Identifikasi defek genetik pada sepuluh keluarga menunjukkan *linkage interval* yang bervariasi besarnya dari 20 Mb hingga 121 Mb dengan skor LOD 0,17 hingga 3,3, penyimpangan inaktivasi kromosom-X pada empat keluarga, dan tidak terdapat mutasi pada kandidat gen. Analisis STR markers bermanfaat untuk menentukan linkage interval, mempersempit daerah yang akan diteliti, dan untuk konseling genetika.

Kata kunci: *X-linked mental retardation*, defek genetik, analisis *linkage*, analisis mutasi

IDENTIFICATION OF GENETIC DEFECTS INVOLVED IN X-LINKED MENTAL RETARDATION

Backgrounds: X-linked mental retardation (XLMR) has been the focus of MR research because of 40% excess of males with MR. Genetic defects are estimated to account for 50% MR cases. There are still 56 non-syndromic (MRX) and 35 syndromic XLMR (MRXS) loci with unknown causative genes.

Aims: Identification of the genetic defects in XLMR families.

Methods: Four MRXS and 6 MRX families were studied. Clinical dysmorphologic examination and conventional cytogenetic analysis were performed followed by Fragile-X exclusion. Linkage analysis was conducted with highly polymorphic STR-markers on the X-chromosome followed by LOD scores calculation. An *FMR1* X-inactivation assay was performed in 15 females from all families, followed by *AR* method if the result were uninformative for *FMR1*. Candidate genes were selected in linkage interval and mutation analysis was performed.

Results: Gross numerical chromosomal abnormalities and Fragile-X were excluded in all 10 families. Ten XLMR families showed intervals varying from 20 Mb to 121 Mb. Family W92-053 (mental retardation and hypomyelination) showed no mutation in *HSD17B10, UBQLN2, SYP, ARGHEF*. Two families with MR and congenital hydrocephalus (P03-0452 and 13753/HC) showed no mutations in *SLITRK2* and *SLITRK4*. Family DF27004 (MR and overgrowth features) showed no mutations in *GPC3*. Family W092-053, PO3-0452, DF27004, and W08-2152 showed skewed X-inactivation in the obligate carrier female.

Conclusions: Genetic defects identification in ten families showed varying linkage intervals from 20 Mb to 121 Mb with varying LOD scores from 0,17 to 3.3, skewed X-inactivation in 4 families, and no mutation in the candidate genes. STR markers analysis was useful in determining linkage intervals, narrowing down the region of interest for further studies, and genetic counselling.

Keywords: X-linked mental retardation, genetic defects, linkage analysis, mutation analysis

CHAPTER I
INTRODUCTION

I.1 Backgrounds

Mental Retardation (MR) is defined by IQ below 70 and adaptive behavior limitations, which manifest before 18 years of age (Schalock *et al.* 2007). The prevalence of mental retardation is estimated to be about 1 to 3% of the general population (Brosco *et al.* 2006). Mental retardation is the most common reason for referral to genetic services and one of the important unsolved problems in healthcare (de Vries *et al.* 1997; Yeargin-Allsopp *et al.* 1997). Genetic defects are estimated to account for approximately 50% of cases (Leonard and Wen, 2002). Genetic defects in MR consist of chromosomal abnormalities (structural and numerical), single gene disorders, and multifactorial defect (Basel-Vanegeite, 2008).

X-linked mental retardation (XLMR) is characterized as mental retardation with a distinctive pattern of inheritance, associated with X-chromosome (Ijntema, 2001). XLMR has been the focus of MR research for over three decades because of the fact that there is 40% excess of MR males (Yeargin-Allsopp *et al.* 1997; Leonard and Wen, 2002). In this case, linkage to the X-chromosome can be established in families with only 2 male patients and one obligate female carrier, such as nephew uncle families. In addition, instead of the whole genome, only X-chromosome needs to be considered (de Brouwer *et al.* 2007). XLMR is a heterogeneous disorder for which many of the causative genes are still unknown, although 69 genes have been identified as causing syndromic XLMR and 33 genes as causing non syndromic XLMR (18 causing both syndromic and non-syndromic XLMR) (Greenwood Genetic Center, 2010). However, there are still 56 non syndromic and 35 syndromic XLMR loci for which the gene defect is still unknown (Gecz *et al.* 2009). It is assumed that most of these loci represent separate, novel XMLR genes, suggesting that there are still more than 80 MR genes to be disclosed on the X chromosome alone (de Brouwer *et al.* 2007).

Genetic defect identification in MR is a challenging process. The first step in elucidating genetic defect in MR is a thorough clinical work-up, which could screen acquired factor from anamnesis and also largely known syndrome for example Down Syndrome and Fragile X from the clinical features (Lugtenberg *et al.* 2006). The pedigree taken also could show the possible mode of inheritance of the disorder. The next step is exclusion other cause of mental retardation. Considering the large role of chromosomal aberration in MR (11%; Stevenson *et al.* 2003), in the following step patients are routinely screened for large chromosomal aberration by conventional karyotyping. In addition, considering that Fragile X is the most common inherited MR syndrome with incidence of 1/3000 male, in the next step all the patients are screened for CGG repeat expansion in 5' untranslated region of *FMR1* that cause Fragile X syndrome (de Vries *et al.* 1997). Before researchers could identify and finally sequence the gene responsible for a disease, the gene location first must be mapped in the genome. Linkage analysis is a method that allow to rule out regions of chromosomes that are likely to contain a risk gene in the linkage interval, and determine areas where there is a low chance of finding a risk gene (Massanet, 2009). This approach use the polymorphism character of microsatellite markers (STRs: short tandem repeats) -a short blocks (often less than 150 bp) of simple repetitive sequences (1-4 bp) dispersed randomly across all chromosomes- for mapping the linkage interval (Stopps and McDonald, 1998). Once a linkage interval is located in a chromosome, candidate genes within the interval could be selected based on its characteristics, for example its expression in brain, inactivation status by X chromosome inactivation (XCI) mechanism, homology to known MR genes, etc (Lugtenberg *et al.* 2006). This approach is called positional candidate gene analysis approach, which is assumed to be able to largely reduce cost needed compared to screening the whole genomes (Stratchan and Read, 1999).

National Survey, SUSENAS in 2000 reported 384.818 person with MR in Indonesia (0,19% (National Survey, 2000)). Underreporting is likely considering the widespread stigma (Komardjaja, 2005; Gabel, 2004) and discrimination (Kats, 2008; Croot, 2008) that people with disabilities and their families have to endure

in Asian cultures and the lack of research on mental retardation in Indonesia. As a developing country, there is no health care insurance system for all citizens making costs for medical care for MR patients unbearable for their families. Associated mental impairment, high risk of recurrence, and no therapy available makes genetic counseling essential for families with mental retardation to prevent recurrence of similarly affected children in the family. The understanding of the molecular basis of MR will lead to improvement in diagnostic testing, genetic counselling and also future therapeutics (Basel-Vanagaite, 2008). In contrast to the extensive research performed on XLMR in European countries, so far there are only a few XLMR studies performed in the Indonesian population, for example Fragile X screening by Faradz et al in 1998 and subtelomeric duplication screening by Mundhofir et al 2008, and no study about other XLMR. Currently, there are no researchprotocols nor diagnostic workflows for XLMR in general in Indonesia. Thus, this study aims to identify genetic defects involved in XLMR by using positional candidate gene analysis approach which can be used for genetic counselling purposes and to set up a basic workflow for future XLMR studies in Indonesia.

I.2 Research questions

I.2.1. General research question

What genetic defects found in the XLMR families of this study?

I.2.2. Specific research questions

1. What linkage intervals do we observe in the XLMR families?
2. What is the X-inactivation status of the females carriers within these families?
3. What are the best candidate genes within the linkage intervals in these families?

I.3 Research purposes

I.3.1. General research purposes

Identification of genetic defects in X-linked mental retardation in families.

I.3.2. Specific research purposes

1. Identify linkage intervals within XLMR families.
2. Measure X-inactivation status of the females carriers in these families.
3. Select new candidate genes within the linkage intervals in these families.

I.4 Research advantages

1. This study will contribute to the understanding of the molecular basis of MR in XLMR families
2. Considering the current absence of an XLMR workflow in research and diagnostics in Indonesia, this study will establish a starting point for XLMR research and diagnostics by developing the first steps for a diagnostic workflow for XLMR in Indonesia.
3. Linkage result from this study will help to show potential carrier which can be used to perform genetic counselling prevent recurrence of similarly affected children in the family
4. The technical knowledge, gained by this research will help to introduce new techniques in genetic research in Semarang: e.g linkage analysis by STR-markers.
5. Regarding the limited XLMR research in Indonesia, this study will encourage other researchers to perform further studies in mental retardation in Indonesia.

I.5 Research originality

1. To our knowledge this is the first study in Indonesia to identify the genetic defects that cause X-linked mental retardation in Indonesian patients by using linkage analysis.
2. Screening of Fragile-X and conventional cytogenetic abnormalities in individual with mental retardation in Indonesia have been performed by Faradz et al in 1998, but no other study known about other XLMR in Indonesia.

3. Mundhofir et al.in 2008 performed population screening of chromosomal abnormalities, CGG repeat expansion in FMR1, subtelomeric deletion and duplication (STD), and Prader Willy/Angelman Syndrome in mentally retarded pupil in Semarang. No other study known about other MR in Indonesia.

Table1. Research originality in matriks form

Title	Author	Method	Result
Fragile X Mental Retardation and Fragile X Chromosome in Indonesian Population	Faradz et al, 1998	Descriptive study on large population to screen chromosomal abnormality, CGG repeat of FMR1 in Indonesian population.	Fragile X prevalence in Indonesia is about 1.6-2%, which is significantly different from Kaukasian population
X-Linked Mental Retardation: A clinical and molecular study	Hamel et al., 1999	Descriptive study on 13 Dutch large thre degree families with XLMR	From 13 families, FRAXE was segregated in one family, one mutation of RaBGD11 was found in one family, LOD score more than two was found in the rest of the family.
Mutation Frequencies of X-linked Mental Retardation Genes in Families from the EuroMRX Consortium	De Brouwer et al., 2007	Descriptive study about mutation analysis in 400 XLMR families from EuroMRX Consortium.	For 42% of the families with obligate female carriers MR phenotype could be explained by a mutation. There was no difference between families with (lod score >2) or without (lod score <2) significant linkage to the X chromosome.

Title	Author	Method	Result
Cytogenetics, molecular and clinical studies among mentally retarded individual in Semarang	Mundhofir et al., 2008	Description study about population screening of chromosomal abnormality, CGG repeat of FMR1, subtelomeric deletion and duplication (STD), Prader Willy and Angelman Syndrome (PW/AS) using MLPA in Semarang.	From 122 mentally retarded pupils, 1 patient showed fully CGG repeat expansion , 13 patients showed STD, and none of patients showed PW/AS

CHAPTER II
LITERATURE REVIEW

II.1. Mental Retardation

II.1.1. Definition

Mental retardation has been classified as disease category three decades ago. According to DSM-IV in 1994, American Psychiatric Association defines mental retardation as sub average intellectual functioning and concurrent deficits or impairments in present adaptive functioning in at least two of the following skill areas: communication, self-care, home living, social/interpersonal skills, use of community resources, self-direction, functional academic skills, work, leisure, health, and safety with onset before 18 years (APA, 1994). In 1996, WHO in the guideline ICD-10, described mental retardation as a reduced level of intellectual functioning, which decrease the ability to adapt with the daily needs of normal social environment (WHO, 1996). Nowadays, we used definition developed by AAMR on the 2002 AAMR Manual, which describe mental retardation as significant limitation in both intellectual functioning and adaptive behavior, which presents before age of 18 (Luckasson *et al.* 2002). Lately, term of "intellectual disability" is increasingly being used instead of "mental retardation" (Schallock *et al.* 2007).

II.1.2. Classification

There are several classification system of intellectual disability that are currently used. The first classification is developed by WHO on 1996, and summarized in ICD-10. WHO classified intellectual disability in axis I of five axes of ICD-10 as: mild (IQ 50-69), moderate (IQ 35-49), severe (IQ 20-34), and profound (IQ under 20) (WHO, 1996). The second classification is developed by American Psychiatric Association on 2002 in multiaxial system DSM-IV TR, which divided intellectual disability as mild (IQ 50-55 to 70), moderate (IQ 35-40 to 50-55), severe (IQ 20-25 to 35-40), profound (IQ below 20 or 25), and severity

unspecified (strong presumption of MR but the intellegence untestable by standard test) (First, 2004).

Based on the clinical features, intellectual disability could also be divided into syndromic and non syndromic form. Syndromic forms of MR are characterized by MR accompanied by either malformations, dysmorphic features, or neurological abnormalities. Non syndromic MR are characterized by MR without any additional features (Basel-Vanagaite, 2008). Nowadays, the boundary between syndromic and non syndromic forms of MR is becoming blurred due to the finding that in several genes, different mutations in the same gene can result in both syndromic and non syndromic form of MR (Frints, 2002).

II.1.3 Prevalence

In 2002, Leonard et al made a meta-analysis about MR prevalence, and estimated that MR is affecting 1-3% of general population (Leonard and Wen, 2002). The prevalence of intellectual disability in Asia seemed to be consistent with Western population, which account about 0, 06-1,3% of total population, except for China (6,68%) (Jeevanandam, 2009).

II.1.4. Etiology

The etiology factors of mental retardation is heterogeneous. In 2003, Stevenson et al, based on a study cohort of 10,997 individuals with MR, found that, a specific cause for the MR could be found in 43.5% of the cohort and that genetic causes accounted for 28% of all cases and 63% of cases in which the cause could be identified (Figure 1; Stevenson *et al.* 2003)

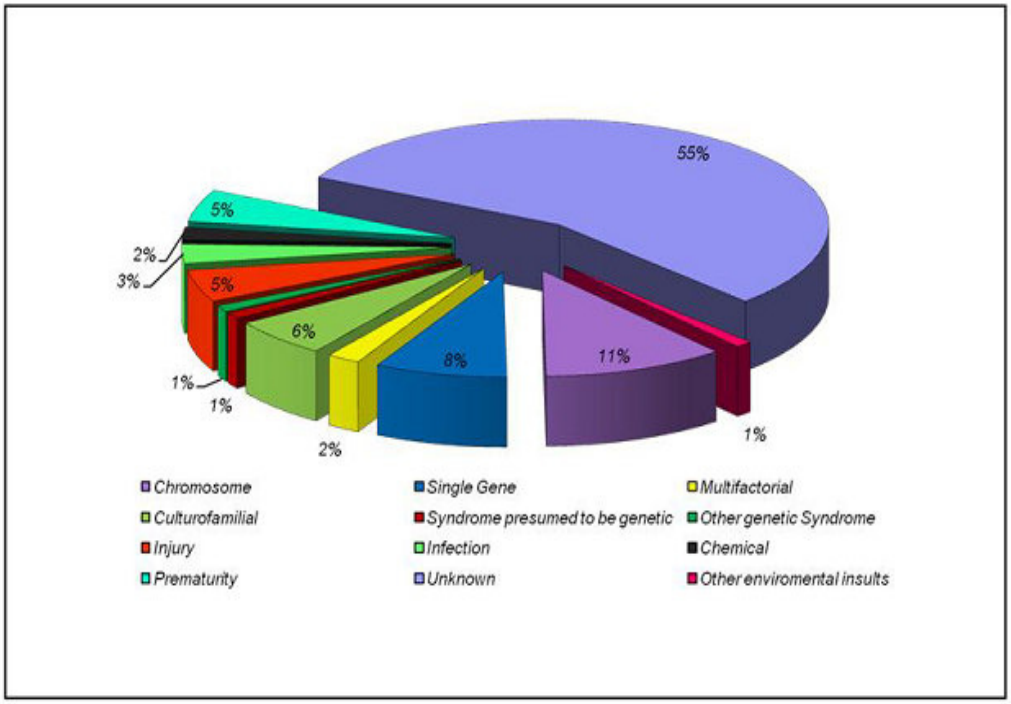


Figure 1. Etiology of mental retardation (adopted from Stevenson et al, 2003)

The etiology of intellectual disability are basically categorized as genetic, acquired, and unknown causes (Moog, 2005). Acquired causes of MR can be divided based on the timing of defect as: prenatal (for example: fetal alcohol syndrome, teratogen exposure, toxoplasmosis), perinatal (for example: intrapartum metabolic acidosis, early onset severe neonatal encepalopathy, perinatal distress), and post natal (for example: traumatic brain damage, lead intoxication) (Moog, 2005). Genetic causes of MR include chromosomal abnormalities, monogenic disorder, and multifactorial causes (Figure 2; Moog, 2005; Basel-Vanegeite, 2008).

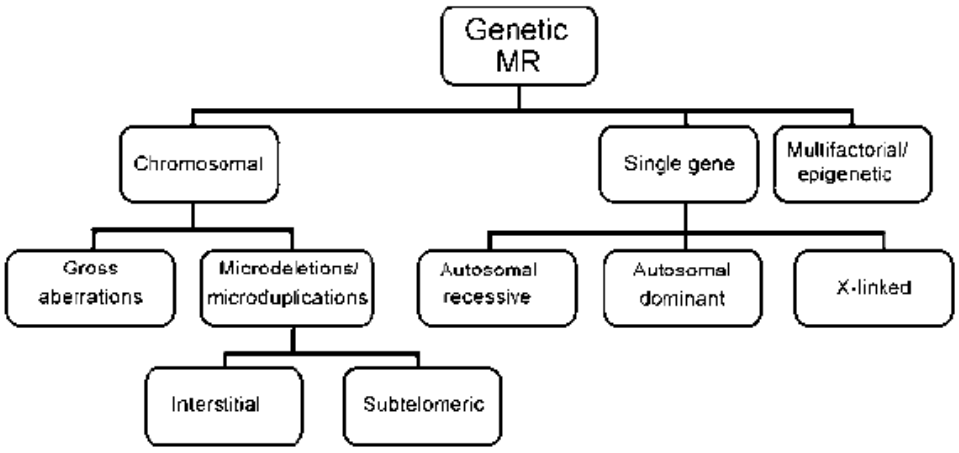


Figure 2. Main genetic causes of mental retardation (Adopted from Basel-Vanegeite, 2008)

Chromosomal abnormalities as a cause of intellectual disability has been recognized for many years. Trisomy 21 that cause Down syndrome is one of recurrent chromosomal abnormality that cause mental retardation with the incidence of 1/600 newborns (Hulten et al. 2008). Chromosomal abnormalities cause cognitive impairment, which is also frequently with defects of heart formation and dysmorphic features (Raymond and Tarpey, 2006), which represent the most frequent cause of syndromic MR (Basel-Vanegeite, 2008). Chromosome abnormalities with size of 3-5 megabases (Mb) can be detected by conventional microscopic analysis of chromosomes isolated from peripheral blood lymphocytes in ~5% of patients with unexplained MR (Anderson *et al.* 1996; de Vries *et al.* 1997). In the early 1990s, with the introduction of fluorescence in situ hybridization (FISH), recurrent small microdeletions of the genome (with maximum resolutin of 150 kb) not visible by light microscopy were identified associated with characteristic syndromic MR (Raymond and Tarpey, 2006). By the development of molecular cytogenetic techniques, such as FISH and multiplex ligation-dependent probe amplification (MLPA) (Schouten *et al.* 2002), it is shown that causative submicroscopic rearrangements of the subtelomeric regions can be found in ~5% of patients with human malformations and MR (Koolen et

al. 2004). In 2005, Van Karnebeek estimated that the frequency of deteced chromosomal abnormalities is about 10%, ranging from 2% to 50% depending on the variation in the study design among published report. (Van Karnebeek, 2005). Nowadays, the focus of MR research has been shifted to identify smaller chromosome abnormalities associated with disease, especially after introduction of high-resolution array. During the past three years, numerous copy number variations (CNVs) have been identified that are associated with MR and developmental delay (Stankiewicz and Beaudet, 2007). Zahir and Friedman in 2007 estimated that pathogenic CNVs can be found in 10–15% of individuals with idiopathic mental retardation (Zahir and Friedman, 2007). By the development of array technology, even it is assumed that up to 25% of all cases of MR may be explained by copy number-dependent gene dosage variations, although not all of these variants will be fully penetrant, which create a challenge in clinical interpretation (Vissers *et al.* 2009).

Monogenic disorders include autosomal dominant disorders, autosomal recessive disorders, and X-linked disorders. Single-gene disorders have been increasingly recognized to cause MR over the past half century. Searching in McKusick catalogue of genes and phenotypes (Online Mendelian Inheritance in Man (OMIM); OMIM, 2010) on January 2010 show 1629 entries associated with "mental retardation". In cohort study of 10,997 individuals with MR in 2003 by Stevenson et al, it was found that 8% of MR in the cohort was caused by single-gene disorders.

II.2. X-Linked Mental Retardation

II.2.1. Definition

XLMR is defined as proportion of mental retardation indicating distinctive pattern of inheritance associated with X-chromosome (Ijntema, 2001). General characteristic of XLMR recessive inheritance are demonstrating the following pattern: (Kingston, 2002)

- Only male affected almost exclusively.
- Transmission through carrier females.

- No male to male transmission.
- All daughters of affected males will be carriers.

II.2.2. Prevalence

Contribution of X-chromosome mutations to the spectrum of mental retardation has become subject of interest for many years. It was Penrose in 1938 who reported for the first time that mental retardation is significantly more common in males than in females, with the ratio of affected males to females being 1.3:1 (Penrose, 1938). Following studies described large families with X-linked inheritance pattern arising concept that X-linked genetic defects play an important role in the etiology of MR. It was predicted that XLMR (including monogenic and multiple gene XLMR) might be contribute to up to 20-25% of mental retardation (Turner, 1996). In 2005, Roper and Hamel predicted that monogenic XLMR might be contribute to up to 10-15% of mental retardation (Ropers and Hamel, 2005). In 1980, Herbst and Miller estimate that the prevalence of XLMR was about 1.83/1000 males (Herbst and Miller, 1980), with the fragile-X syndrome being considered as the most prevalent condition (20% of all XLMR cases) (Fishburn,1983). Later on, the estimation was reduced into 10–12% of all MR cases in males by the finding of a much smaller contribution of individual genes other than FMR1, to XLMR (Mandel and Chelly, 2004; Ropper and Hamel, 2005).

II.2.3. Classification of XLMR

Kerr in 1991 suggested classification of XLMR into syndromic (MRXS) and non-syndromic (MRX) (Kerr, 1991). Syndromic MRXS refers to condition associated with distinctive clinical features. Nonsyndromic MRX is associated with nonprogressive condition that affects cognitive function without any other distinctive features (Gecz and Mulley, 2000). Trinucleotide repeat expansion on FMR1 gene that cause Fragile X syndrome is generally regarded as the most common cause of XLMR with the prevalence of 1/4000-1/8000 (Hagerman, 2008). Nowadays, at least 215 different monogenic X-linked mental retardation

disorders have been described : 149 with specific clinical findings, including 98 syndromes and 51 neuromuscular conditions, and 66 nonspecific (MRX) forms (Chiurazzi *et al.* 2008). More than 90 XLMR-associated genes have been identified, which at least 53 were for syndromic, 27 for nonsyndromic, and 11 for both syndromic and nonsyndromic forms of mental retardation, which show the heterogeneity of XLMR (Figure 3; Table 2; Chiurazzi *et al.,* 2008; Tarpey *et al.,* 2009; Greenwood Genetic Center 2010; XLMR Website 2010).

II.2.4. Identification of genetic defects involved in XLMR

The effort to identification genetic defects involved in XLMR has been developed since many years ago. There are several methods developed to address this effort, namely positional cloning, candidate gene, mutation analysis of the known gene, array method, and the newest, next generation sequencing.

II.2.4.1. Positional Cloning

Positional cloning is intended to localize determinants of disease susceptibility in the DNA sequence prior to determining their function (Maniatis *et al*., 2004). This method identifies a disease gene based on no information except its approximate chromosomal location. Linkage mapping is routinely used to get the position information. In this method, it is important to define the candidate region as tightly as possible, considering the disadvantage of this method of being expensive and time- and resource- consuming (Strachan and Read, 1999; Zhu and Zhao, 2007).

Later on, it was found that chromosomal aberrations can provide a useful short-cut to locating a disease gene. Translocation could give a chance to clone the X-chromosome gene which is disrupted by the translocation (Strachan and Read, 1999). Small-scale deletions (microdeletions) are also valuable for positional cloning in which the deletion could encompass gene that cause XLMR. Using positional cloning methods, several MRX genes have been identified, for example: *FMR2* (Knight et al, 1994), *ZNF81*(Kleefstra *et al.,* 2004)*, OPHN1, TM4SF2, IL1RAPL1,* and *ARHGEF6* (Ijntema 2001).

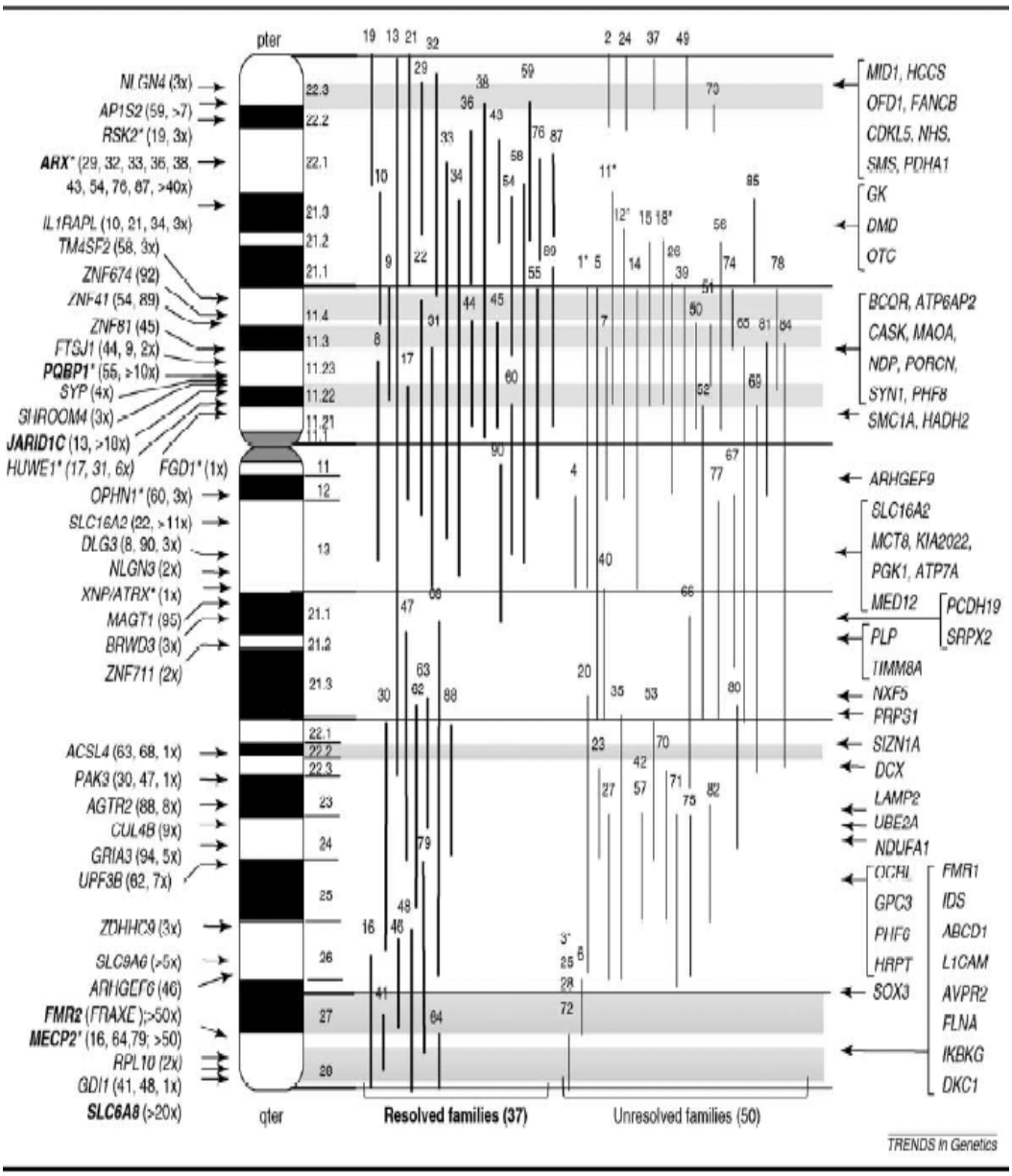


Figure 3. Ideogram of human X-chromosome showing genetic heterogeneity of XLMR. Genes in left side are currently known to be mutated in NS-XLMR (n=38). Genes in the right side are known to be mutated in syndromic XLMR (n=52). Vertical lines shows linkage interval in MRX families. Asterisks sign near the gene names show genes which is mutated in both syndromic and non-syndromic XLMR (n=11) (adopted from Gecz, 2009).

Table 2. Genes known to be mutated in non syndromic XLMR

Gene Symbol	Gene Name	Protein Function	Years Found	References
AFF2 (FMR2)	FMR2 family, member 2	Transcription regulation	1996	Gecz *et a*l. 1996 Bensaid *et al*. 2009
OPHN1	Oligophrenin 1	Axon guidance, signal transduction, Rho-GTPase-activating protein	1998	Billuart *et al.* 1998
PAK3	p21 protein (Cdc42/Rsc)-activated kinase 3	Axon guidance, signal transduction, actin cytoskeleton regulation	1998	Allen *et al*. 1998
GDI1	GDP dissociation inhibitor 1	Signal transduction, regulation of GTPase activity	1998	D'Adamo *et al*. 1998
RPS6KA3	Ribosomal protein S6 kinase, 90kDa, polypeptida 3	Kinase, post-transitional modification	1999	Merienne *et al.* 1999
MECP2	Methyl-CpG binding protein 2	Transcription regulation	1999	Amir *et al.* 1999 Orrico et al. 2000
IL1RAPL1	Interleukin 1 receptor accessory protein-like 1	Signal transduction; innate immune response	1999	Carrie et al. 1999
ATRX	α-thalassemia-mental retardation, X-linked	Transcription regulation, chromatin remodelling protein	2000	Gibbons and Higgs, 2000
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor 6	Actin cytoskeleton regulation	2000	Kutsche et al. 2000
TM4SF2	Transmembrane 4 superfamily member 2	Signal transduction, neurite outgrowth, integrin binding	2000	Zemni et al. 2000
SLC6A8	Solute carrier family 6 (creatine), member 8	Sodium ion transport, neurotransmitter transport, muscle contraction	2002	Hahn et al. 2002
FGD1	FYVE, RhoGEF and PH-domain-containing 1	Actin cytoskeleton regulation, Rho/Rac guanine nucleotide exchange factor	2002	Lebel et al. 2002
ARX	Aristaless-related homeobox	Transcription regulation	2002	Stromme et al. 2002
ACSL4	Acyl-CoA synthetase long chain family member 4	Fatty acid metabolism, fatty-acid-coenzyme A ligase	2002	Meloni et al. 2002
AGTR2	Angiotensin II receptor type 2	G-protein signalling, renin angiotensin system	2002	Vervoort et al. 2002
ZNF41	Zinc finger protein 41	Transcription regulation	2003	Shoichet et al. 2003

Gene Symbol	Gene Name	Protein Function	Years Found	References
PQBP1	Polyglutamine-binding protein 1	Transcription regulation	2003	Kalscheuer et al. 2003
NLGN4X	Neuroligin 4, X-linked	Cell adhesion molecule	2003 2004	Jamain et al. 2003
NLGN3	Neuroligin 3	Cell adhesion molecule, synaptic transmission	2003	Laumonnier et al. 2004
ZNF81	Zinc finger protein 81	Transcription regulation	2004	Kleefstra et al. 2004
DLG3	Dics, large homolog 3	Signal transduction, kinase, NMDA receptor localization	2004	Tarpey et al. 2004
FTSJ1	Ftsj homolog 1	Nucleolar protein, modification of rRNA	2004	Freude et al. 2004
JARID1C	Jumonji, AT rich interact domain 1C	Transcription regulation, chromatine modifier	2005	Jensen et al. 2005
SHROOM4	Shroom family member 4	Cytoskeletal architecture, protein-protein interaction	2006	Hagens et al. 2006
ZNF674	Zinc finger family member 674	Transcription regulation	2006	Lugtenberg et al. 2006
AP1S2	Adaptor-related protein complex 1. sigma 2 subunit	Recruits clathrin to vesicular membranes	2006	Tarpey et al. 2006
RPL10	Ribosomal protein L10	Assembly of large ribosomal subunit, protein synthesis	2005	Klauck et al. 2006
CUL4B	Cullin 4B	E3 ubiquitin ligase, proteolysis of DNA replication regulator	2007	Tarpey et al. 2007
ZDHHC9	Zinc finger, DHHC-type containing 9	Kinase, post-translational modification	2007	Raymond et al. 2007
BRWD3	Bromodomain and WD repeat domain containing 3	Intracellular signaling pathways affecting cell proliferation	2007	Field et al. 2007
UPF3B	UPF3 regulator of nonsense transcripts homolog B	Nucleotide binding, nonsense-mediated mRNA decay	2007	Tarpey et al. 2007
GRIA3	Glutamine receptor, ionotrophic, AMPA3	Signal transduction, excitatory neurotransmitter receptor	2007	Wu et al. 2007
HUWE1	HECT, UBA-and WWE-domain containing 1	E3 ubiquitin ligase, p53 associated regulation of neuronal cell cycle	2008	Froyen et al. 2008
SLC9A6	Solute carrier family 9 (sodium-hydrogen exchanger)	Sodium ion transport, pH regulation	2008	Gilfillan et al. 2008

Gene Symbol	Gene Name	Protein Function	Years Found	References
MAGT1	Magnesium transporter 1	N-Glycosylation of neuronal cell adhesion molecules	2008	Molinari et al. 2008
ZNF711	Zinc finger protein 711	DNA replication	2009	Molinari et al. 2008
CASK	Calcium/calmodulin-dependent serine protein kinase	Kinase, post-translational modification	2009	Tarpey et al. 2009
SYP	Synaptophysin	Synaptic vesicle maturation and membrane organization	2009	Tarpey et al. 2009

(adopted from Gecz, 2009)

II.2.4.2. Positional Candidate Gene Analysis

A purely positional approach is often inefficient because candidate regions identified by positional cloning usually contain dozens of genes, which will be time-consuming and labour-consuming to screen them all. This matter can be resolved by combining both positional and non positional information in a positional candidate gene approach. This method uses mutation analysis of the most promising functional candidate genes encompassed by linkage intervals (Strachan and Read, 1999). Several genes that have been found by this approach are: *GDI1*, *PAK3*, and *RSK2* (Mulley, 2008). This method brings advantages for being effective and economical method for direct gene discovery. However, the practicability of this approach is limited by its reliance on prior knowledge about the known or presumed biology of the phenotype under investigation, necessity of discrete phenotypic differences, and also necessity of highly subjective in the process of choosing specific candidates from numbers of potential possibilities. (Zhu and Zhao, 2007).

Nowadays, the technology development emerges several bioinformatic tools that could help in candidate gene prioritization. This tools use concept of data-fusion (Figure 4) which prioritizing candidate genes based on combined information from many sources, including converging actual experimental data, web database-based resources (including literature-based resources and biological ontology resources) or the theoretical assembling of molecular features or

molecular interaction principles, e.g., gene structure variation, homologs, orthologs, SNPs data, protein-DNA interactions, protein-protein interactions (interactome), molecular module, pathway and gene regulatory network (Aerts et al. 2006). Several bioinformatic tools that use data-fusion for prioritizing candidate genes are Endeavour (Aerts *et al.* 2006) and ToppGene (Chen, 2007).

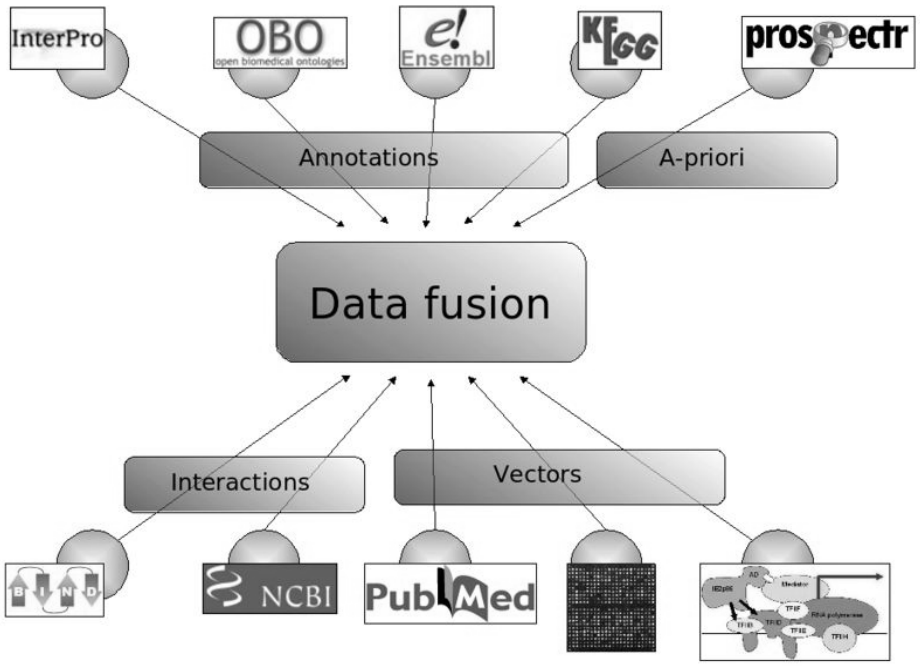


Figure 4. Concept of data fusion (adopted from KU Leuven, 2010).

Both Endeavour and ToppGene use the training genes, genes already known to be involved in the process under study, as model. Then, the model is used to score the candidate genes based on the similarity and rank them according to their score (Chen, 2007). The basic difference of those software is that Endeavour use Blast, cis-element and transcriptional motifs in sequence features and annotation, while ToppGene not. The other difference is that ToppGene use mouse phenotype in the annotation, and PubMed ID for literature information, while Endeavour not using mouse phenotype and use keywords in abstract for literature data (Table 3; Chen, 2007). In 2008, Endeavour extend the framework to several model organism, so it can be performed for *M. musculus*, *R. norvegicus*

and *C. elegans*, and also developing the versions for *D. rerio* and *D. Melanogaster* (Tranchevent et al., 2008). However, it is important to remember that prioritizing candidate genes is only worked for syndromic MR.

Table 3. Comparisson between Endeavour and ToppGene

Feature type	**ENDEAVOUR**	**ToppGene**
Sequence Features & Annotations	Blast *cis*-element Transcriptional motifs	Not used
Gene Annotations	Gene Ontology	Gene Ontology Mouse Phenotype
Transcript Features	Gene expression EST expression	Gene expression
Protein Features	Protein domains Protein interactions Pathways	Protein domains Protein interactions Pathways
Literature	Keywords in abstracts	Co-citation (PMIDs)

(adopted from Chen et al., 2007)

II.2.4.3. Mutation analysis of known gene

XLMR is a clinically complex and genetically heterogeneous disorder arising from many mutations along the X chromosome. Lately, two large studies by de Brouwer et al and Raymond et al showing the contribution of point mutations to XLMR. Brouwer et al in 2007 screened 90 known and candidate XLMR genes in a cohort of 600 families of varying size and identified 73 mutations in 21 genes, resolving 42% of the families (de Brouwer et al., 2007). Fascinatingly, this study also showed that there was no significant difference between the proportion of resolved large families with LOD >2.0 and smaller families with LOD <2.0. Tarpey et al in 2009 screened the coding regions of 718 genes in probands from 208 families and detected 1,858 different coding sequence variants (Tarpey et al., 2009). In this study, the proportion of resolved brother pairs and larger families were quite similar, 21% versus 23%, which indicating that a considerable proportion of affected brother pairs might result from X-linked mutations.

II.2.4.4. Array technology

This technology appeared seven years ago, when Veltman et al described microarray-based copy number analysis of all human telomeres in patients with mental retardation (Veltman et al., 2002). Later on, microarrays have developed and target not only the telomeres, but even entire genome at varying resolution levels (Menten et al., 2006). In the beginning, the array technology used clone-based genomic microarrays was only available to researchers with dedicated microarray facilities. Nowadays, these microarrays have been replaced by commercially available microarrays using oligonucleotide probes with higher genome coverage that can easily be put into practice in clinical diagnostic laboratories (Koolen et al., 2009). Increasing resolution of the different array platforms open up the possibility to detect smaller and smaller genomic copy number variations (CNVs) (Vissers et al.,2009). There were several different chromosome X specific DNA microarrays developed and applied for screening of XLMR families in search for new causative mutations (Bashiardes et al., 2009). The first chromosome X-specific array CGH study using tiling resolution BAC array gave causal hit in 3 of 40 patients with nonspecifix-XLMR (Lugtenberg et al.,2006*a*), later followed by identification of novel nonspecific XLMR gene by this approach (Lugtenberg et al, 2006*b*), indicating that this method is useful in XLMR. However, the array practice is still hampered by the high cost needed and challenging interpretation of the CNV results. Nowadays, 2.7M array is available, With unbiased, whole-genome coverage and the density of 2.7 million copy number markers, this array enables detection of the smallest submicroscopic aberrations, including those that would have been missed with classical array techniques (Affymetrix).

II.2.4.5. Next generation sequencing

The newest advances in DNA sequencing technologies, called next-generation sequencing (NGS) technologies, are now enabling the comprehensive analysis of whole genomes, transcriptomes and interactomes. This method capable of detecting both single base mutations and structural variation (Visser et

al., 2009), with capability of reading 400 K—4 M sequences compared with the traditional 96 capillary, and reading length from 25 to 450 basepairs, depending on the platform (Mardis, 2008). In shotgun sequencing, the genome is cut up into smaller fragments of DNA which can be massively sequenced in parallel. Subsequently, the sequenced fragments are assembled into contigs based on the overlap in the sequence reads or, alternatively, aligned and compared to a reference genome which will bring to disease-gene identification. This promising method, however, still limited by its high cost. Clinical and biological interpretation the variants resulted from this method will require large international and multidisciplinary collaborative efforts (Visser et al., 2009)

II.3. X-Chromosome Linkage Analysis

Before researchers could elucidate and finally sequence the gene responsible for a disease, it must be first mapped, located in the Genome. Genetic linkage analysis plays role in identification regions of the genome containing genes (locus) that predispose to disease by use of observations of related individuals (Teare and Barret, 2005). This method works using short tandem repeat (STR)-markers or microsatellite, a well-characterized regions of DNA that consist of multiple repeats of a short sequence (typically 2–8 bp) and highly show genetic variation (polymorphism) in nature (Weber, 1990). Researcher are looking for a marker that is consistently present in those that are affected, and is not present in non-affected relatives, assuming that a causative genetic variant is likely to lie close to that marker (Burton et al. 2005). Linkage analysis work on the principle of cosegregation of stretches of DNA in families rearranged by recombination events in meiosis. The probability of recombination between two loci at meiosis is called *recombination fraction* (Θ), which can be utilized as a stochastic measure for the genetic distance between two genes (Massanet, 2009). The further apart two loci are from each other on a chromosome, the greater the probability is that a recombination will occur between them(hypothesis null assumes no linkage, or Θ=0.5) (Teare and Barret, 2005). Two loci segregate together more often if they are located close enough together on the same

chromosome (in other words the chance of recombination is less than 50%, or alternative hypothesis assumes linkage exists (Θ<1/2)) (Burton et al. 2005). The expected numbers of recombination occurring between two loci on a single chromatid during meiosis is called genetic map distance (in units of Morgans) (Teare and Barret, 2005). Linkage is described in linkage interval and scored in logarithm of the odds (LOD) score, a function of the recombination which indicates how much higher the likelihood of the data is under linkage than under the absence of linkage (Massanet, 2009).

$$LOD(\theta) = \log_{10} \frac{likelihood(\theta)}{likelihood(\theta = 1/2)}$$

Morton (1955) proposed a critical value of LOD score=2 for significant linkage in X-linked locus (Morton, 1955). Linkage can be excluded from the region if the LOD score is below -2. This approach is called *exclusion mapping* (Massanet, 2009). In mental retardation, linkage analysis is often used as first stage to narrow down region of interest into linkage interval in effort to find evidence of containing a disease gene (Teare and Barret, 2005). Candidate gene present in the linkage interval can be used as a target of sequencing to find the disease causing genes (Lugtenberg et al., 2006).

II.4. X-Chromosome Inactivation

X-chromosome inactivation (XCI) is described as the transcriptional silencing of one of the two X-chromosomes in female mammalians (Orstavik, 2009). Males have one copy whereas females have two copies of the X chromosome, and this potential dosage difference from the two X-chromosomes in females is equalized by inactivating one X in humans and other mammals at 1N (Agrelo and Wutz, 2009; Nora and Heard, 2009). As the result, females are mosaics for two cell populations cells with either the paternal or the maternal X in the active form (Kristiansen et al., 2005). This mechanism occurs in early embryonic life at the preimplantation stage following early whole-genome activation, and is stochastic and permanent for all descendants of a cell (Berg et al., 2009). This event is orchestrated by the X-inactivation center (Xic) located on

the X-chromosome (Royce-Tolland and Panning, 2008). The silencing mechanism of the X-chromosome is a complex mechanism involving interplay between noncoding transcripts such as Xist, chromatin modifiers, and factors involved in nuclear organization (Chow and Heard, 2009). Most of the X-chromosome, with exception of pseudoautosomal regions at Xpter and Xqter, participates in the inactivation (Miller et al., 1995).

Generally, X-chromosome inactivation is a random process, which result in 50% of cells expressing the paternal and the remaining 50% expressing the maternal genes (Migeon, 2007). Once this ratio is established, it remains fixed for all descendants of a particular cell. This random inactivation is altered in the presence of certain gene mutations and genomic alternations, where the chromosome bearing the mutated gene or region is preferentially inactivated. If there is a marked deviation from this 50:50 ratio, then it will be called skewing of XCI, arbitrarily defined as preferential inactivation of either the maternally or paternally inherited X-chromosome in 30:70 or more of cells (Plenge et al., 2002). A ratio of X-inactivation of >90:10 is defined as marked skewing of X-inactivation (Stevenson and Schwartz, 2009).

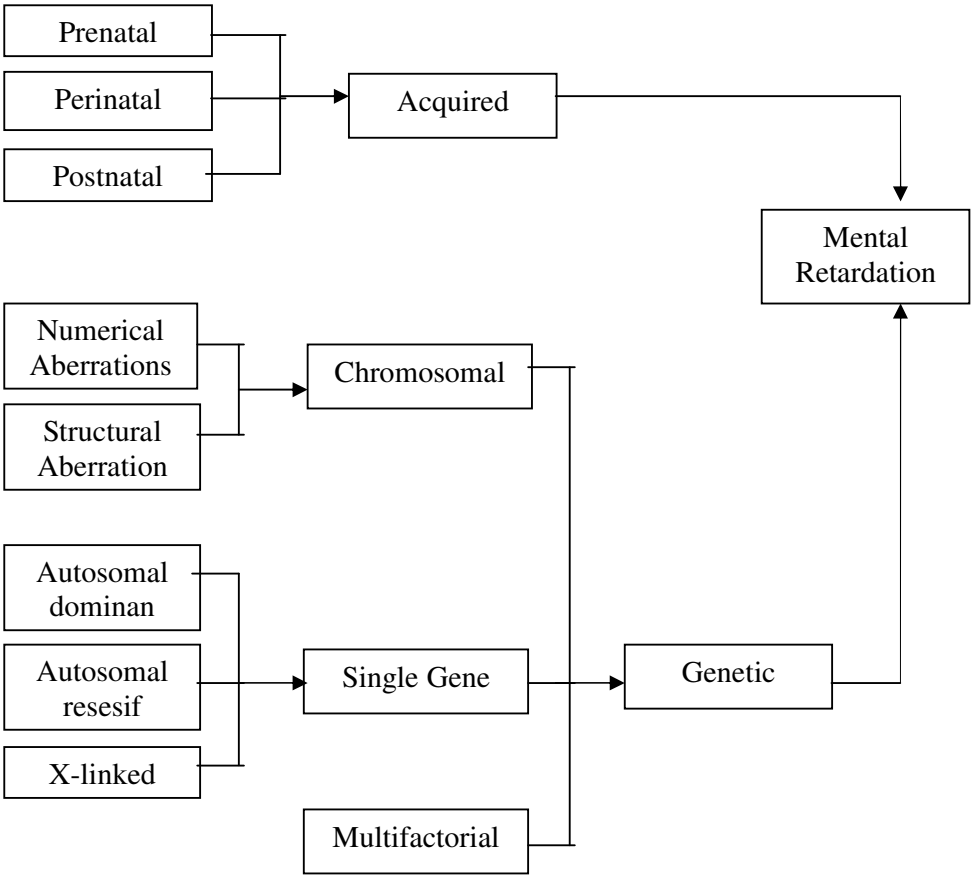
In XLMR, skewed X-chromosome inactivation is often observed in phenotypically normal females who carry the mutant gene. This phenomena is presumed to work as selection against cells that express the mutant allele during early development and the degree of skewing can vary between different tissues (Muers, 2007). Previous studies of families with XLMR indicated skewed XCI in all carriers in three of 19 (Raynaud et al., 2000) and four of 20 families (Plenge et al., 2002). Skewed X-chromosome inactivation is more or less consistently seen in carriers of genomic duplications and X-linked alpha-thalassemia mental retardation syndrome (ATRX) mutations. Also, marked skewing of X-inactivation is less consistently present in carriers of other XLID disorders (Plenge et al.,2002). So, skewed XCI in the mother of an affected male may indicate the presence of XLMR. However, random XCI does not exclude the possibility of an X-linked disorder (Orstavik et al., 2009).

II.5. Genetic Counselling

The implication of a genetic diagnosis on an individual will also affect the entire family. Thus, genetic counselling is crucial in genetic condition. Genetic counseling is described as "... the process of helping people understand and adapt to the medical, psychological and familial implications of genetic contributions to disease,". (National Society of Genetic Counselors' Definition Task Force et al. 2006). In this process, genetic counselors play pivotal roles in risk assessment and patient counseling, consultation and case management, and education for patients and providers (O'Daniel, 2009). Risk assessment is important for prospective parents, especially couples who already have a child with mental retardation. Parents are keen to know the risk of their next child being affected. This information may help them make informed decisions about having the next child. (WHO, 2010).

Despite the importance and advantages of genetic counselling, many children who should be receiving genetic counseling and testing often do not receive all of the services they require (Wang and Watts, 2007). Data from American Academy of Paediatric have indicated that families of children with mental retardation perceive significantly higher need for genetic counselling compared to other children with special need. Data from 2005–2006 National Survey of Children With Special Health Care Needs also showed that access to genetic counselling services is affected by several barriers: the lack of a medical home, the lack of insurance, low family income and low education attainment (McGrath et al., 2009). There are also several factors influencing transmission of genetic counselling information inside family members. First-degree family members are more frequently informed compared to second- or third-degree family member (Claes et al., 2003). Gender is also play role in this process, as women are more likely to communicate (d'Agincourt-Canning, 2001). Intrafamily mode of communication and emotional bond, mode of inheritance of the genetic condition, positive family history and the perception ofthe ability to act on the genetic information are also affecting the transmission of information (Forrest et al., 2008).

II.6. Theoritical Framework



CHAPTER III
RESEARCH METHOD

III.1. Research Aspect

III.1. 1. Research Field

This research was in the field of Molecular Genetics, intercorrelated with Clinical Genetics.

III.1.2. Research Location

Indonesian families from patients and several special schools for intellectual disabilited people in Semarang and patients were collected and examined. Conventional cytogenetic analysis for Indonesian patients was carried out in the Molecular and Cytogenetic Laboratory of Center of Biomedical Research, Faculty of Medicine Diponegoro University Semarang. Dutch families were collected from available DNA from the Radboud University Nijmegen Medical Center (RUNMC), the Netherlands. DNA analysis for Fragile-X syndrome, linkage analysis, X-chromosome inactivation status and sequencing analysis of candidate gene were performed in the department of Human Genetics, Radboud University Nijmegen Medical Center (RUNMC), the Netherlands.

III.1.3. Research Period

Sample collection ,conventional cytogenetic analysis and DNA extraction: 6 months. Molecular analysis: 12 months.

III.1.4. Research Design

This was a descriptive study.

III.1.5. Variables

- Dependent variable: X-linked mental retardation

Scale: Nominal

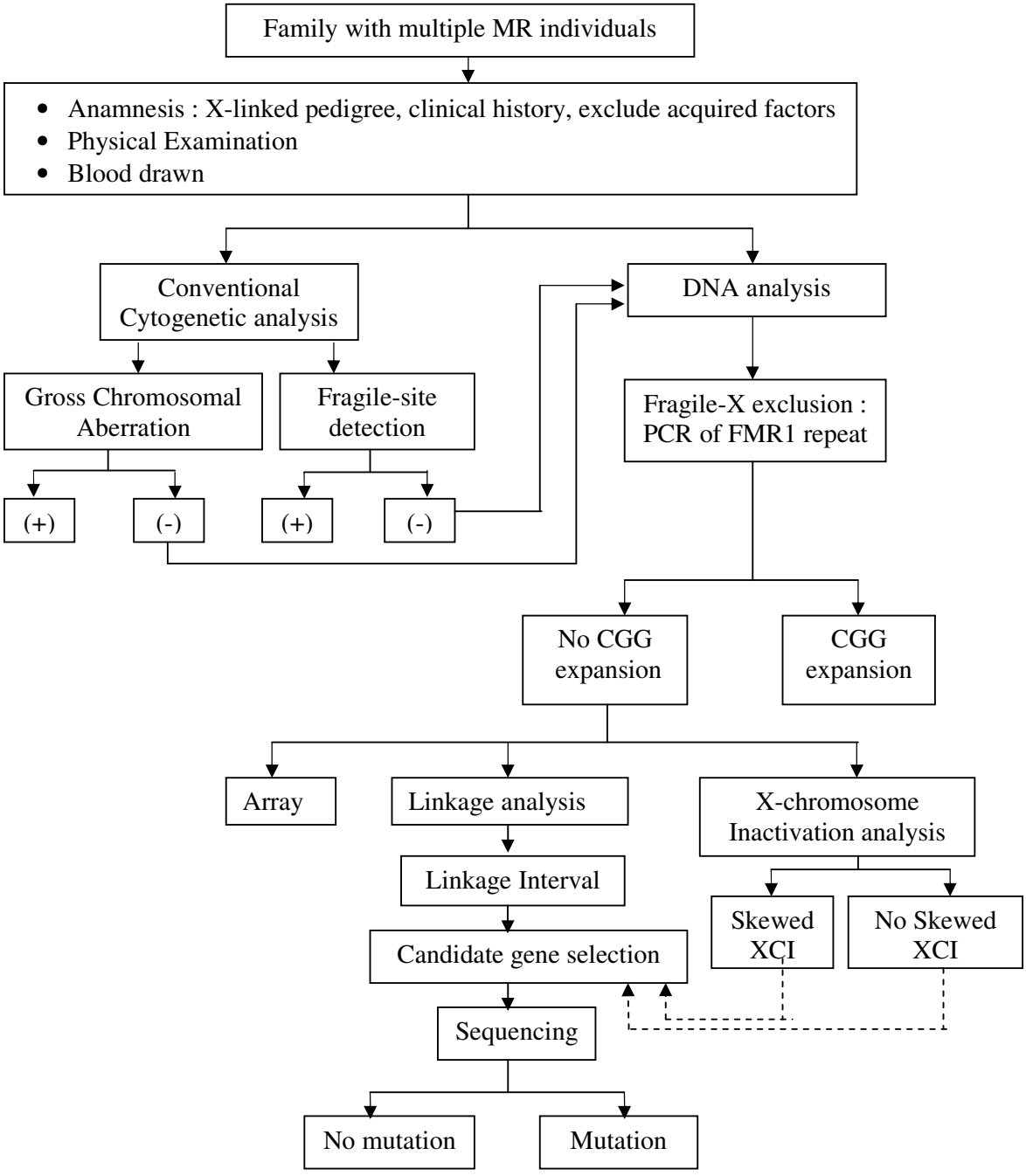
- Independent variable: Cytogenetic and molecular result

Scale: Nominal

III.1.6. Operational Definition

- **Phenotype** : all clinical features
- **Genotype** : all genetic defects found in molecular analysis
- **Mental Retardation:** According to American Association on Intellectual Developmental Disabilities :
    - IQ<70
    - concomitant limitations in two or more areas of adaptive skills
    - Onset before the age of 18
- **X-Linked Mental Retardation:** Families with a pedigree suggestive of X-linked inheritance:
    - at least two males with mental retardation with or without additional clinical findings for the Indonesian families
    - at least two males with syndromic mental retardation for the Dutch families
    - predominant sparing of carrier females
    - no evidence of male-to-male transmission of mental retardation

III.1.7. Research Protocol



III.2 . Method

III.2.1. Population

Families with multiple individuals of mentally retarded were included in this study. Families originated from Indonesia and the Netherlands.

III.2.2. Samples

Samples were collected from family members of the Indonesian and Dutch families which showing X-linked inheritance from the pedigree.

III.2.2.1 Inclusion Criteria

- Families with a pedigree suggestive of X-linked inheritance:
    * at least two males with mental retardation with or without additional clinical findings
    * predominant sparing of carrier females
    * no evidence of male-to-male transmission of mental retardation.
- DNA available from two or more affected family members and parents.
- written informed consent obtained

III.2.2.2 Exclusion Criteria

- X-linked families with clinical suspicion of known MR syndromes, for example: Down Syndrome.

III.2.2.3. Clinical Examination

Indonesian families: patients was clinically examined, according to the RUNMC form, by a medical doctor from CEBIOR Semarang. Clinical photograph was taken from the affected children.

Dutch families: patients were clinically examined by a clinical geneticist form the department of Human Genetics, RUNMC, the Netherlands.

III.2.2.4. Sample Collection

Indonesian families: For all patients, siblings and parents, 5 mL heparinized blood was obtained for conventional cytogenetics and 5-10 mL EDTA blood was obtained for DNA isolation.

Dutch families: for all patients heparinized blood was obtained for EBV transformation of lymphocytes and EDTA blood was obtained for DNA isolation.

III.2.2.5 Minimum samples required

No minimal number of samples required, as this study is a molecular study (not a population study).

III.3. Work-flow

III.3.1. General

Figure 5 illustrates general workflow of this research. The first step was a thorough clinical work-up, which could exclude acquired factor from anamnesis. Pedigree of the family was drawn to describe the mode of inheritance in the family. Physical examination with special attention on clinical dysmorphologic examination was performed as described above to exclude known syndrome for example Down Syndrome (Appendix 1). Blood samples was taken from all families for cytogenetic preparation and DNA isolation. Conventional cytogenetic analysis was performed in all families to exclude gross chromosomal abnormalities and also by paying special attention to fragile-site, followed by analysis of CGG repeat to exclude Fragile-X. Linkage analysis was conducted with highly polymorphic STR-markers evenly spread over the X-chromosome to find the linkage interval. An *FMR1* X-chromosome inactivation assay was performed to determine the X-inactivation status of carrier females from all families. Females that were uninformative for *FMR1* were analyzed for X-chromosome inactivation status by using the CAG repeat of *AR*. Promising candidate genes were selected in linkage intervals on the X-

chromosome using bioinformatic tools (ToppGene and Endeavour; Chen *et al*, 2007; Tranchevent *et al*, 2008) and by manual selection based on the expression in brain/neuronal tissues, homology with known MR genes, involvement in the same protein network as already known MR genes, and X-inactivation status of the genes. Mutation analysis of the most promising candidate genes was performed. 2.7M array was performed on one affected of each syndromic XLMR families. More details about chromosomal preparation procedures, DNA Isolation, FMR1 gene amplification, X-Chromosomal Linkage Analysis, X-Chromosome inactivation analysis and candidate gene selection procedures can be found in the appendix section.

III.4 Collected Data

III.4.1 Primary Data:

MR patients including personal data: date of birth and pedigree.

III.4.2. Secondary Data:

Medical records from special schools and medical record from the RUNMC.

III.5 Data analysis

Data was analyzed with the descriptive method and presented in tables and graphics.

III.6. Ethical Implication

- This research involved affected person which unable to give consent. Informed consent will be obtained from the parents. Parents were given right to decline their involvement in this research. Informed consent form is attached in the Appendix 1.
- All data including patients and family identity, clinical, and laboratory data was confidential.

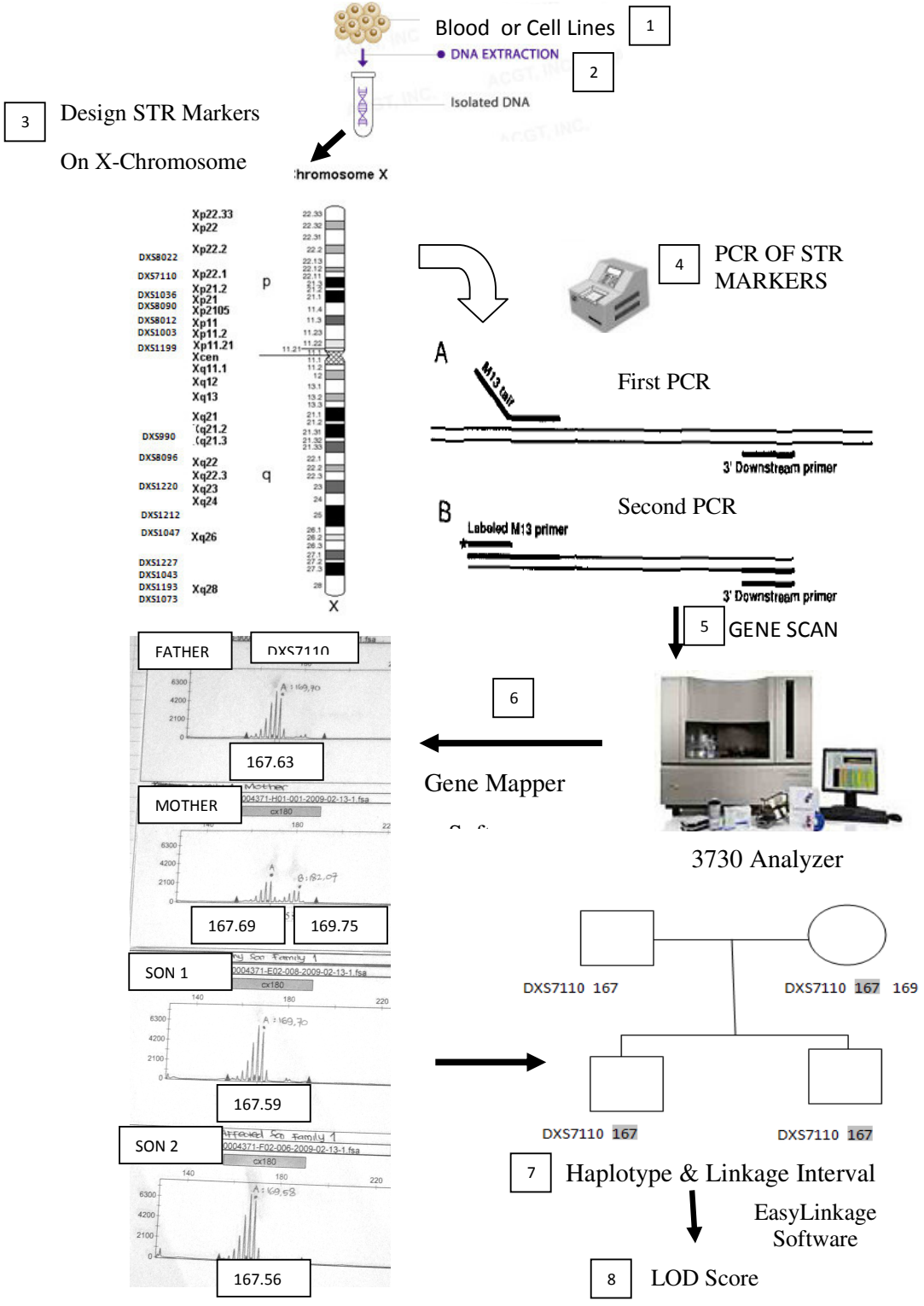


Figure 5. Multiple steps in linkage analysis

CHAPTER IV
RESULTS AND DISCUSSION

IV.1 Clinical Findings

Clinical examination was performed in Indonesian samples based on standarized protocols from Radboud University of Nijmegen (Appendix 1). A detailed description of antropometry parameters and dysmorphisms also described in the form. In four families, the pedigree and clinical examinations were compatible with syndromic XLMR (table 3). Six families presented with non syndromic XLMR. Main clinical features in the syndromic XLMR families were hydrocephalus (family P03-0452 and 13753/HC), hypomyelination (family W092-053) and overgrowth features (family DF27004) (table 4).

Table 4 . Summary of Dysmorphological Features

Family Number	Indonesian /Dutch	Syndromic/ Non Syndromic	Dysmorphological Features
P03-0452	Dutch	Syndromic	mental retardation, congenital hydrocephalus, short stature, obesity, hypogonadism
13753/HC	Dutch	Syndromic	mental retardation, congenital hydrocephalus
W92-053	Dutch	Syndromic	mental retardation, blindness, convulsion, spasticity, early death, hypomyelination
DF27004	Dutch	Syndromic	Mental retardation, macrocephaly, hepatomegaly, kidney enlargement
W09-0071	Indonesian	Non Syndromic	mental retardation, sandal gap, flat foot, high arched palate, tappering pad, short third toe
W09-0072	Indonesian	Non Syndromic	mental retardation, prominent ear, long face, broad nasal bridge
W09-0074	Indonesian	Non Syndromic	mental retardation, prominent ear, macroorchidism
W09-0078	Indonesian	Non Syndromic	Mental retardation, long face, heavy eyebrow, prominent ears, sandal gap, pes planus
W08-2152	Dutch	Non Syndromic	Mental retardation, autism, epilepsy, long narrow face, deep set eyes, high nasal bridge, macroorchidism, short fifth metatarsal, long finger and toes

Family Number	Indonesian /Dutch	Syndromic/ Non Syndromic	Dysmorphological Features
W07-604	Dutch	Non Syndromic	Mild to moderate mental retardation, behavioral problems

IV.2 Conventional Cytogenetic analysis

Conventional cytogenetic analysis was performed in all Indonesian and Dutch samples. None of the affected probands showed any macroscopic choromosomal abnormalities.

IV.3 Fragile-X exclusion test

PCR analysis of the CGG repeat in the promoter region of *FMR1* was performed in all Indonesian samples. None of the affected probands showed any CGG repeat more than 55 (Table 5). In all Dutch families Fragile-X syndrome was already excluded previously.

Table 5. CGG repeat sizes in subject screened by *FMR1* analysis

Family Number	Patient Number	Gender	CGG Repeat	Remarks
W09-0071	50156	Male	28 repeat	Normal
W09-0072	WB70	Male	36 repeat	Normal
W09-0072	35/NK/08	Male	29 repeat	Normal
W09-0072	32/NK/08	Female	29 repeat 32 repeat	Normal

IV.4 Linkage Analysis

Linkage analysis was performed in eight families. Linkage analysis in family W92-053 and W07-604 had already performed in other center before. Main interval size was varied from 8 Mb to 121 Mb (Table 6).

Table 6. Linkage analysis result in all families

Family Number	Indonesian /Dutch	Interval	Interval Size	LOD Score
P03-0452	Dutch	Xq22.2-Xq27.2	37 Mb	0.2
		Xq27.2-Xq28	12 Mb	
13753/HC	Dutch	Xp21.1-Xq28	121 Mb	0.6
W92-053	Dutch	Xp11.3-q12	20 Mb	3.30

Family Number	Indonesian /Dutch	Interval	Interval Size	LOD Score
DF27004	Dutch	Xp22.2-Xq21.32	79 Mb	1.62
		Xq26.3-Xq28	17 Mb	
W09-0071	Indonesian	Xp22.2-Xp22.11	8 Mb	0.75
		Xp11.4-Xq25	81 Mb	
W09-0072	Indonesian	Xp22.2-Xp11.3	32 Mb	0.91
W09-0074	Indonesian	Xp22.2-xp11.3	32 Mb	0.3
		Xq25-Xq28	31 Mb	
W09-0078	Indonesian	Xq23-q27.3	45 Mb	1.39
W08-2152	Dutch	Xq25-Xq28	31 Mb	1.36
W07-604	Dutch	Xp21.1-Xp22.2	28 Mb	

IV.5 X-Chromosome Inactivation Analysis

XCI status was found to be informative in 13 of the 18 female carriers for analysis of the CGG repeat in the promoter region of *FMR1*. Five females carriers that were uninformative for *FMR1* then were examined by analysis of the CAG repeat in the promoter region of *AR*. Skewed XCI (> 80% skewing) was observed in four (patient 50165 from family W09-0072, patient 51677 from family W08-2152, patient 29301 from family P03-0452, and patient 3485 from family W92-053; Table 7) of the eighteen female carriers.

Table 7. XCI status for Females

Family Number	Sample Number	% XCI		Remarks
		FMR1	*AR*	
W09-0071	50157	Uninformative	58%	No Skewing
	50161	Uninformative	46%	No Skewing
W09-0072	50165	100%		Skewing
	50169	59%		No Skewing
	50170	42%		No Skewing
	50172	Uninformative	64%	No Skewing
W09-0074	50174	56%		No Skewing
W09-0078	50207	36%		No Skewing
	50198	39%		No Skewing
	50209	Uninformative	74%	No Skewing
W08-2152	51677	100%		Skewing
W07-604	42721	41%		No Skewing
P03-0452	29301	Uninformative	0%	Skewing
13753/HC	26744	65%		No Skewing
	27003	54%		No Skewing

Family Number	Sample Number	% XCI		Remarks
		FMR1	AR	
W92-053	3485	100%		Skewing
DF27004	4992	41%		No Skewing
	4984	28%		No Skewing

IV.6 Mutation analysis in Candidate Genes

Due to specifical clinical features, candidate gene can only be selected in four syndromic XLMR families. Mutation analysis was performed in candidate genes and revealed no mutation (Table 8).

Table 8. Candidate genes sequencing

Family	Patient	Candidate Gene	Sequencing Relust
W92-053	3485	*HSD17B10*	No mutation
		SYP	No mutation
		SYN1	No mutation
		UBQLN2	No mutation
		ARHGEF9	No mutation
P03-0452	28558	*SLITRK2*	No mutation
		SLITRK4	No mutation
13753/HC	26857	*SLITRK2*	No mutation
		SLITRK4	No mutation
DF27004	33431	*GPC3*	No mutation

IV.7. Results and discussion for each family

Family W92-053 (XLMR and hypomyelination family)

Clinical Examination

Dutch family W92-053 has been reported before by Hamel *et al.* in 1999 (Hamel *et al*., 1999). From the history, it was described that for IV.5, IV.7, and IV.8, pregnancy and delivery had been uneventful and all were born with bilateral pes calcaneovalgus. Since the age of 3 months, the onset was started with gradual loss of vision, spastic tetraplegia and scoliosis, convulsions, secondary microcephaly, unexplained, febrile episodes, severe mental retardation, and failure to thrive. Hearing was normal. They never sat, crawled, nor spoke. Proband IV.5 died at the age of 29 months during a febrile episode. Proband IV.7 died at the age of 16½ months from aspiration pneumonia. Proband IV.8 died at the age of 26 months of aspiration pneumonia. Obligate and possible female carrier showed no abnormality.

Laboratory Analysis

Ophthalmological examination showed pale fundi in patient IV.5. The EEG showed an epileptic focus in the left frontotemporal region. The skeletal age was retarded as well. In patient IV.8, ophthalmological examination showed pale fundi as well, whereas the EMG was normal. Metabolic analysis showed no abnormalities (1971). Lysosomal enzymes (in 1971) were normal.

Patients IV.7 and IV.8 showed small brain for age, thin gyri, hypomyelination (Hamel *et al*., 1999). Patient IV.7 showed delicate optic nerves. Slightly enlarged lateral ventricles were found in family member IV.8.

Genetic Analysis

Conventional cytogenetic analysis showed no gross chromosomal aberration in IV.5, IV.7, and IV.8 (Hamel *et al*., 1999). Linkage analysis pointed to a 20 Mb linkage interval at Xp11.3-q12, with maximum lod score of 3.30 at θ=0.0 in marker DXS1204, with DXS337 and PGK1P1 as flanking markers (Hamel *et al*., 1999). Haplotypes in the family member are shown in Figure 6. X-Chromosome inactivation analysis on III.9 showed skewing X-chromosome inactivation.

Candidate Gene Selection

We checked the genes contents in the linkage interval using UCSC Genome Browser. There were 215 genes in the 20 Mb linkage interval. We performed candidate gene selection using manual selection and ENDEAVOUR, a bioinformatic tool that can be used to prioritize candidate genes.

We used 128 genes associated with demyelination and 14 genes associated with hypomyelination from NCBI Entrez Gene as a training set, and 215 genes in the linkage interval as test set for candidate gene prioritization with ENDEAVOUR (Fig 7). We did not choose other top genes such as *AR*, *UXT, ALAS2* for candidate genes because previous reports showed that mutation found in those genes were not associated with mental retardation features. Instead,we selecteded *HSD17B10, SYP, SYN1, UBQLN2* and *ARHGEF9* as candidate genes.

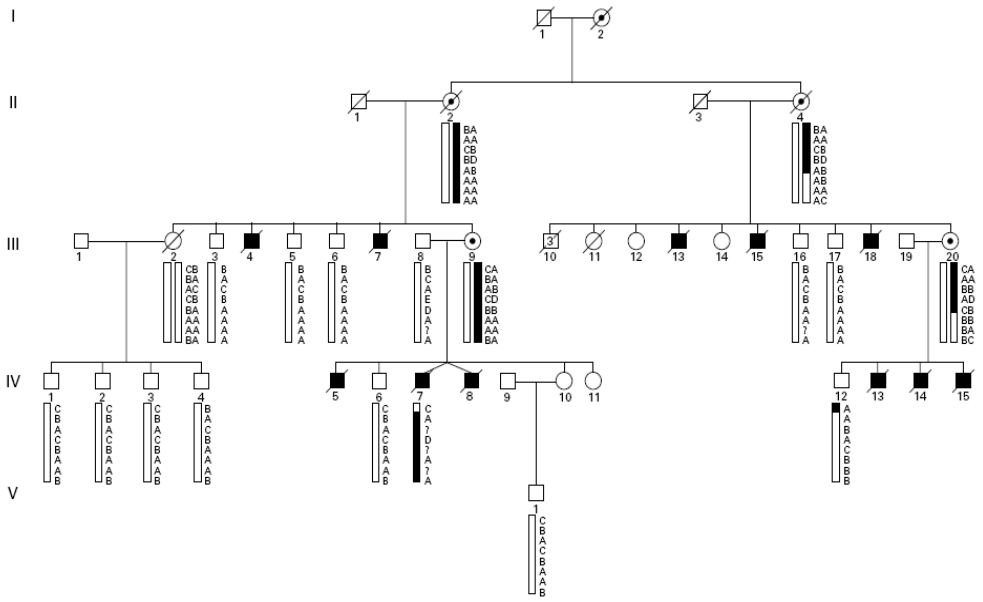


Figure 6. Haplotypes within family W92-053 as described by Hamel *et al* (adopted from Hamel *et al*., 1999). The cosegregating haplotype has been marked by a black bar. Filled symbols represent male patients with MR.

HSD17B10 encodes 17-beta-hydroxysteroid dehydrogenase X, a multifunctional mitochondrial enzyme that acts on a wide spectrum of substrates. Mutation in *HSD17B10* associated with HSD10 deficiency, which is characterized by normal or mildly delayed development in the first months or year of life, followed by regression of previously acquired motor and mental skills (Korman, 2006). Additional clinical features include myoclonic or other seizures, hypotonia, optic atrophy, pigmentary or non-pigmentary retinopathy, sensorineural deafness, ataxia, dystonia, choreoathetosis, spastic di-/tetra-plegia, cardiomyopathy frontotemporal or frontoparietal atrophy with enlarged ventricles parieto-occipital periventricular white matter and mild dysmorphism (Olpin *et al*.,2002; Sutton *et al*.,2003; Poll The *et al*., 2004; Perez-Cerda *et al*., 2005). *SYP* encodes synaptophysin, an integral membrane protein of small synaptic vesicles which are found in brain and endocrine cells. Previous reported mutation in *SYP* were associated with X-linked mental retardation and epilepsy (Tarpey *et al*., 2009). *SYN1* encodes synapsin I, a neuronal phosphoprotein associated with the membranes of small synaptic vesicles which may have a role in the regulation of neurotransmitter release. Previous reported mutation on this gene were associated with mental retardation, behaviour problems, and epilepsy (Garcia *et al*., 2004). *UBQLN2* encodes an ubiquitin-like protein (ubiquilin), which are thought to functionally link the ubiquitination machinery to the proteasome to affect in vivo protein degradation. *ARHGEF9* is a member family of Rho-like GTPases that act as key regulators of the actin cytoskeleton and are involved in cell signaling. *ARHGEF9* disruption is associated with X linked mental retardation and sensory hyperarousal (Marco *et al*., 2008). Direct DNA sequencing of *HSD17B10, SYP, SYN1, UBQLN2* and *ARGHEF9* in patient III.9 revealed no mutation.

Genetic Counselling

No candidate gene mutation was found in this family. However, linkage analysis revealed that this family shared clear X-linked inheritance and that patient II.1, II.2, III.9, III.20 (female) and IV.7 (male) shared similar risk haplotype which can affect their children. The rest of living male did not share

risk haplotype, thus there are no risk of developing this disorders in their next generation. This finding can be used for genetic counselling purpose

Discussion

We analysed *HSD17B10, SYP, SYN1, UBQLN2* and *ARGHEF9* in patient III.9 but found no mutation. Previously, there were already several XLMR syndrome associated with neurological features and early death according to Online Mendelian Inheritance in Man (OMIM; table 9). However, none of the conditions linked to the pericentromeric region.

hypodemyeanddemyelinization.txt **Test Set** **Results** **SprintPlot**

	Gene Symbol	Gene main ...	Precalculat...	Precalculat...	Blast	Interaction_...	Interaction_...	Interaction_...	Interaction_...	Interaction_...	Motif	Express
1	HSD17B10	ENSG0000...	0.452	0.234	3.464E-9.0	Infinity	1.103	2.0	Infinity	Infinity	0.957	0.124
2	AR	ENSG0000...	0.533	0.206	1.183E-12.0	3.333	2.056	3.0	2.58	5.5	0.889	0.028
3	UXT	ENSG0000...	0.612	0.719	29.12	5.0	1.266	2.0	1.25	2.0	0.931	0.097
4	ALAS2	ENSG0000...	0.281	0.243	517.687	Infinity	1.348	Infinity	1.0	Infinity	0.94	0.291
5	ARAF	ENSG0000...	0.392	0.646	4.243E-18.0	Infinity	1.305	5.333	2.667	12.0	0.911	0.378
6	PQBP1	ENSG0000...	0.528	0.703	618.87	Infinity	2.1	2.25	2.167	4.0	0.939	0.094
7	HDAC6	ENSG0000...	0.482	0.712	18.347	3.0	2.111	5.0	4.0	1.143	0.937	0.56
8	CFP	ENSG0000...	0.346	0.294	23.664	Infinity	1.212	Infinity	Infinity	Infinity	0.937	0.091
9	APEX2	ENSG0000...	0.561	0.298	314.986	Infinity	1.318	1.667	3.0	2.0	0.785	0.377
10	PLP2	ENSG0000...	0.563	0.423	565.685	Infinity	1.606	1.667	2.0	Infinity	0.862	0.376
11	ELK1	ENSG0000...	0.482	0.39	249.8	Infinity	Infinity	Infinity	2.5	1.5	0.921	0.376
12	SYP	ENSG0000...	0.392	0.682	466.905	Infinity	2.167	Infinity	1.5	Infinity	0.918	0.102
13	TFE3	ENSG0000...	0.392	0.305	45.299	Infinity	2.167	10.0	4.5	Infinity	0.963	0.103
14	FGD1	ENSG0000...	0.567	0.331	1.0E-163.0	Infinity	2.182	Infinity	2.5	Infinity	0.935	0.377
15	WAS	ENSG0000...	0.566	0.297	634.035	Infinity	1.889	9.0	2.286	7.0	0.88	0.532
16	SYN1	ENSG0000...	0.447	0.372	773.951	Infinity	1.786	3.0	1.786	Infinity	0.999	0.546
17	UBQLN2	ENSG0000...	0.482	□	446.279	1.5	Infinity	Infinity	9.0	4.0	0.853	0.097
18	OPHN1	ENSG0000...	0.325	0.292	514.896	Infinity	3.0	Infinity	4.0	Infinity	0.959	0.022
19	GATA1	ENSG0000...	0.482	0.3	90.648	Infinity	1.44	Infinity	2.0	1.5	0.941	0.102
20	HUWE1	ENSG0000...	0.325	0.461	64.738	Infinity	1.231	4.0	1.667	Infinity	0.943	0.69
21	MAGED2	ENSG0000...	0.567	0.684	13.491	2.0	Infinity	2.0	2.0	Infinity	0.96	0.095
22	CACNA1F	ENSG0000...	0.482	0.228	437.035	Infinity	6.0	Infinity	Infinity	Infinity	0.712	0.377
23	PIM2	ENSG0000...	0.482	0.745	3.464E-11.0	Infinity	2.75	6.0	4.5	Infinity	0.751	0.285
24	CLCN5	ENSG0000...	0.567	0.297	444.376	Infinity	5.167	Infinity	1.333	2.0	0.887	0.43
25	MSN	ENSG0000...	0.393	0.563	13.439	Infinity	1.634	2.25	3.0	Infinity	0.941	0.581

Figure 7. Top 25 candidate genes according to candidate gene prioritization with ENDEAVOUR for family W92-053.

The clinical presentations of patients in family W92-053 resemble HSD10 deficiency (formerly called 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency), which is predominantly characterized by neurodegenerative phenotype. In HSD10 deficiency, there is an increase in excretion of 2-methyl-3-hydroxybutyric acid (2M3HBA) and tiglylglycine (TG) and absence of 2-methylacetoacetic acid (2MAA) (Korman, 2006). However, in family W92-053,

there were no metabolic abnormalities found, considering the time of examination in 1971.

Table 9. XLMR syndromes associated with neurological features and early death

Name	MIM	Locus
Lubs X-linked mental retardation syndrome; MRXSL	300260	Xq28
Spinal muscular atrophy, X-linked 2; SMAX2	301830	Xp11.23
Adrenomyodystrophy	300270	-
Adrenoleukodystrophy; ALD	300100	Xq28
Rett Syndrome	312750	Xq28
Arts Syndrome	301835	Xq22-q24, Xq21.2-q24
Spastic Paraplegia 2 (SPG2)	312920	Xq22
Cantu	308830	-
Gustavson Syndrome	309555	Xq26
Mental retardation-hypotonic facies syndrome, X-linked, 1	309580	Xq13
HSAS	308840	Xq28
Microphthalmia, syndromic 1	309800	Xq27-q28
Lowe Syndrome	309000	Xq26.1
Lesch-Nyhan Syndrome	300322	Xq26-q27.2
Menkes Syndrome	309400	Xq12-q13
Paiene-Seemanova Syndrome	311400	-
Pelizaeus-Merzbacher Disease; PMD	312080	Xq22
Pyruvate decarboxylase deficiency	312170	Xp22.2-p22.1
Spinocerebellar ataxia, X-linked 3	301790	-
VACTERL with hydrocephalus	314390	-
Wittwer	300421	Xp22.3
Hoyeraal-Hreidarsson Syndrome	300240	Xq28
Mucopolysaccharidosis type II	309900	Xq28
Allan-Herndon-Dudley Syndrome	300523	Xq13.2

In genetic basis, HSD10 deficiency is associated with mutation in *HSD17B10* (formerly the *HADH2*) which encodes hydroxysteroid (17β) dehydrogenase 10 (HSD10) (Korman, 2006). HSD10 plays role as a mitochondrial multifunctional enzyme which catalyze the oxidation of steroid modulators of γ-aminobutyric acid type A (GABAA) receptors, steroid hormones, and xenobiotics and degradation of isoleucine (Yu Yang *et al*., 2009). There are six mutation reported in *HSD17B10* (Korman *et al*., 2007; Yu Yang *et al*., 2007; Lenski *et al*., 2007). Yu Yang *et al.* in 2009 describe the clinical spectrum of mutation in *HSD17B10* (Yu Yang *et al.,* 2009). The clinical comparisson between patients of Yu Yang *et al.* and patients in W92-053 are depicted in Table 10.

Table 10. Clinical Comparisson between Family W92-053 and previous patients with mutation in *HSD17B10*

	YUYANG *et al.* PATIENT 1	**YUYANG *et al.* PATIENT 2**	**MRX10**	**W92-053**
Mutation	c.419C>T	c.776G>C.	c.605C>A	?
Onset	24 months	6 years	1 years	3 month
Clinical picture				
• MR	(+)	(+)	(+)	(+)
• Regression	(+)	(+)		(+)
• Gradl visual loss	(+)			(+)
• Seizure	(+)			(+)
• Early death	(+)			(+)
• Other		disarthria	Disarthria Coreoathetosis Abn behaviour	
Ophtalmology	Cortical blindness pale optic disk	normal	normal	Pale fundi
Neurology	mild truncal hypotonia	Gait, rigidity	Gait, mild spastic hypertonia with hyperflexia	spastic tetraplegia
EEG	Myoclonical seizure	Left focal epileptiform	nonspecific slow dysrhythmia	left frontotemporal focal
Brain		CT scan: Brain atrophy Arnold-Chiari type I	Normal	Autopsy: brain atrophy, mild hypomyelination, thyn gyri, delicate optic nerves&corpus callosum
Metabolism	3-hydroxy 2methylbutyril-CoA dehydrogenase activity(-)	- 3-kethotiolase deficiency - CSF lactate ↑ - ↓complexI	Normal	Skeletal age retarded normal

The previous patients with mutation in *HSD17B10* described by Yu Yang *et al.* had a somewhat more severe clinical course than MRXS10 (table 9). This indicates that mutations in *HSD17B10* result in a wide clinical spectrum of disease in males, ranging from the more severe presentation to the much milder clinical course, which may be explained by the difference in mutation type. Our patients in family W92-053 showed a more severe clinical course than the previous patients with a mutation in *HSD17B10*, but have the same features. However, we did not find any mutations. Still, *HSD17B10* might be the gene, since it is possible that these female carrier had complete exon deletion, which would not be seen on the sequencing results or that a mutation is located within the intronic region, which was not covered by the PCR. This may result in exon skipping, activation of cryptic splice sites, creation of a pseudo-exon within an intron, or intron retention. RT-PCR will help to show if there is an aberrant transcript. It is also possible that the mutation is located in the promoter region of the gene which was not covered by the primer we used. Other possibilities include defects in other regulatory elements, genetic or epigenetic, involved in the regulation of transcription. This might be assessed by quantitative real-time PCR. Alternatively, *HSD17B10* is not involved in this MR family and other candidate genes have to be considered. In fact, the region is quite large (20 Mb) and contains 215 genes including several known XLMR genes. Development of array technology will be able to help to reveal any pathogenic copy number variation on genes inside interval. Intervals resulted from linkage analysis also could become target of high troughput sequencing which will reveal any gene mutation in these intervals.

In conclussion, we report on a family with mental retardation, spasticity, blindness, hypomyelination, early death with a 20 Mb linkage interval in Xp11.3-q12. One obligate carrier showed extremely skewing XCI. No mutation was found on sequencing genomic DNA of *HSD17B10, SYP, SYN1, UBQLN2* and *ARGHEF9.*

Family P03-0452 and 13753/HC (XLMR with Hydrocephalus families)

Clinical Examination

Dutch families P03-0452 and 13753/HC were referred to Moleculer Genetic Division RUNMC with congenital hydrocephalus and mental retardation. Patient 5037 and 28558 of family P03-0452 also presented with short stature, obesity and hypogonadism. Pedigree of family P03-0452 and W05-111 are described in Figure 8 and 9 respectively, both showing X-linked inheritance.

Figure 8. Pedigree and haplotypes within family P03-0452. The cosegregating haplotype has been marked by a black bar. Filled symbols represent male patients with MR.

Linkage Analysis

We performed linkage analysis with 16 markers on the X chromosome. In family P03-0452, a maximum two-point LOD score 0.2 was obtained for marker DXS1220 (Table 11). It was possible to exclude part of Xp22.2-Xq22.2 (flanked by DXS8022 to DXS8096; Table 11) of the X chromosome from linkage (LOD score <-2). The interval in region Xq22.2-Xq28 is flanked by marker DXS8096 and DXS1073. This 51 Mb interval contains 249 annotated genes (NCBI Map Viewer build 36.3).

Table 11. Two-Point LOD Scores for 16 X-Chromosomal Markers of family P03-0452

Marker	Position (cM)	θ = 0.0	θ =0.05	θ =0.1	θ =0.15	θ =0.2
DXS8022	22.18	-4.69	-0.73	-0.46	-0.32	-0.22
DXS7110	29.22	0.00	0.00	0.00	0.00	0.00
DXS1036	33.54	-4.69	-0.73	-0.46	-0.32	-0.22
DXS8012	42.21	-4.69	-0.73	-0.46	-0.3	-0.22
DXS1003	47.08	-4.39	-0.72	-0.44	-0.29	-0.19
DXS1199	52.50	0.00	0.00	0.00	0.00	0.00
DXS990	60.62	-4.39	-0.72	-0.44	-0.29	-0.19
DXS8096	68.74	-4.39	-0.72	-0.44	-0.29	-0.19
DXS1220	70.91	0.17	0.14	0.12	0.10	0.08

DXS1212	77.15	0.00	0.00	0.00	0.00	0.00
DXS1047	82.84	0.00	0.00	0.00	0.00	0.00
DXS1227	88.33	-4.69	-0.73	-0.46	-0.32	-0.22
DXS8043	94.22	0.00	0.00	0.00	0.00	0.00
DXS1193	97.89	0.00	0.00	0.00	0.00	0.00
DXS1073	102.35	0.00	0.00	0.00	0.00	0.00

In family 13753/HC, a maximum two-point LOD score 0.6 was obtained for marker DXS8012 (Table 12). It was possible to exclude Xp22.33-Xp22.2 of the X chromosome from linkage (LOD score <-2). The interval is flanked by marker DXS1036 and DXS1073. The 122 Mb interval located in Xp21.1-Xq28 (UCSC Genome Browser). This is a very large interval covering almost the whole X-chromosome.

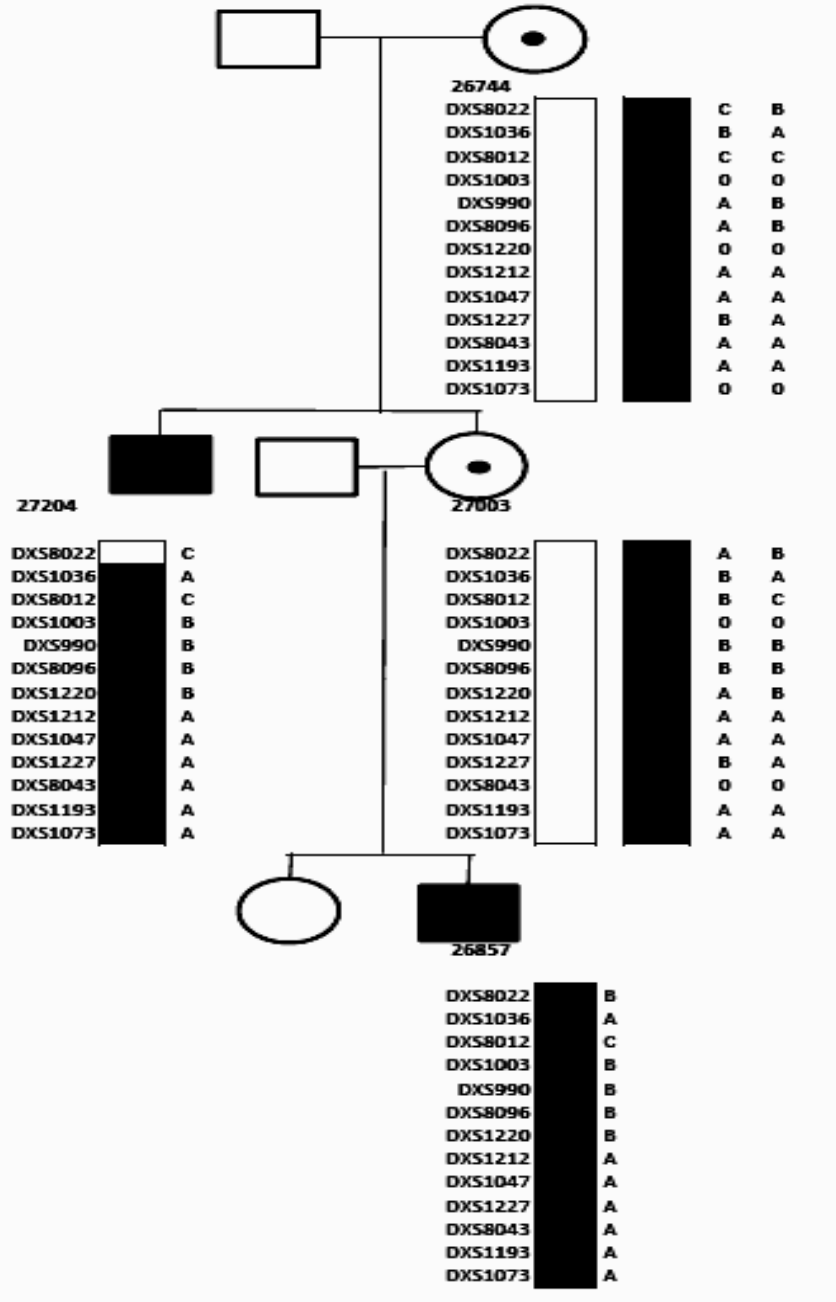


Figure 9. Pedigree and haplotype within family 13753/HC. The cosegregating haplotype has been marked by a black bar. Filled symbols represent male patients with MR.

Table 12. Two-Point LOD Scores for 16 X-Chromosomal Markers of family 13753/HC

Marker	Position (cM)	θ = 0.0	θ =0.05	θ =0.1	θ =0.15	θ =0.2
DXS8022	22.18	-4.39	-1.72	-1.14	-0.81	-0.59
DXS1036	33.54	0.00	-0.05	-0.10	-0.12	-0.13
DXS8012	42.21	0.60	0.53	0.47	0.40	0.33
DXS990	60.62	0.30	0.25	0.21	0.17	0.13
DXS8096	68.74	0.30	0.25	0.21	0.17	0.13
DXS1212	77.15	0.00	0.00	0.00	0.00	0.00
DXS1047	82.84	0.00	0.00	0.00	0.00	0.00
DXS1227	88.33	0.00	-0.05	-0.10	-0.12	-0.13
DXS8043	94.22	0.00	0.00	0.00	0.00	0.00
DXS1193	97.89	0.00	0.00	0.00	0.00	0.00
DXS1073	102.35	0.00	0.00	0.00	0.00	0.00

X-Chromosome Inactivation Analysis

X-Chromosome inactivation analysis using analysis of the CGG repeat in the promoter region of *FMR1* in female 29301 on family P03-0452 was uninformative for *FMR1*. XCI analysis using analysis of the CAG repeat in the promoter region of *AR* showed extremely skewing XCI in this female. XCI analysis of female 26744 and 27003 in family 13753/HC showed no skewing XCI with *FMR1* method which might indicate different genetic defect in this family.

Overlapping Interval

Previous molecular genetic studies showed X-linked human congenital hydrocephalus can be caused by mutations in *L1CAM* (L1 protein) at Xq28 [Jouet *et al.,* 1993] and in *AP1S2* at Xp22 (Saillour *et al*., 2007). However, both family P03-0452 and family 13753/HC have been sequenced for *L1CAM*, which revealed no mutation. Linkage analysis now also excludes *AP1S2*. Strain *et al*. in 1994 reported an interesting X-linked aqueductal stenosis case, in which linkage was established outside Xq28, flanked by DXS548 and FRAXA loci in Xq27.3 (Strain *et al*., 1994). This overlaps with the intervals in family P02-0453 and family

13753/HC.The overlapping region is 7 Mb and contains 36 annotated genes (NCBI Map Viewer build 36.3; Figure 10).

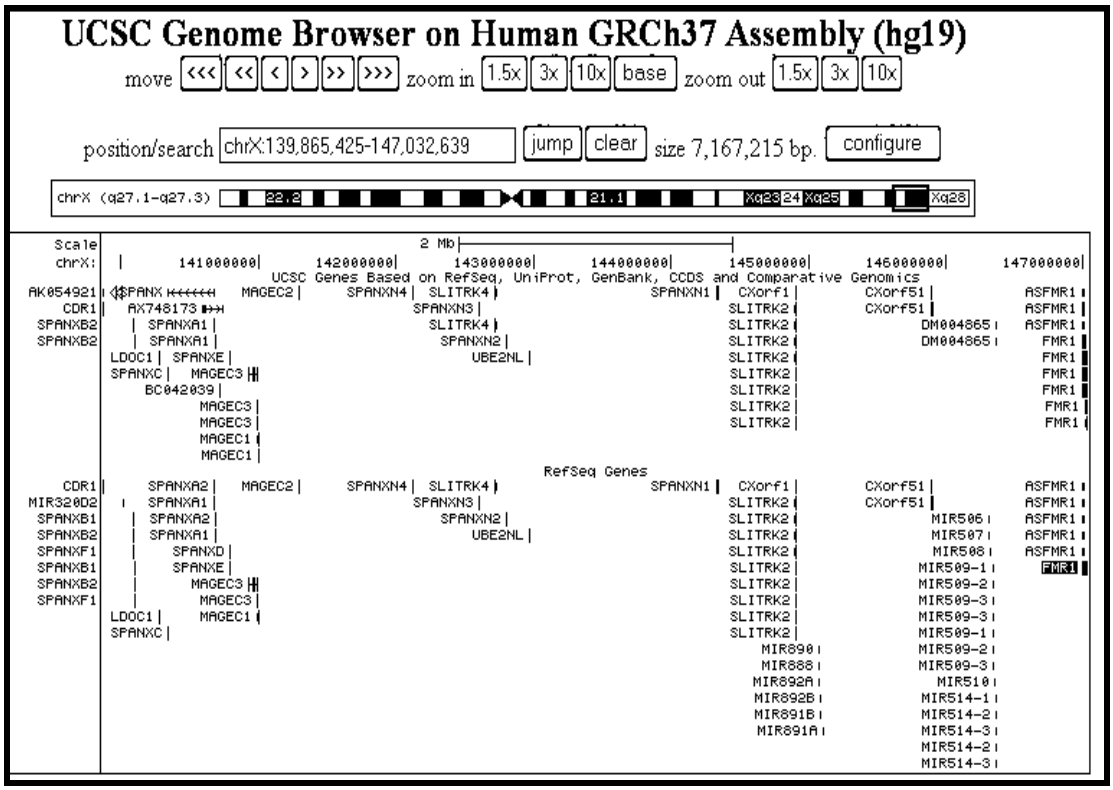


Figure 10. Candidate genes in the overlapping interval between family P03-0452, 13753/HC, and previous reported family by Strain *et al*.

Candidate Gene Selection

We performed candidate gene prioritization using manual selection and bioinformatic tool (ToppGene, available at http://toppgene.cchmc.org). We used known genes for hydrocephalus in human, *L1CAM, AP1S2* and *NPH*, and the human orthologous genes of hydrocephalus mouse genes *HYDIN, a-SNAP, RFX4, FREAC-3, DNAHS, OTX2, MSX1, SOCS7*, and *MYH10*, as training gene and 36 genes in overlapping interval as test gene. Result of candidate gene prioritization with ToppGene are described in Figure 11. *FMR1* was shown as a top candidate gene. However, mutations in this gene were associated with Fragile-X syndrome,

and no previous reports associated with hydrocephalus. Thus we did not choose *FMR1* as our candidate gene.

SLITRK2 and *SLITRK4* are belong to SLITRK family, which was identified as neuronal transmembrane proteins that play role in regulating neurite outgrowth (Aruga and Mikoshiba, 2003). *SLITRK2* and *SLITRK 4* were all predominantly expressed in the brain (Aruga, Yokota, and Mikoshiba, 2003). Slitrk2 is strongly expressed in the ventricular layer and in neuroepithelium adjacent to the third ventricle. Slitrk4 is most strongly expressed in subventricular zone and the lateral part of the periaqueductal gray matter (Aruga and Mikoshiba, 2003). Neurite outgrowth in the ventricle and aquaductus could cause stenosis that led into hydrocephalus. Thus, both *SLITRK2* and *SLITRK4* are good candidate genes for hydrocephalus. *SLITRK2* and *SLITRK4* were selected as candidate genes and sequenced. No mutation was found in *SLITRK2* and *SLITRK4* on patient 28558 and patient 26857 of family P03-0452 and 13753/HC respectively.

Genetic Counselling

No candidate gene mutation was found in these families. However, linkage analysis revealed that patient 29301, 5037 and 28558 in family P03-0452 shared similar risk haplotype. This finding support X-linked inheritance in this family. Thus, each male child of patient 29301 has 50% risk to develop the disorder and also, each female child of 29301 has 50% risk to be a carrier. Linkage analysis also showed that patient 26744, 27204, 27003, 26857. This finding support X-linked inheritance in this family. Thus, sister of 26857 has 50% risk to be a carrier. This finding can be used for genetic counselling purpose.

Discussion

Hydrocephalus is described by abnormalities in the flow or resorption of cerebrospinal fluid (CSF), resulting in ventricular dilatation. Hydrocephalus is categorized as congenital, which is present at birth and often associated with developmental defects; and acquired, which occurs after development of the brain and ventricles (Mori, 1995). Forty percents of hydrocephalus cases are estimated to be caused by genetic factors (Haverkamp *et al.*, 1999; table 13).

Although there is strong evidence for genetic causes, only two X-linked hydrocephalus genes has been identified so far in humans, which are *L1CAM* and *AP1S2*. In this study, we reported two family with X-linked congenital hydrocephalus and mental retardation. In both family, *L1CAM* had already been excluded by sequencing and *AP1S2* had already been excluded by linkage interval. Strain *et al* in 1994 reported an interesting X-linked aqueductal stenosis case, in which linkage was established outside region *L1CAM* and *AP1S2*, flanked by DXS548 and FRAXA loci in Xq27.3 (Strain *et al*., 1994). Thus, we checked the shared intervals in family P03-0452, W05-111, and family from Strain et al, which results in a 7 Mb overlapping intervals containing 36 genesWe used manual selection and bioinformatic tool (ToppGene, available at http://toppgene.cchmc.org) to do candidate gene prioritization. ToppGene has an advantageous feature of using mouse phenotype from Mammalian Phenotype (MP) Ontology (Chen et al., 2007), so in this case we could also include human orthologous genes of a mouse genes that were known associated with hydrocephalus in mouse model as training genes. *SLITRK2, SLITRK4* and *CDR1* were selected as candidate genes.

Rank (net)	Gene Symbol	Gene ID	GO: Biological Process		Mouse Phenotype		Average score	Overall P-value
			Score	pValue	Score	pValue		
1	FMR1	2332	0.5431664	0.0265096	0.4585751	0.19	0	0.0316865
2	SLITRK2	84631	0.9561065	0.005891			0	0.0359861
3	SLITRK4	139065	0.9561065	0.005891			0	0.0359861
4	CXorf1	9142	0	0.5257732			0	0.8620884
5	LDOC1	23641	0	0.5257732			0	0.8620884
6	SPANXA1	30014	0	0.5257732			0	0.8620884
7	SPANXB2	100133171	0	0.5257732			0	0.8620884
8	UBE2NL	389898	0	0.5257732			0	0.8620884
9	SPANXC	64663	0	0.5257732			0	0.8620884
10	SPANXN4	441525					-1	0.9999875
11	SPANXN2	494119					-1	0.9999875
12	SPANXN1	494118					-1	0.9999875
13	CDR1	1038					-1	0.9999875
14	SPANXN3	139067					-1	0.9999875
15	MAGEC2	51438					-1	0.9999875
16	MAGEC1	9947					-1	0.9999875
17	ASFMR1	100126270					-1	0.9999875
18	SPANXA2	728712					-1	0.9999875
19	CXorf51	100129239					-1	0.9999875
20	SPANXE	171489					-1	0.9999875

Figure 11. Top 20 candidate genes according to candidate gene prioritization with ToppGene for hydrocephalus families.

Table 13. Summary of known loci of hydrocephalus in vertebrates (adopted from Zhang *et al*.,2006)

Species	Strain	Clinical form	Trait[a]	Locus	Chromosome	Human syntenic region	Human Gene
Human		C	AR	Unknown	unknown		
Human		C	AD	Unknown	8q12.2–21.2 or unknown		
Human		AO	AD	VPH	unknown		
Human		C	X-linked	L1cam	X	X	L1CAM
Human		AO	X-linked	Unknown	X	X	
Rat	HTX	C	QTL	D9Rat2	9q38	5q21.1, 18p11.22–31	
Rat	HTX	C	QTL	D10Rat136, D10Rat135	10q32.1–10q32.3	1/q21.3–q25.3	
Rat	HTX	C	QTL	D11Arb2, D11Rat46	11q23	3q27–28, 22q11.21, 10p12.2	
Rat	HTX	C	QTL	D17Mit4, D17Rat154	17q12.1	1q43, 10p11.21–p13	
Rat	LEW/Jms	C	AR, (QTL)	unknown	unknown		
Mouse	C57BL/6J	C	QTL	Vent8a	8	8p11–23, 13q11–34	
Mouse	C57BL/6J	C	QTL	Vent4b	4	6p, 9	
Mouse	C57BL/6J	C	QTL	Vent7c	7	19q10–13	
Mouse	lhy1	C	AR	unknown	unknown		
Mouse	lhy2	C	AR	unknown	unknown		
Mouse	lhy3	C	AR	Hydin	8	16q22.2	HYDIN
Mouse	C57BL/10J	C	AR	lhyh	7	19q13.3	a-SNAP
Mouse	C57BL6/J	C	AD	Rfx4	10	12q24	RFX4
Mouse	BALB/cHeA	C	AR	lhly	12	14q32	
Mouse	dh	C	AR	Mf1	13	6p25	FREAC-3
Mouse	STOCK 1b	C	AR	oh	unknown		
Mouse	C57BL/6 *CBA/J	C	AR	Mdnah5	15	5p15.2	DNAH5
Mouse	C57BL/CBA	C	AD	Otx2	14	14q21–q22	OTX2
Mouse	129P2/OlaHsd	C	AR	Msx1	5	4p16.3–p16.1	MSX1
Mouse	C57BL/6	C	AR	Socs7	11	17q12	SOCS7
Mouse	C57BL/6J	C	AR	Myh9c-b	11	17q13	MYH10
Zebrafish	m404/m491	C	AR	apo	unknown		
Zebrafish	m409/m432	C	AR	cudak	unknown		
Zebrafish	m691	C	AR	eagle	unknown		
Zebrafish	m591	C	AR	endeavor	unknown		
Zebrafish	m584	C	AR	enterprise	unknown		
Zebrafish	m492/m510	C	AR	galileo	unknown		
Zebrafish	m445/m585/m700	C	AR	gurnowy	unknown		
Zebrafish	m727	C	AR	hubble	unknown		
Zebrafish	m221/m470/m680	C	AR	interrail	unknown		
Zebrafish	m733	C	AR	kepler	unknown		

Direct DNA sequencing of *SLITRK2* and *SLITRK4* on patient 28558 and patient 26857 of family P03-0452 and 13753/HC respectively revealed no mutation. Still, we can not exclude the presence of deep intronic or promoter mutations in *SLITRK2* and *SLITRK4*. Moreover, we did not investigate the presence of intragenic rearrangements by techniques such as multiplex qPCR, MLPA or target microarray. Other possibilities, *SLITRK2* and *SLITRK4* is not involved in this MR family. Development of array technology will be able to help to reveal any pathogenic copy number variation on genes inside interval. Intervals resulted from linkage analysis also could become target of high troughput

sequencing which will reveal any gene mutation in these intervals. This findings suggest the etiological heterogeneity in x-linked hydrocephalus.

Family W07-604

History

This Norwegian family was referred to Moleculer Genetic Division RUNMC with mental retardation and behavioural problems. The pedigree of this family is shown in figure 2 Conventional cytogenetic and Fragile-X exclusion had already been performed in Norway and showed no abnormalities. Linkage analysis that had been performed in Norway indicated linkage to Xp21.1-Xp22.2.

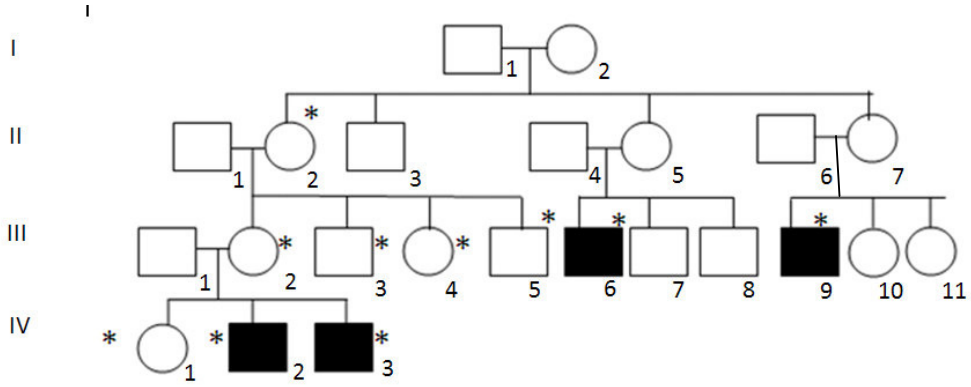


Figure 12. Pedigree of family W07-604.

X-Chromosome Inactivation Analysis

Skewing of X inactivation was investigated via analysis of the CGG repeat in the promoter region of *FMR1*. The tested carrier females (IV.1) showing no skewing XCI with *FMR1* method.

250K SNP Array Analysis

Affymetrix 250K SNP Array analysis (Figure 13) of patients III.9 identified 4.6 Mb deletion of the pter of chromosome 9 (last deleted SNP: SNP_A-2236672) and 5 Mb duplication of pter chromosome 2 (pTer; SNP_A-1786649 and a small piece right next to this duplicated region: SNP_A-1917548;

SNP_A-4199698; first or last duplicated SNPs given), which suggested a unbalanced translocation t(2;9)(p25.2;p24.2)

Discussion

We reported a family with mental retardation and behavioural problems. Conventional cytogenetic analysis did not show any abnormalities.This family was assumed to be X-linked due to the type of inheritance showed by pedigree. Linkage analysis had been performed in this family, showing linkage interval on Xp21.1-Xp22.2. However, we identified a deletion of chromosome 9p and duplication of chromosome 2p, suggesting an unbalanced translocation t(2;9)(p25.2;p24.2).

There have been a number of studies about 9p deletion syndrome that reported several clinical features for this syndrome including mental retardation, trigonocephaly, low set ears and dysmorphic facial features, such as up-slanting palpebral fissures and a long philtrum (Huret *et al*., 1988; Swinkels *et al*., 2008; Hauge *et al*., 2008). Our patients only showed mental retardation and behavioural problems, which also were reported in the previous 9p deletion syndrome cases (table 14).

About the consequences of a duplication of chromosome 2p less is known. There were rare reports about partial trisomy 2p, which indicate serious complications including: diaphragmatic hernia (2p23-p25), neural tube defects (2p24), broncho-pulmonary anomalies (2p21-p25), and congenital heart defects (2p23-p24). However, in most cases the duplication involves larger regions from 2pter up to band 2p21 (Lurie *et al*., 1995).

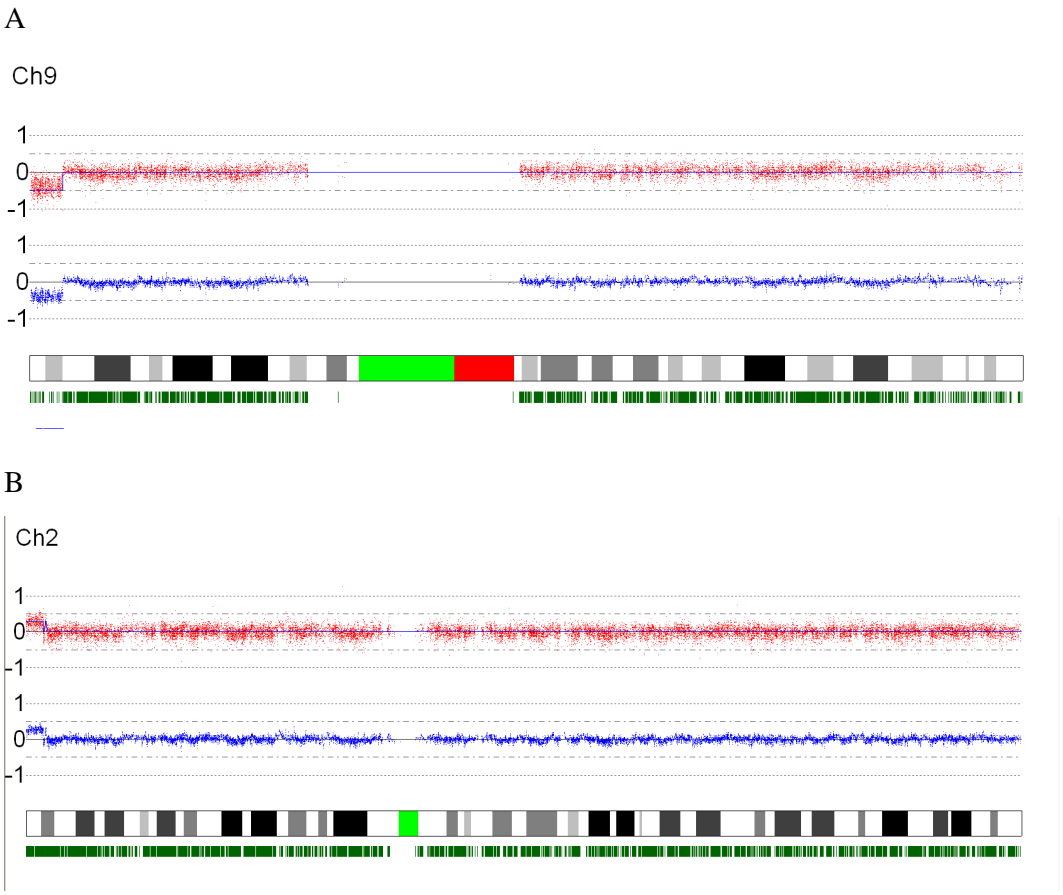


Figure 13. 250K SNP array data on the family W07-604 patient III.9 showing a (A) 4.6 Mb deletion of chromosome 9 involving region 9p24.3-p24.2 and (B) 5 Mb duplication within 2p25.3-2p25.2 (Figure 13.B).

To our knowledge, there was only one pure duplication case with a duplication as distal as in the patient reported here (Wakita *et al.*, 1985). In that case, patient showed trigonocephaly, hypertelorism, mongoloid slant of palpebral fissures, right exophthalmos, anteverted nostrils, low-set and malformed ears, arachnodactyly, and contractures of the elbow joints and interphalangeal joints of fingers II-V, and normal psychomotor development. None of those clinical features were found in our patient. Altogether, the duplication of the terminal part

of chromosome 2p may only mildly contribute to the phenotype of the patient described here.

Tabel 14. Comparisson of clinical features of patient IV.9 from family W07-604 with previous reported cases of 9p deletion syndromes.

Clinical Features	**Huret et al**	**Swinkels et al**		**Hauge et al**					**Our patient**
		12	13	1	2	3	4	5	
MR	36/36	+	+	+	+	+	+	+	+
Behaviour	U			+	+	+	+	+	+
Head	Trigonocephaly 32/32	-	-	-	-	prominent	trigonocephaly	-	-
Ears	low set ears 27/30	-	-	-	+	+	+	-	-
Long philtrum	32/32	+	-	-	+	+	U	+	-
Midface hypoplasia	6/7	+	-	-	-	+	U	+	-
Arching eyebrows	9/12	-	-	-	+	Medial flare	U	-	-
Broad internipple	31/31	U	U	-	-	U	+	U	-
Hand/foot									-
Genitourinary	15/36	+	+	-	U	U	APA	-	-
Cardiovascular	16/35	-	-	U	-	U	R Ao Arch	-	-

Family anamnesis suggested that the genetic defect in family W07-604 was linked to the X chromosome. However, additional STR markers analysis in healthy male III.4 conducted after finishing this study excluded the original linkage interval. By affymetrix 250K SNP Array analysis of patients III.9, we

now identified a 4.6 Mb deletion of the pter of chromosome 9 and 5 Mb duplication of pter chromosome 2 which suggests there is an unbalanced translocation t(2;9)(p25.2;p24.2). Further examinations are needed to confirmed the deletion and duplication in patients, also to check whether this aberration de novo or also segregated in parents. FISH analysis will be able to show balanced translocation in patients and unbalanced translocation in carrier parents. Another affected member of family also need to be checked to see whether they also shared similar unbalanced translocation. FISH, MLPA and qPCR could help to check the translocation in other affected family members. It is still possible that the MR in other family members cannot be explained by the unbalanced translocation in patient IV.9.

Family DF27004 (MR and Overgrowth Features)

Clinical Examination

Dutch families DF27004 (Fig. 14) were referred to Moleculer Genetic Division RUNMC with mental retardation and overgrowth syndrome. Pedigree of this four generation family showed X-linked inheritance. The propositus, 33431, showed mental retardation, macrocephaly, hepatomegaly, kidney enlargement. Other affected boy, 91105 showed similar features. Obligate carriers were said to be normal.

Genetic Analysis

Gross cytogenetic abnormalities and fragile-X already excluded before. Following the hypothesis of an X-linked disorder, linkage analysis was then performed with sixteen highly polymorphic markers spanning the entire X-chromosome were used for linkage analysis (Table 15). This family showed intervals of 37 Mb and 9.5 Mb in Xp22.2-Xq11.2 and Xq27.3-Xq28 respectively (maximum LOD score of 1.62 at θ=0,0 for DXS8022) that segregated with the disease. X-chromosome inactivation analysis in obligate carrier 4984, showed no skewing XCI.

The overgrowth features (macrocephaly, hepatomegaly, and renal enlargement) in this cases lead suspicion to Simpson-Golabi-Behmel syndrome (SGBS) -primarily characterized by overgrowth- as a differential diagnosis. SGBS is caused by mutation in GPC3 gene and OFD1 gene. However, sequence analysis of GPC3 coding region in patient 33431 did not revealed any pathogenic point mutation.

Table 15. Two-Point LOD Scores for 16 X-Chromosomal Markers of family DF27004

Marker	Position (cM)	θ = 0.0	θ =0.05	θ =0.1	θ =0.15	θ =0.2
DXS7108	18.37	-0.65	-0.36	-0.21	-0.12	-0.06
DXS7104	20.27	-0.02	-0.02	-0.02	-0.02	-0.01
DXS8022	22.18	1.62	1.45	1.28	1.11	0.92
DXS7110	29.22	1.32	1.19	1.05	0.91	0.76
DXS1036	33.54	-0.67	-0.62	-0.51	-0.40	-0.30
DXS8090	36.79	-0.67	-0.49	-0.28	-0.12	-0.02
DXS8012	42.21	0.44	0.41	0.36	0.32	0.28
DXS1003	47.08	0.00	0.00	0.00	0.00	0.00
DXS1199	52.50	0.00	0.00	0.00	0.00	0.00
DXS990	60.62	-2.37	-1.27	-0.86	-0.59	-0.39
DXS8020	65.50	0.04	0.02	0.00	-0.01	-0.01
DXS8096	68.74	-0.67	-0.52	-0.32	-0.18	-0.07
DXS1059	68.75	-0.55	-0.39	-0.28	-0.20	-0.14
DXS1220	70.91	-0.67	-0.58	-0.44	-0.32	-0.22
DXS1212	77.15	-0.63	-0.40	-0.27	-0.17	-0.11
DXS1047	82.84	0.18	0.17	0.16	0.14	0.13
DXS8094	82.85	-0.37	-0.32	-0.22	-0.14	-0.07
DXS1192	83.92	-0.37	-0.38	-0.36	-0.29	-0.22
DXS984	85.55	-0.22	-0.17	-0.13	-0.09	-0.06
DXS1227	88.33	0.00	0.00	0.00	0.00	0.00
DXS8028	95.13	0.46	0.42	0.37	0.31	0.26
DXS8091	97.89	1.03	0.89	0.75	0.62	0.49
DXS1193	97.89	0.21	0.20	0.19	0.17	0.15
DXS1073	102.35	-0.37	-0.37	-0.33	-0.28	-0.22

Discussion

We report a three generation family in which an X-linked trait seems to segregate. This family present with overgrowth features and mental retardation. There are several overgrowth syndromes which show overlapping clinical and molecular features, such as: Beckwith-Wiedemann, Pallister-Killian, Soto, Perlman, and Simpson-Golabi-Behmel syndrome (Vora and Bianchi, 2009). Among those syndromes, only Simpson-Golabi-Behmel syndrome (SGBS) shows X-linked inheritance. Patients with SGBS have pre- and postnatal overgrowth, coarse facies, congenitalheart defects, cleft lip and palate, enlarged and dysplastic kidneys, skeletal abnormalities, and an increased risk of embryonal tumours (Hughes *et al*., 1992). This syndrome can be caused by mutations in *GPC3* or *OFD1*. *GPC3* is a membrane associated heparan sulphate proteoglycan, which is a member of the glypican related integral membrane proteoglycans (GRIPS). This gene are known to modulate the interaction between growth factors and receptors (Pilia *et al*., 1996). Functional data confirm *GPC3* as an excellent candidate gene, as *Gpc3*-deficient mice show developmental overgrowth (Cano-Gauci *et al*., 1999). We thus performed *GPC3* mutational screening in patient 33431, but we could not find any mutations. Still, *GPC3* might be the gene, since it is possible that a mutation is located within the intronic region, which was not covered by the PCR. This may result in exon skipping, activation of cryptic splice sites, creation of a pseudo-exon within an intron, or intron retention. RT-PCR will help to show if there is an aberrant transcript. It is also possible that the mutation is located in the promoter region of the gene which was not covered by the primer we used. Other possibilities include defects in other regulatory elements, genetic or epigenetic, involved in the regulation of transcription. This might be assessed by quantitative real-time PCR. Alternatively, *GPC3* is not involved in this MR family and other candidate genes have to be considered. In fact, the region is quite large (37.5 Mb and 9 Mb) and contains hundreds genes including several known XLMR genes. Development of array technology will be able to help to reveal any pathogenic copy number variation on genes inside interval. Intervals resulted from

linkage analysis also could become target of high troughput sequencing which will reveal any gene mutation in these intervals.

In summary, we reported a family with mental retardation and overgrowth features with intervals of 37 Mb and 9.5 Mb in Xp22.2-Xq11.2 and Xq27.3-Xq28 respectively. One obligate carrier showed no skewing XCI. No mutation was found on sequencing genomic DNA of *GPC3.*

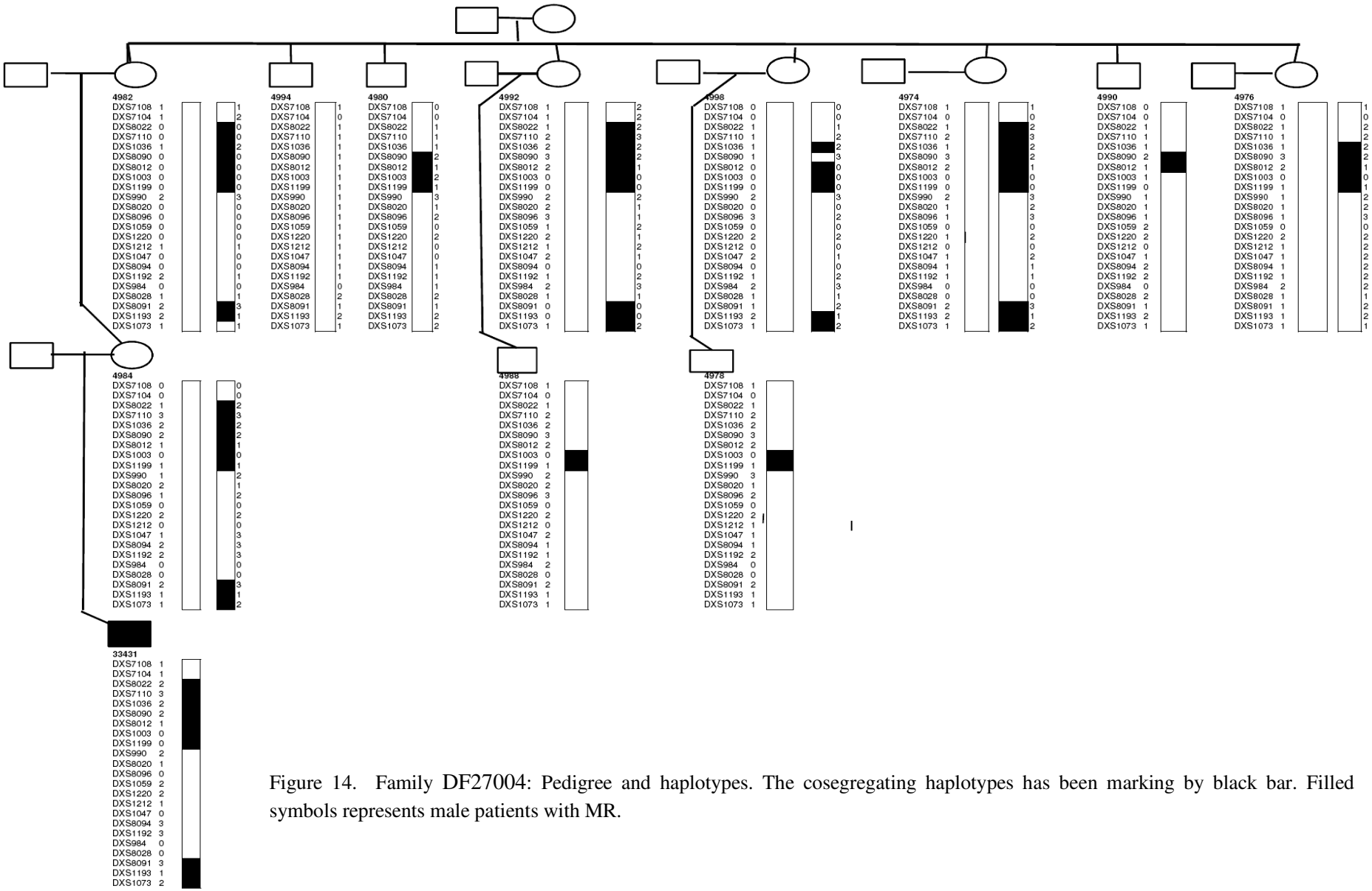


Figure 14. Family DF27004: Pedigree and haplotypes. The cosegregating haplotypes has been marking by black bar. Filled symbols represents male patients with MR.

Family W09-0071, W09-0072, W09-0074, W009-0079, and W08-2152

W09-0071 Clinical Reports

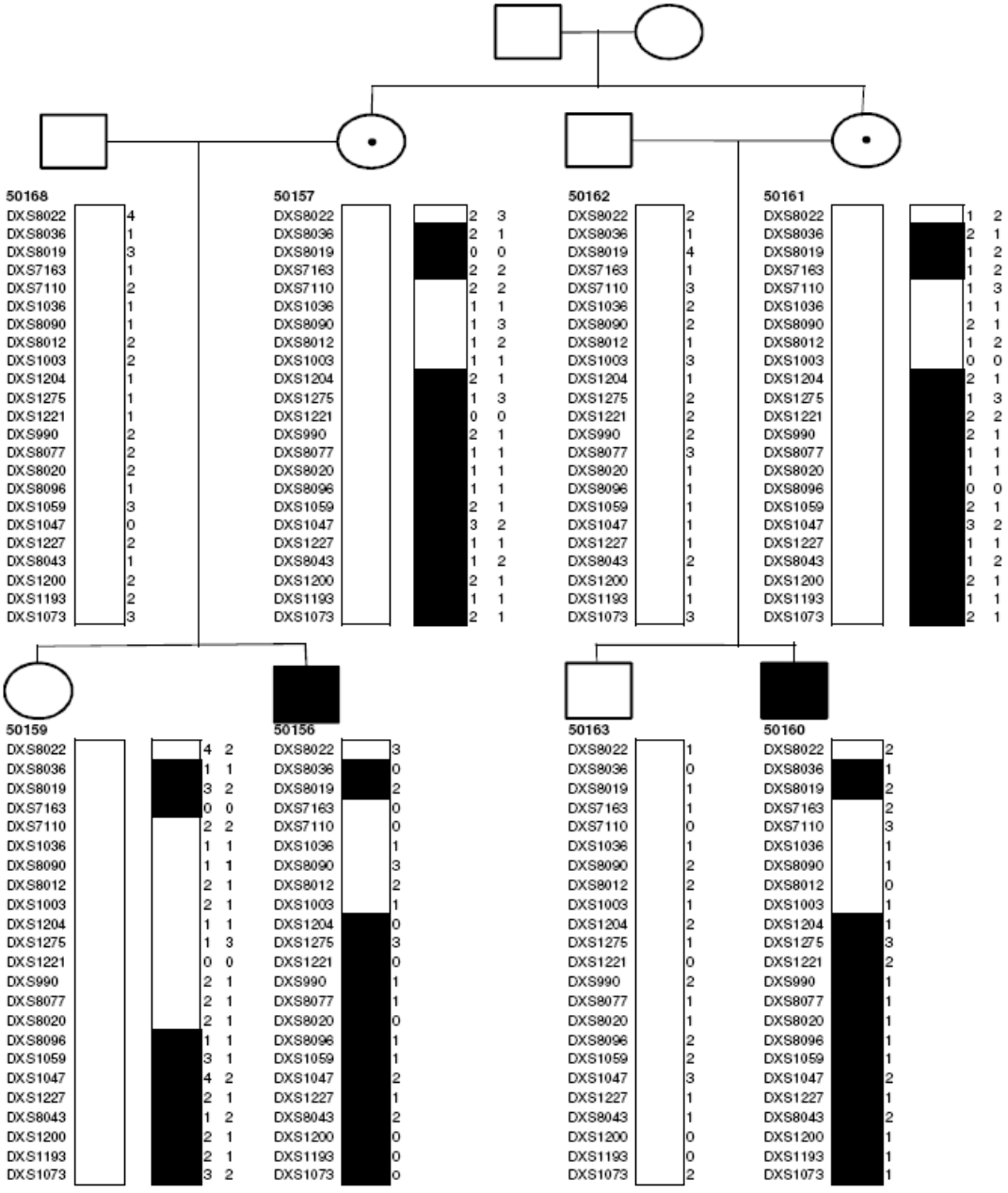
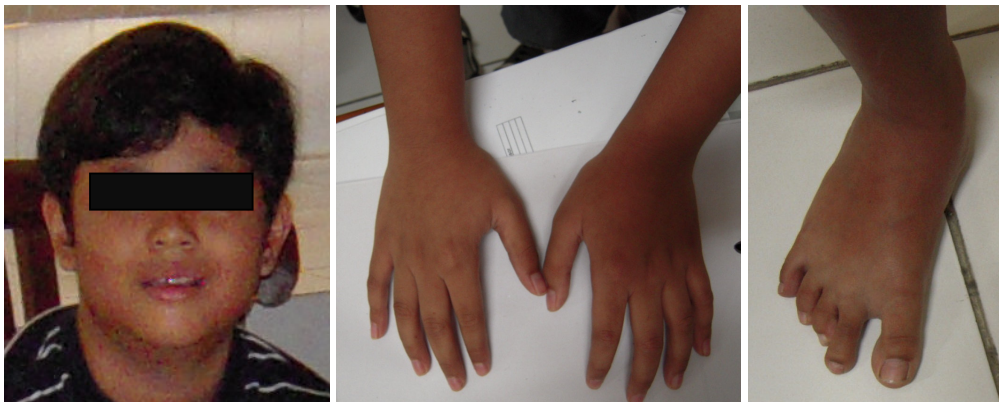


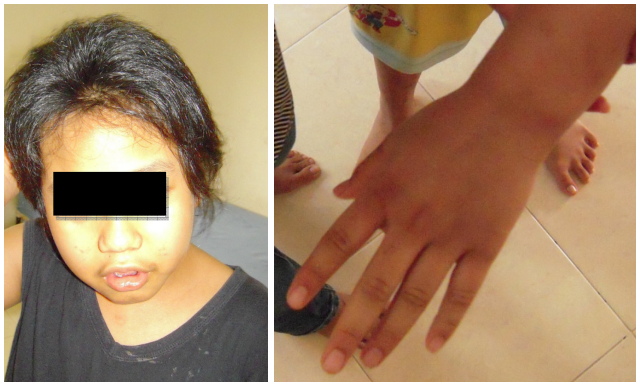
Figure 15. Family W090-0071: Pedigree and haplotype. The cosegregating haplotypes has been marking by black bar. Filled symbols represents male patients with MR.

Family W09-0071 (Fig.15) was ascertained by purposive sampling in a special school. The pedigree of this family was compatible with X-linked inheritance. Not much is known about the past medical history of the patients. Patient 50156 was attending a special school, while patient 50160 was kept at home by his parents. Obligate carriers were said to be normal.

The propositus, 50156, was examined at age 8 years. He had high arched palate, tappering pad, sandal gap, soft fleshy hands and short third toe (Fig .16). His behaviour was unremarkable. Patient 50160 was seen at the age of 11 years. Soft fleshy hands, sandal gap, pes planus and macroorchidism were noted (Fig.16). His behaviour was difficult to control.



A B C



D E

Figure 16. Clinical pictures of family W09-00071. (A) facial appearance of Patient 50156. This patient showed (B) shoft fleshy hands and (C) short third toe. (D) facial appearance of patient 50160. This patient showed (E) shoft fleshy hand.

The overall intellectual capacities of the tested patients (50156 and 50160) were rated as below average with the onset of before age of 18. Therefore both patient 50156 and 50160 were classified as mentally retarded.

Microscopic cytogenetic analysis in patient 50156 and 50160 showed no chromosomal abnormalities. Analysis of CGG repeat on the promoter region of *FMR1* on patient 50156 showed no CGG repeat expansion. Twenty-three highly polymorphic markers were used for linkage analysis (Table 16). This family showed two intervals of 8 Mb and 81 Mb in region Xp22.2-Xp22.11 and Xp11.4-Xq25 (maximum LOD score of 0.75 at θ=0,0 for DXS1047) that segregated with the disease. It was possible to exclude Xp22.33-Xp22.22 and Xp21.1-Xp11.4 of the X-chromosome from linkage (LOD score <-2), marked by marker DXS8022 and DXS8090. X-chromosome inactivation analysis in obligate carrier 50157 and 50161 showed no skewing XCI.

Table 16. Two-Point LOD Scores for X-Chromosomal Markers of family W09-071

Marker	Position (cM)	θ = 0.0	θ =0.05	θ =0.1	θ =0.15	θ =0.2
DXS8022	22.18	-5.80	-1.95	-1.39	-1.01	-0.73
DXS8036	22.72	0.12	0.11	0.10	0.09	0.07
DXS8019	23.26	0.52	0.43	0.34	0.25	0.17
DXS7163	23.26	-1.11	-1.14	-1.11	-0.98	-0.81
DXS7110	29.22	0.00	0.00	0.00	0.00	0.00
DXS1036	33.54	0.02	0.02	0.01	0.01	0.01
DXS8090	36.79	-5.81	-1.95	-1.39	-1.01	-0.73
DXS8012	42.21	-1.58	-0.58	-0.34	-0.21	-0.14
DXS1003	47.08	0.06	0.04	0.03	0.02	0.01
DXS1204	52.50	0.41	0.36	0.31	0.25	0.20
DXS1275	55.75	0.73	0.64	0.55	0.46	0.37
DXS1221	57.37	0.00	0.00	0.00	0.00	0.00
DXS990	60.62	0.72	0.63	0.53	0.44	0.35
DXS8077	62.52	0.07	0.05	0.04	0.03	0.02
DXS8020	65.50	0.04	0.03	0.02	0.02	0.01
DXS8096	68.74	-1.10	-1.14	-1.10	-0.98	-0.81
DXS1059	68.75	0.74	0.66	0.57	0.48	0.39
DXS1047	82.84	0.75	0.66	0.57	0.48	0.39

Marker	Position (cM)	θ = 0.0	θ =0.05	θ =0.1	θ =0.15	θ =0.2
DXS1227	88.33	0.04	0.03	0.02	0.02	0.01
DXS8043	94.22	0.72	0.63	0.54	0.46	0.37
DXS1200	96.94	0.12	0.10	0.08	0.07	0.05
DXS1193	97.89	0.05	0.04	0.03	0.02	0.02
DXS1073	102.35	0.47	0.40	0.32	0.26	0.19

Genetic Counselling

Although the linkage interval found in this family is quite big, still it can be used to show risk haplotype in this family. It was found that patient 50157, 50161, 50159, 50156 and 50160 shared risk haplotype. This finding support X-linked inheritance in this family. The mother shared risk haplotype with their affected son. Interestingly, healthy female 50159 shared similar risk haplotype with the other affected male, but in narrower scale. Thus, there is a chance that she is a carrier, except if the genetic defect lie in between DXS1204 and DXS8020. This finding was used for genetic counselling purposes.

Parents in this family come from high education and high income background. The first information whas shared with carrier mother 50157, considering that the daughter 50159 shared risk haplotype. Additional information from carrier mother 50157 revealed more affected male family members from maternal side. This information supports X-linked inheritance in this family. Despite of her high educational background, carrier mother 50157 refused to share the information to other family members. From previous experience in Indonesia, it seems that Indonesian parents with higher educational profile are less willing to cooperate in carrier testing. Genetic counselling in such family often lead to divorce and aversion from the counsellor (Faradz et al., 2010).

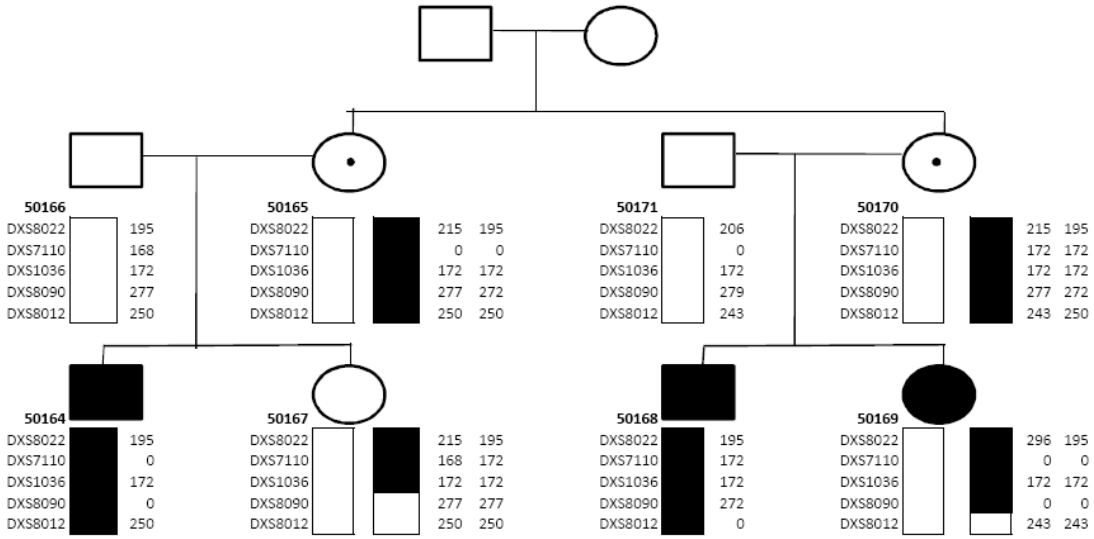
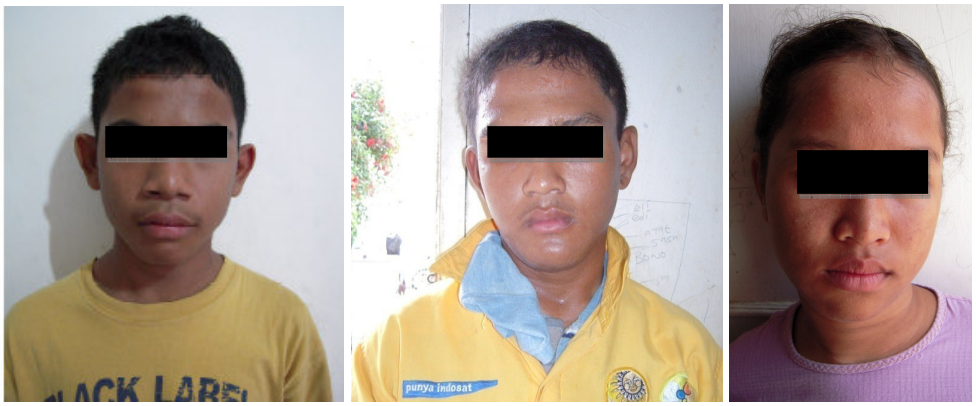


Figure 17. Family W090-0072: Pedigree and haplotypes. The cosegregating haplotypes has been marking by black bar. Filled symbols represents male patients with MR.

The family W09-0072 (Fig. 17) was was ascertained by purposive sampling in a special school. The pedigree of this family was compatible with X-linked inheritance. Not much is known about the past medical history of the patients. Patient 50164 and 50168 were attending special school, while patient 50169 was kept at home by his parents. Obligate carriers were said to be normal.

The propositus, 50164 (Fig. 18A), was examined at age 13 years. He had long face, heavy eyebrows, broad nasal bridge, prominent ears, sandal gaps, and pes planus. Patient 50168 (Fig 18B) was examined at age 16 years. He had long face, heavy eyebrows, broad nasal bridge, and prominent ears. Patient 50169 (Fig 18C) was seen at the age of 11 years. No facial dysmorphism was noted in this patient. Their behaviour were unremarkable.



A B C

Figure 18. Clinical picture of family W09-072: This patient showed long face, heavy eyebrows, broad nasal bridge, prominent ears in patient 50164 (A) and patient 50168 (B). No facial dysmorphism in patient 50169 (C).

Microscopic cytogenetic analysis in patient 50164, 50168 and 50169 showed no chromosomal abnormalities. Analysis of CGG repeat on the promoter region of *FMR1* on patient 50164 showed no CGG repeat expansion. Fifteen highly polymorphic markers were used for linkage analysis (Table 17). This family showed an intervals of 32 Mb in region Xp22.2-Xp11.3 (maximum LOD score of 0.91 at θ=0,0 for DXS8022) that segregated with the disease. It was possible to exclude Xp11.4-Xq28 of the X-chromosome from linkage (LOD score <-2), marked by marker DXS8012 to DXS1193. X-chromosome inactivation analysis showerd skewing XCI in obligate carrier 50165 and no skewing XCI in carrier 50170.

Genetic Counselling

Although the linkage interval found in this family is quite big, still it can be used to show risk haplotype in this family. It was found that patient 50165, 50170, 50164, 50167, 50168, and 50169 shared risk haplotype. This finding support X-linked inheritance in this family. The mother shared risk haplotype with their affected son. Interestingly, healthy female 50167 shared similar risk haplotype with the other affected male and female, but in narrower scale. Thus,

there is a chance that she is a carrier, except if the genetic defect lie in between DXS8090 and DXS8020. This finding can be used for genetic counselling purposes.

Table 17. Two-Point LOD Scores for 16 X-Chromosomal Markers of family W09-072

Marker	Position (cM)	θ = 0.0	θ =0.05	θ =0.1	θ =0.15	θ =0.2
DXS8022	22.18	0.91	0.81	0.71	0.61	0.50
DXS7110	29.22	-0.01	-0.01	-0.01	-0.01	-0.01
DXS1036	33.54	0.00	0.00	0.00	0.00	0.00
DXS8090	36.79	0.36	0.31	0.26	0.20	0.16
DXS8012	42.21	-2.86	-0.72	-0.45	-0.31	-0.21
DXS1003	47.08	-2.86	-0.72	-0.45	-0.31	-0.21
DXS1199	52.50	-8.97	-1.5	-0.98	-0.66	-0.44
DXS990	60.62	-4.87	-0.41	-0.18	-0.08	-0.03
DXS8096	68.74	-4.82	-0.65	-0.39	-0.25	-0.16
DXS1212	77.15	-8.97	-1.57	-0.98	-0.66	-0.44
DXS1047	82.84	-0.02	-0.01	-0.01	-0.01	-0.01
DXS1227	88.33	-4.57	-0.85	-0.54	-0.36	-0.24
DXS8043	94.22	-1.89	-0.66	-0.40	-0.26	-0.17
DXS1193	97.89	-2.57	-0.62	-0.38	-0.25	-0.17
DXS1073	102.35	0.22	0.19	0.15	0.11	0.08

W09-0074 Clinical Reports

The family W09-0074 (Fig. 19) was ascertained by purposive sampling in a special school. The pedigree of this family was compatible with X-linked inheritance. Not much is known about the past medical history of the patients. Patient 50176 and 50177 were attending special school. Obligate carriers were said to be normal.

The propositus, 50176 (Fig 20A), was examined at age 14 years. Patient 50177 (Fig 20B) was examined at age 12 years. Both patients showed large prominent ears and macroorchidism. Their behaviour were unremarkable.

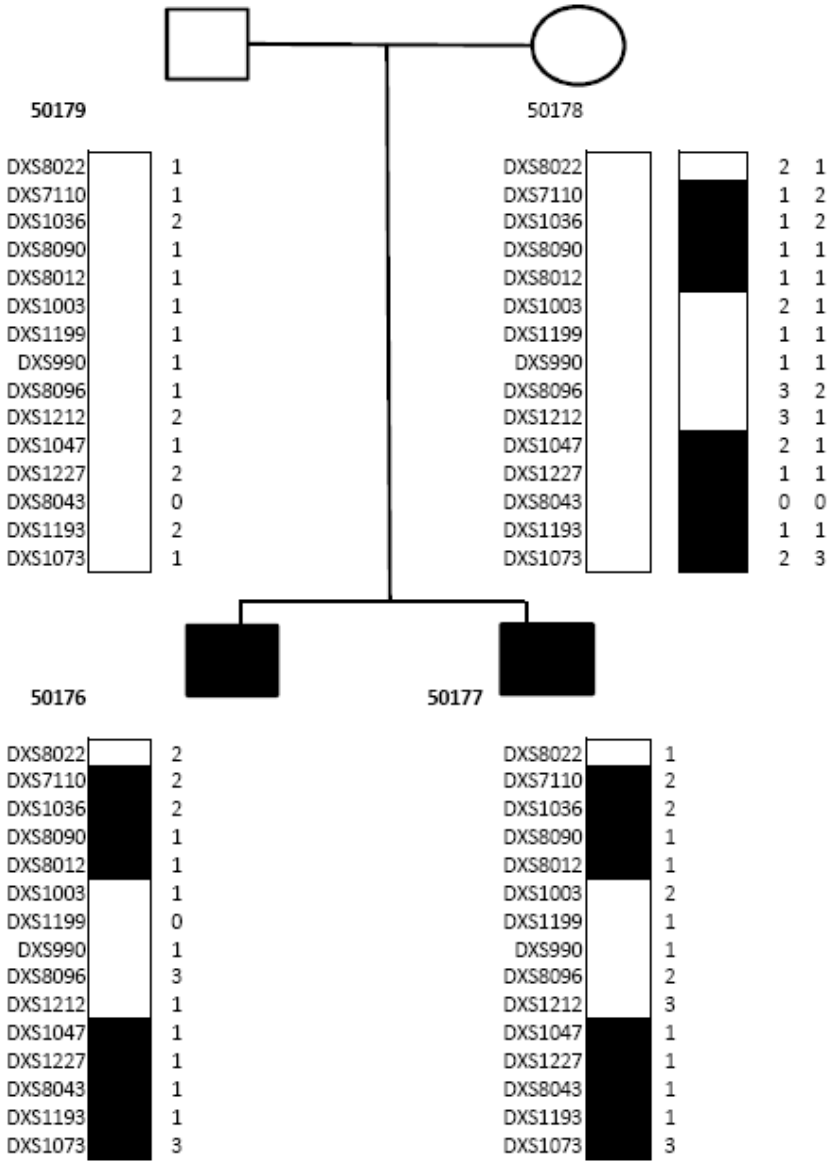
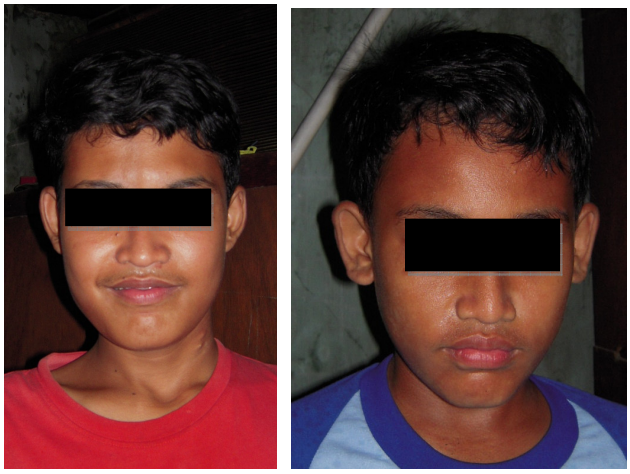


Figure 19. Family W090-0074: Pedigree and haplotype. The cosegregating haplotypes has been marking by black bar. Filled symbols represents male patients with MR.



A B

Figure 20. Pasien 50176 (A) and patient 50177 (B). Those patients showed prominent ears.

Microscopic cytogenetic analysis in patient 50176 and 50177 showed no chromosomal abnormalities. Analysis of CGG repeat on the promoter region of *FMR1* on both patients showed no CGG repeat expansion. Previous MLPA with subtelomeric probes showed no subtelomeric rearrangement. Fifteen highly polymorphic markers were used for linkage analysis (Table 18). This family showed two intervals of 32 Mb and 31 Mb in region Xp22.2-xp11.3 and Xq25-Xq28 (maximum LOD score of 0.3 at θ=0,0) that segregated with the disease. It was possible to exclude Xp22.33-Xp22.22 and Xq22.2-Xq25 from linkage (LOD score <-2). X-chromosome inactivation analysis in obligate carrier 50178 showed no skewing XCI.

Genetic Counselling

Although the linkage interval found in this family is quite big, still it can be used to show risk haplotype in this family. It was found that patient 50157, 50178, 50177, and 50176 shared risk haplotype. This finding support X-linked inheritance in this family. The mother shared risk haplotype with their affected son. This finding can be used for genetic counselling purposes.

Table 18. Two-Point LOD Scores for 16 X-Chromosomal Markers of family W09-074

Marker	Position (cM)	θ = 0.0	θ =0.05	θ =0.1	θ =0.15	θ =0.2
DXS8022	22.18	-4.69	-0.72	-0.44	-0.29	-0.19
DXS7110	29.22	0.30	0.25	0.21	0.17	0.13
DXS1036	33.54	0.30	0.25	0.21	0.17	0.13
DXS8090	36.79	0.00	0.00	0.00	0.00	0.00
DXS8012	42.21	0.00	0.00	0.00	0.00	0.00
DXS1003	47.08	-4.69	-0.72	-0.44	-0.29	-0.19
DXS1199	52.50	0.00	0.00	0.00	0.00	0.00
DXS990	60.62	0.00	0.00	0.00	0.00	0.00
DXS8096	68.74	-4.69	-0.72	-0.44	-0.29	-0.19
DXS1212	77.15	-4.69	-0.72	-0.44	-0.29	-0.19
DXS1047	82.84	0.30	0.25	0.21	0.17	0.13
DXS1227	88.33	0.00	0.00	0.00	0.00	0.00
DXS8043	94.22	0.00	0.00	0.00	0.00	0.00
DXS1193	97.89	0.00	0.00	0.00	0.00	0.00
DXS1073	102.35	0.30	0.25	0.21	0.17	0.13

W09-0078 Clinical Reports

The family W09-0078 (Fig. 21) was ascertained by purposive sampling in a special school. The pedigree of this family was compatible with X-linked inheritance. Not much is known about the past medical history of the patients. Patient 50215, 50196 and 50122 were attending special school. Obligate carriers were said to be normal.

The propositus, 50215 (Fig 22), was examined at age 12 years. He showed long face, heavy eyebrow, prominent ears, soft fleshy hands, sandal gap, and pes planus. His behaviour was unremarkable.

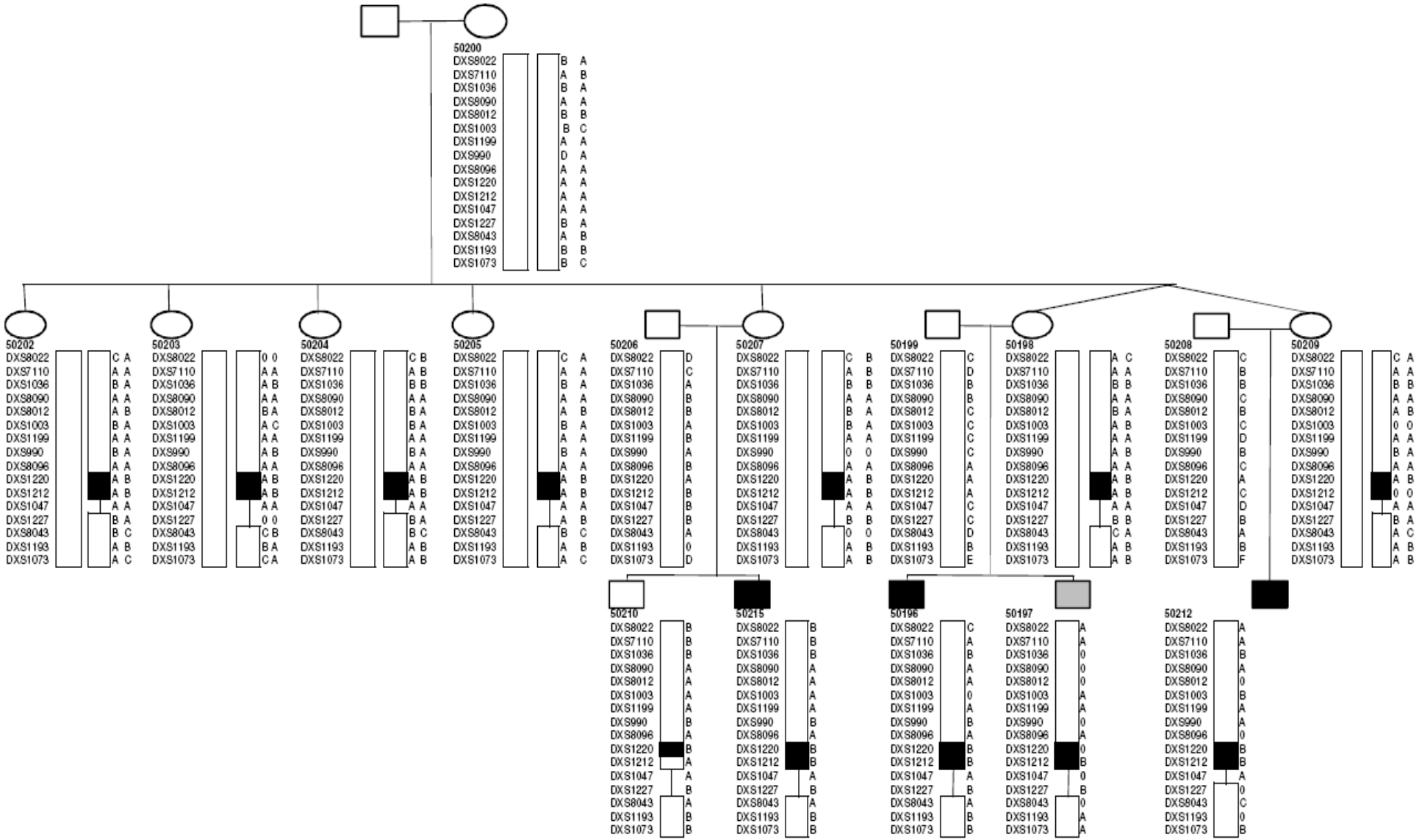
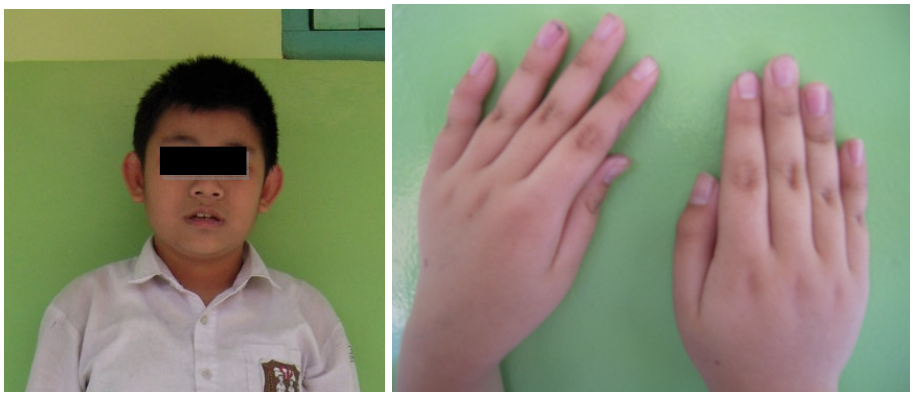


Figure 21. Family W090-0078: Pedigree and haplotypes. The cosegregating haplotypes has been marking by black bar. Filled symbols represents male patients with MR.



A B

Fig 22. Patient 50196 from Family W09-078: This patient showed long face, heavy eyebrow, prominent ears (A), and soft fleshy hands (B).

Microscopic cytogenetic analysis in patient 50215, 50196 and 50122 showed no chromosomal abnormalities. Analysis of CGG repeat on the promoter region of *FMR1* on patient 50196 showed no CGG repeat expansion. Previous MLPA with subtelomeric probes showed no subtelomeric rearrangement. Sixteen highly polymorphic markers were used for linkage analysis (Table 19). This family showed two intervals of 32 Mb and 31 Mb in region Xp22.2-xp11.3 and Xq25-Xq28 (maximum LOD score of 1.39 at θ=0,0 for DXS1212) that segregated with the diseaseX-chromosome inactivation analysis in obligate carrier 50207, 50198, and 50209 showed no skewing XCI.

Table 19. Two-Point LOD Scores for X-Chromosomal Markers of family W09-078

Marker	**Position (cM)**	**θ = 0.0**	**θ =0.05**	**θ =0.1**	**θ =0.15**	**θ =0.2**
DXS8022	22.18	-2.22	-2.03	-1.27	-0.84	-0.56
DXS7110	29.22	-4.81	-1.31	-0.82	-0.55	-0.36
DXS1036	33.54	0.58	0.52	0.45	0.38	0.30
DXS8090	36.79	0.00	0.00	0.00	0.00	0.00
DXS8012	42.21	-2.51	-1.46	-1.01	-0.74	-0.54
DXS1003	47.08	-1.62	0.07	0.25	0.31	0.31
DXS1199	52.50	0.00	0.00	0.00	0.00	0.00
Marker	**Position**	**θ = 0.0**	**θ =0.05**	**θ =0.1**	**θ =0.15**	**θ =0.2**

	(cM)					
DXS990	60.62	-1.65	-1.19	-0.68	-0.42	-0.26
DXS8096	68.74	0.00	0.00	0.00	0.00	0.00
DXS1220	70.91	-2.21	-1.4	-1.20	-0.98	-0.78
DXS1212	77.15	1.39	1.23	1.07	0.90	0.73
DXS1047	82.84	0.00	0.00	0.00	0.00	0.00
DXS1227	88.33	-1.41	-0.65	-0.41	-0.27	-0.18
DXS8043	94.22	-1.22	-0.84	-0.40	-0.18	-0.06
DXS1193	97.89	-1.11	-0.38	-0.17	-0.05	0.01
DXS1073	102.35	-0.81	-0.12	0.04	0.10	0.12

Genetic Counselling

Although the linkage interval found in this family is quite big, still it can be used to show risk haplotype in this family. It was found that obligate carrier 50207, 50198 and 50209 shared similar risk haplotype with their affected son. This finding support X-linked inheritance in this family. The mother shared risk haplotype with their affected son. Interestingly, females 50202,50203, 50204, 50205, shared similar risk haplotype with obligate carrier and affected male. Thus they can be a carrier, except if the genetic defect lie in between DXS1212 and DXS8043 which is uninformative in this family. This finding can be used for genetic counselling purposes.

W08-2152 Clinical Reports

Dutch families W08-2152 (Fig. 23) were referred to Moleculer Genetic Division RUNMC with mental retardation and behavioural problem. The pedigree of this family was compatible with X-linked inheritance. Not much is known about the past medical history of the patients. Obligate carriers were said to be normal. The propositus, 49744, showed mental retardation, autism, epilepsy, long narrow face, deep set eyes, high nasal bridge, macroorchidism, short fifth metatarsal, long finger and toes. Other affected boys, 49745 and 49746, showed similar features.

Gross cytogenetic abnormalities and fragile-X already excluded before. Sixteen highly polymorphic markers were used for linkage analysis (Table 20). This family showed one interval of 31 Mb in region Xq25-Xq28 (maximum LOD

score of 1.36 at θ=0,0 for DXS1193) that segregated with the disease. It was possible to exclude Xp22.2-Xq22.2 (flanked by DXS1060 and DXS1106) from linkage (LOD score <-2). X-chromosome inactivation analysis in obligate carrier 51677, showed skewing XCI.

Table 20. Two-Point LOD Scores for X-Chromosomal Markers of family W08-2152

Marker	**Position (cM)**	**θ = 0.0**	**θ =0.05**	**θ =0.1**	**θ =0.15**	**θ =0.2**
DXS1060	15.12	-11.62	-1.65	-1.31	-1.03	-0.78
DXS8051	17.29	-6.84	-0.57	-0.38	-0.29	-0.25
DXS8022	22.18	-14.72	-1.11	-0.87	-0.71	-0.57
DXS987	22.18	-9.62	-1.65	-1.32	-1.04	-0.79
DXS1226	27.59	-9.62	-1.46	-0.94	-0.62	-0.41
DXS7110	29.22	-9.32	-1.37	-0.82	-0.50	-0.30
DXS1214	33.54	-6.84	-0.57	-0.37	-0.29	-0.25
DXS1036	33.54	-9.32	-1.37	-0.82	-0.50	-0.30
DXS8090	36.79	-9.32	-1.37	-0.82	-0.50	-0.30
DXS1068	37.33	0.00	0.00	0.00	0.00	0.00
DXS993	42.21	-9.62	-1.45	-0.94	-0.62	-0.41
DXS8012	42.21	-9.32	-1.37	-0.82	-0.50	-0.30
DXS1003	47.08	-9.02	-1.11	-0.88	-0.74	-0.62
DXS1199	52.50	-0.76	-0.70	-0.56	-0.42	-0.30
DXS991	52.50	-11.62	-1.65	-1.31	-1.03	-0.78
DXS986	57.38	-11.62	-1.65	-1.31	-1.03	-0.78
DXS990	60.62	-0.62	-0.55	-0.42	-0.31	-0.22
DXS1106	68.74	-9.92	-0.85	-0.43	-0.23	-0.13
DXS1220	70.91	0.00	0.00	0.00	0.00	0.00
DXS1001	75.79	1.16	1.05	0.94	0.81	0.68
DXS1047	82.84	-0.32	-0.38	-0.45	-0.49	-0.49
DXS1227	88.33	0.00	0.00	0.00	0.00	0.00
DXS8043	94.22	0.00	0.00	0.00	0.00	0.00
DXS8091	97.89	-0.62	-0.65	-0.63	-0.55	-0.45
DXS1193	97.89	1.36	1.23	1.09	0.95	0.80
DXS1073	102.35	-0.62	0.03	0.18	0.23	0.23

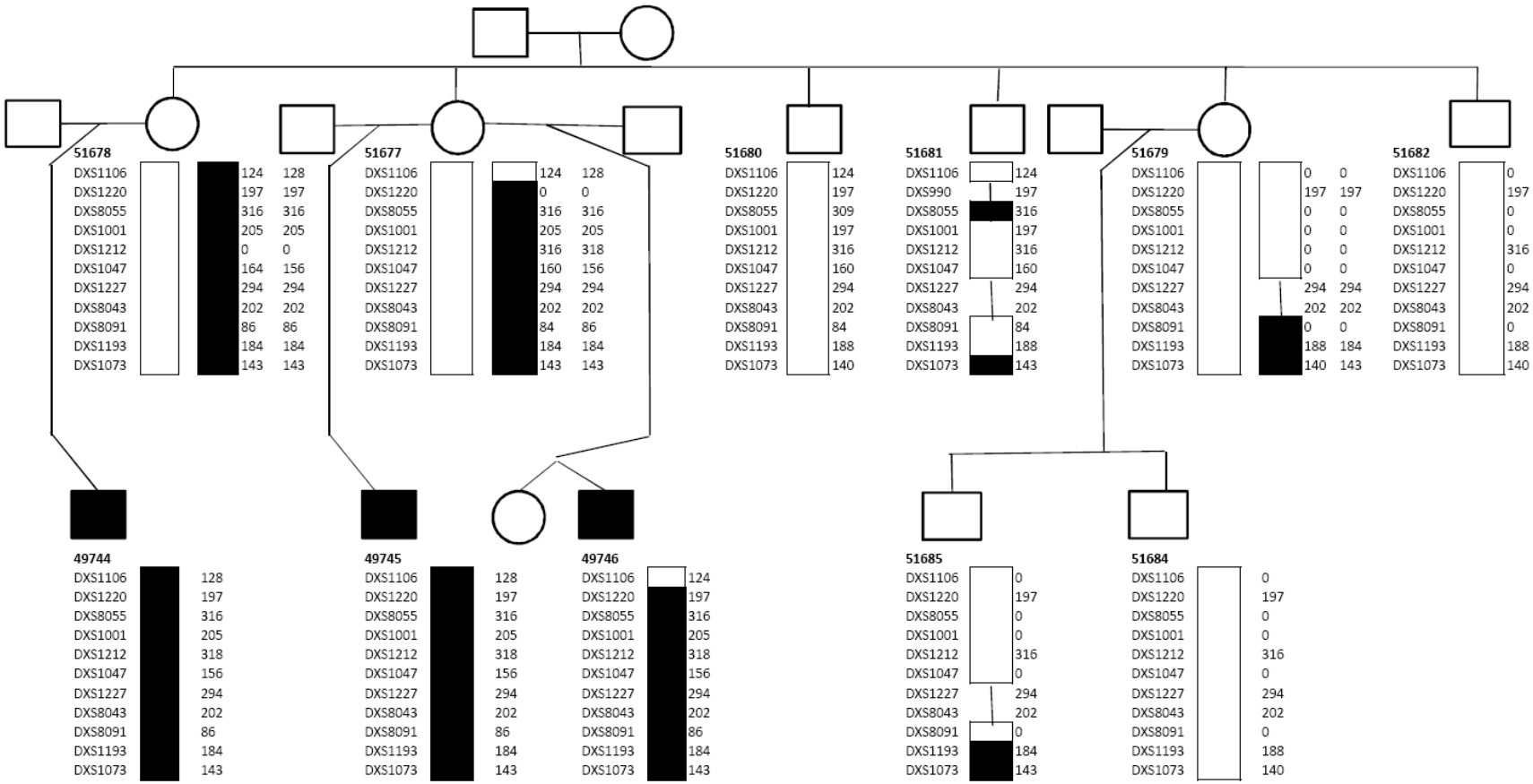


Figure 23. Family W08-2152: Pedigree and haplotypes. The cosegregating haplotypes has been marking by black bar. Filled symbols represents male patients with MR.

Discussion

We presented 5 families with non specific XLMR. All affected males in each of the families did not show any consistent clinical findings apart from MR, which lead to nonspesific XLMR as an appropriate diagnosis. Obligate carriers in all families showed normal intelligence. Based on three criteria of mental retardation all affected male from the 5 families were classified as mentally retarded. The degree of mental retardation showed variation within each family, ranging from mild to profound.

We have found interval to specific regions of the X-chromosome in all those 5 families with X-linked mental retardation. However, LOD score from those families are below 2, and interval found were large containing high number of genes. Addition of other family members, especially the affected family members, will help to narrow down the interval. Linkage result from this families can be used for genetic counselling purpose, considering that linkage will show risk haplotype in probable carrier.

Due to non specific clinical phenotype and large interval found, it is difficult to choose the candidate gene in the intervals region. Development of array technology will help to reveal any pathogenic copy number variation on genes inside interval. Intervals resulted from linkage analysis also could become target of high troughput sequencing which will help to reveal any gene mutation in these intervals.

CHAPTER V
CONCLUSION AND SUGGESTION

V.1. Conclusion

1. Identification of genetic defect in four syndromic and six non-syndromic XLMR families showed no macroscopic chromosomal abnormalities nor a CGG expansion in 5' untranslated region of FMR1. Ten XLMR families studied showed linkage intervals varying in size from 20 Mb to 121 Mb with varying LOD scores from 0.7 to 3.3.
2. Four families showed skewed X-inactivation in the obligate carrier female.
3. Candidate genes were selected in four syndromic XLMR families. No mutation was found in the those candidate genes. Candidate selection is easier to perform in the syndromic- compared to non-syndromic XLMR families, although no causative gene was found yet.

V.2. Suggestion

In all 10 families STR markers analysis was useful to determine linkage intervals. Although these are still large linkage intervals, we could narrow down the region of interest for further studies, such as next generation sequencing of the X-chromosome. In future studies, a more detailed clinical work-up need to be considered in order to find specific clinical features that could help in selection of candidate genes. Addition of DNA from other family members will help to establish smaller linkage intervals and to increase LOD scores. In addition, linkage interval found in this study can be useful for genetic counselling purpose, especially to diagnose carrier.This study also presents a workflow that will be very useful to implement in future studies of XLMR cases in Indonesia (Appendix VII). In addition, workflow used in two cases of X-linked hydrocephalus and MR in this study can be used for future studies of X-linked hydrocephalus and MR in Indonesia (Appendix VIII).

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APPENDIX I:

Chromosomal Preparation

Methods:

Ten drops of heparinized blood were cultured into two different 5 mL media (TC99 and MEM), to each 5% Fetal Bovine Serum (FBS) and 0,025 mL Phytohemaglutinin-P (Gibco) was added and incubated at 37°C for 72 hours. 0.1 mL thymidine (final concentration of 0.3 µg/m) was added to MEM media tube 24 hour before further processing, followed by addition of 3 drops of colchicine (final concentration of 1 µg/mL) twenty five minutes before further processing. 3 drops of colchicine (concentration of 1 µg/mL) were added to TC199 tubes 25 minutes before further processing. The cultured tubes were centrifuged at 1000 rpm for 10 minutes, followed by removal of supernatan. Warm (37°C) hypotonic solution KCL 0.075M was added to the cell pellet followed by resuspending of the solution and incubation at 37°C in waterbath for 15-30 minutes. Subsequently, the cell suspension was centrifuged at 1000 rpm for 10 minutes, followed by removal of suppernation and slow addition of 5 mL Carnoy's solution (3:1 methanol:acetic acid glacial) through the tube wall, then shaken well. These steps were repeated continuously untill a clear precipitation appeared. Subsequently, the fresh Carnoy's solution was added to suspend the residue. After that, two drops of cell suspension were droppled onto a glass slide of 20 cm, then stored for 72 hours. The aged slide was rinsed in water, put into a warm (37°C) Hanks solution, then moved into a 0.1% trypsin solution (in warm Hanks buffer) for 10-25 seconds, and finally rinsed again with water. Subsequently, the slide was submerged with Giemsa 10% staining in buffer phosphate pH 6.8 for 1 minute for GTG banding staining.

Appendix II:
DNA Isolation

Methods:

DNA extraction was performed by the Salting Out methods as described by Miller et al (Miller *et al*., 1988). EDTA frozen blood was transfered into 50 mL tube. A total of 5-10 ml NH4CL lysis buffer was added to the blood samples and then left for incubation by room temperature for 10-30 minutes. The tube was centrifuged for 5 minutes, 3000-3500 RPM. Supernatan was removed subsequently NH4Cl lysis buffer was added. This steps was repeated three times. Two mililiter of TE lysis buffer, Proteinase-K 10 mg/mL and 100 ul 10% SDS was added to the white palet, followed by incubation at 50^{o}C for 24 hours. Approximately one third volume NaCl 6M suspension was added followed by centrifuge at 4000 RPM for 10 minutes. Supernatant was collected in a new tube, followed by addition of 100% ethanol, twice the volume of the supernatant. DNA, present as a white substance, was taken for washing with 70% ethanol, then transferred into a 1.5 mL tube. The tube was left open for at least one hours to evaporate the excess of ethanol. DNA was resuspended in TE buffer.

Appendix III:
FMR1 gene amplification

Methods:

Primers were designed in the (CGG)n flanking sequences of the *FMR1* as described by Fu *et al*. (Fu *et al*, 1991). The forward primer was unlabelled and the reverse primer was 5'- labelled with FAM. Primer sequences are forward primer: 5'-GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT-3', and reverse primer: Fam 5'-AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA-3'. Approximately 100 nanogram DNA was amplified in a reaction mix containing 2 µL of 10x Pfx amplification buffer, 0.6 µL of 50 Mm MgSO4, 8 µL of enhancer solution, 0.5 µL of 10 mM dNTPs, 0.6 µL of forward primer, 0.6 µL of reverse primer, 0.3 µL of Platinum Taq polymerase, and 6.4 µL milliQ in a 20 µL final reaction volume. The reaction was initiated by denaturation for 3 minutes at 95°C, followed by 31 cycles of 15 seconds denaturation at 95°C, 2 minutes annealing at 64°C, and 2 minutes elongation at 75°C. A final elongation step was set at 75°C for 10 minutes. 5 µL loading dye was mixed with 5 µL of PCR product and loaded on a 2% agarose gel for 3 hours at 120 volt. All samples gave PCR product, so Southern Blot was not performed.

Fragment length analysis:

Principle:

In DNA fragment analysis, a mixture of fluorescent labeled DNA fragments were resolved into its constituent parts on the basis of molecular weight. After this, a profile was created.

Method:

One microliter of PCR product was mixed with 0.3 µL LIZ 500 size standard marker and 8.7 µL formamide. The samples were denatured by heating at 100C for two minutes. Then, the fragment size was measured by capillary electrophoresis on the ABI 3730 Analyzer. After the run, the data

were automatically be sent to the BioLIMS Database. Data was analyzed using Gene Mapper software version 4.0 (Apache Software). The fragment length were calculated by the following formula:

CGG repeat = {(length of the peaks-282)/3} +23

Appendix: IV

X-Chromosome Linkage Analysis

Principle:

Particular set of alleles at linked loci (haplotypes) of the X-chromosome in families were determined by use of highly polymorphic markers. This method based on automated systems using fluorescently labeled polymerase chain reaction (PCR) fragments that allow very precise allele calling. This method used one primer that is 5' end labeled with a fluorescent dye. Then, forward primer with a 19-bp extension at its 5' end, identical to the sequence of an M13 sequencing primer, a regular reverse primer and a third universal fluorescent labeled M13 primer were used to reduce the costs of genotyping with fluorescently labeled microsatellites This primer "tail" gave a complementary sequence to the universal fluorescent primer starting from the third PCR cycle, producing a fluorescent product that can be detected on an automatic DNA sequencer. In this case, instead of synthesizing one specific labeled forward primer for each microsatellite marker, only one universal labeled primer was needed (Figure 5; Oetting *et al*., 1995). This approach was known as multiplexing with tailed primers.

These short-tandem-repeat markers were selected with an average distance of 10 cM between markers and a heterozygosity score of at least 0.7. Primers and location of the STR markers are described in Table 3. First, genomic DNA was amplified using the specific CA-repeat primers (in the first PCR), subsequently in the second PCR 1µl of the first PCR reaction was amplified with an M13 forward primer, labeled with one of four fluorophores (FAM/VIC/NED/PET) at the 5' end (Oetting *et al.,* 1995) and a M13 reverse primer with a 5'-GTTTCTT-3' added to its 5' end to reduced tailing. Samples were pooled and analyzed by the 3730 Analyzer (Applied Biosystems). Differences in length of the CA-repeats was measured and raw data was analyzed with Genemapper software (Applied

Biosystems) to determine the haplotypes that are inherited together in the families. Two-point LOD scores was calculated by easyLINKAGE software (Hoffman and Lindner, 2005).

Table 21. Markers sequences used for X-Chromosome Linkage analysis

Markers	Forward primer 5'>3'	Reverse primer 5'>3'	Location
DXS8022	Tggaaactaatgcagcatgtc	aagtcccattttagccaacc	Xp22.2
DXS7110	Gcacaaaggaggaaccaacc	tcggcttgtttaaatggtcct	Xp22.11
DXS1036	Tgcagtttattatgtttccacg	gccattgataagtgccagat	Xp21.1
DXS8090	Atcccccaaagaaccaagaa	caagggtgaaattccatcaca	Xp21.1
DXS8012	Tttggaaggcggacataaac	aacaagaagcttagcaagccc	Xp11.4
DXS1003	tgtgtgtgagtgagggagagag	agaagccgttattggtggac	Xp11.3
DXS1199	ggtgactgactctgtggc	tggagtgaaatcaacatttaacata	Xp11.22
DXS990	agctatatgaccagtacaaacatac	gacagaagggacatcaactc	Xq21.32
DXS8096	attgggaaggtcatctcag	tcatgtgagccagttcttg	Xq22.2
DXS1220	agcgagagtctgacccac	ggggcctataaaatggag	Xq23
DXS1212	Aacagctcattttgtgtcatgg	tgacccagagaagtggaacc	Xq25
DXS1047	Ccggctacaagtgatgtcta	cctaggtaacatagtgagaccttg	Xq25
DXS1227	agaggtccgagtcttccac	ataagggtttactcccccaa	Xq27.2
DXS8043	Ttggcaaagagtacaggcag	tctcagaaacatttggttaggc	Xq27.3
DXS1193	aattctgactctggggc	ttattttaaggtgagtatggtgtgt	Xq28
DXS1073	ggctgactccagaggc	ccgagttattacaaagaagcac	Xq28

First PCR: (protocol by Zonneveld, 2009)

For the first PCR, an input of 40 ng of genomic DNA was used. A reaction mixture was added containing 2.5 µL 10x PCR Buffer, 1 µL $MgCl_2$ 50 mM (final concentration of 2 mM), 0.5 uL dNTPs 10 mM, 0.5 µL forward primer, 0.5 µL reverse primer, 0.1 µL Invitrogen Taq polymerase and 17.4 µL milliQ. The reaction was initiated by denaturation for 5 minutes at 95°C, followed by 30 cycles of 15 seconds denaturation at 95°C, 15 seconds annealing at 58°C, and 45 seconds elongation at 72°C. A final elongation step was set at 72°C for 10 minutes. Five microliter of each

sample was loaded on an 1,5% agarose gel to confirm amplification of the desired fragment.

Second PCR: (protocol by Oostrik, 2009)

Every CA-repeat marker was fluorescently labeled in the second PCR with a labeled forward primer (FAM/NED/VIC/PET). CA-repeat markers labeled with the same fluorophore, differed at least 40 bp in amplicon size. Two microliter of PCR product for the first PCR was taken for the second PCR. A reaction mix consisting of 1 µL 10x PCR Buffer, 0.4 µL $MgCl_2$ 50 mM (with final concentration of 2 mM), 0.2 uL dNTP 10 mM, 0.2 µL fluorescent labeled forward primer (FAM/VIC/NED/PET), 0.2 µL M-13 pigtail reverse primer, 0.04 µL Invitrogen Taq polymerase and 5.96 µL milliQ. The reaction was initiated by denaturation for 5 minutes at 95°C, followed by 15 cycles of 15 seconds denaturation at 95°C, 15 seconds annealing at 50°C, and 45 seconds elongation at 72°C. A final elongation step was set at 72°C for 10 minutes. Five microliters of each samples was mixed with 5 µl loading buffer and loaded on an 1,5% agarose gel to confirm amplification.

Up to four different markers of the same sample were pooled (1 µl of each marker). Seven point five microliters formamide and 0.5 µl of LIZ standard was added to 2 µl of pooled sample of the second PCR, final volume of 10µl. Samples were analyzed with the 3730 Analyzer (Applied Biosystems). Raw data was analyzed by Gene Mapper software (Apache Software) to determine haplotypes. Two-point LOD scores were calculated by Superlink (Fishelson and Geiger, 2002), and exclusion mapping was performed with GeneHunter PLUS v. 1.2 (Kong and Cox, 1997) integrated in easyLINKAGE (Hoffman and Lindner, 2005). Inheritance mode was set at recessive inheritance, disease allele frequency was set at 0.001, and full penetrance was assumed.

Appendix V:
X-Chromosome inactivation (XCI) analysis

Methods:

Assays of XCI may be performed by direct approach using expression analysis that requires RNA, or indirect approach with DNA-based methylation analysis (Allen *et al.* 1992). Numerous methylation assays of XCI have been described with numerous technical approach, for example: conventional polymorphisms in the phosphoglycerate kinase (*PGK*) and hypoxanthine phosphoribosyl transferase (*HPRT*) genes (Vogelstein et al. 1987 and Maestrini *et al.* 1992) and others more informative VNTR in the DXS255 locus recognized by the probe M27P (Boyd and Fraser, 1990) or microsatellite (Allen *et al.* 1992) markers. All of these methods rely on two basic assumptions: the presence of a polymorphism to discriminate between the maternal and the paternal X-Chromosome and a different methylation pattern on the active versus the inactive X-Chromosome. However, considering that allelic difference are needed in these test, assay with highly polymorphic loci are desirable. Thus, methylation study in *FMR1* and *AR* loci were used in this study considering the high heterozygosity (more than 90% heterozygosity of *AR* loci polymorphism in females (Allen *et al.* 1992) and -65% heterozygosity for the *FMRl* CGG repeat (Fu *et al.* 1991)) compared to other locus (30% heterozygous in *PGK* probe, 18% heterozygous in *HPRT* probe and 90% heterozygous in M27I probe in those situations that provide enough DNA to perform both Southern analysis and PCR) previously described (Allen *et al.* 1992).

X-Chromosome inactivation analysis: *FMR1*

Principle: (Carrel and Willard, 1996)

An assay based on methylation status at the fragile X mental retardation gene, *FMRl,* was used to examine the pattern of X-chromosomal inactivation. Digestion of genomic DNA with BamHI was used to cut

genomic DNA in order to improve PCR efficiency. This was followed by digestion with HhaI. Digestion of genomic DNA with this methylation-sensitive enzyme cleaved two restriction sites near the CGG repeat of the *FMRl*-repeat of unmethylated (active X chromosome), but did not digest these sites on the methylated X (inactive X-chromosome). After digestion, a PCR, using primers that flank the CGG repeat site of the *FMRl* gene, will result in amplification of only undigested alleles, (the methylated inactive X-chromosomes) (figure 24). Amplification of the hypervariable CGG repeat made differention of alleles in heterozygous samples possible, while the relative signal ratio of alleles was used to assess the randomness of X-chromosome inactivation. X-chromosome inactivation patterns would be defined as skewed if there is >80% skewing.

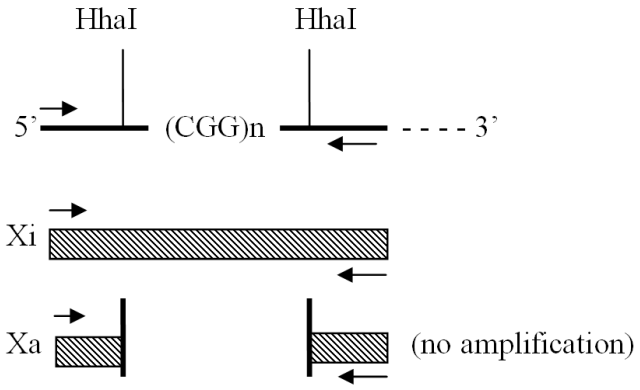


Figure 24. Methylation assay of X-chromosome inactivation based on PCR analysis of the 5' untranslated part of *FMR1*. HhaI is not capable to cut the methylated sites on the inactive X-chromosome (Xi), thus allowing PCR amplification. On the other hand, digestion at either or both sites on the active X-chromosome will not result in amplification.

Procedure:

First, informativity of *FMR1* was checked, by PCR of the *FMR1* trinucleotide repeat as described in III.3.4. The next steps were performed if a sample was informative for the *FMR1* trinucleotide repeat site. One

hundred twenty five nanogram of DNA from carrier females and a male control was digested overnight by 37°C with a combination of BamHI and methylation-sensitive restriction enzyme HhaI as well as BamHI alone as a control. The first reaction included 125 ng of DNA with the following reaction mix: 0.75 µL BamHI, 0.5 µL HhaI, 0.25 µL Bovine Serum Albumine (BSA), 2.5 µL of buffer NEB3, and 8.5 uL milliQ incubated overnight by 37°C. The control reaction included 12.5 µL of DNA mixed with a reaction mix of 1 µL BamHI, 0.25 µL Bovine Serum Albumine (BSA), 2.5 µL buffer NEB3, and 8.75 uL milliQ incubated overnight by 37°C. Then, complete digestion was ensured by redigestion of the DNA by addition of half the amount of the previously described BamHI and HhaI reaction mixtures for 4 hours by 37°C. Subsequently, the enzymes were inactivated for 20 minutes by 65°C. Five microliters of each digested sample was mixed with 5 µL of loading dye loaded on a 1,5% agarose gel to confirm the digestion.

PCR amplification of CGG repeat was performed with 5 µL digested sample mixed with 2 µL of 10x Pfx amplification buffer (Invitrogen, Breda, The Netherlands), 0.6 µL of 50 Mm MgSO4, 8 µL of enhancer solution, 0.5 µL of 10 mM dNTPs (Invitrogen, Breda, The Netherlands), 0.6 µL of forward primer (5'-GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT-3') ,0.6 µL of reverse primer (Fam 5'-AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA-3'), 0.3 µL of Platinum Taq polymerase, and 2.4 µL milliQ, 20 µL final volume. The reaction was initiated by denaturation for 3 minutes at 95°C, followed by 32 cycles of amplification by denaturation at 95°C for 15 seconds, annealing at 64°C for 2 minutes, and elongation at 75°C for 2 minutes. A final elongation step was done at 75°C for 10 minutes. 5 µL loading dye was mixed with 5 µL of PCR product and loaded on a 2% agarose gel (120 volt for 3 hours) to confirm amplification.

Two microliter of PCR products was mixed with 0,5 µL LIZ 500 size standard marker and 7,5 µL formamide and analyzed on the ABI 3730

analyzer. The raw data was analyzed using Gene Mapper software version 4.0 (Apache Software).

X-Chromosome inactivation analysis: *AR* method

Principles: (Allen *et al*., 1992)

This method was a modification of the human androgen receptor (*AR*) gene assay described by Allen et al. Inactivation status of the X-chromosome were assessed by taking advantage of the favorable characteristics of highly polymorphic trinucleotide repeat in the first exon of the human *AR* locus at Xq11–q12. The principle was similar with *FMR1* method, only the repeat is more informative in some cases. HhaI will digest unmethylated DNA in the *AR* (CAG)n repeat region of the active X-chromosomes and will not cut methylated sites of the inactive X-chromosomes.

Procedure :

Primers were designed in the (CAG)n flanking sequence of the first exon of *AR* gene as described by Allen et al. The forward primer was 5' labelled (Fam). Primer sequences are: forward primer, Fam 5' TCC AGA ATC TGT TCC AGA GCG TGC 3' and reverse primer, 5' GCT GTG AAG GTT GCT GTT CCT CAT 3'.

First we checked *AR* informativity. PCR was performed as follows: 1 µg DNA in 3 µL (AB) Buffer, 0.5 µL dNTPs, 0.6 µL forward primer, 0.6 µL reverse primer, 0.3 µL ampliTaq, 2.5µL DMSO, 21.5 µL water. The reaction was initiated by denaturation for 2 minutes at 95°C, followed by 30 cycles of 30 seconds denaturation at 93°C, 30 seconds annealing at 55°C, and 30 seconds elongation at 73°C. A final elongation step was set at 73°C for 10 minutes. The next steps were performed when the *AR* repeat turned out to be informative.

Three micrograms of DNA dissolved in 70 uL milliQ from carrier females and a male control were digested for 6 hours by 37° with a combination of

BamHI and methylation-sensitive restriction enzyme HhaI, as well as BamHI alone as a control. First, digestion of 35 µL of DNA with a reaction mix, containing 1.5 µL BamHI, 1 µL HhaI, 0.4 µL 20 mM spermidine, 4 µL React4 was initiated by incubation for 6 hours by 37°C. The control reaction conditions were: digestion of 35 µL of DNA with a reaction mix, containing 2 µL BamHI, 0.4 µL 20 mM Spermidine, 4 µL React4 incubated for 6 hours by 37°C. Digestion was confirmed by gel electrophorese:4 µL digested sample and 2 µL bromphenol blue 10x were loaded on an 0.8% agarose ME Seakem gel (1 hour, 120 volt).

PCR amplification was performed on 3 µL of digested sample under the following conditions: 3µL (AB) buffer, 0.5 µL dNTPs, 0.6 µL forward primer, 0.6 µL reverse primer, 0.3 µL Taq (PE), 2.5 µL DMSO, 19.5 µL milliQ. The reaction was initiated by denaturation for 2 minutes at 95°C, followed by 30 cycles of 30 seconds denaturation at 93°C, 30 seconds annealing at 55°C, and 30 seconds elongation at 73°C. A final elongation step was set at 73°C for 10 minutes.

Five microliter of orange G loading buffer was mixed with 5 µL of PCR product and loaded on an 1% agarose gel (ME Seakem) (200 volt for 45 minutes in 0,5 TBE) to confirm amplification of the *AR* repeat.

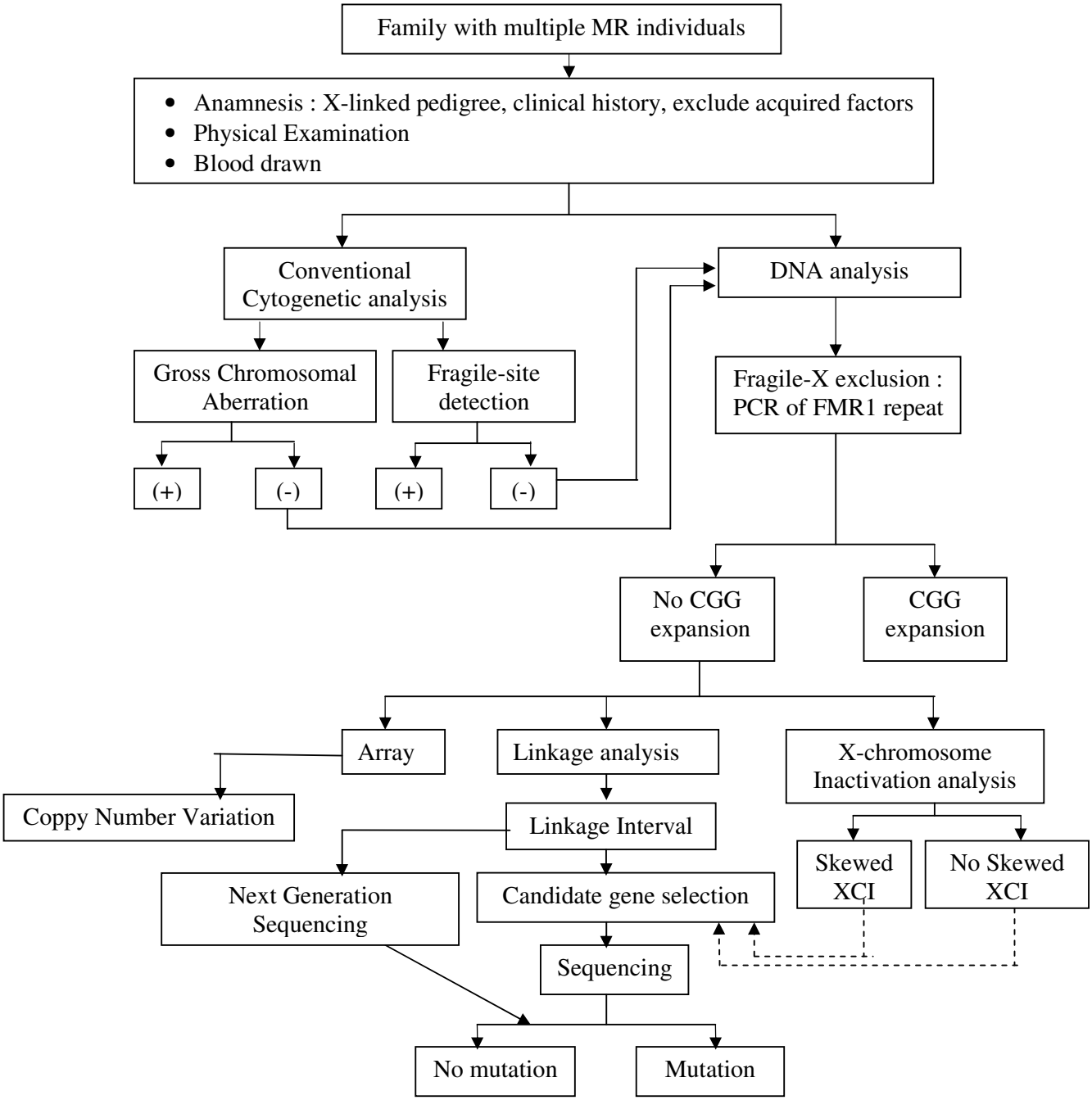
One microliter PCR products was mixed with 0,3 µL LIZ 500 size standard marker and 9 µL formamide and analyzed on the ABI 3730 analyzer. The raw data were analyzed using Gene Mapper software version 4.0 (Apache Software).

Appendix VI:
Candidate Gene Selection

Promising candidate genes were selected in linkage intervals on the X-chromosome. The UCSC Genome browser database was used to extract all UCSC genes in the linkage intervals. Prioritizing candidate genes was performed by use of two independent bioinformatics tools (ToppGene {available at at: http://toppgene.cchmc.org/} and Endeavour {available at: http://homes.esat.kuleuven.be/~bioiuser/endeavour/index.php}; Chen et al, 2007; Tranchevent et al, 2008) and secondly, by manual selection based on the expression in brain/neuronal tissues, homology with known MR genes, involvement in the same protein network as already known MR genes, and gene methylation status.

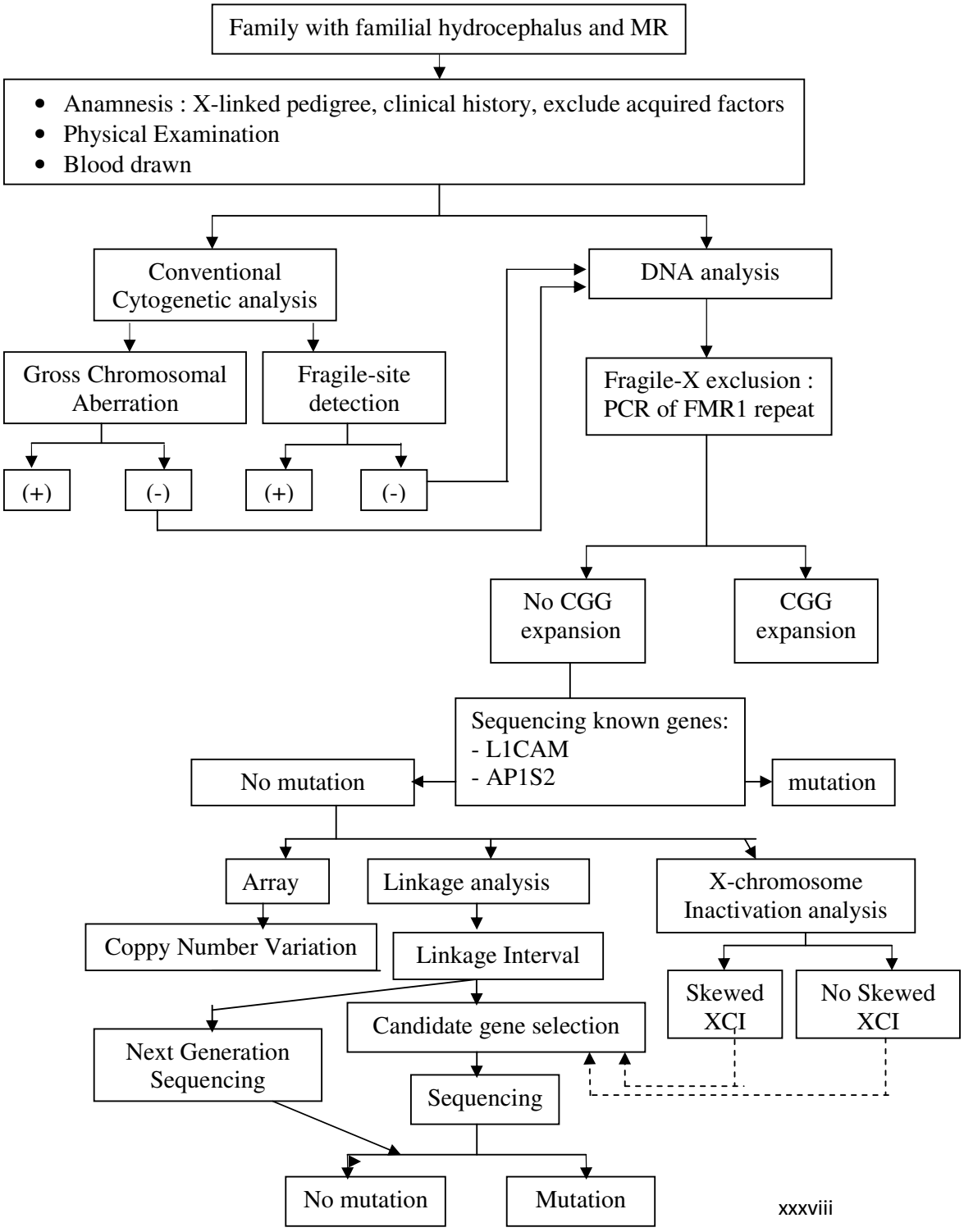
APPENDIX VII:

Proposed Workflow for XLMR Studies



APPENDIX VIII:

Proposed Workflow for X-linked Hydrocephalus and MR



APPENDIX IX:

Physical Examination Form

Dr: DATE: LOCATION :

Family/proband			
Gene	Mutation	DNA/fam.nr.	Laboratory

Clinical photographs yes/no	Archived where	Consent patiënt/parents for use in teaching and/or scientific publications/meetings

Clinical genetic conclusion

	Diagnosis	Recurrence risk	Remarks
1			
2			
3			

Relevant patiënt organisation:

Literature given to patiënt/parents:

NAME AND ADDRESS OF REFERRING/TREATING DOCTORS

	Name	Specialism	Address
1.			
2.			
3.			

Date	Name supervisor	Signature of supervisor

PEDIGREE

Consanguinity yes/no

HISTORY

Conception

Exposition by profession/recreational?

Profession of man/father: Profession of woman/mother:

Pregnancy:

fluxus
fever
skin problems
infections
trauma
toxicosis
diabetes mellitus
medicines
smoking
alcohol
X-ray/radiation
other intoxications

Prenatal care from ……weeks GA by:
Prenatal diagnosis (indication and results):
Ultrasound examination (indication and results):

Delivery: by whom where
gestational age spontaneous
induction artificial labour
position duration Apgar score
amniotic fluid umbilical cord placenta
W (P:) L: (P:) OFC: (P:)
asphyxia icterus
artificial ventilation: how long in hospital:

Neonatal period:
feeding problems
hypotonia

Psychomotor development:
laughing grasping
making noises sitting with help
standing walking
social contact
behaviour
regression yes/no
rolling over
sitting without help
speech
school

Past ilnesses/admissions/operations

Paramedical treatment (physiotherapy, speech therapy etc.)

Prescribed medicines

SPECIFIC HISTORY

EXAMINATION Date: Age at examination:

General aspects

Bodily habitus: Developmental level
motor :
cognitive:

Stature in proportion: yes/no

Measurements		P/SD				P/SD
weight			Ear length	AD AS		
length/height			Nipple distance			
OFC						
spanwidth			Chest circumference			
US/LS						
Sitting height			Penile length			
ICD			Testis volume	ri le		
OCD			Foot length	ri le		
IPD						
Palp. fissures			Hand length	ri le		
Corneadiameter	OD OS		Palml ength	ri le		
fontanel			finger III length	ri le		

HEAD

General:		form	micro/retrognathia
		forehead	mimics
		mid face	
Eyes	:	position	form
		hypo/hypertelorism blepharophimosis ri/le microphthalmos ri/le	tele/epicanthus ptosis ri/le iris coloboma
		cornea	eye lids
		eye colour	eye movements
Ears	:	position	fistula
		form	appendages
Nose	:	form	philtrum
		choanae	
Mouth	:	size	palate (uvula)
		lips	teeth
		tongue	gingiva
Neck :	:	webbing	hairline
		fistula	movements

TRUNK

Thorax	:	form	heart
		mammae	lungs
		nipples	
Abdomen	:	liver	spleen

kidneys	hernia
diastasis mm. recti	abdominal wall

Back : kyphosis/lordosis/scoliosis spina bifida

sacrale dimple anus

Genitalia : puberty stages (Tanner) A M P G

testis

LIMBS

Arms : proportions upper arm

muscle tone under arm

hands: syn/poly/clino/camptodactyly

palm creases

Legs : proportions upper leg

spiertonus lower leg

feet: syn/poly/clino/camptodactyly

pes cavus/planus

Hypermobility score: thumb to under arm 5th finger > 90^{o}

elbows > 10^{o}

knees > 10^{o} hands to floor

Total ...../9

Contractures?

SKIN

Hair (incl.eyebrows, eyelashes) sweating

elasticity nails

pigment changes others

bullae/ichthyosis/hyperkeratosis

vascular abnormalities

NERVOUS SYSTEM

Summary

Preliminary conclusion and differential diagnosis

Additionnal investigations/management

Results of additionnal investigations (with dates!)

biochemistry/clinical chemistry

cytogenetics

DNA

imaging

IQ

consultands